Technische Universität München

Lehrstuhl für Ernährungsphysiologie

Re-assessing intestinal sugar transport by use of mice lacking the sodium-dependent glucose transporter SGLT1, the glucose transporter GLUT2 or the fructose transporter GLUT5

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. M. Klingenspor
Prüfer der Dissertation:	
	1. Univ.-Prof. Dr. H. Daniel
	2. Univ.-Prof. Dr. H. Witt

Die Dissertation wurde am 25.03.2014 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 18.06.2014 angenommen.

Der Fortschritt der Wissenschaft besteht in der Verschiebung der Wissenslücken.

Prof. Dr. Hans-Jürgen Quadbeck-Seeger

Danksagung

Obwohl unter dem Titel dieser Arbeit nur ein Name steht, so haben doch weitaus mehr Leute direkt und indirekt zu ihrer Entstehung beigetragen und hiermit möchte ich ganz herzlich DANKE sagen.

Der größte Dank gilt Frau Prof. Hannelore Daniel. Sie haben mir diese Arbeit ermöglicht und zu Ihrer Entstehung und Vollendung maßgeblich beigetragen. Vielen Dank für Ihre Betreuung, Unterstützung, Begeisterung und all die Anregungen und Ratschläge, die Sie mir während zahlreicher Diskussionen gegeben haben.
Mein Dank gilt auch Herrn Prof. Witt als meinem Zweitgutachter sowie Herrn Prof. Klingenspor als Prüfungsvorsitzendem.

Besonderer Dank gebührt Tamara und Kerstin. Ihr standet mir immer mit Rat und Tat zur Seite, habt mit mir all meine Fragen und Probleme diskutiert und mich motiviert. Ich kann euch gar nicht genug danken.

Vielen Dank an Irmi, Ronny und Margot für eure Hilfe mit all den Western blots, Mäusen und Immunos.

Danke an das ALIA Projekt der Agence Nationale de la Recherche (ANR) und die Deutsche Forschungsgemeinschaft (DFG), die meine Arbeit finanziert hat.

Lieben Dank an meine beiden Würfelkolleginnen Katrin und Julia. Ihr wart immer für mich da. Ich werde unseren Würfel – und natürlich euch ☺ – vermissen.

Mein Dank geht natürlich auch an alle Kollegen der AG Daniel, die da waren und die da sind, vor allem Mena, Veronika, Ramona, Christine und Manu. Ihr alle seid/wart tolle Kollegen und ich habe mich sehr wohl bei euch gefühlt.

Vielen Dank an das Sekretariat, Brigitte, Cordula und Dorothea, für eure Hilfe in allen Lebenslagen und -fragen.

Großer Dank gebührt auch meinem Freund Basti, du bist mein Fels in der Brandung, mein Ruhepol, meine Quelle der Kraft. Danke, dass du immer für mich da bist. Und natürlich meiner Familie, danke für eure Unterstützung und dass ihr immer für mich da seid.

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Summary

Although carbohydrates constitute the majority of our diet and are under suspicion to contribute to obesity and/or diabetes mellitus type 2, the mechanisms underlying intestinal sugar absorption are not completely unveiled yet. According to physiology text books, uptake of glucose across the apical membrane of enterocytes is mediated by the sodium-dependent glucose transporter SGLT1 whereas GLUT5 is responsible for fructose absorption. Glucose and fructose are then released from the cell into circulation by the glucose transporter GLUT2 located in the basolateral membrane. Recent studies – mainly in rats – suggested that GLUT2 may be inserted into the apical membrane at high luminal glucose concentrations, allowing bulk absorption of glucose in addition to SGLT1 and at the same time to function as an intestinal glucose sensor. In humans suffering from an established fructose malabsorption the simultaneous ingestion of glucose prevents fructose malabsorption in a dose-dependent manner. The underlying mechanisms for this clinical finding are not known but may include the involvement of both, SGLT1 and GLUT2.

Taking advantage of mice that lack SGLT1, GLUT2 or GLUT5, we aimed to re-assess the contribution of the respective transporter to intestinal sugar transport by using *in vitro* methods as well as oral administration of radiolabeled glucose and fructose, respectively, combined with Western blot analyses. Our comparative studies revealed a drastically reduced absorption of glucose and a concomitant abolished GIP- and GLP-1 secretory response as well as diminished insulin secretion in the absence of SGLT1. In contrast, radiolabeled glucose accumulated in intestinal tissues of GLUT2-deficient mice in which glucose-induced insulin but not incretin secretion was impaired. Loss of GLUT5 resulted in significantly reduced fructose absorption that was not improved when glucose was co-administered.

The studies described here are the first to use *in vivo* a tracer gavage of glucose or fructose to assess intestinal sugar transport under standardized conditions. The data obtained confirm the “classical” roles of SGLT1 and GLUT2 in mediating transepithelial glucose transport and demonstrate that GLUT5 is the prime fructose transporter. However, we did not find evidence for any apical GLUT2 and its involvement in either luminal glucose or fructose absorption.

Zusammenfassung

Obwohl Kohlenhydrate den Hauptteil unserer Nahrungsenergie stellen und im Verdacht stehen, zu Fettleibigkeit und/oder Diabetes mellitus Typ 2 beizutragen, sind die zugrunde liegenden Mechanismen der Kohlenhydrataufnahme im Darm bisher noch nicht vollständig geklärt. Nach allgemeiner Auffassung erfolgt die Glukoseaufnahme durch die apikale Membran der Darmepithelzellen durch den Natrium-abhängigen Glukosetransporter SGLT1 und die Aufnahme der Fruktose durch GLUT5. Glucose und Fruktose werden anschließend durch GLUT2 in der basolateralen Membran in den Blutkreislauf gebracht. Neuere Studien – vor allem in Ratten – deuteten darauf hin, dass bei hohen luminalen Glukosekonzentrationen GLUT2 an die apikale Membran rekrutiert wird, um zusätzlich zu SGLT1 den Großteil der Glukoseaufnahme zu vermitteln und gleichzeitig als intestinaler Glukosesensor zu fungieren. In Menschen, die an einer Fruktosemalabsorption leiden, kann die gleichzeitige Zufuhr von Glukose die Malabsorption aufheben und zu einer deutlichen Verbesserung der klinischen Symptomatik führen. Die diesem Phänomen zugrunde liegenden Mechanismen sind bisher noch nicht geklärt, es könnten jedoch SGLT1 und/oder GLUT2 beteiligt sein.

Durch die Verfügbarkeit von Mäusen mit einer SGLT1-, GLUT2- oder GLUT5-Defizienz prüften wir die Bedeutung dieser Proteine in der Glucose und Fruktoseresorption, indem wir *in vitro* Methoden und oral verabreichte radiomarkierte Glukose und Fruktose verwendeten sowie Western blot Analysen zum Nachweis der Transporter in der apikalen Membran durchführten. Unsere Vergleichsstudien offenbarten eine drastisch reduzierte Glukoseaufnahme, einen gänzlichen Verlust der GIP- und GLP-1-Sekretion sowie eine verringerte Insulinausschüttung in der Abwesenheit von SGLT1. Im Gegensatz dazu reicherte sich Glukose im Darmgewebe von GLUT2-defizienten Mäusen an, die zudem eine Störung der Glukose-bedingten Insulin-, nicht aber Inkretinsekretion aufwiesen. Der Verlust von GLUT5 führte zu einer deutlich verminderten Fruktoseaufnahme, die in der Gegenwart von Glukose nicht verbessert wurde.

Die hier beschriebenen Untersuchungen waren die ersten, bei denen unter standardisierten Bedingungen radiomarkierte Substrate *in vivo* eingesetzt wurden,

um die Monosaccharidaufnahme im Darm zu quantifizieren. Unsere Studien bestätigen die „klassischen" Rollen von SGLT1 und GLUT2 hinsichtlich der Vermittlung des epithelialen Glukoseein- beziehungsweise -ausstroms sowie von GLUT5 als den für die Fruktoseaufnahme verantwortlichen Transporter. In unseren Studien haben wir jedoch keine Hinweise auf eine Rekrutierung von GLUT2 in die apikale Membran und damit für eine Beteiligung an der Glukose- und/oder Fruktoseaufnahme aus dem Lumen gefunden.

1. Introduction

1.1. Sugar transport in the small intestine

Carbohydrates are the main energy source for humans and herbivores although they are not essential for survival [1].

Due to the worldwide increase in obesity and diabetes mellitus type 2 [2] there is an emerging interest in the mechanisms underlying sugar transport in the intestine and possible adaptation mechanisms promoting development of diabetes mellitus type 2.

Nutrient absorption takes place in the small intestine which is divided from proximal to distal into duodenum, jejunum and ileum, each part being more or less distinguishable by morphology, composition of cell types and function. The inner mucosal layer of the small intestine is pleated. Kerckring folds exhibit finger-like extensions (villi) which are lined by epithelial cells. Intestinal epithelial cells, also called enterocytes, are polarized cells with an apical membrane facing the intestinal lumen – also called brush border membrane due to finger-like extensions (microvilli) – and a blood-facing basolateral membrane.

Carbohydrates can be classified as simple, namely mono- and disaccharides, and complex sugars such as oligo- and polysaccharides. In particular a high intake of sucrose and fructose are suspected of promote diet-mediated diseases [3-7], whereas a proper intake of polysaccharides seems to provide health benefits [8, 9]. Ingested complex carbohydrates such as starch are broken down by several digestive enzymes, namely by α-amylase in the lumen as well as by glucoamylase and maltase at the surface of enterocytes resulting in the release of glucose. Dissacharides such as lactose and sucrose need only the surface bound enzymes for hydrolysis releasing glucose, galactose and fructose. These monosaccharides are then taken up by the epithelial cell via two types of sugar transporters located in the brush border membrane: the sodium-dependent glucose co-transporter 1 SGLT1 (SLC5A1) mediating transport of glucose and galactose and the GLUT5 protein (SLC2A5) transporting fructose. All three sugars enter the blood circulation across the basolateral side of the enterocyte through the facilitated glucose transporter GLUT2 (SLC2A2) [10].

1.1.2. Active and passive transporters

There are two major forms of membrane transporters. Active transporter processes are energy-dependent and utilize ATP to overcome electrochemical or concentration gradients in order to transport larger molecules or ions. These active transport systems are subdivided into primary, secondary and tertiary active mechanisms. Primary transport systems use the energy provided directly by hydrolysis of ATP to ADP and inorganic phosphate – which is performed by ATPases such as the sodium-potassium-ATPase or by proteins such as multi-drug transporter 1 (MDR1) as a representative of a large number of xenobiotic transporters. Secondary active transport systems utilize the energy of an electrochemical gradient, generated and maintained by ion pumps, to transport molecules in the same (symport, e.g. SGLT1) or the opposite direction (antiport, e.g. the sodium-proton exchanger NHE3 (SLC9A3)). This type of transport also requires a second solute – mostly an ion – with sodium ions or protons being the most common ones. The di- and tripeptide transporter PEPT1 (SLC15A1) is an example for a tertiary active transport mechanism combining the primary (Na^+/K^+-ATPase) and secondary active system (Na^+/H^+-exchanger) for provision of protons for the proton-coupled electrogenic influx of peptides.

Passive transporters do not depend on energy but carry molecules and ions downstream of a concentration gradient or the membrane potential. By enabling a facilitated diffusion, small molecules, either polar or non-polar pass the membrane to equilibrate the concentration gradient. Passive diffusion is mediated by either channel proteins or carrier-type proteins but there may as well be intermediate forms. The carrier proteins are substrate-specific and possess usually one or two substrate binding sites. Binding of the substrate leads to a change in the carrier’s conformation which in turn allows the molecules to pass a pore-like structure formed by the proteins domains in the membrane. Uniporters such as the members of the GLUT family belong to the one-binding site carriers whereas symporters, e.g. the lactose permease, or antiporters such as the chloride/bicarbonate exchanger require the binding of two different substrates [11].

1.2. The sodium-dependent glucose co-transporter 1 (SGLT1)

1.2.1. Molecular structure, mRNA and protein abundance of SGLT1

SGLT1 is the first protein of twelve members within the solute carrier family 5 (SLC5). The human *SLC5A1* gene is located on chromosome *22.q12.3* and encodes for an integral membrane protein consisting of 664 amino acids and a molecular mass of 73.5 kDa. The transporter has 14 membrane spanning *α*-helices, one putative N-linked glycosylation site at position 248 and both, N- and C-terminus, facing the extracellular space [12-14] (Figure 1).

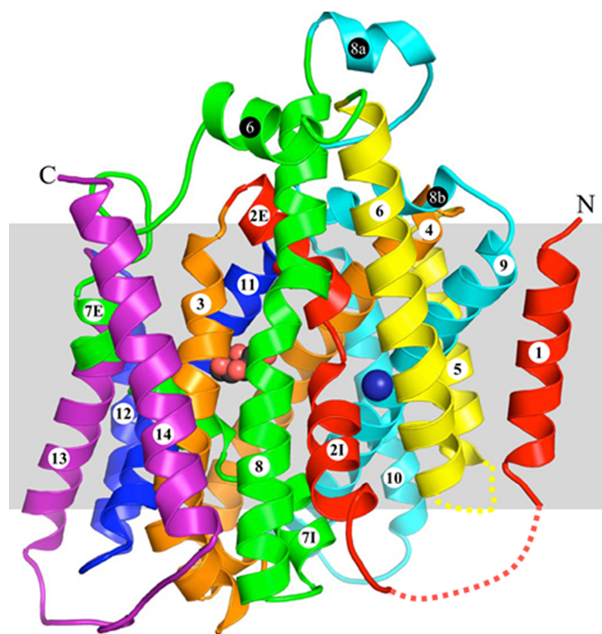


Figure 1: Crystal structure of SGLT1 from *Vibrio parahaemolyticus*. Structure viewed in the membrane plane. Adopted from Faham *et al.* (2008) [15].

Mutations in the *SLC5A1* gene resulting in a dysfunctional protein [16] and/or defective trafficking of SGLT1 to the membrane [17] can manifest in the congenital glucose-galactose malabsorption syndrome (GGM; OMIM #606824). This rare autosomal-recessive disease can be lethal due to severe diarrhea that is associated with dehydration [17-20]. For patients suffering from GGM survival is ensured on diets excluding glucose and galactose but also the disaccharide lactose including mother's milk [20].

In mice, the *slc5a1* gene is located on chromosome 5 and the encoded protein consists of 665 amino acids and has a molecular weight of 73.3 kDa [13]. Deletion of the gene results in a phenotype similar to human GGM. In contrast to humans, suckling *sglt1* knockout mice tolerate mother's milk but do not survive on glucose-containing diets after weaning. However, *sglt1* knockout mice kept on a glucose-/ galactose-free diet are viable, healthy and fertile [21].

SGLT1 mRNA and protein are mainly expressed in the small intestine but also in the proximal straight tubule (S3 segment) of the nephron [22] where the protein contributes to glucose re-absorption from the glomerular filtrate [23]. The intestinal SGLT1 protein is located at the apical side of mature enterocytes [24, 25]. There are segmental differences in the abundance of SGLT1 mRNA as well as protein along the small intestine. SGLT1 mRNA is expressed throughout the whole small intestine slightly decreasing towards the distal part [26, 27]. SGLT1 protein as detected by immunohistochemistry is most abundant in rat jejunum and similar in duodenum and ileum [28] whereas protein expression assessed by Western blot after *in vivo* perfusion with glucose is comparable in rat duodenum and proximal jejunum [29].

1.2.2. Substrate specificity, mode of transport and inhibitors

SGLT1 does not only transport glucose or galactose along with sodium ions in a stoichiometry of 1:2 [30] but all pyranoses with equatorial hydroxyl-groups, preferably at position 2. This includes the natural sugars D-glucose and D-galactose as well as the non-metabolizable glucose analogues *α*-methyl-D-glucopyranoside (*α*-MDG) and 3-*O*-methyl-D-glucose (3-OMG) [31]. Data on apparent K_m values for those substrates are available mainly for *in vitro* studies, i.e. the expression of SGLT1 in oocytes from *X. laevis* but little information comes from studies in brush border membrane vesicles (BBMV) or from *in vivo* estimations as for example from intestinal perfusion studies in rats (Table 1). There are obvious differences in the apparent K_m values obtained in injected oocytes compared brush border membrane vesicle preparations and especially compared to *in vivo* estimations that are most likely due to unstirred layer effects [32]. In addition, the difference between the values obtained in BBMVs and in the perfused intestine might be explained by isolated cells that lack hormonal and neural stimuli *versus* an intact organism that may affect substrate uptake [33, 34].

Table 1: Apparent K_m values for SGLT1

Model organism	Substrate	K_m value	References
human SGLT1 expressed in oocytes	glucose	0.5 mM 0.6 mM	[35] [36]
	galactose	0.6 mM	[35]
	α-MDG	0.5 mM 0.3 – 0.5 mM 0.7 mM	[37] [38] [35]
	3-OMG	6 mM	[35]
rabbit SGLT1 expressed in oocytes	*α*-MDG	0.2 mM	[37]
rat SGLT1 expressed in oocytes	*α*-MDG	0.3 mM	[37]
BBMV (guinea pig)	glucose	0.5 mM 0.7 mM	[39] [40]
	α-MDG	2.0 mM 2.4 mM	[39] [40]
BBMV (rat)	glucose	0.13 – 0.30 mM	[41]
rat intestine perfused *in vivo*	glucose	23 mM 27 mM	[42, 43] [44]
	galactose	32 mM	[42, 43]
	α-MDG	31 mM	[42, 43]

The mode of transport by SGLT1 is secondary active: sodium is transported along its electrochemical gradient – which is maintained by the basolateral Na^+-K^+-ATPase – allowing glucose and galactose to bind and get translocated [45]. Binding of sodium to the negatively charged SGLT1 protein leads to a conformational change in the structure of the protein making the sugar binding site accessible which in turn results in sugar transport [46] (Figure 2).

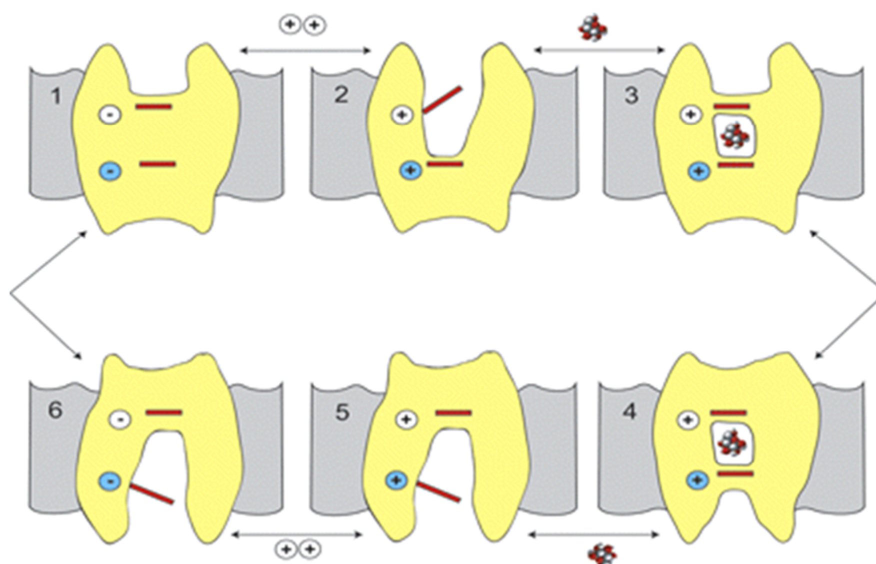


Figure 2: A 6-state model of SGLT1 during transport. State 1: outward-facing conformation without ligands bound. State 2: extracellular sodium ions bind to their binding site and open the external sugar binding site. State 3: glucose/galactose bind to their binding site which in turn closes the external gate, resulting in the outward-facing closed conformation. State 4: conformational change resulting in the inward-facing closed conformation. State 5: opening of the internal gate, thereby releasing first glucose/galactose then the sodium ions into the cell interior. State 6: inward-facing open conformation without ligands bound. Conformational change to outward-facing ligand-free state completes the cycle. Adopted from Sala-Rabanal *et al.* (2012) [38].

The widely used and most potent inhibitor of SGLT1 is phlorizin [2′-(*β*-D-glucopyranosyloxy)-4′, 6′-dihydroxy-3-(4-hydroxyphenyl)-propiophenon], a *β*-glycoside naturally occurring in the bark of fruit trees such as apple, pear and cherry. The mode of inhibition is competitive [47]: phlorizin binds sodium-dependently to its specific binding site within the C-terminal region of the SGLT1 protein which in turn blocks the conformational change that is necessary for the subsequent sugar binding [38, 48, 49]. When phlorizin is administered orally, the lactase-phlorizin hydrolase, a *β*-glucosidase located in the brush border membrane, converts phlorizin into its aglycon phloretin [50]. Phloretin, a non-competitive inhibitor of SGLT1, blocks glucose absorption with an apparent K_i of 15-50 µM [48]. Besides phlorizin and phloretin other glycosides namely quercetin-3-*O*-glucoside [51-53], quercetin-4'-glucoside [52] and kaempferol 3-*O*-*α*-rhamnoside [54] are putative inhibitors of SGLT1.

1.2.3. Regulation of SGLT1 mRNA and protein expression

Several factors are involved in the regulation of SGLT1 including the hepatocyte nuclear factor 1 (HNF-1), members of the Sp1 family [55] and the RS1 protein [56-60]. Further important modulators of SGLT1 expression levels are dietary carbohydrates that appear to lead to an up-regulation of SGLT1 mRNA and/or protein during short-term [21, 29, 61] and long-term administration [62-64]. Furthermore, sweet receptors and thus the receptor subunit T1R3 – which is part of the heterodimeric sweet taste receptor T1R2+3 as well as the G protein *α*-gustducin have been shown to affect SGLT1 expression [29, 63]. Diurnal rhythm [65, 66] and hormones, in particular cholecystokinin [67], glucagon-like-peptide 2 [41], epidermal growth factor [68], the thyroid hormone T3 [69] and leptin [70, 71], seem to be involved in the regulation of SGLT1 gene expression and/or control of protein synthesis.

1.2.4. SGLT1 and glucose-induced secretion of GIP and GLP-1

Besides its expression in epithelial cells, SGLT1 was shown by immunofluorescence to be co-localized with two subsets of enteroendocrine cells (EECs) namely glucose-dependent insulinotropic peptide (GIP)-secreting K-cells [21, 72] and glucagon-like-peptide 1 (GLP-1)-secreting L-cells [21, 73]. EECs represent only ~1 % of the total intestinal cell population and comprise also gastrin-secreting G-cells, somatostatin-secreting δ-cells, cholecystokinin-secreting I-cells as well as peptide YY (PYY)- and glucagon-like-peptide 2 (GLP-2)-secreting L-cells [74].

K-cells are mainly located in the proximal part of the small intestine [75, 76] and release GIP in response to fat and carbohydrates, e.g. glucose [77]. These nutrients also stimulate GLP-1 secretion from L-cells [77] that are located in higher density in the distal small intestine [78]. Both hormones represent the so-called incretins that mediate the incretin effect [79-82] as the phenomenon that orally administered glucose causes a greater release of insulin from pancreatic *β*-cells compared to intravenous administration causing identical plasma glucose concentrations [83-85].

Glucose-induced GIP and GLP-1 secretion from K- and L-cells is initiated by the influx of glucose and sodium through SGLT1 which results in an elevation of cyclic adenosine monophosphate (cAMP), closure of ATP-sensitive K^+-channels followed by membrane depolarization and consequent opening of voltage-dependent Ca^{2+}-channels. The increase in the intracellular calcium concentration finally results in the

release of GIP- and GLP-1 by promoting vesicle trafficking and membrane fusion [72, 73, 86, 87]. The prime importance of SGLT1 for glucose-induced incretin secretion is demonstrated by the absence of proper GIP- and GLP-1-responses in the presence of phlorizin [88] and in *sglt1* knockout mice [21].

1.3. The facilitative glucose transporter GLUT2

1.3.1. Molecular structure, mRNA and protein abundance of GLUT2

GLUT2 belongs to the solute carrier family 2 (SLC 2) whose 14 members are divided based on sequence similarities into three subclasses: class I comprises GLUT1-GLUT4, class II consists of GLUT5, GLUT7, GLUT9 and GLUT11 and class III contains GLUT6, GLUT8, GLUT10, GLUT12 as well as the H^+/myoinositol transporter HMIT [89]. All GLUT proteins are supposed to share a similar molecular structure with 12 transmembrane spanning *α*-helixes and both, the N- and C-terminus exposed to the cytoplasm [90] (Figure 3).

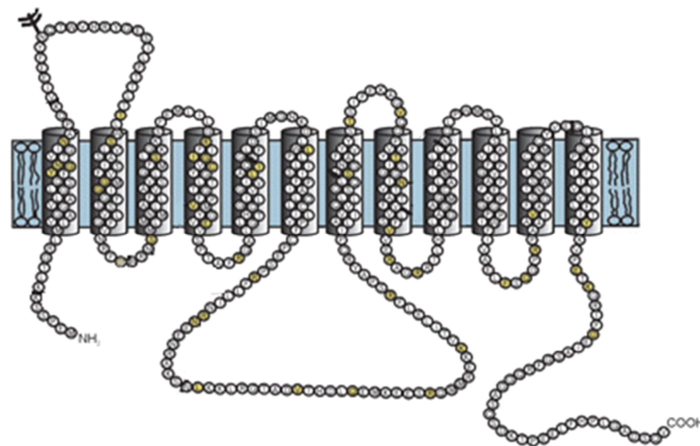


Figure 3: Schematic structure of GLUT1 representative for all GLUTs. Adopted from Mueckler *et al.* (1985) [90].

GLUT2, a member of the subclass I, is encoded by the human *SLC2A2* gene located on chromosome *3q26.2.* This isoform contains 524 amino acids and has a molecular mass of 57.5 kDa. There is a putative N-linked glycosylation site at position 62 which is thought to stabilize and retain the transporter at the cell surface of the pancreatic *β*-cells [13, 14].

The Fanconi-Bickel-syndrome (FBS; OMIM #227810) is a glycogen storage disease that is attributed to defective GLUT2 resulting from mutations in the *SLC2A2* gene [91, 92]. This disorder is characterized by abnormal hepatic glycogen accumulation, tubular nephropathy, fasting hypoglycaemia and ketonuria but postprandial hyperglycaemia as well as physical impairments (short stature, protuberant abdomen due to hepatomegaly) [93].

In mice, the *slc2a2* gene is located on chromosome 3 and encodes a protein consisting of 523 amino acids and with a molecular weight of 57.1 kDa [13]. Deletion of the gene leads to a non-insulin-dependent diabetes-like phenotype characterized by hyperglycemia, hypoinsulinemia and impaired glucose tolerance, loss of the first-phase of glucose-stimulated insulin secretion (GSIS) and an inversion of the pancreatic *α*- to *β*-cell ratio. These mice die within the first three weeks of life due to the impaired glucose-induced insulin secretion [94]. However, re-expression of either GLUT1 (in RIPGLUT1xGLUT2$^{-/-}$ mice) or GLUT2 (in RIPGLUT2xGLUT2$^{-/-}$ animals) in *β*-cells restores first-phase insulin secretion and consequently glucose tolerance and rescues the *glut2* knockout mice from early death [95].
GLUT2 mRNA and protein is mainly expressed in the sinusoidal membrane of hepatocytes and the plasma membrane of pancreatic *β*-cells but also in the basolateral membrane of renal proximal tubules of the S1 segment as well as of enterocytes lining the small intestine [96]. Intestinal mRNA expression as well as protein abundance is similar to the expression patterns of SGLT1 showing higher levels in duodenum and jejunum compared to ileum [26, 76, 97].

1.3.2. Substrate specificity, mode of transport and inhibitors

In the intestine, basolateral GLUT2 provides a common exit pathway for D-glucose, D-galactose and D-fructose down the concentration gradient into system circulation [1, 96]. GLUT2 is also capable of transporting D-glucosamine [98] as well as the non-metabolizable glucose analogues 3-OMG [99, 100] and 2-deoxy-D-glucose (2-DG) [101-103]. Several groups have measured apparent K_m values in GLUT2-injected oocytes of *Xenopus laevis* but only one group determined the K_m *in vivo* using intestinal perfusion of rat intestine (Table 2).

Table 2: Apparent K_m values for GLUT2

Model organism	Substrate	K_m value	References
human GLUT2 expressed in oocytes	glucose	17 – 20 mM	[98]
	fructose	67 mM 76 mM	[102] [104]
	galactose	86 mM 92 mM	[102] [104]
	glucosamine	0.8 mM	[98]
	3-OMG	21 mM 35 mM	[105] [100]
	2-DG	11 mM 17 mM	[102] [101]
rat intestine perfused *in vivo*	glucose	56 mM	[44]

Similar for all GLUT proteins, the mode of transport is a carrier-mediated facilitated diffusion during which the GLUT protein undergoes a conformational change for transport of its specific substrate across the membrane (Figure 4). In case of GLUT2, glucose transport can be inhibited by phloretin [44, 106] and other flavonoids – especially by quercetin [107, 108] but also by cytochalasin B [109], a mycotoxin that non-specifically inhibits glucose uptake via several facilitative transporters [110, 111]. It is not clear, however, whether this occurs in a competitive [112, 113] or non-competitive manner [111, 114].

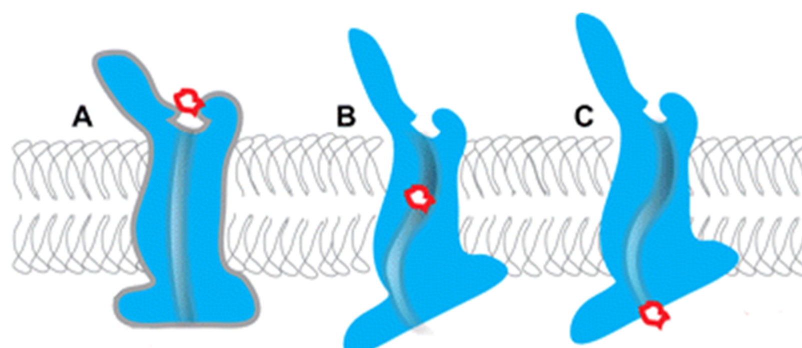


Figure 4: Carrier-mediated facilitated diffusion. State A: outward-facing conformation without substrate bound. State B: substrate binds to its internal binding site, resulting in conformational change of the carrier. State C: release of the substrate into the cell interior. Adopted and modified from Leturque *et al.* (2009) [115].

1.3.3. Regulation of GLUT2 mRNA and protein expression

There are different factors modifying tissue-specific GLUT2 abundance. Intestinal GLUT2 mRNA and/or protein expression is regulated by short-term and long-term exposure to glucose and fructose content of the diet [62, 109, 116], it shows developmental changes in expression [76, 97, 117] and a diurnal rhythm [76, 118].

1.4. The history of sugar transport

1.4.1. Two systems mediating intestinal glucose transport

Intestinal glucose absorption has always been a matter of debate. At the beginning of the last century, the contentious point concerned the mechanism of glucose absorption. As early as in 1924, there were two fractions; one suggesting the brush border membrane may have “some special physiological function” going beyond diffusion and filtration whereas the others postulated a “purely mechanical scheme” of absorption have “some special physiological function” going beyond diffusion and filtration whereas the others postulated a “purely mechanical scheme” of absorption [119]. *In vivo* experiments with phlorizin, a plant-derived flavonoid that is nowadays known as the most potent inhibitor of SGLT1, classified the mechanism of sugar absorption into a purely physical, phlorizin-insensitive basic process pertaining for all sugars and an additional one for “biologically important” sugars such as glucose and galactose. The latter process was described as to be non-physical and inhibited by phlorizin [120]. In the following years, further evidence was found for two distinct mechanisms, especially when high glucose concentrations are present in the intestinal lumen. Using an intestinal perfusion system, glucose absorption at high luminal concentrations (290 mosm/l) was shown to consist of a constant and a variable amount, the latter being proportional to the water absorption rate [121]. Measurements of the transmural potential difference and radiolabeled sugar uptake substantiated two components contributing to intestinal glucose absorption: the “electrogenic active component” and the “diffusive component”. The first induced differences in the transmural potential, was saturable and could be inhibited by phlorizin whereas the second one was the exact opposite: non-electrogenic, non-saturable and phlorizin-insensitive [42]. The “electrogenic active component” is meanwhile known as SGLT1 with its sodium-dependency for sugar transport already described in 1958 by Riklis and Quastel [122], its sodium co-transporting mechanism

proposed by Crane in 1960 [123] and its identification by cloning as SGLT1 by Hediger *et al*. in 1987 [124].

1.4.2. The "diffusive" component

In contrast to the active component, the "diffusive" component is still under debate. In the same year of SGLT1's cloning, Pappenheimer and Reiss suggested paracellular flow ("solvent drag") as the major route for enlarged glucose absorption. Since the active transport of sodium and glucose generates an osmotic force leading to fluid absorption it in turn entrains hydrophilic molecules like glucose through cell-connecting tight junctions [125]. Cytoskeletal changes were proposed to lead to a widening of the tight junctions resulting in bulk absorption of glucose [126]. However, Pappenheimer's proposal of paracellular flow/solvent drag constituting the diffusive component was conceptually supported only by very few groups [127, 128] whereas others explained the increase in glucose absorption at higher glucose concentrations by adaption of apical transporters [129], an active transcellular [130, 131] or a carrier-mediated process without specifying the carrier [132]. However, Kellett and Helliwell very precisely designated the carrier being responsible for bulk glucose absorption. In 2000, they used *in vivo* perfusion studies in rats to show the translocation of the basolateral located facilitative glucose transporter GLUT2 into the apical membrane which in addition to SGLT1 would then support glucose absorption [44].

1.4.3. Working model of GLUT2 insertion into the apical membrane

Subsequent work on GLUT2 trafficking revealed a SGLT1-dependent process that is regulated by the activation of protein kinase C βII (PKC βII) [33, 44] as well as the extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) [34] and AMP-activated protein kinase (AMPK) signaling pathways [133].

The mechanism of GLUT2 insertion into the apical membrane is thought to be initiated by the co-transport of glucose and sodium ions through SGLT1. Influx of sodium depolarizes the membrane followed by entry of calcium ions through the voltage-gated L-type calcium channel $Ca_v1.3$ [134, 135]. Intracellular Ca^{2+} ions and the regulatory protein calmodulin form a complex that stimulates the myosin-light chain kinase (MLCK) to phosphorylate the regulatory light chain (RLC_{20}) of myosin II

in the terminal web. The subsequent re-arrangement of the cytoskeleton allows the insertion of GLUT2 from intracellular storage vesicles into the apical membrane [134] (see Figure 2). GLUT2 recruitment is stimulated by glucagon-like-peptide 2 [136] and inhibited by stress, glucocorticoids (dexamethasone) [137] and inactivation of PKC βII including an incision of the small intestine [33, 44] (Figure 5).

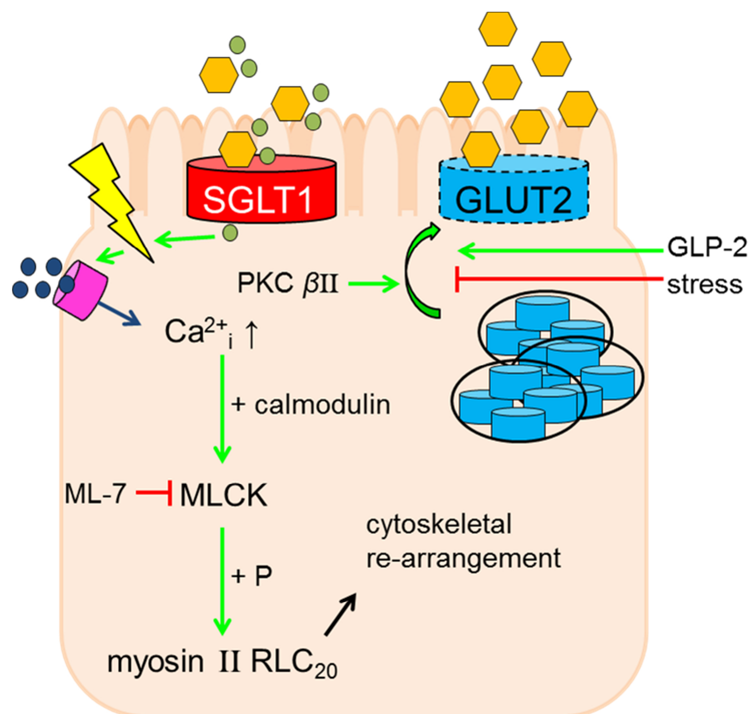


Figure 5: Proposed mechanism of GLUT2 trafficking into the apical membrane. Glucose ( ) and sodium ions ( ) are transported by SGLT1, thereby depolarizing the membrane which in turn opens the voltage-gated calcium channel $Ca_v1.3$ ( ). The influx of Ca^{2+} ions ( ) activates a signaling cascade involving myosin-light chain kinase (MLCK), phosphorylation of the Ser^{19} residue of the myosin II regulatory light chain (RLC_{20}), cytoskeletal re-arrangement resulting in fusion of GLUT2-containing vesicles ( ) with the brush border membrane.
Stimulation Inhibition

1.5. The facilitative fructose transporter GLUT5

1.5.1. Molecular structure, mRNA and protein abundance of GLUT5

GLUT5 belongs to the subclass II of the SLC2 family and is encoded by the *SLC2A5* gene which is located on chromosome *1p36.23* in humans. The protein consists of 501 amino acids resulting in a molecular weight of 55.0 kDa. Furthermore, there is a putative N-linked glycosylation site at position 51 [13, 14]. So far, mutations in the *SLC2A5* gene resulting in a GLUT5-defect and consequently in the inability to absorb fructose are not known [138].

The murine *slc2a5* gene is located on chromosome 4 and encodes for a protein of 501 amino acids and a molecular mass of 55.4 kDa [13]. Deletion of the *slc2a5* gene in mice does not cause any obvious phenotype as long as the *glut5* knockout mice are kept on a conventional diet. However, when *glut5* knockout mice are kept on a high-fructose diet, they lose weight, suffer from malabsorption symptoms and hypotension followed by hypovolemic shock and death [139].
GLUT5 mRNA and/or protein is found in the small intestine [140], the renal S3 segment [141], adipose tissue [142] and skeletal muscle [143, 144], in testis and spermatozoa [140] as well as in the brain [145]. In the small intestine, GLUT5 is located at the brush border membrane [117] but there is also some evidence for GLUT5 protein abundance in the basolateral membrane of humans [146]. Similar to SGLT1 and GLUT2, expression of GLUT5 mRNA is higher in proximal as compared to distal parts in the human [142], rat [147, 148] and murine small intestine [26].

1.5.2. Substrate specificity, mode of transport and regulation

When GLUT5 was discovered in 1990, it was described as a glucose transporter sensitive to cytochalasin B [142]. However, two years later, GLUT5 was demonstrated to be a high-affinity fructose transporter insensitive to cytochalasin B [140] that transports fructose in a carrier-mediated facilitated manner. Apparent K_m values for fructose are around 6-15 mM in GLUT5-expressing oocytes [140, 149-151] and around 10 mM in human erythrocytes [152].

GLUT5 mRNA and/or protein levels in the intestine are mainly regulated by fructose during acute [153, 154] and chronic [62, 155, 156] fructose exposition as well as by development [117, 148], glucocorticoids [157] and diurnal rhythm [118, 148].

1.5.3. Fructose malabsorption and metabolic disorders caused by excessive fructose intake

Fructose intake has dramatically increased due to the application of high-fructose corn syrup in the USA (HFCS) and higher consumption of sweetened foods (invert sugar) and beverages, mainly fruit juices [158]. Whether increased fructose consumption is associated with an increasing incidence of intestinal fructose malabsorption seems plausible but is not unequivocally proven. It is certain, however, that the limited capacity to absorb fructose is a common phenomenon in humans [159-164]. Since there are no known mutations in the *GLUT5* gene [138] resulting in dysfunctional GLUT5 the cause for limitations in fructose transport in some individuals remains unclear. In fact, mRNA and protein expression of GLUT5 but also of GLUT2 – the latter suggested to mediate fructose absorption, too [33, 34, 109] – are almost identical in healthy people and patients suffering from symptomatic fructose malabsorption [165].

Intestinal fructose malabsorption as determined during a breath hydrogen test is present when hydrogen levels rise more than 20 ppm over basal levels after ingestion of a 10 % fructose solution [159, 161, 163, 164]. There is a large variety in human fructose absorption capacity ranging from less than 5 g to more than 50 g fructose [161] and not all patients suffering from fructose malabsorption display symptoms such as abdominal pain, bloating, flatulence and diarrhea after ingestion of fructose which arise in a dose-dependent manner [159-164]. Both, fructose absorption and associated symptoms were shown to be improved (or could even completely be prevented) when glucose was added simultaneously with the best effects observed at a 1:1 ratio [159-162]. So far, the mechanism underlying this improvement is not clear. Hypotheses include activation of GLUT5 by SGLT1 [160], a glucose-dependent fructose co-transport [161], a disaccharidase-related system that jointly transports glucose and fructose [166], promotion of solvent drag [167, 168] or a GLUT2 trafficking into the apical membrane to mediate fructose absorption [33, 34].

There is also evidence that elevated/excessive fructose consumption is associated with metabolic disorders such as insulin [169] and leptin resistance [170], increased

body fat [171-173], dyslipidemia [174], non-alcoholic fatty liver disease [175] and hypertension [176].

1.6. Facilitative sugar transporters and glucose-induced incretin secretion

Besides SGLT1, GLUT5 and GLUT2 may also be involved in the secretion of the incretins since not only glucose but also fructose stimulates at least GLP-1 secretion [177-179]. The underlying mechanism is thought to be similar to that described for the glucose-induced release of insulin from pancreatic *β*-cells [180] and in the case of fructose-induced secretion of GLP-1 requires fructose metabolism [87]: glucose and fructose metabolism drive the production of ATP resulting in an increased ATP/ADP-ratio which in turn leads to the closure of ATP-sensitive K^+-channels. The subsequent decrease in the magnitude of the outward directed K^+ current elicits depolarization of the membrane and opening of voltage-dependent Ca^{2+}- channels. This is followed by an elevation of the intracellular calcium concentration that in the end results in the fusion of GLP-1-containing vesicles with the membrane and the subsequent release of their content [87, 181].

Aim of the project

The increasing consumption of simple sugars such glucose and fructose is associated not only with obesity and diabetes mellitus type 2 but also with other diet-related diseases. This has brought emerging interest concerning the absorption of monosaccharides by the intestine that has been studied mainly in rats using intestinal perfusion.

Therefore, we aimed to assess intestinal sugar absorption in comparative studies taking advantage of mice that lack SGLT1, GLUT2 or GLUT5.

In the first part of the project, the role of SGLT1 and GLUT2 in intestinal glucose absorption as well as the contribution of the respective transporter to glucose-induced hormone secretion of GIP, GLP-1 and insulin were determined at high luminal glucose concentrations in mice being deficient of either SGLT1 or GLUT2.

To accomplish this, we applied the *in vitro* method of everted gut rings as well as oral administration of radiolabeled glucose in combination with Western blot analysis.

The second part focused on intestinal fructose absorption and here in particular on the phenomenon of improved fructose absorption when glucose is ingested simultaneously as found in humans suffering from fructose malabsorption. These studies were performed in mice lacking SGLT1, GLUT2 or GLUT5 including gavage of radiolabeled fructose and Western blot analysis as well as feeding a fructose-containing diet.

2. Materials and Methods

2.1. Materials

If not stated otherwise, all reagents were purchased from Merck KGaA (Darmstadt, Germany), Carl Roth GmbH + Co. KG (Karlsruhe, Germany) and Sigma Aldrich Co. LLC (Germany).

2.1.1. Buffers

Brush border membrane preparation buffers M100 and M300

Preparation of the buffers happened at the day of the experiment. Buffers were kept on ice for at least one hour. Tris(hydroxymethyl)-aminomethan was used to set pH 7.4. Protease inhibitor (1 tablet/200 ml; Roche Diagnostics, Deutschland Holding GmbH) was added right before starting the membrane preparation.

Table 3: Composition of M100 and M300 buffer

	M100	M300
Mannitol	100 mM	300 mM
HEPES	2 mM	20 mM

Citrate buffer

The buffer was always made fresh. Using NaOH pH was set to 6.0.

Table 4: Composition of citrate buffer

Citric acid	1.8 mM
Tri-sodium citrate dihydrate	8.2 mM

Krebs buffer (KB)

Krebs buffer was prepared freshly right before the experiment. The buffer was gassed with 95 % O_2 / 5 % CO_2 for at least one hour before the pH was set to 7.4.

Table 5: Composition of Krebs buffer

NaCl	119 mM
KCl	4.7 mM
$CaCl_2$	2.5 mM
$MgSO_4$	1.2 mM
KH_2PO_4	1.2 mM
$NaHCO_3$	25 mM

2.1.2. Antibodies for Western blot and immunohistochemistry

Primary antibodies were purchased from Santa Cruz Biotechnology, Inc (Dallas, USA) and abcam® (Cambridge, UK). SGLT1 antibody was custom-made (Pineda, Berlin, Germany) against rat amino acids 586-601 confirmed for specificity in *sglt1* knockout mice that lacked the band at 90 kDa.

Table 6: Primary antibodies for Western blot

	Species	Concentration	Product name	Manufacturer
anti-*β*-actin	goat	1:5000	C-11: sc-1615	Santa Cruz
anti-Na-K-ATPase	rabbit	1:50000	EP1845Y	abcam
anti-GLUT2	goat	1:250	C-19: sc-7580	Santa Cruz
anti-SGLT1	rabbit	1:15000	-	Pineda

Table 7: Primary antibodies for immunofluorescence

	Species	Concentration	Product name	Manufacturer
anti-GLUT2	goat	1:200	C-19: sc7580	Santa Cruz
anti-SGLT1	rabbit	1:250	-	Pineda

Secondary antibodies for Western blots were IRDye® infrared dyes purchased from LI-COR® Biosciences, Inc. (Lincoln, USA). Secondary antibodies for immunofluorescence were cyanin-conjugated fluorescent dyes (Jackson ImmunoResearch (UK). DAPI was obtained from Sigma Aldrich.

Table 8: Secondary antibodies for Western blot

	Species	Concentration	Label
anti-goat IgG	donkey	1:12000	IRDye® 800CW
anti-rabbit IgG	donkey	1:12000	IRDye® 680RD

Table 9: Secondary antibodies for immunofluorescence

	Species		Label
DAPI		1:1000	
anti-goat IgG	donkey	1:400	Cy3
anti-rabbit IgG	donkey	1:400	Cy3

2.1.3. Radiolabeled chemicals

All radiolabeled chemicals were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, USA).

Table 10: Radiolabeled chemicals

	Product name	Label	Specific activity	Concentration
α-methyl-D-glucopyranoside	ARC 0131A	[^{14}C(U)]	300 mCi/mmol	1 mCi/ml
D-Fructose	ARC 0116A	[^{14}C(U)]	300 mCi/mmol	0.1 mCi/ml
D-Glucose	ARC 0122D	[^{14}C(U)]	10 mCi/mmol	1 mCi/ml
D-Mannitol	ARC 0118	[1-^{3}H(N)]	20 Ci/mmol	1 mCi/ml

2.2. Methods

2.3. Mice

2.3.1. Animals and husbandry

Three different knockout mouse models lacking SGLT1, GLUT2 or GLUT5 (Table 11) were used to examine the contribution of the respective transporter to intestinal sugar absorption, enteroendocrine hormone secretion and transporter interactions.

Sglt1 wild type and knockout mice were bred and kept in individually ventilated cages (IVC) in a specific pathogen free (SPF) animal facility at 22 ± 1 °C, a constant humidity of 55 % and a 12:12 hour light/dark cycle.

Glut2 wild type and RIPGLUT1xGLUT2$^{-/-}$ mice (in this work referred to as *glut2*$^{-/-}$mice) were bred and kept in a conventional animal house at 22 ± 2 °C, a constant humidity of 55 % and a 12:12 hour light/dark cycle.

Glut5 knockout mice were bred and kept in a conventional animal house at 22 ± 2 °C, a constant humidity of 55 % and a 12:12 hour light/dark cycle. Since we lacked appropriate wild type littermates to the *glut5* knockout animals we used *glut2* wild type mice due to similar housing conditions.

Table 11: Mouse models

Model	Embryonic stem cells	Background	References
Sglt1	129/OLA	C57BL/6J	Gorboulev *et al.* [21]
Glut2	129/Sv	C57BL/6J	Thorens *et al.* [95]
Glut5	129/SvEv	C57BL/6J	Wu *et al.* [182]

2.3.2. Diets

All animals had free access to water and were fed a standard chow diet (V1534, ssniff Spezialdiäten GmbH, Soest, Germany) except for the *sglt1*$^{-/-}$ mice. Similar to humans suffering from the glucose/galactose malabsorption syndrome, *sglt1*

knockout mice are not able to absorb glucose, lose weight and die within a few days when kept on conventional diets containing glucose [21]. Therefore, weaned animals received a sugar-free diet (C 1000 modified glucose-deficient diet, Altromin Spezialfutter GmbH & Co. KG, Lage, Germany; Figure 6A). To exclude diet-specific effects, *sglt1* wild type, *glut2* wild type and knockout as well as *glut5* knockout mice were fed the sugar-free diet one week prior to experiments.
Some animals were fed a fructose-containing diet (S6745-E010, ssniff; Figure 6B) in order to assess diet-induced changes on fructose absorption.

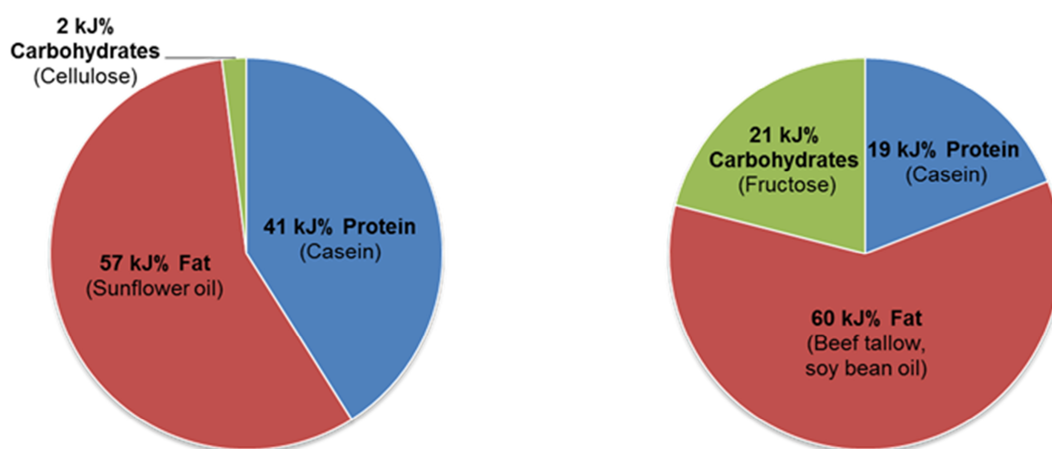


Figure 6: Composition of the diets. (A) Sugar-free diet. (B) Fructose-containing diet.

2.3.3. General procedures

Since all three transporters are regulated by development 18-20 week old animals of both genders were used for the majority of the experiments. Only short-term interventions and long-term high-fat feeding studies investigating the effects on hormone secretion were performed with male mice to avoid impairments due to female cycle fluctuations. Whenever it was possible, littermates were used.
On the day of the experiment, mice were deprived of food between 7 and 8 a.m. for 6 hours. Right before the experiment, animals were weighed and fasting blood glucose was measured at the tail vain using a glucometer (FreeStyleLite, Abbott Laboratories, Illinois, USA). Mice were killed without or with previous anesthesia by cervical dislocation. Those receiving a gavage were killed after 15 minutes.
All experiments and treatments were performed according to the recommendations of the Federation of European Laboratory Animal Science Associations and approved by the state ethics committee (permit number 55.2-1-54-2531-39-10).

2.4. Biochemistry / Molecular biology

2.4.1. Preparation of brush border membranes (BBM)

Buffers, instruments and tubes were pre-cooled and ice cold conditions were maintained throughout the procedure. For preparation of BBM, frozen tissue was given to 10 ml of ice cold M100 buffer (see 2.1.1. Table 3) mixed with 1 % PMSF and homogenized for 1 x 30 seconds and 2 x 10 seconds using an Ultra-Turrax. Samples were put back on ice between first and second homogenization. Homogenizates were centrifugated at 3600 x g for 5 minutes at 4 °C to remove cell debris before supernatants were filled up to 30 ml and incubated with 20 mM $MgCl_2$ for 15 minutes on ice to precipitate basolateral membrane fractions. Subsequent centrifugation at 2500 x g for 15 minutes at 4 °C separated apical (supernatant) and basolateral (pellet) membrane fractions. Supernatants were centrifugated at 36000 x g for 30 minutes at 4 °C before pellets were resuspended in 1 ml M300 buffer (see 2.1.1. Table 3) plus 1 % PMSF followed by homogenization using a 24 G' needle. The suspensions were filled up to 30 ml with M300 buffer mixed with 1 % PMSF. Incubation with $MgCl_2$ as well as subsequent centrifugations were performed as described above. Final pellets containing brush border membranes were resuspended in 150 µl M300 buffer plus 1 % PMSF and homogenized with a 24' G needle. Aliquots were snap-frozen until use for Western blot.

2.4.2. SDS PAGE and Western blot

Gels for SDS PAGE consisted of a 9 % running gel (Table 12) and a 5 % stacking gel (Table 13).

Table 12: Composition of running gel (9 ml)

Component	Volume [ml]
1.12 M Tris-HCl + 0.3 % SDS, pH 8.8	3
30 % acrylamide	2.7
distilled water	3.3
10 % ammonium persulfate (APS)	0.1
Tetramethylethylenediamine (TEMED)	0.005

Table 13: Composition of stacking gel (4 ml)

Component	Volume [ml]
1.14 M Tris-HCl + 0.11 % SDS, pH 6.8	3.4
30 % acrylamide	0.6
10 % ammonium persulfate (APS)	0.025
Tetramethylethylenediamine (TEMED)	0.005

Running buffer

Table 14: Composition of running buffer

960 mM Glycine
125 mM Tris
5 % SDS

Transfer buffer

Table 15: Composition of transfer buffer

150 mM Glycine
20 mM Tris
20 % Methanol
0.02 % SDS

30 µg of brush border membrane protein were mixed with Laemmli buffer without heating the samples. Electrophoresis was conducted at a constant voltage of 120 V for 15 minutes followed by 200 V for ~45 minutes. The gels were washed quickly in VE water and wet blotting on a nitrocellulose membrane (Whatman Optitran BA-S 85 Reinforced NC, 0.45 µM, Whatman GmbH, Germany) was performed at a constant electric current of 0.36 A for 20 minutes. Afterwards, membranes were blocked in 1 % BSA (fraction V) diluted in PBS for 1 hour at room temperature. Incubation with primary antibodies diluted in 1 % BSA in PBS-T (0.05 % Tween-20) was performed overnight at 4 °C. The next day, membranes were washed three times with PBS-T and subsequently incubated with secondary antibodies in PBS-T for 1.5-2 hours at room temperature, gently agitating. After washing with PBS, membranes were visualized and analyzed by the Odyssey® Infrared Imaging System (LI-COR® Biosciences, Inc., Lincoln, USA).

2.4.3. Glucose-induced hormone secretion

Concentrations of GIP, GLP-1 and insulin in the plasma were analyzed using commercial ELISA kits: total GIP levels were determined using rat/mouse total GIP ELISA kit (EMD Millipore Corporation, Massachusetts, USA); active GLP-1 levels were measured with a high sensitivity GLP-1 active chemiluminescent ELISA kit (EMD Millipore Corporation, Massachusetts, USA); insulin levels were determined with an ultra-sensitive mouse insulin ELISA kit (Crystal Chem, Inc., Illinois, USA).

2.4.4. Luminal glucose concentrations after high glucose gavage

Wild type mice received a glucose gavage of 4 g/kg body weight and were killed after 15 minutes. The whole intestine was removed and luminal contents were collected in Eppendorf tubes by placing the intestine in the tube and cutting it open vertically where luminal contents accumulated. The contents were centrifugated at 0.5 x g for 10 seconds to sediment intestinal solids. Glucose concentrations were determined using Glucose Hexokinase FS* assay kit (DiaSys Diagnostic Systems GmbH, Holzheim, Germany).

2.5. Immunohistochemistry

2.5.1. Preparation for immunofluorescence

The intestine was removed and placed on an ice cold glass plate. A piece of ~5 mm was cut off 13 cm downwards the pylorus and fixed in 4 % paraformaldehyde in PBS pH 7.4 at room temperature for 6-10 hours while gently shaking. The fixed tissues were dehydrated automatically and embedded in paraffin. Sections of 6 µm thickness were cut and dried on SuperFrost Ultra Plus® slides (Thermo Scientific, Braunschweig, Germany) over night at 37 °C. Slides were stored at 4 °C.

2.5.2. Immunofluorescence staining

Paraffin-embedded tissue samples were deparaffinized in 100 % xylene for 2 x 5 minutes followed by rehydration in descending concentrations of ethanol: 100 % for 2 x 5 minutes and 1 x 2 minutes, 96 % for 2 x 2 minutes, 80 % for 1 x 2 minutes. Slides were rinsed with running tap water for 3 minutes. For antigen retrieval, deparaffinized and rehydrated sections were boiled (~ 110 °C) in citrate buffer (see 2.1.1. Table 2) for 5 minutes using a pressure cooker in a microwave oven (AEG Micromat 15) at maximal power (1150 W). After cooling down, slides were blocked in 1 % BSA in PBS at room temperature for 30 minutes. Sections were incubated with primary antibodies diluted in PBS-T (0.05 % Tween-20) in a humified chamber over night at 4 °C. The next day, slides were washed three times with PBS before incubation with secondary antibodies diluted in PBS-T in the dark at room temperature for 1 hour. Slides were washed again three times with PBS, mounted using fluorescent mounting media (Dako Deutschland GmbH, Hamburg, Germany) and sealed with a cover slip, avoiding formation of air bubbles. They were allowed to

air dry in the dark and examined using a confocal microscope (FluoView FV10i-DOC, Olympus; 60x oil lens).

2.6. Transport functions assessed *ex vivo*

2.6.1. Influx studies of radiolabeled substrates into everted gut rings

In order to investigate whether high concentrations of luminal glucose induce GLUT2 trafficking into the apical membrane some of the mice received a 40 % glucose gavage to a final dose of 2 g/kg body weight. After 15 minutes, mice were killed and the whole small intestine was excised. The intestine was everted cautiously by means of a metal rod and washed thoroughly in ice cold Krebs buffer (see 2.1.1. Table 3) pre-gassed with 95 % O_2 / 5 % CO_2. It was placed on an ice cold plexiglas plate and by means of razor blades being fixed on a rod at an interval of exactly 1 cm intestinal rings with the luminal side facing outwards were produced (Figure 7A). Three rings each were incubated in 1 ml pre-gassed Krebs buffer pH 7.4 containing 1 mM glucose mixed with 11.1 kBq/ml ^{14}C-D-glucose and 1.5 kBq/ml ^{3}H-D-mannitol; the latter used as a space marker to allow for correction for unabsorbed, adherent radiolabeled substrate. Tissues were incubated in the absence and presence of either 200 µM phloridzin or 2 mM phloretin for 2 minutes at 37 °C in a 12-well plate while agitating (Figure 7B). Following the incubation, rings were washed thoroughly in ice cold Krebs buffer and blotted dry on absorptive tissue (Figure 7C). Each ring was placed in a scintillation vial and solubilized with 100 µl Biosol® Tissue Solubilizer (National Diagnostics, Atlanta, USA) in a shaking incubator at 50 °C overnight. The digested tissue was decolorized with 20 µl hydrogen peroxide for 1 hour at room temperature. Finally, 3 ml of Bioscint® Scintillation solution (National Diagnostics, Atlanta, USA) were added and the mixture vortexed well. Incorporated radiolabeled substrate was quantified by a liquid scintillation counter (PerkinElmer Inc., USA). An aliquot (10 µl) of the labeled uptake solution was taken to calculate the concentration of the incorporated radioactivity in nmol/cm*min.

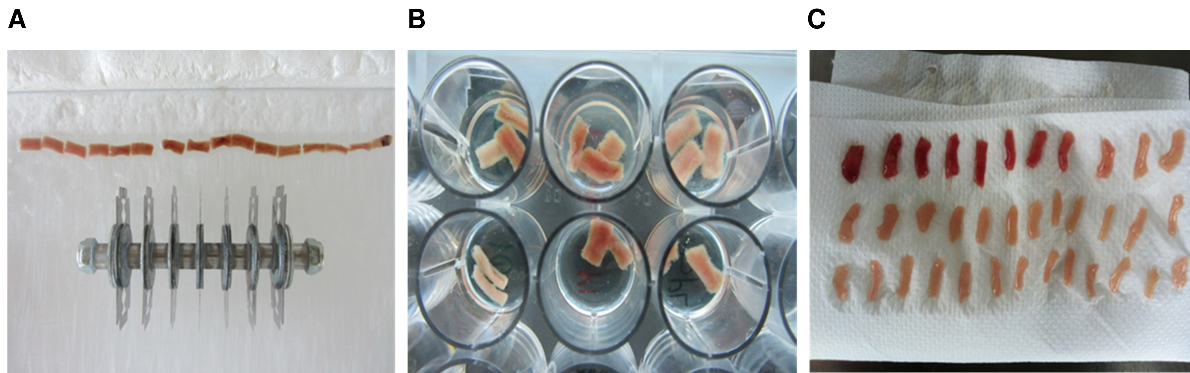


Figure 7: Preparation of everted gut rings. (A) Generation of 1 cm intestinal rings using razor blades fixed in a 1 cm interval on a rod. (B) Incubation of everted gut rings. (C) Intestinal rings.

2.7. Transport functions assessed *in vivo*

2.7.1. Intragastric glucose gavage for measurements of glucose-induced hormone secretion and for Western blot analysis

Sglt1$^{+/+}$ and *sglt1*$^{-/-}$ as well as *glut2*$^{+/+}$ and *glut2*$^{-/-}$ mice were randomly divided into two groups: the fasting state (basal) and the glucose-treated group which received a 40 % glucose gavage to a final dose of 4 g/kg body weight. Blood glucose was measured prior to and 15 minutes after the bolus if administered. Animals were killed with CO_2 for blood taking from the *vena cava inferior* using a 22 G' needle (B. Braun Melsungen AG, Melsungen, Germany) fixed on a 1 ml syringe (B. Braun Melsungen AG, Melsungen, Germany). Blood for hormone measurements was collected in ice cold Na-EDTA tubes (Sarstedt AG & Co, Nümbrecht, Germany) and immediately mixed with 10 µl/500 µl DPP-IV inhibitor (EMD Millipore Corporation, Massachusetts, USA) to prevent cleavage of GLP-1. Blood was kept on ice for 15 minutes and then centrifugated at 1200 x g for 20 minutes at 4 °C. Plasma was aliquoted, snap-frozen and stored at -80 °C until measured. For Western blot, the entire small intestine was removed, washed thoroughly in ice cold M100 buffer mixed with 1 % PMSF. In the 4 °C room, mucosa was scraped off on an ice cold glass plate using an ice cold glass slide and snap-frozen. Samples were stored at -80 °C until further processing.

2.7.2. Intragastric gavage of radiolabeled glucose for the assessment of glucose transport *in vivo*

Sglt1$^{+/+}$ and *sglt1*$^{-/-}$ as well as *glut2*$^{+/+}$ and *glut2*$^{-/-}$ mice received a 40 % glucose gavage to a final dose of 4 g/kg body weight mixed with 37 kBq/100 µl ^{14}C-D-glucose

and 37 kBq/100 µl ^{3}H-D-mannitol to correct for unabsorbed, adherent radiolabeled substrate. Blood glucose was measured before and 15 minutes after the gavage from the tail vain. Mice were anesthetized with isoflurane and blood was collected from the retro-orbital venous plexus for measurement of glucose tracer counts in the plasma. After cervical dislocation, the whole intestine was removed, washed thoroughly and blotted dry on absorptive tissue. Intestinal segments were produced and processed as described under 2.6.1., while maintaining the physiological order. The concentration of the incorporated radioactivity was expressed in nmol/cm over 15 minutes. For statistical analyses the average amount of radiolabeled glucose per cm as the sum of all segments divided by the number of segments was calculated.

2.7.3. Intragastric gavage of radiolabeled fructose to assess fructose absorption in the presence of mannitol, *α*-MDG or glucose

Sglt1 wild type and knockout, *glut2* wild type and knockout as well as *glut5* knockout mice were administered a 40 % gavage (final dose: 2 g/kg body weight) consisting of a 20 % fructose solution mixed with a 20 % mannitol, glucose or *α*-MDG solution (Table 16). Glucose and *α*-MDG, respectively, were added to stimulate SGLT1; unlabeled mannitol served as an osmotic control. After 15 minutes, blood glucose was measured before mice were killed by cervical dislocation. All other procedures were applied as described under 2.7.2.

Table 16: Composition of the radiolabeled fructose gavage

1 g/kg fructose + 37 kBq/100 µl [^{14}C(U)]-D-fructose + 37 kBq/100 µl [1-^{3}H(N)]-D-mannitol		
+ 1 g/kg D-mannitol	+ 1 g/kg D-glucose	+ 1 g/kg *α*-MDG

2.7.4. Fructose feeding and intragastric gavage of radiolabeled fructose-glucose combination

Sglt1 wild type and knockout were kept in the conventional animal house and fed either the sugar-free or the fructose-containing diet (see 2.3.2. Figure 7). Likewise, *glut2* wild type and knockout as well as *glut5* knockout mice were fed the fructose diet. After one week, all animals received the above described fructose-glucose combination per gavage and underwent the same procedures as described under 2.7.2.

3. Results

3.1. The role of SGLT1 and GLUT2 in glucose transport and sensing

3.1.1. *Ex vivo* approach – glucose influx into everted gut rings

Intestinal glucose influx is mainly mediated by SGLT1 whereas GLUT2 is thought to provide exit from cells into the blood stream. However, it was proposed that at high luminal glucose concentrations GLUT2 is inserted into the apical membrane in which it could in the capacity as a uniporter mediate bulk absorption of glucose [44]. To investigate whether a glucose gavage providing high luminal glucose concentrations can increase absorption, *sglt1* wild type and knockout mice received a glucose gavage of 2 g/kg body weight. After 15 minutes, glucose influx into everted gut rings was assessed in the absence and presence of phloretin and phlorizin.

Glucose influx into everted gut rings of non-gavaged $sglt1^{+/+}$ mice (basal) was significantly reduced by phloretin by almost 70 % (4.1 ± 0.6 nmol/cm*min *vs.* 1.3 ± 0.4 nmol/cm*min, $p < 0.001$) and was abolished by phloridzin (4.1 ± 0.6 nmol/cm*min *vs.* 0.008 ± 0.005 nmol/cm*min, $p < 0.001$). Glucose absorption was completely absent in $sglt1^{-/-}$ animals ($p < 0.001$; Figure 8).

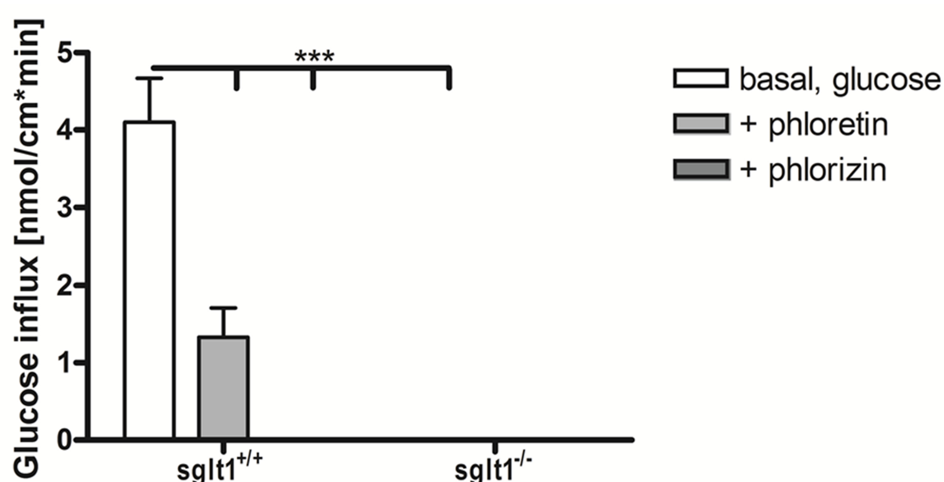


Figure 8: Glucose influx into everted gut rings in the absence and presence of phloretin and phlorizin in non-gavaged *sglt1* wild type and knockout mice. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. *** $p < 0.001$. N = 4-5 mice per group.

After the gavage, glucose uptake was less in *sglt1*$^{+/+}$ animals (2.2 ± 0.2 nmol/cm*min) and still absent in *sglt1*$^{-/-}$ littermates ($p < 0.001$). The decline was most probably attributed to adherent unlabeled glucose from the gavage that attenuated the uptake of radiolabeled glucose due to dilution. Yet, phloretin decreased uptake by 55 % (2.2 ± 0.2 nmol/cm*min *vs.* 1.0 ± 0.1 nmol/cm*min, $p < 0.001$) and phlorizin again completely inhibited absorption (2.2 ± 0.2 nmol/cm*min *vs.* 0.02 ± 0.02 nmol/cm*min, $p < 0.001$; Figure 9).

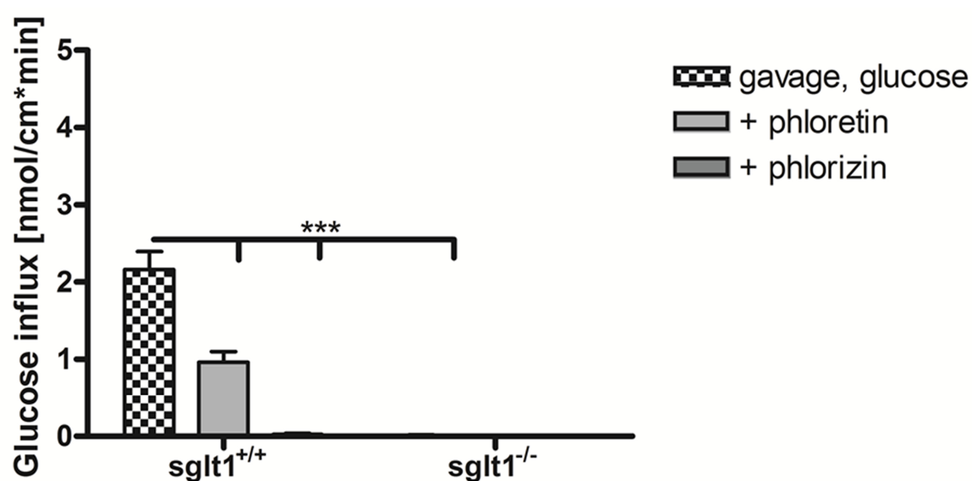


Figure 9: Glucose influx into everted gut rings in the absence and presence of phloretin and phlorizin in *sglt1* wild type and knockout mice after receiving the glucose gavage. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. *** $p < 0.001$. N = 4-5 mice per group.

In *sglt1*$^{-/-}$ mice, the rise in blood glucose levels following the glucose gavage was significantly diminished compared to *sglt1*$^{+/+}$ littermates (72 ± 12 mg/dl *vs.* 21 ± 11 mg/dl, $p < 0.01$; Figure 10).

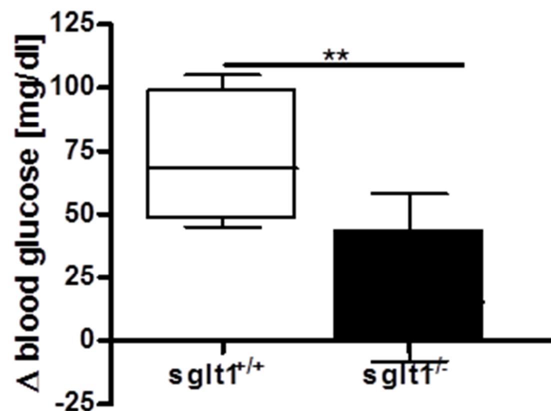


Figure 10: Rise in blood glucose after the glucose gavage in *sglt1* wild type and knockout mice. Values are expressed as mean ± SEM. Statistical analyses were performed using unpaired t-test. ** $p < 0.01$. N = 4-5 mice per group.

3.1.2. Assessment of glucose transport *in vivo* using intragastric gavage of radiolabeled glucose

To avoid any dilution of glucose tracer but also prevent inactivation of PKC βII whose functionality was shown to be crucial for GLUT2 trafficking [33, 34], *sglt1* wild type and knockout as well as *glut2* wild type and knockout animals received a radiolabeled glucose gavage to assess glucose transport at high luminal glucose concentrations *in vivo*. The glucose gavage was administered to a final dose of 4 g/kg body weight which accounted for 160 ± 23 mM in intestinal luminal fluids when measured.

At 15 minutes after the gavage, *sglt1*$^{-/-}$ mice exhibited significantly reduced glucose tracer contents in all segments along the entire small intestine compared to *sglt1*$^{+/+}$ mice (Figure 11).

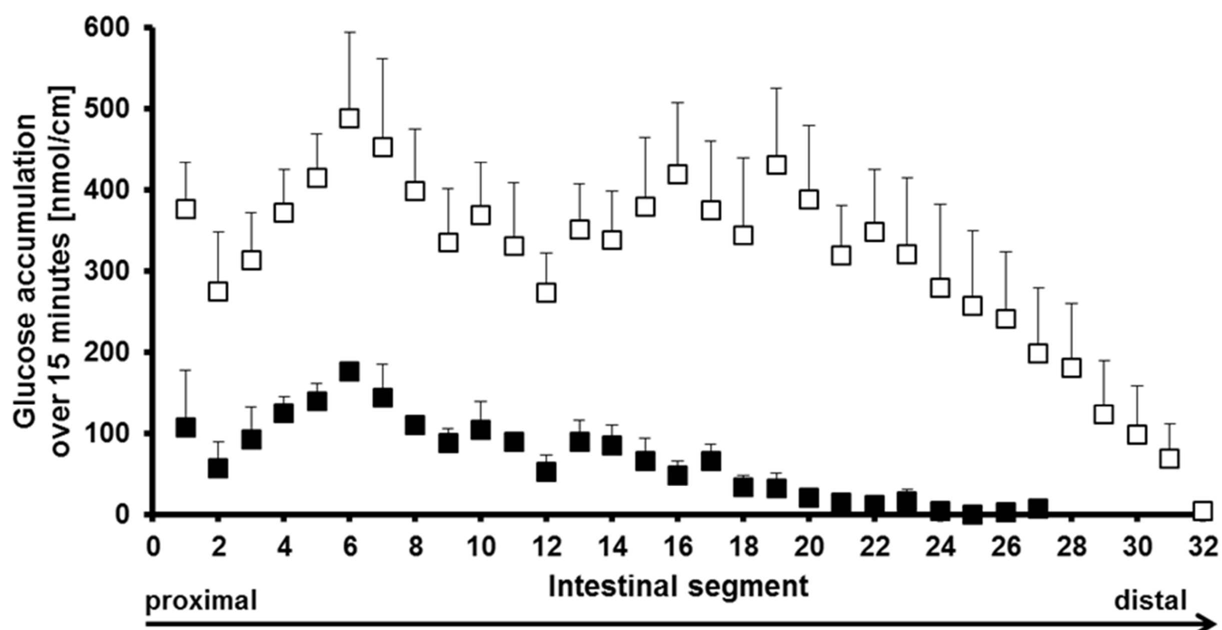


Figure 11: Glucose tracer contents in intestinal 1 cm segments of the entire small intestine of *sglt1*$^{+/+}$ (white squares) and *sglt1*$^{-/-}$ (black squares) mice 15 minutes after the glucose gavage.

The strong diminution in glucose absorption in the absence of SGLT1 accounting for 80% (282.9 ± 49.5 nmol/cm *vs.* 56.6 ± 3.0 nmol/cm, $p < 0.01$; Figure 12) confirmed the prime role of SGLT1 in glucose absorption even at high luminal glucose concentrations.

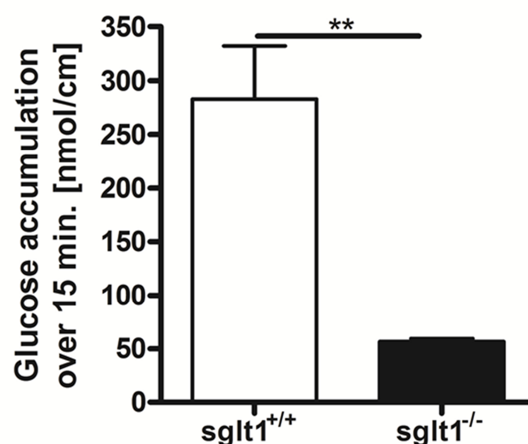


Figure 12: Average amount of glucose tracer in intestinal tissue samples of *sglt1* wild type and knockout mice collected 15 minutes after the gavage. Statistical analysis was performed using unpaired t-test with Welch's correction. ** $p < 0.01$. N = 4-5 mice per group.

As a consequence of decreased glucose influx in the absence of SGLT1 glucose tracer levels in the plasma were significantly lowered by 73 % in *sglt1* knockout compared to wild type animals (25.03 ± 3.17 nmol/cm *vs.* 93.49 ± 13.71 nmol/cm, $p < 0.01$; Figure 13A) as was the rise in blood glucose levels following glucose administration by 35 % (116 ± 20 mg/dl *vs.* 178 ± 23 mg/dl, $p < 0.05$; Figure 13B).

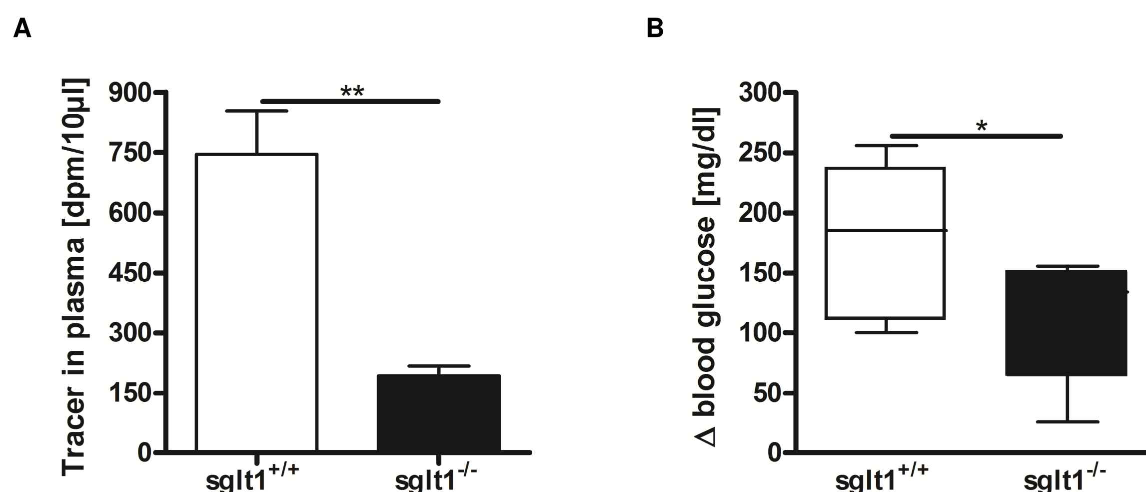


Figure 13: Glucose tracer contents in plasma and rise in blood glucose levels in *sglt1* wild type and knockout animals. (A) Glucose tracer in the plasma. (B) Increase in blood glucose after the glucose gavage. Values are expressed as mean ± SEM. Statistical analyses were performed using unpaired t-test with Welch's correction for comparison of tracer in plasma. Unpaired t-test was used to compare rises in blood glucose. * $p < 0.05$, ** $p < 0.01$. N = 4-5 mice per group.

Animals lacking GLUT2 displayed significantly elevated levels of glucose radiotracer in intestinal tissue samples as compared to wild type littermates (Figure 14), confirming that GLUT2 plays a critical role in the efflux of glucose into circulation as demonstrated by a strongly reduced efflux that is observed in the absence of GLUT2.

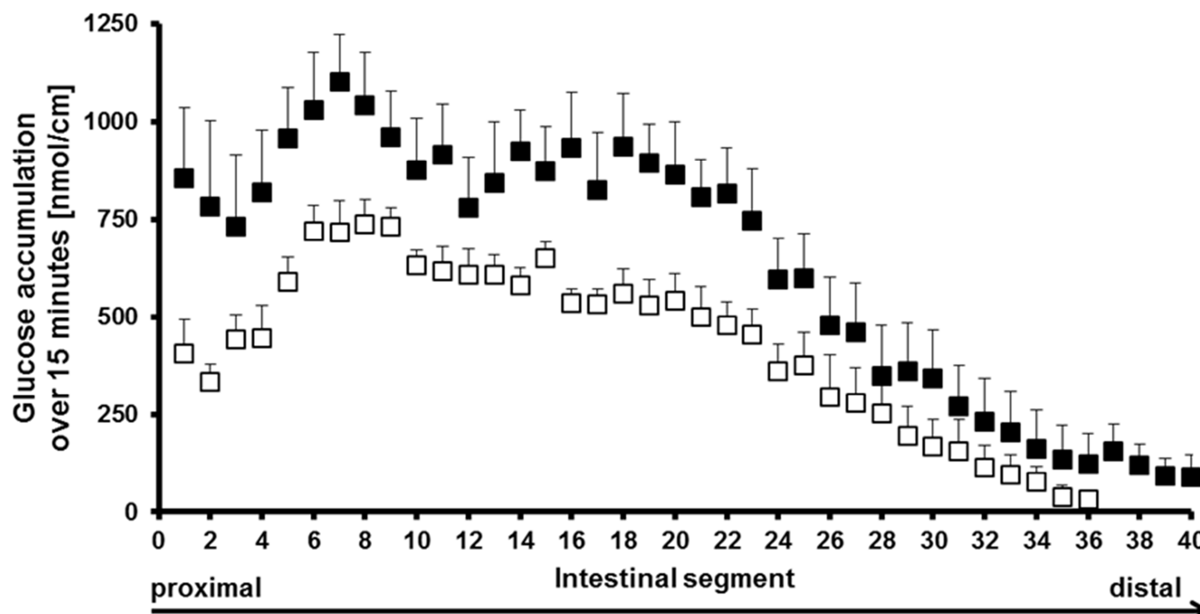


Figure 14: Glucose tracer amounts in intestinal 1 cm segments along the small intestine of *glut2*$^{+/+}$ (white squares) and *glut2*$^{-/-}$ (black squares) mice 15 minutes after the glucose bolus.

In GLUT2-deficient mice the mean of radiotracer accumulation in intestinal tissues increased by 56 % from 403.0 ± 46.8 nmol/cm to 627.1 ± 80.0 nmol/cm ($p < 0.05$; Figure 15).

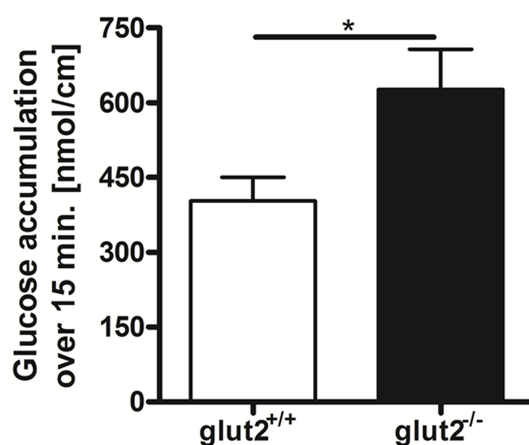


Figure 15: Average glucose tracer contents in intestinal tissues of *glut2* wild type and knockout animals. Values are expressed as mean ± SEM. Statistical analysis was performed using unpaired t-test. * $p < 0.05$. N = 5-6 mice per group.

The observed impairment of glucose exit from the gut resulted also in significantly diminished amounts of labeled glucose in plasma of *glut2* knockout compared to wild type mice (87.50 ± 10.76 nmol/10µl *vs.* 146.50 ± 11.28 nmol/10µl, $p < 0.01$; Figure 16A) and also in a lower increase in blood glucose after the glucose gavage (94 ± 13 mg/dl *vs.* 147 ± 17 mg/dl, $p < 0.05$; Figure 16B).

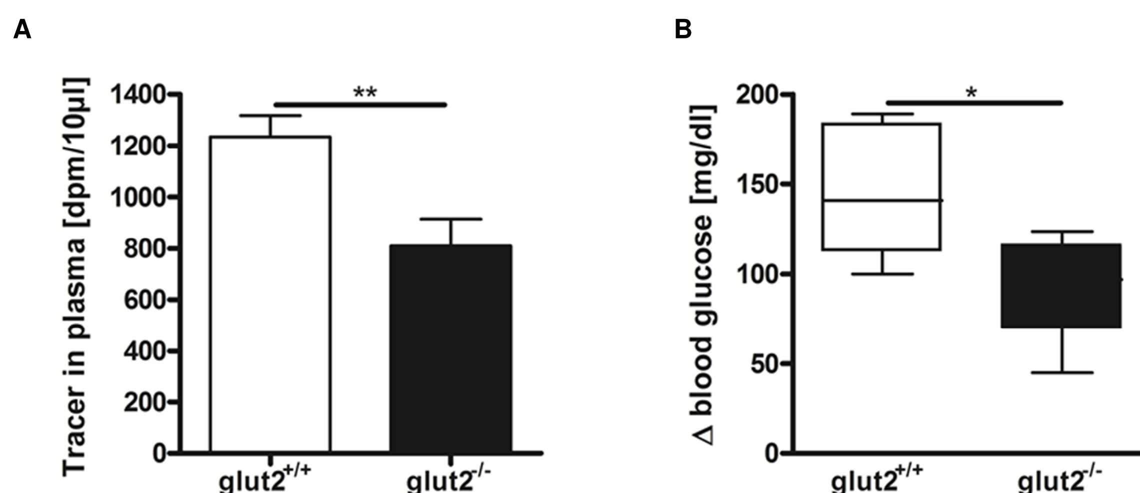


Figure 16: Radiotracer content in plasma and increases in blood glucose in *glut2* wild type and knockout animals. (A) Radiotracer in the plasma. (C) Rises in blood glucose levels after the glucose gavage. Values are expressed as mean ± SEM. Statistical analyses were performed using unpaired t-test. * $p < 0.05$, ** $p < 0.01$. N = 5-6 mice per group.

3.1.3. Glucose-induced hormone secretion

After the glucose gavage, plasma GIP levels in *sglt1* wild type mice increased more than 5-fold (67.8 ± 5.6 *vs.* 366.0 ± 56.1 pg/ml, $p < 0.01$) whereas in knockout animals the response was abolished (48.9 ± 8.0 and 62.8 ± 9.8 pg/ml; Figure 17A). GLP-1 concentrations in the plasma of *sglt1*$^{+/+}$ mice were enhanced 10-fold (0.3 ± 0.0 *vs.* 3.4 ± 0.9 pM, $p < 0.05$) but did not rise significantly in *sglt1*$^{-/-}$ animals (0.5 ± 0.1 and 0.8 ± 0.1 pM; Figure 17B). Plasma insulin levels following the glucose load were similarly enhanced around 1.7-fold in *sglt1*$^{+/+}$ (1.0 ± 0.2 *vs.* 1.7 ± 0.1 ng/ml, $p < 0.01$) and 1.6-fold in *sglt1*$^{-/-}$ mice (0.7 ± 0.1 *vs.* 1.1 ± 0.2 ng/ml) albeit this was statistically not significant in the latter ones (Figure 17C). While glucose-induced GIP- and GLP-1 responses were reduced by 83 % ($p< 0.001$) and 76 % ($p < 0.05$), respectively, in *sglt1* knockout animals compared to wild type littermates, insulin concentrations were reduced by only 36 % ($p < 0.01$) in *sglt1*$^{-/-}$ animals.

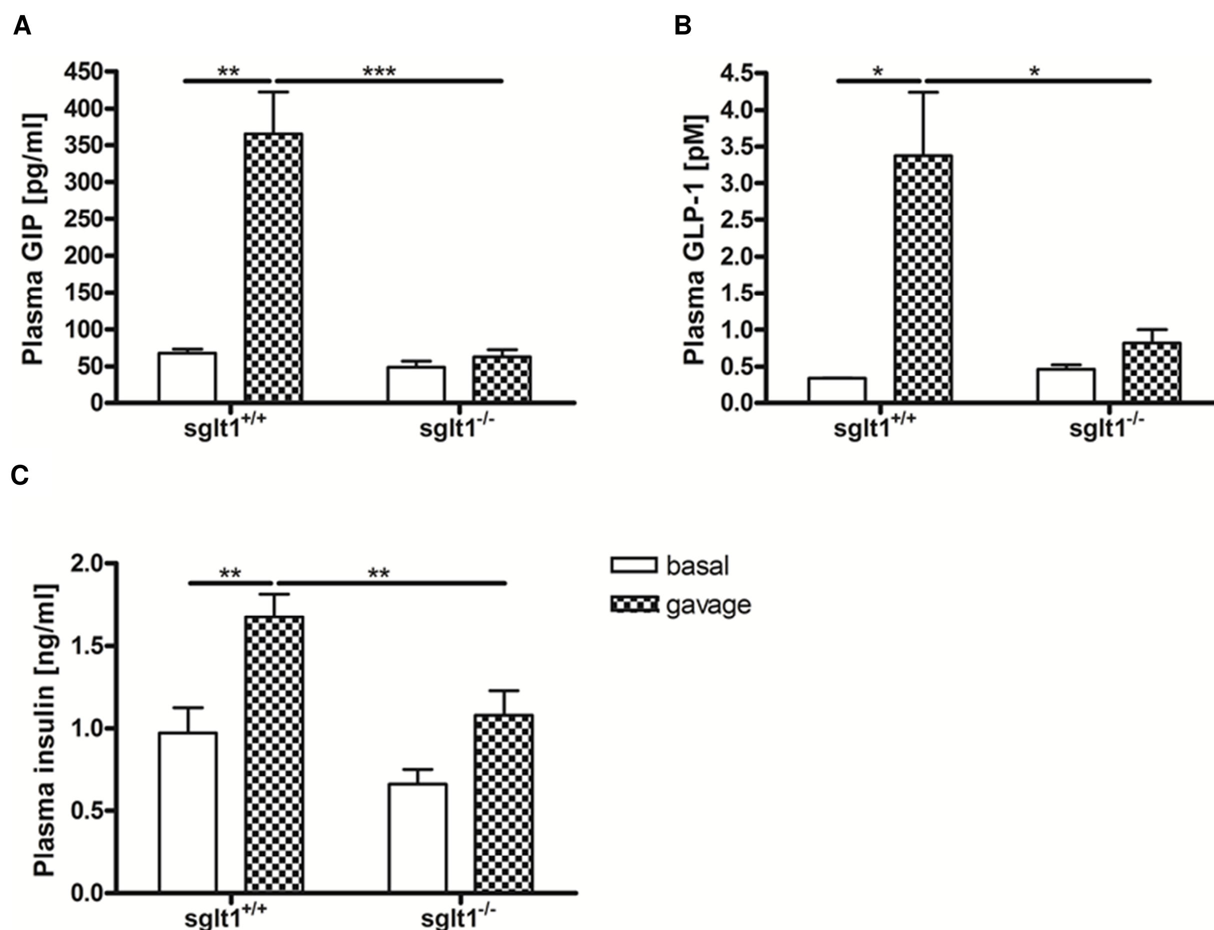


Figure 17: Plasma incretin and insulin levels in *sglt1* wild type and knockout mice. (A) Total GIP concentrations in the fasting state (basal, white bars) and after glucose gavage (gavage, checkered bars). (B) Active GLP-1 concentrations. (C) Insulin concentrations. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N = 3-8 mice per group.

In *glut2* wild type mice, the glucose bolus elevated plasma GIP concentrations as well more than 11-fold (57.2 ± 18.8 *vs.* 646.4 ± 84.6 pg/ml, $p < 0.001$) whereas the increase in knockout animals was only 7-fold (64.3 ± 9.0 *vs.* 451.6 ± 54.9 pg/ml, $p < 0.001$; Figure 18A). There was an around 3-fold increase in GLP-1 levels in both, in *glut2*$^{+/+}$ (1.8 ± 0.8 *vs.* 5.3 ± 1.3 pM, $p < 0.05$) and *glut2*$^{-/-}$ animals (1.8 ± 0.4 *vs.* 5.3 ± 1.9 pM, $p < 0.05$) when comparing basal and glucose-induced concentrations, respectively (Figure 18B). Plasma insulin levels were increased over 3-fold (from 0.49 ± 0.08 to 1.61 ± 0.09 ng/ml, $p < 0.001$) in *glut2*$^{+/+}$ mice, but only 2-fold and not significant (from 0.16 ± 0.00 to 0.35 ± 0.4 ng/ml), in *glut2*$^{-/-}$ littermates after glucose gavage (Figure 18C). Basal insulin levels in *glut2* knockout animals were reduced by 67 % ($p < 0.05$) compared to wild type littermates; this reduction was even more pronounced after the glucose bolus, then accounting for around 75 % ($p < 0.001$).

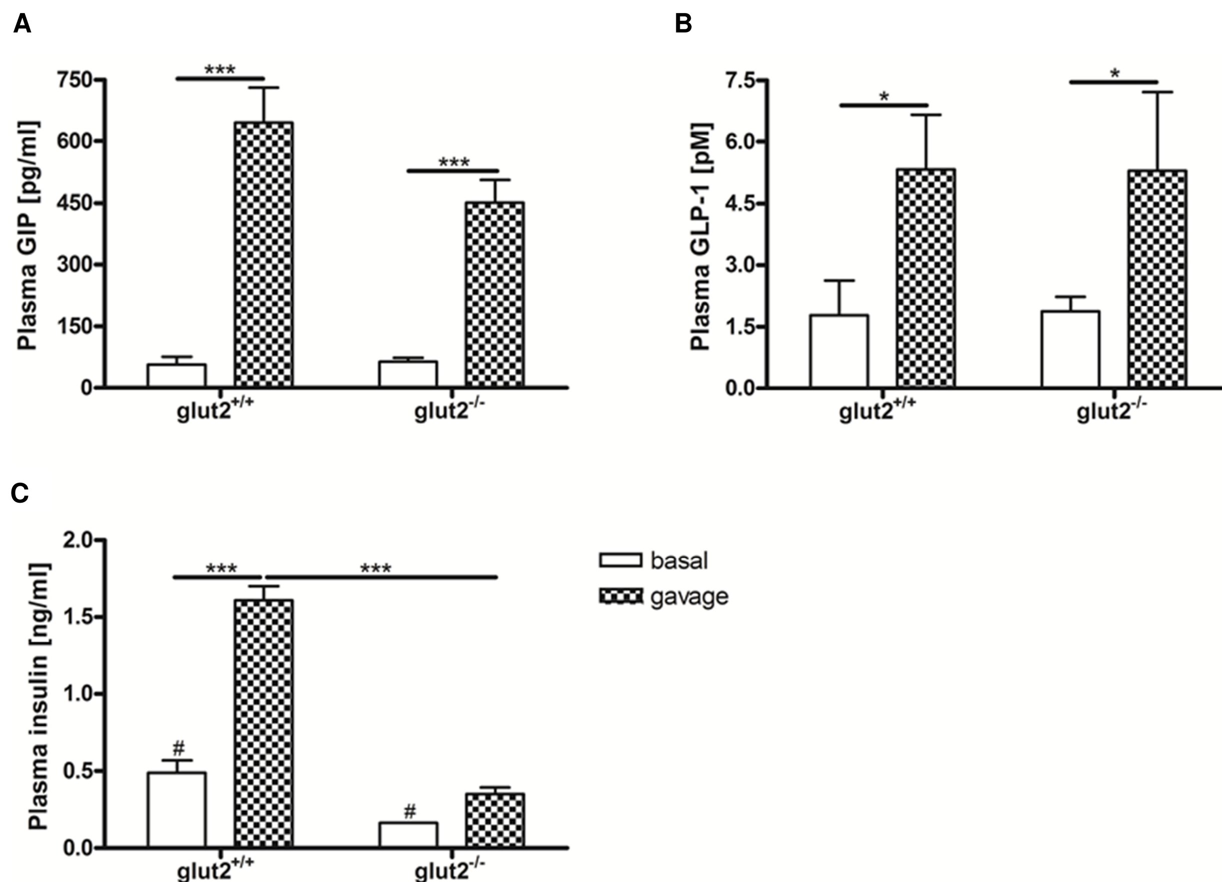


Figure 18: Plasma incretin and insulin levels in *glut2* wild type and knockout animals. (A) Total GIP concentrations in the fasting state (basal, white bars) and after glucose gavage (squared bars). (B) Active GLP-1 concentrations. (C) Insulin concentrations. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests for comparison of GIP and insulin concentrations. $^{\#}$ $p < 0.05$, *** $p < 0.001$. 2-way ANOVA was used for comparison of GLP-1 levels. * $p < 0.05$. N = 3-4 mice per group.

3.1.4. Protein levels in isolated brush border membranes (BBM)

Western blot analysis was performed to assess whether the abundance of SGLT1 and GLUT2 protein in brush border membranes changed in response to the glucose administration. BBM were isolated from mucosal scrapings before (fasting basal level) and after glucose gavage of 4 g/kg body weight.

As shown in Figure 19A, protein abundance of SGLT1 remained unchanged after the glucose gavage compared to the basal expression in *sglt1*$^{+/+}$ mice (fold change: 0.9 ± 0.05 and 1.0 ± 0.10) and was absent in knockout littermates. SGLT1 expression was also not altered by glucose compared to basal levels in either *glut2*$^{+/+}$ (fold change: 1.0 ± 0.14 and 1.0 ± 0.14) or *glut2*$^{-/-}$ animals (0.8 ± 0.08 and 0.9 ± 0.17; Figure 19B).

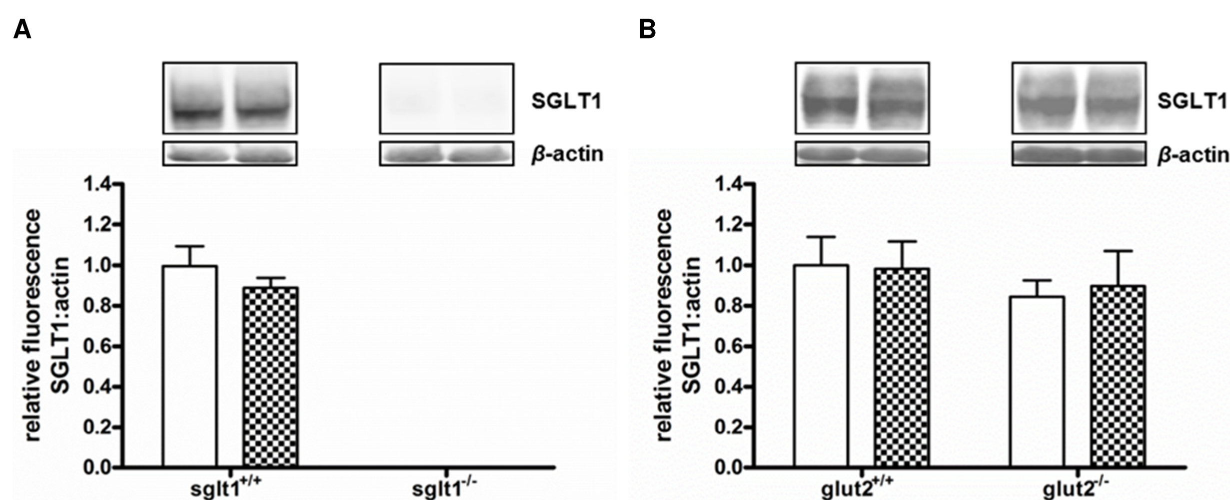


Figure 19: SGLT1 protein levels in BBM isolated from *sglt1* and *glut2* wild type and appropriate knockout mice. (A) SGLT1 abundance in *sglt1*$^{+/+}$ and *sglt1*$^{-/-}$ mice before (basal, white bars) and after glucose gavage (squared bars). (B) SGLT1 protein expression in *glut2*$^{+/+}$ and *glut2*$^{-/-}$ animals before and after gavage. Values are given in fold change expressed as mean ± SEM. Statistical analysis was performed using unpaired t-test for comparison of SGLT1 protein abundance before and after glucose gavage, respectively, in wild type and appropriate knockout mice. N = 3 mice per group.

The GLUT2 immunoreactivity respectively protein found in the BBM derived mainly from cross-contaminations with basolateral membranes since there were also considerable amounts of the basolateral marker Na-K-ATPase detected in all preparations (Figures 20A and B). The proportion of this basolateral carry-over was comparable in all experiments. Regardless of the contamination of the brush border membranes with basolateral membranes, GLUT2 levels were not changed by glucose administration when compared to the basal abundance in *sglt1*$^{+/+}$ (fold change: 0.6 ± 0.02 and 1.0 ± 0.27) and *sglt1*$^{-/-}$ mice (fold change: 0.4 ± 0.02 and 0.5 ± 0.13; Figure 20A). In *glut2*$^{+/+}$ animals, GLUT2 levels were also not altered by orally administered glucose when compared to basal protein levels (fold change: 0.7 ± 0.09 and 1.0 ± 0.24) and was absent in *glut2*$^{-/-}$ littermates (Figure 20B).

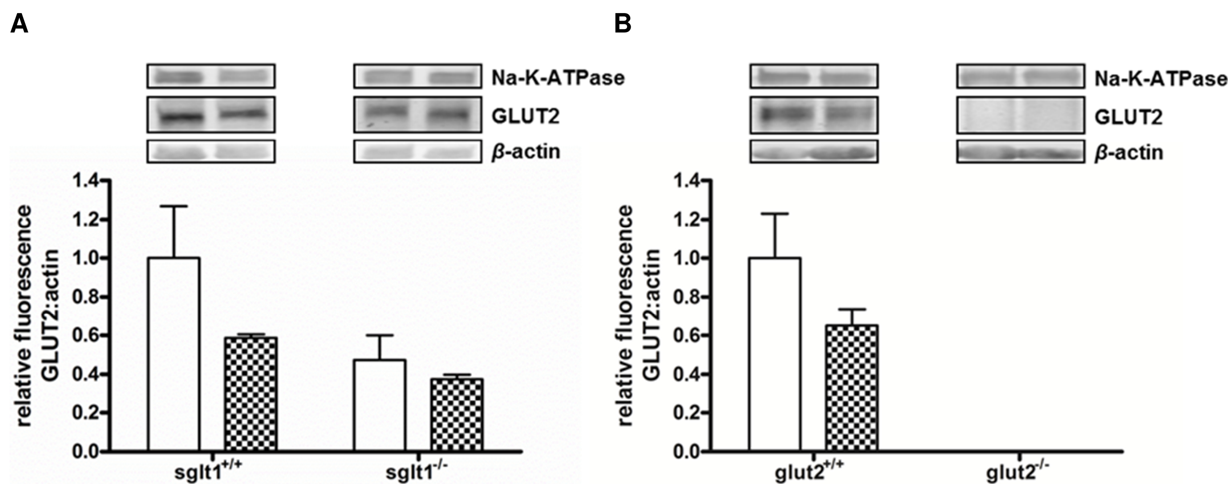


Figure 20: GLUT2 protein abundance in BBM isolated from *sglt1* and *glut2* wild type and appropriate knockout animals. (A) GLUT2 protein levels in *sglt1*$^{+/+}$ and *sglt1*$^{-/-}$ mice before (basal, white bars) and after glucose gavage (squared bars). (B) GLUT2 abundance in *glut2*$^{+/+}$ and *glut2*$^{-/-}$ animals. Values are given in fold change expressed as mean ± SEM. Statistical analysis was performed using unpaired t-test with Welch's correction for comparison of GLUT2 expression in *sglt1* wild type animals before and after glucose gavage. Unpaired t-test was used for comparing GLUT2 abundance before and after glucose gavage in *sglt1* knockout, *glut2* wild type and knockout, respectively. N = 3 mice per group.

3.1.5. Immunofluorescence staining of SGLT1 and GLUT2

Paraformaldehyde fixed and paraffin-embedded jejunal sections from *sglt1* and *glut2* wild type mice as well as their respective knockout littermates were stained for SGLT1 and GLUT2 immunoreactivity to define their localization in the mucosa and whether their densities change after the glucose gavage of 4 g/kg body weight.

As expected, SGLT1 immunoreactivity was found in the apical membrane of *sglt1* wild type mice with no obvious difference in staining between fasted animals and those receiving the glucose bolus (Figures 21A and B). In *sglt1* knockout littermates, SGLT1 staining was absent (Figure 21C).

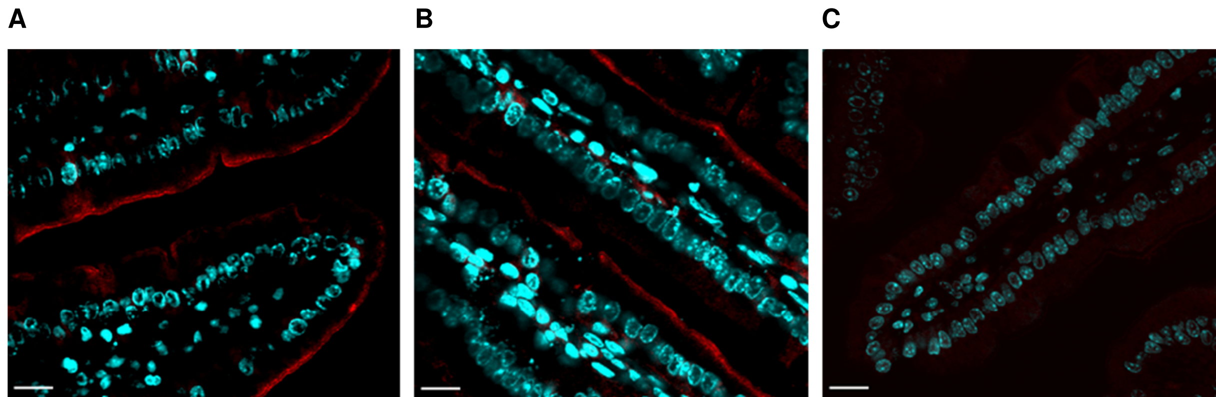


Figure 21: Immunohistochemical localization of SGLT1 (red) in *sglt1* wild type and knockout mice. Nuclei were stained with DAPI (blue). Jejunal sections from *sglt1*$^{+/+}$ animals in the (A) basal state and (B) after glucose gavage. (C) SGLT1 staining in *sglt1*$^{-/-}$ littermates. Scale bar: 20 µm.

GLUT2 immunoreactivity was found in the basolateral membrane of fasted *glut2* wild type animals with no evidence for insertion of GLUT2 into the apical membrane following the glucose gavage (Figures 22A and B). This finding further supports our assumption that the GLUT2 protein detected in brush border membranes was derived from basolateral cross-contaminations. Confirming the specificity of the GLUT2 antibody, staining was absent in *glut2* knockout mice (Figure 22C).

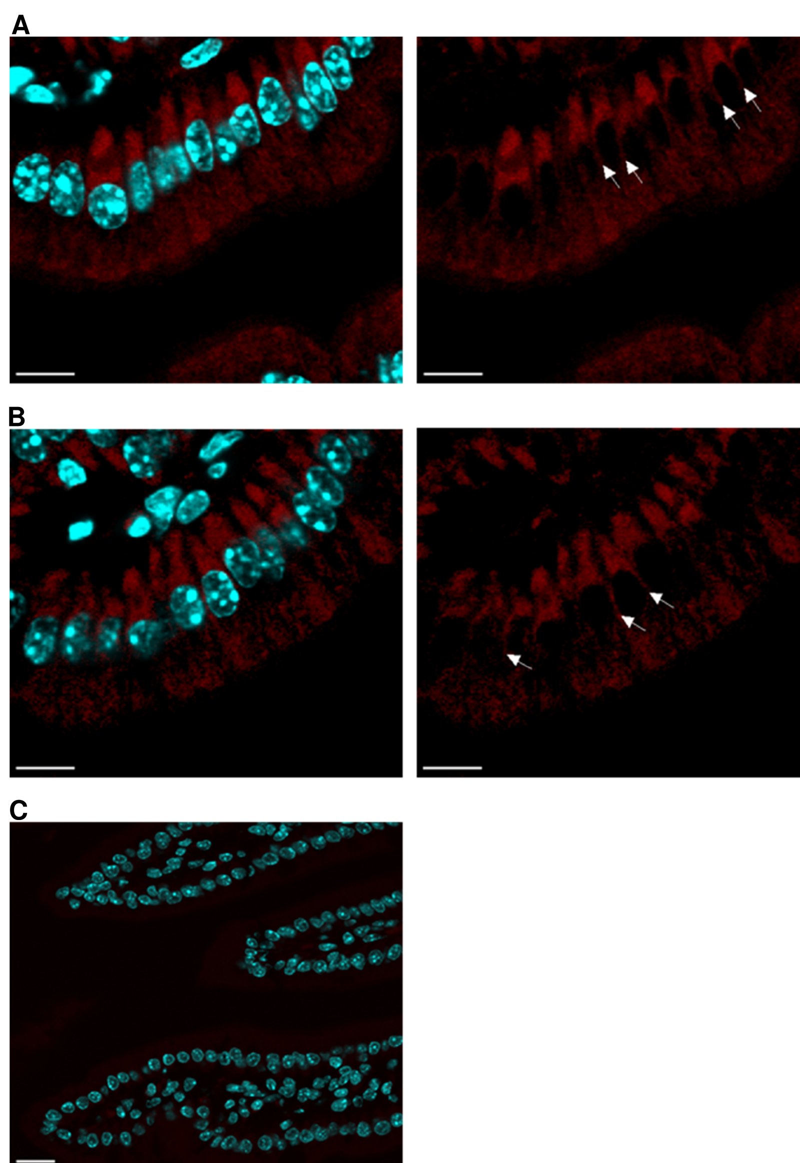


Figure 22: Immunohistochemical localization of GLUT2 (red) in *glut2* wild type and knockout animals. Nuclei were stained with DAPI (blue). Jejunal sections from *glut2*$^{+/+}$ mice (A) before and (B) after glucose gavage. (C) Staining of GLUT2 in *glut2*$^{-/-}$ animals. Scale bar: 20 µm.

3.2. Fructose absorption in mice that lack SGLT1, GLUT2 or GLUT5

3.2.1. Intestinal fructose absorption in mice fed the sugar-free diet

In humans, intestinal fructose absorption is improved when glucose is administered together with fructose and the maximum is achieved with an equal amount of glucose [159-162]. Despite this well-documented effect, the underlying mechanism is unknown and whether intestinal transporters such as SGLT1 or GLUT2 contribute to this phenomenon is also not known. We used *sglt1* and *glut2* wild type and their respective knockout littermates as well as *glut5* knockout mice and administered a gavage of radiolabeled fructose in combination with an equal amount of mannitol (as an osmotic control), *α*-MDG or glucose to a final dose of 2 g/kg body weight.

3.2.1.1. Intestinal fructose absorption in *sglt1* wild type and knockout animals fed the sugar-free diet

At 15 minutes after the fructose gavage, radiotracer contents from the labeled fructose in intestinal tissues of $sglt1^{+/+}$ mice were significantly increased in the presence of glucose by 73 % compared to mannitol as control (307.3 ± 20.0 nmol/cm *vs.* 177.9 ± 11.6 nmol/cm, $p < 0.001$) whereas the addition of *α*-MDG did not alter the amount of fructose tracer when compared to mannitol (149.5 ± 38.7 nmol/cm and 177.9 ± 11.6 nmol/cm). In $sglt1^{-/-}$ animals, fructose tracer contents in intestinal tissues were similar regardless of whether mannitol, *α*-MDG or glucose was added (134.5 ± 41.1 nmol/cm, 218.4 ± 24.2 nmol/cm and 156.1 ± 24.8 nmol/cm, respectively). However, when compared to wild type animals, tracer amounts were significantly lower in *sglt1* knockout (156.1 ± 24.8 nmol/cm *vs.* 307.3 ± 20.0 nmol/cm, $p < 0.001$; Figure 23) when glucose was administered with the fructose gavage indicating that glucose influx *via* SGLT1 is required to enhance fructose uptake. Thus, SGLT1 seems to be indirectly involved in fructose absorption.

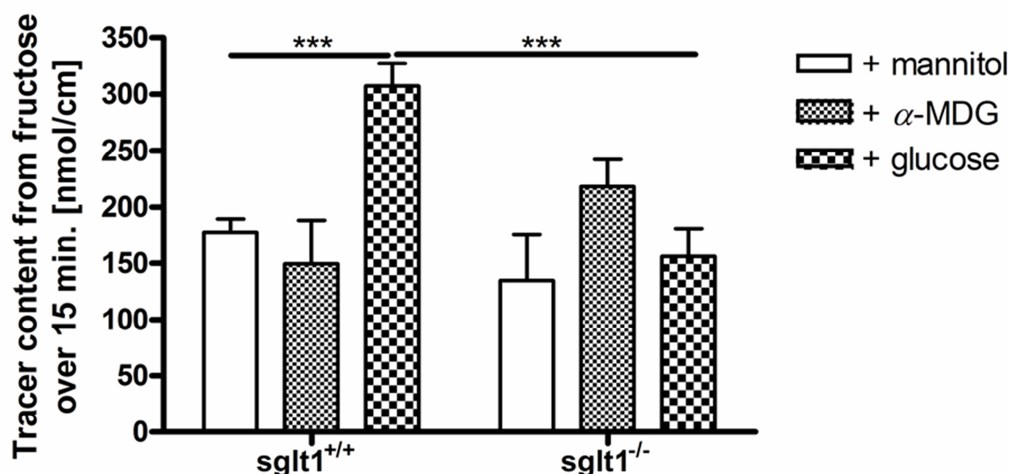


Figure 23: Mean tracer contents from fructose administration in intestinal tissues of *sglt1* wild type and knockout mice. Average fructose tracer contents per 1 cm segment in the presence of mannitol, α-MDG or glucose in the fructose gavage solution. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. *** $p < 0.001$. N = 4-8 mice per group.

As expected, there was a rise in blood glucose levels in *sglt1*$^{+/+}$ mice when glucose was added to the fructose gavage as compared to the addition of mannitol (83 ± 11 mg/dl *vs.* 22 ± 7 mg/dl, $p < 0.001$). In *sglt1*$^{-/-}$ littermates the increase in blood glucose was similar in the presence of mannitol and glucose (39 ± 5 mg/dl and 35 ± 12 mg/dl, respectively) but markedly reduced when compared to wild type animals (83 ± 11 mg/dl *vs.* 35 ± 12 mg/dl, $p < 0.01$; Figure 24).

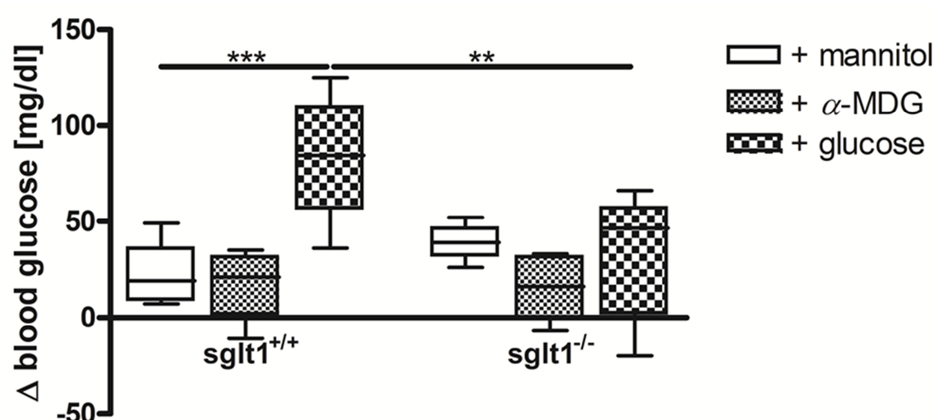


Figure 24: Changes in blood glucose concentrations after gavage of fructose in combination with mannitol, α-MDG or glucose in *sglt1* wild type and knockout mice. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. ** $p < 0.01$, *** $p < 0.001$. N = 4-8 mice per group.

To assess whether the increased fructose absorption in the presence of glucose was due to changes at transporter protein levels, brush border membranes were isolated from *sglt1* wild type and knockout mice that received the fructose gavage either in combination with mannitol or glucose.

SGLT1 protein expression following the fructose-glucose combination was 1.4-fold higher than in case of the fructose-mannitol combination (fold change: 1.4 ± 0.06 *vs.* 1.0 ± 0.04, $p < 0.01$; Figure 25A). Such a short-term regulation by a glucose administration was previously described [21, 29, 61] and also supports the notion that SGLT1 might play a role in fructose absorption. Unfortunately, a GLUT5-specific antibody was not available to be able to determine whether its levels also changed in the apical membrane.

When testing for GLUT2 protein abundance, similar levels were found after the fructose-mannitol and fructose-glucose combinations in $sglt1^{+/+}$ (fold change: 1.0 ± 0.15 and 1.2 ± 0.55) and $sglt1^{-/-}$ mice (fold change: 1.1 ± 0.05 and 1.1 ± 0.37; Figure 25B). This finding matches crossly with unchanged GLUT2 levels after the 4 g/kg glucose gavage reported in the first part of the present study and again does not provide any evidence for a contribution of apical GLUT2 in fructose transport in the presence of glucose.

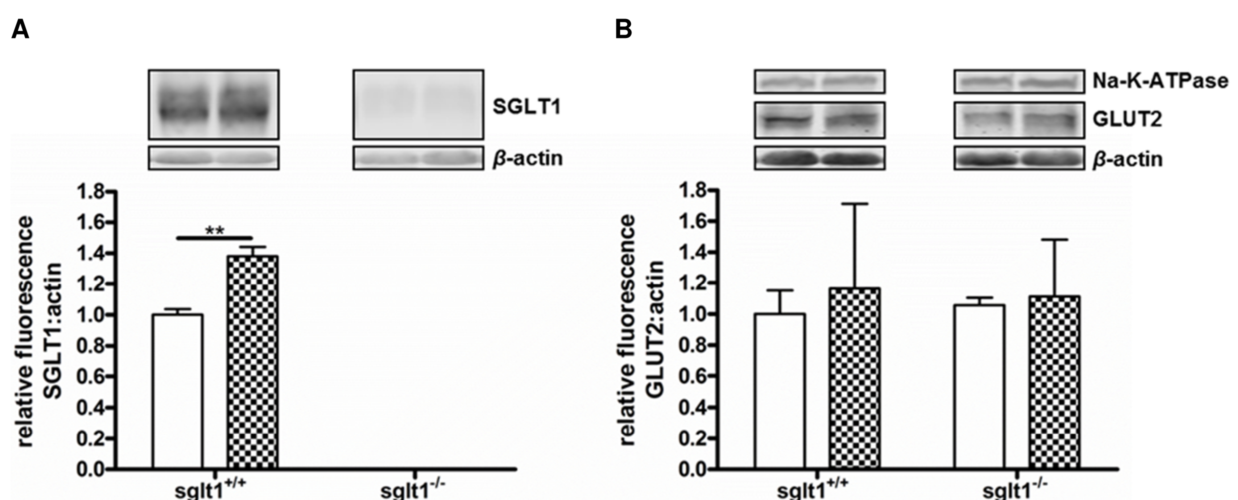


Figure 25: SGLT1 and GLUT2 protein abundance in BBM isolated from *sglt1* wild type and knockout mice after gavage of the fructose-mannitol (white bars) or fructose-glucose combination (checkered bars). (A) SGLT1 protein levels. (B) Expression of GLUT2. Values are given in fold change expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests for comparison of GLUT2 abundance. Unpaired t-test was used to compare SGLT1 expression. ** $p < 0.01$. N = 3 mice per group.

3.2.1.2. Intestinal fructose absorption in *glut2* wild type and knockout animals fed the sugar-free diet

In contrast to *sglt1* wild type animals, the amount of tracer from fructose in intestinal tissues of *glut2*$^{+/+}$ mice was similar regardless of whether mannitol, α-MDG or glucose was combined with fructose in the gavage solution (208.2 ± 18.1 nmol/cm, 233.8 ± 23.1 nmol/cm and 205.2 ± 29.0 nmol/cm, respectively) and very similar results were obtained in *glut2*$^{-/-}$ mice (208.7 ± 36.4 nmol/cm, 204.2 ± 23.3 nmol/cm and 245.9 ± 28.7 nmol/cm, respectively; Figure 26). Since GLUT2 is thought to provide the exit for glucose but also for fructose across the basolateral membrane [10] one might have expected an increased accumulation of fructose tracer in the absence of GLUT2 similar to glucose tracer accumulation after the glucose gavage in *glut2* knockout mice as described in the first part of this study. However, this may be explained by the fact that fructose uptake occurs via a uniporter whereas in case of glucose this is an uphill process.

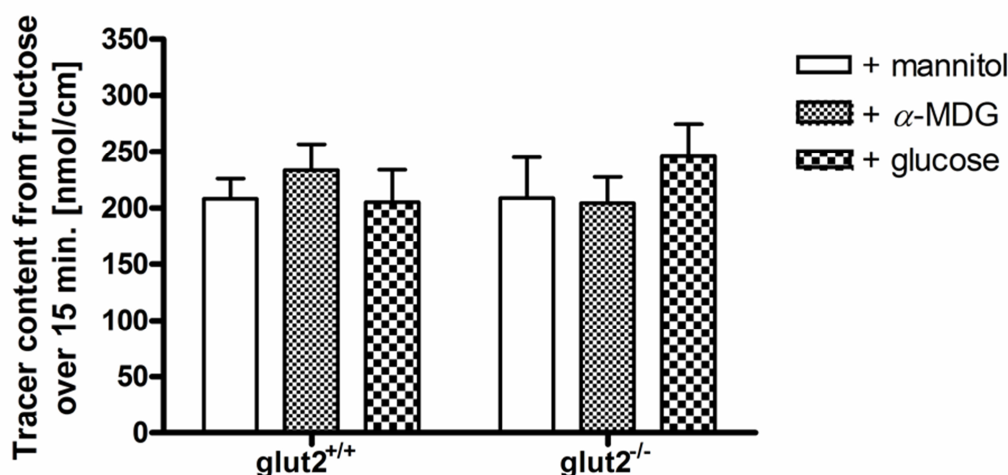


Figure 26: Mean tracer contents from fructose administration in intestinal tissues of *glut2* wild type and knockout mice. Average amounts of radiotracer per 1 cm segment when mannitol, α-MDG or glucose was added to the fructose gavage solution. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. N = 6-8 mice per group.

Like in *sglt1* wild type animals, blood glucose levels rose significantly following the fructose-glucose gavage as compared to the fructose-mannitol combination in *glut2*$^{+/+}$ (97 ± 15 mg/dl *vs.* 43 ± 10 mg/dl, $p < 0.05$) but also in *glut2*$^{-/-}$ mice (74 ± 6 mg/dl *vs.* 22 ± 7 mg/dl, $p < 0.05$) although the increase seemed to be lower

compared to wild type littermates that were gavaged with the fructose-glucose combination (Figure 27).

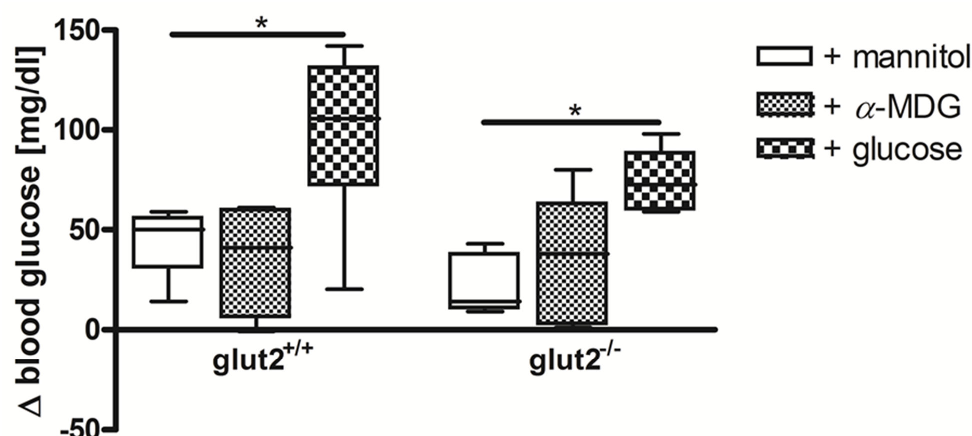


Figure 27: Changes in blood glucose levels after the fructose-mannitol, fructose-α-MDG or fructose-glucose combination provided by gavage in *glut2* wild type and knockout animals. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. * $p < 0.05$. N = 6-8 mice per group.

3.2.1.3. Intestinal fructose absorption in *glut2* wild type and *glut5* knockout animals fed the sugar-free diet

As expected, fructose tracer contents in intestinal tissues of *glut5*$^{-/-}$ mice were significantly reduced when compared to *glut2* wild type animals. The reduction accounted for around 65 % in the presence of mannitol (208.2 ± 18.1 nmol/cm *vs.* 72.1 ± 36.1 nmol/cm, $p < 0.001$), 72 % when *α*-MDG was added (233.8 ± 23.1 nmol/cm *vs.* 64.5 ± 18.1 nmol/cm, $p < 0.001$) and for 94 % when glucose was present with the fructose in the gavage solution (205.2 ± 29.0 nmol/cm *vs.* 12.6 ± 5.2 nmol/cm, $p < 0.001$). However, fructose absorption in *glut5*$^{-/-}$ mice was not altered by the addition of glucose to the fructose gavage when compared to co-administration with mannitol or *α*-MDG (12.6 ± 5.2 nmol/cm, 72.1 ± 36.1 nmol/cm and 64.5 ± 18.1 nmol/cm, respectively; Figure 28).

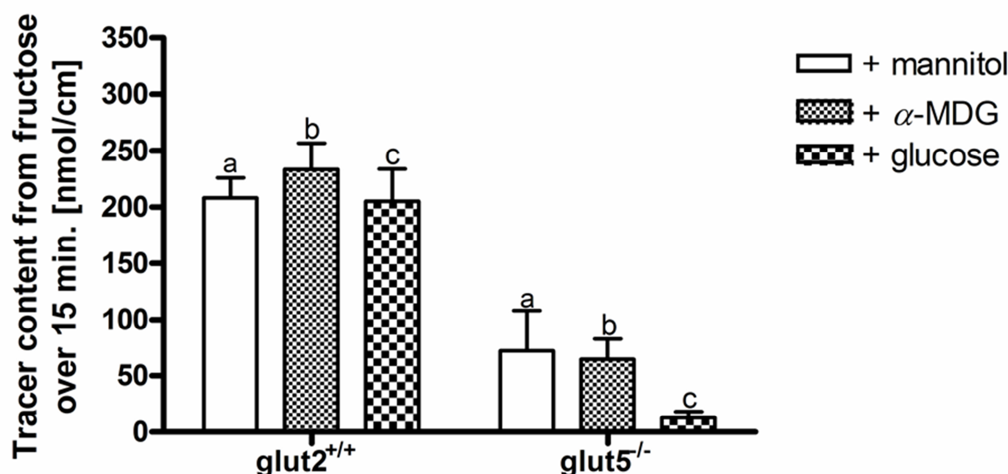


Figure 28: Mean tracer contents from fructose administration in intestinal tissues of *glut2* wild type and *glut5* knockout mice. Average amounts of tracer per 1 cm segment when mannitol, α-MDG or glucose was present in the fructose gavage solution. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. [a,b,c] $p < 0.001$. N = 6-8 mice per group.

The addition of glucose to the fructose gavage solution compared to mannitol resulted in elevated blood glucose concentrations in *glut2*$^{+/+}$ (97 ± 15 mg/dl *vs.* 43 ± 10 mg/dl, $p < 0.05$) but not in *glut5*$^{-/-}$ mice in which the rises were similar for the addition of mannitol, α-MDG or glucose (43 ± 10 mg/dl, 37 ± 10 mg/dl and 50 ± 7 mg/dl, respectively). Since there was no increase in blood glucose levels after the fructose-glucose gavage in animals lacking GLUT5 levels were significantly lower when compared to *glut2* wild type animals (50 ± 7 mg/dl *vs.* 97 ± 15 mg/dl, $p < 0.05$; Figure 29).

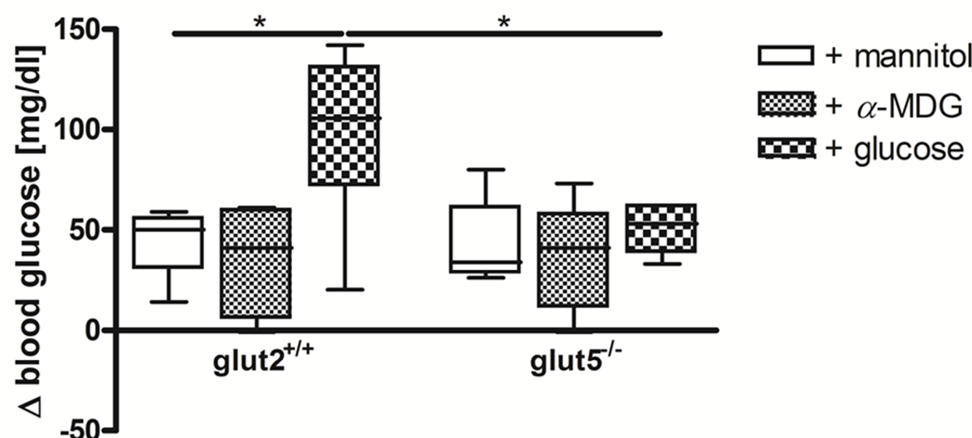


Figure 29: Changes in blood glucose concentrations following the fructose-mannitol, fructose-α-MDG or fructose-glucose combination in *glut2* wild type and *glut5* knockout animals. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. * $p < 0.05$. N = 6-8 mice per group.

Glucose thus did not increase fructose tracer contents in intestinal tissues of *glut2* wild type and *glut5* knockout mice. Moreover, SGLT1 protein abundance was similar after the fructose-mannitol and fructose-glucose gavage combinations in *glut2*$^{+/+}$ (fold change: 1.0 ± 0.09 and 1.0 ± 0.06) and in *glut5*$^{-/-}$ animals (fold change: 1.1 ± 0.14 and 1.0 ± 0.08; Figure 30A). Likewise, GLUT2 protein levels remained unchanged when comparing the fructose-mannitol and the fructose-glucose gavage conditions in *glut2*$^{+/+}$ (fold change: 1.0 ± 0.19 and 1.2 ± 0.13) and *glut5*$^{-/-}$ mice (fold change: 0.5 ± 0.12 and 0.7 ± 0.03), respectively. However, GLUT2 protein levels were significantly lower in *glut5* knockout as compared to *glut2* wild type animals after the fructose-mannitol (0.5 ± 0.12 *vs.* 1.0 ± 0.19, $p < 0.05$) as well as after the fructose-glucose gavage (0.7 ± 0.03 *vs.* 1.2 ± 0.13, $p < 0.05$; Figure 30B).

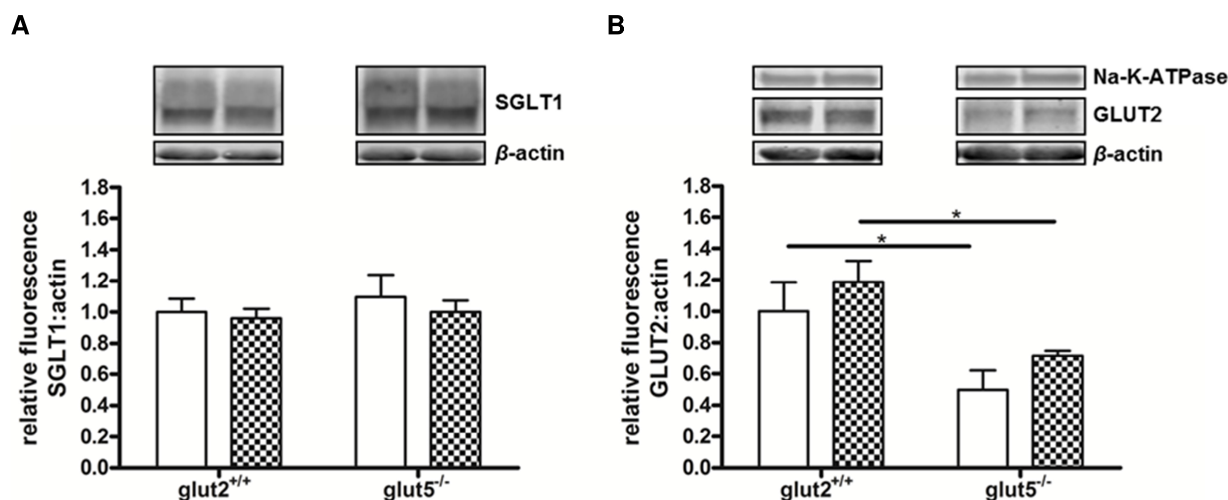


Figure 30: SGLT1 and GLUT2 protein density in BBM prepared from *glut2* wild type and *glut5* knockout mice after the fructose-mannitol (white bars) and the fructose-glucose combination (checkered bars). (A) SGLT1 abundance. (B) GLUT2 protein levels. Values are given in fold change expressed as mean ± SEM. Statistical analysis was performed using unpaired t-test for comparison of GLUT2 expression in *glut2* wild type and *glut5* knockout animals after fructose-mannitol and fructose-glucose gavage, respectively. * $p < 0.05$. N = 3 mice per group.

3.2.2. Intestinal fructose absorption in *sglt1* wild type and knockout mice kept in the conventional animal house

The striking finding that glucose enhanced fructose absorption in *sglt1* wild type mice kept in the SPF facility but not in *glut2* wild type mice housed in the conventional animal facility, it was decided that *sglt1* wild type and knockout mice needed to be moved into the conventional animal facility to assess fructose transport again in the presence of glucose under the same housing conditions as for *glut2* and *glut5* mice.

Animals were housed in the conventional facility for 3-4 weeks before fructose in combination with glucose was administered by gavage again. Intestinal tissues of *sglt1*$^{+/+}$ mice after the fructose-glucose gavage were significantly reduced by 51 % as compared to animals in the SPF facility (150.1 ± 15.6 nmol/cm *vs.* 307.3 ± 20.0 nmol/cm, $p < 0.001$). The amounts were thereby similar to *glut2*$^{+/+}$ animals kept in the conventional facility (205.2 ± 29.0 nmol/cm). In *sglt1*$^{-/-}$ mice, tracer contents after gavage did not differ in animals kept in the conventional compared to the SPF facility (125.6 ± 12.5 nmol/cm and 156.1 ± 24.8 nmol/cm, respectively; Figure 31).

Figure 31: Mean tracer contents from fructose administration in intestinal tissues of *sglt1* wild type and knockout mice kept in the SPF (white bars) or in the conventional animal facility (hatched bars). Average amounts of labeled fructose per 1 cm segment after the fructose-glucose gavage combination. Values are expressed as mean ± SEM. Statistical analyses to compare SPF and conventional housing in sglt1 wild type and knockout mice were performed using 2-way ANOVA with Bonferroni post-tests. *** $p < 0.001$. N = 4-8 mice per group.

Conventional compared to SPF housing did not alter fasting blood glucose levels in *sglt1*$^{+/+}$ (135 ± 14 mg/dl and 136 ± 11 mg/dl) or *sglt1*$^{-/-}$ animals (120 ± 4 md/dl and 129 ± 16 mg/dl; Figure 32A). The lower tracer content in tissues and less pronounced increase in blood glucose levels in response to the fructose-glucose gavage combination in wild type animals kept in the conventional as compared to SPF facility (52 ± 18 mg/dl and 83 ± 11 mg/dl, $p = 0.08$) is striking. In *sglt1*$^{-/-}$ littermates, however, the rise in blood glucose levels after gavage was not affected by housing conditions (57 ± 7 mg/dl and 35 ± 12 mg/dl; Figure 32B).

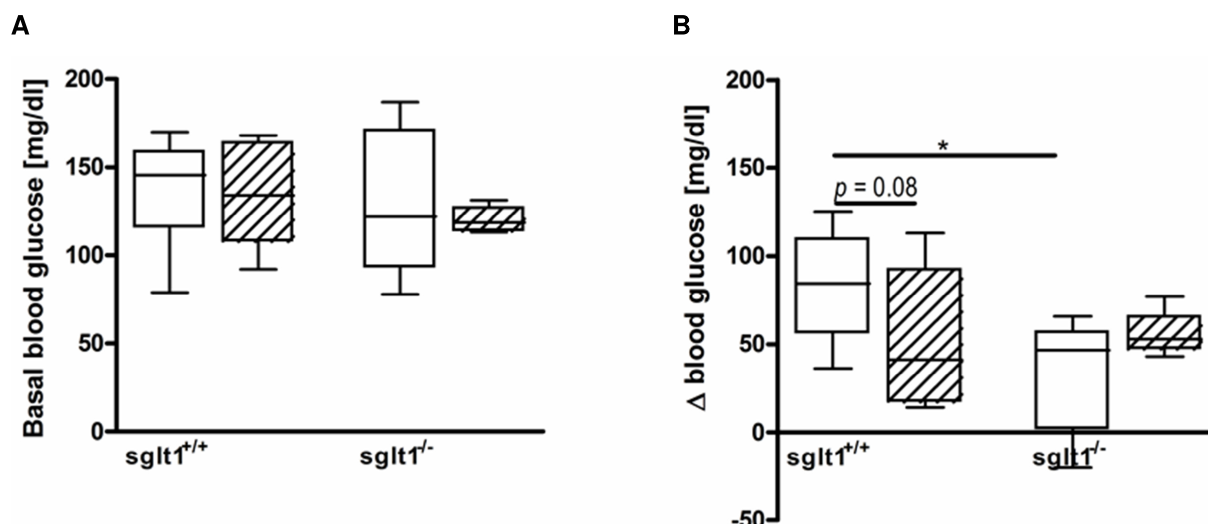


Figure 32: Fasting glucose levels and changes in blood glucose levels in *sglt1* wild type and knockout mice kept in the SPF (white boxes) or in the conventional facility (hatched boxes). (A) Fasting blood glucose concentrations. (B) Increases in blood glucose after the fructose-glucose gavage. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. * $p < 0.05$. Unpaired t-test was used for comparison of rises in blood glucose after the fructose-glucose gavage in *sglt1* wild type animals kept in SPF or conventional facility. N = 4-8 mice per group.

To examine whether the reduction in tracer amounts derived from fructose in intestinal tissues of *sglt1* wild type mice that were moved into the conventional animal house was associated with alterations at transporter protein levels, BBM were prepared from *sglt1* wild type mice that were kept in either the SPF or conventional animal facility. However, we did not find any change in either SGLT1 protein density (fold change: 1.0 ± 0.10 and 1.0 ± 0.13, Figure 33A) or GLUT2 protein levels (fold change: 1.0 ± 0.13 and 1.2 ± 0.04; Figure 33B).

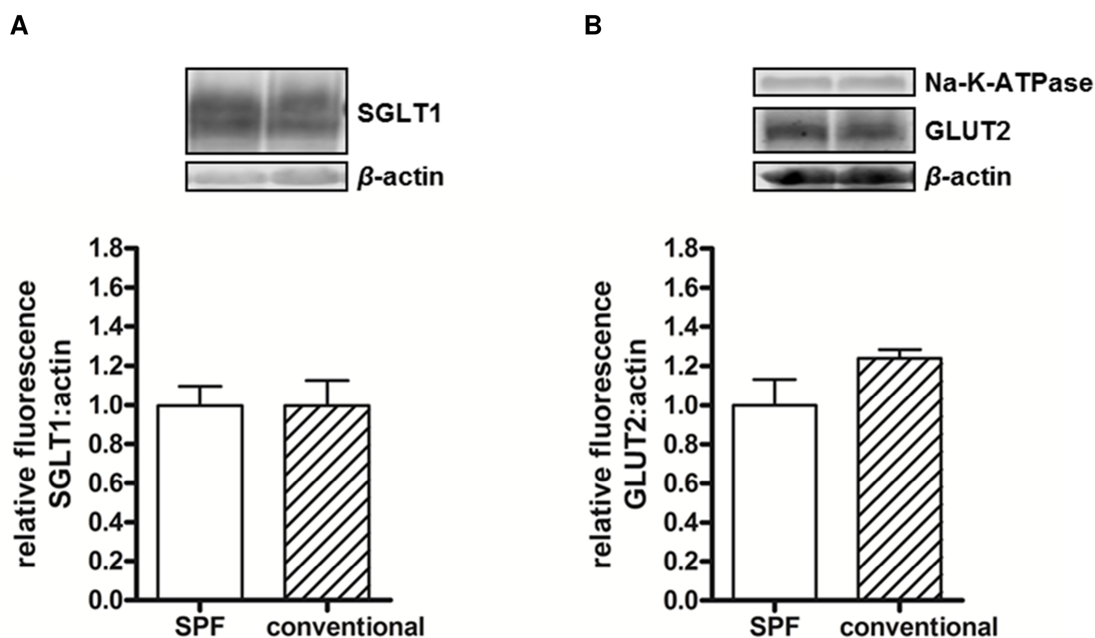


Figure 33: SGLT1 and GLUT2 protein abundance in BBM isolated from *sglt1* wild type mice housed in the SPF (white bars) or in the conventional animal house (checkered bars). (A) SGLT1 expression after the fructose-glucose gavage. (B) GLUT2 protein abundance following the fructose-glucose gavage. Values are given in fold change expressed as mean ± SEM. Statistical analyses were performed using unpaired t-test. N = 3 mice per group.

3.2.3. Intestinal fructose absorption in mice fed the fructose-containing diet

SGLT1, GLUT2 and GLUT5 have been described as to be regulated by quality and quantity of dietary carbohydrates [62]. To test whether such diet-specific effects can be observed and whether those may also explain some of the controversial findings from literature mice were fed a fructose-containing diet and then received the fructose-glucose gavage with radiolabeled fructose to examine the involvement of SGLT1 and GLUT2.

3.2.3.1. Intestinal fructose absorption in *sglt1* wild type and knockout animals on the fructose-containing diet

One week of feeding a fructose-rich diet followed by a fructose-glucose gavage did not alter tracer contents in intestinal tissue of $sglt1^{+/+}$ or $sglt1^{-/-}$ animals when compared to littermates that were fed the sugar-free diet (178.4 ± 12.9 nmol/cm *vs.* 150.1 ± 15.6 nmol/cm and 105.6 ± 16.4 nmol/cm *vs.* 125.6 ± 12.5 nmol/cm, respectively). However, tracer amounts in fructose-fed *sglt1* knockout mice were significantly diminished by over 40 % compared to wild type littermates (178.4 ±

12.9 nmol/cm *vs.* 105.6 ± 16.4 nmol/cm, $p < 0.01$; Figure 34), suggesting diet-specific effects.

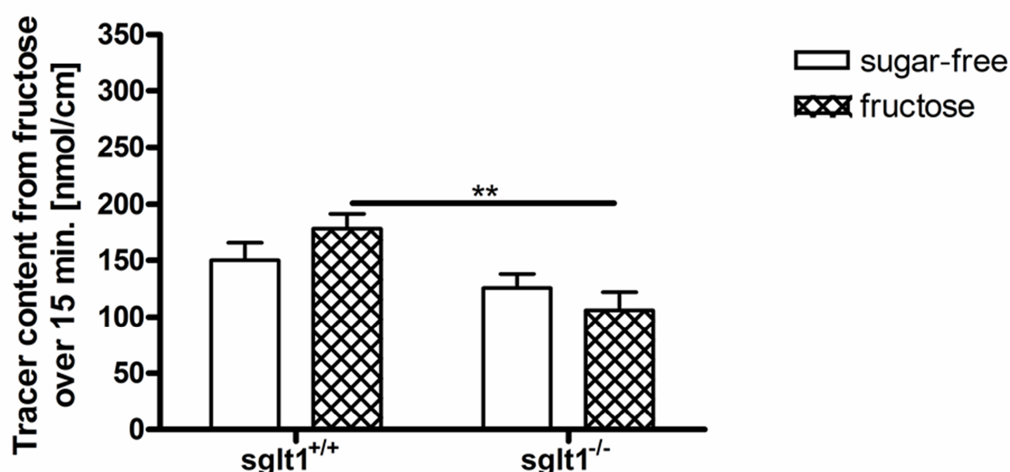


Figure 34: Mean tracer contents from fructose administration in intestinal tissues of *sglt1* wild type and knockout animals fed the sugar-free (white bars) or the fructose-containing diet (diamond-shaped bars). Average amounts of tracer per 1 cm segment after the fructose-glucose gavage. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. ** $p < 0.01$. N = 3-5 mice per group.

Fasting blood glucose concentrations were not altered by feeding a fructose-containing diet when compared to sugar-free feeding in *sglt1*$^{+/+}$ (128 ± 16 mg/dl and 135 ± 14 mg/dl) and *sglt1*$^{-/-}$ mice (112 ± 8 mg/dl and 120 ± 4 mg/dl; Figure 35A). However, increases in blood glucose levels after the fructose-glucose gavage combination were significantly higher in *sglt1*$^{+/+}$ animals fed the fructose-containing diet as compared to the sugar-free diet (141 ± 21 mg/dl *vs.* 52 ± 18 mg/dl, $p < 0.01$) and compared to *sglt1*$^{-/-}$ littermates (141 ± 21 mg/dl *vs.* 61 ± 14 mg/dl, $p < 0.05$) in which the rises were similar for sugar-free and fructose feeding (57 ± 7 mg/dl and 61 ± 14 mg/dl; Figure 35B).

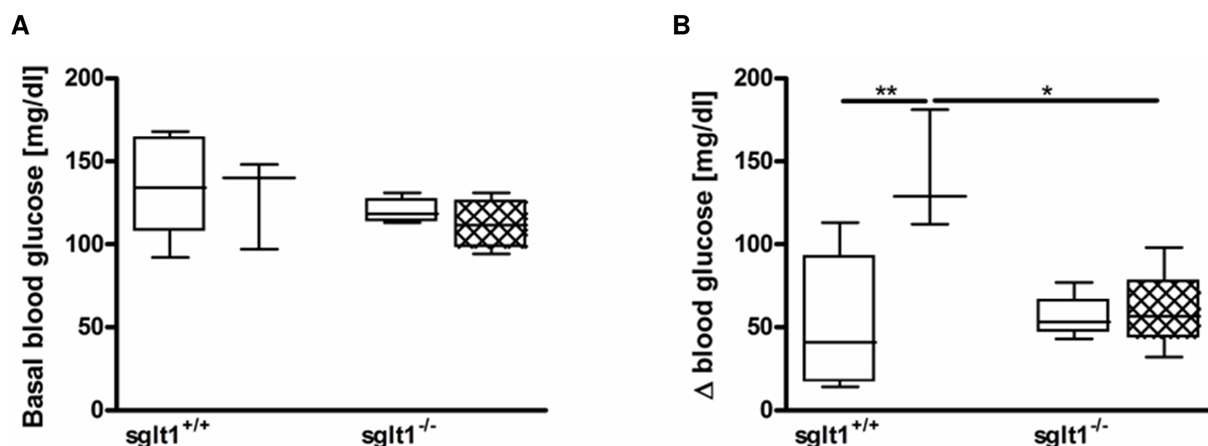


Figure 35: Fasting glucose and changes in blood glucose concentrations in *sglt1* wild type and knockout mice fed the sugar-free (white boxes) or the fructose-containing diet (diamond-shaped boxes). (A) Fasting blood glucose. (B) Increases in blood glucose concentrations after the fructose-glucose gavage. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. * $p < 0.05$, ** $p < 0.01$. N = 3-5 mice per group.

In line with the slightly increased fructose absorption and the significantly higher increase in blood glucose levels following the fructose-glucose gavage combination in *sglt1* wild type mice fed the fructose-containing diet, protein abundance of SGLT1 was increased 1.5-fold compared to littermates fed the sugar-free diet (fold change: 1.5 ± 0.09 *vs.* 1.0 ± 0.09, $p < 0.01$; Figure 36A). GLUT2 expression remained unchanged by diet in $sglt1^{+/+}$ animals (fold change: 0.8 ± 0.16 *vs.* 1.0 ± 0.46) but was significantly lower in fructose-fed $sglt1^{-/-}$ mice compared to fructose-fed $sglt1^{+/+}$ littermates (fold change: 0.3 ± 0.04 *vs.* 0.8 ± 0.16, $p < 0.05$, Figure 36B).

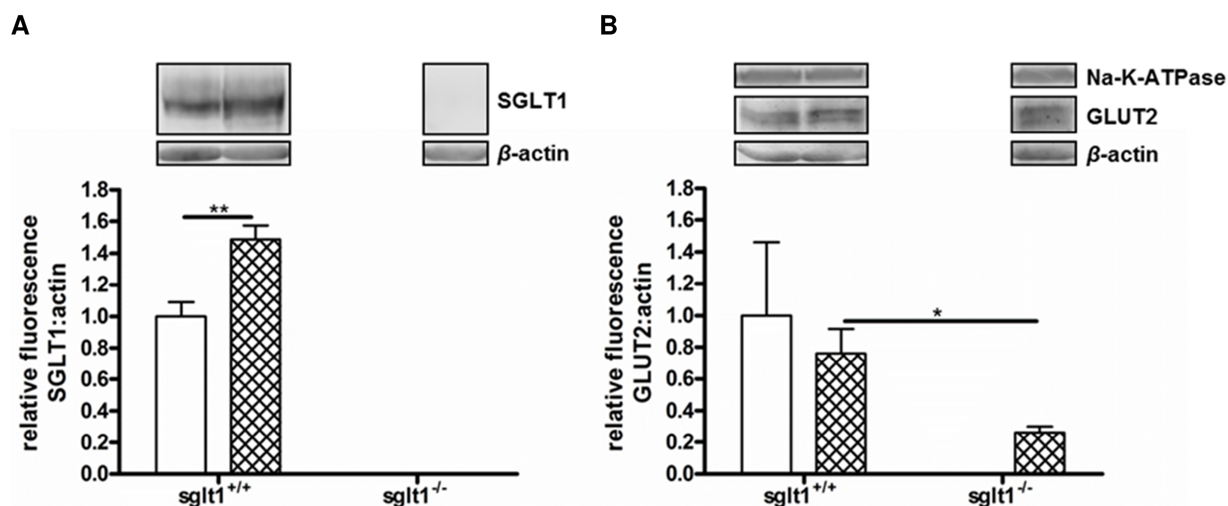


Figure 36: SGLT1 and GLUT2 protein density in BBM isolated from *sglt1* wild type and knockout mice fed the sugar-free (white bars) or the fructose-containing diet (diamond-shaped bars). (A) SGLT1 expression following the fructose-glucose gavage combination. (B) GLUT2 protein levels after the fructose-glucose gavage. Values are given in fold change expressed as mean ± SEM. Statistical analyses were performed using unpaired t-test. * $p < 0.05$, ** $p < 0.01$. N = 3 mice per group.

3.2.3.2. Intestinal fructose absorption in *glut2* wild type and knockout animals on the fructose-containing diet

The amounts of tracer from labeled fructose in intestinal tissues of *glut2*$^{+/+}$ animals were similar when fed the fructose-containing or sugar-free diet (166.7 ± 43.8 nmol/cm and 205.2 ± 29.0 nmol/cm). In *glut2*$^{-/-}$ mice, fructose feeding also did not change tracer contents compared to sugar-free-fed littermates (172.8 ± 17.8 nmol/cm and 245.9 ± 28.7 nmol/cm; Figure 37).

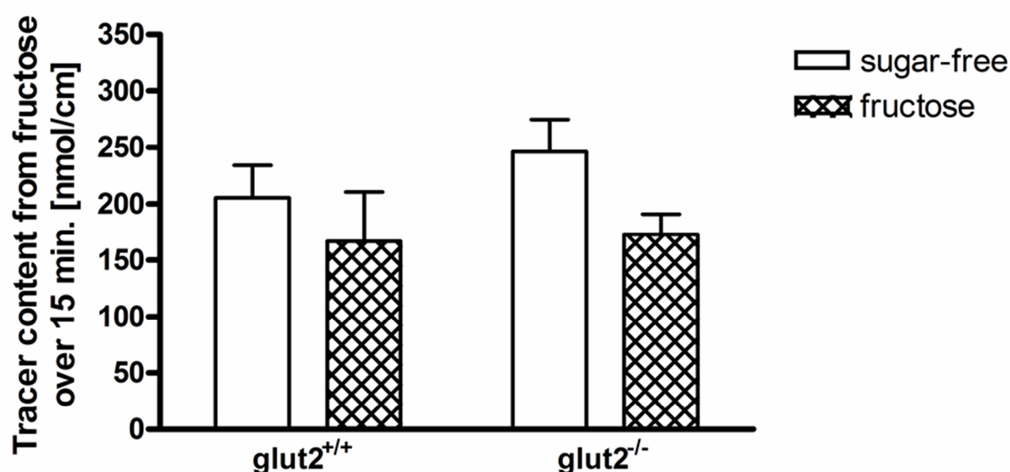


Figure 37: Mean tracer contents from fructose administration in intestinal tissues of *glut2* wild type and knockout animals fed the sugar-free (white bars) or the fructose-containing diet (diamond-shaped bars). Average amounts of radiotracer per 1 cm segment after the fructose-glucose gavage combination Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. N = 5-8 mice per group.

Fructose compared to sugar-free feeding did not alter fasting glucose concentrations in either *glut2*$^{+/+}$ (137 ± 4 mg/dl and 102 ± 12 mg/dl) or *glut2*$^{-/-}$ animals (73 ± 5 mg/dl *vs.* 104 ± 12 mg/dl). However, *glut2* knockout mice fed the fructose-containing diet displayed significantly lower fasting glucose levels compared to wild type animals (73 ± 5 mg/dl *vs.* 137 ± 4 mg/dl, $p < 0.01$; Figure 38A). The changes in blood glucose following the fructose-glucose gavage were similar in fructose-fed compared to sugar-free fed *glut2*$^{+/+}$ (127 ± 11 mg/dl and 97 ± 15 mg/dl) and *glut2*$^{-/-}$ mice (104 ± 22 mg/dl and 74± 6 mg/dl; Figure 38B).

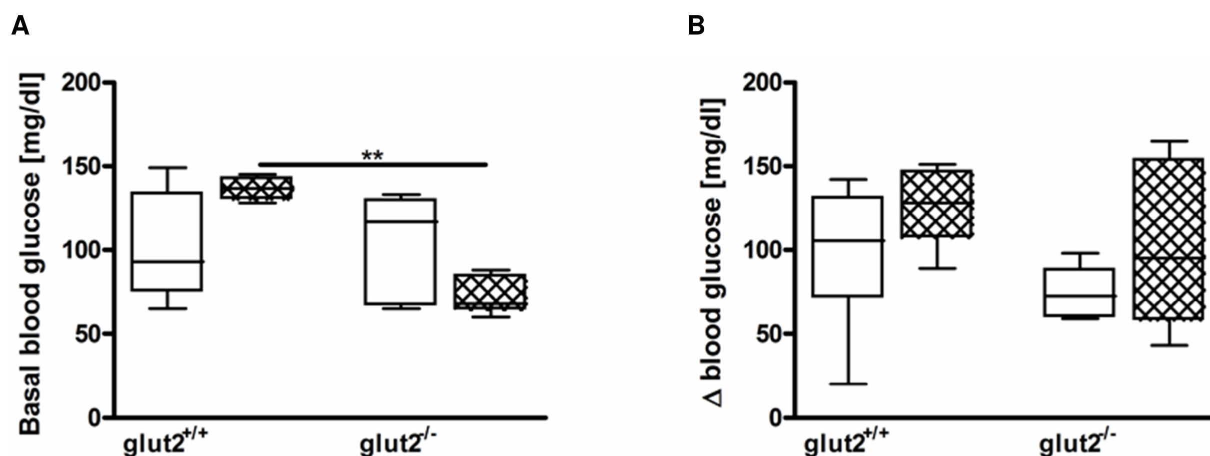


Figure 38: Fasting glucose and changes in blood glucose levels in *glut2* wild type and knockout mice fed the sugar-free (white boxes) or the fructose-containing diet (diamond-shaped boxes). (A) Fasting blood glucose. (B) Changes in blood glucose levels after the fructose-glucose gavage. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. ** $p < 0.01$. N = 5-8 mice per group.

3.2.3.3. Intestinal fructose absorption in *glut2* wild type and *glut5* knockout animals on the fructose-containing diet

After one week on the fructose-containing diet, amounts of tracer from fructose in intestinal tissues of *glut5*$^{-/-}$ mice were not altered compared to littermates kept on the sugar-free diet (12.6 ± 5.2 nmol/cm and 30.3 ± 7.0 nmol/cm). The fact that tracer contents were still significantly lower when compared to *glut2* wild type mice (30.3 ± 7.0 nmol/cm *vs.* 166.7 ± 43.8 nmol/cm, $p < 0.05$; Figure 39) argues against fructose feeding being able to induce GLUT2 translocation into BBM to allow increased fructose absorption [109].

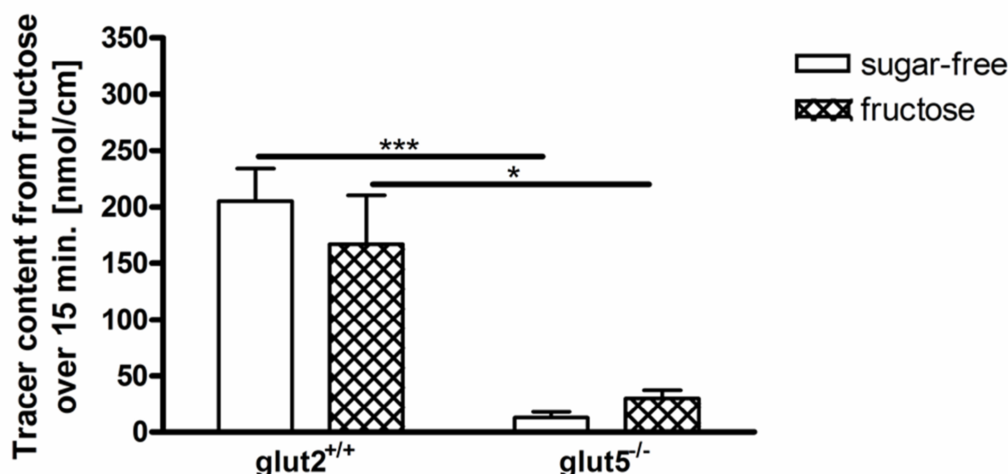


Figure 39: Mean tracer contents from fructose administration in tissues of *glut2* wild type and *glut5* knockout mice fed the sugar-free (white bars) or the fructose-containing diet (diamond-shaped bars). Average amounts of tracer per 1 cm segment after the fructose-glucose gavage combination. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. * $p < 0.05$, *** $p < 0.001$. N = 3-8 mice per group.

Fasting glucose concentrations were significantly higher in *glut5*$^{-/-}$ mice on the fructose-containing diet as compared to the sugar-free diet (106 ± 1 mg/dl *vs.* 80 ± 1 mg/dl, $p < 0.0001$) but lower than in *glut2* wild type animals (106 ± 1 mg/dl *vs.* 137 ± 4 mg/dl, $p < 0.01$; Figure 40A), substantiating again distinct diet-induced effects. Elevations in blood glucose levels were similar in fructose- and sugar-free-fed *glut5* knockout mice (74 ± 8 mg/dl and 67± 16 mg/dl) but lower than in wild type animals on the fructose-containing diet (74 ± 8 mg/dl *vs.* 127 ± 11 mg/dl, $p < 0.01$; Figure 40B).

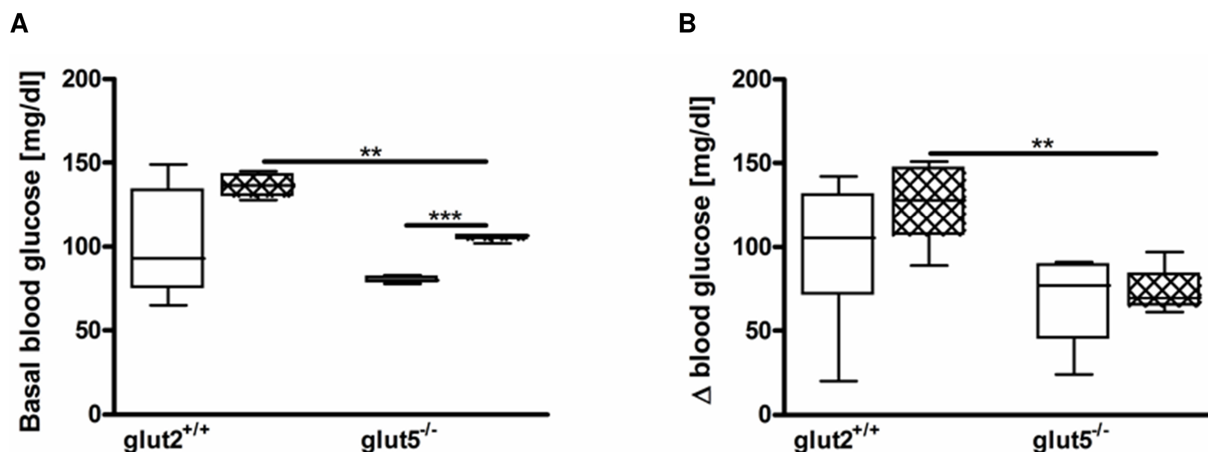


Figure 40: Fasting glucose levels and changes in blood glucose concentrations in *glut2* wild type and *glut5* knockout animals fed the sugar-free (white boxes) or the fructose-containing diet (diamond-shaped boxes). (A) Fasting blood glucose. (B) Increases in blood glucose levels after the fructose-glucose gavage. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. Unpaired t-test was used to compare fasting glucose in sugar-free- and fructose-fed *glut5* knockout animals as well as rises in blood glucose in *glut2* wild type and *glut5* knockout animals on fructose diet. Unpaired t-test with Welch's correction was used for comparison of basal blood glucose levels in fructose-fed *glut2* wild type and *glut5* knockout mice. ** $p < 0.01$, *** $p < 0.0001$. N = 3-8 mice per group.

As previously shown in *sglt1* wild type mice, SGLT1 protein levels in *glut5* knockout animals fed the fructose-containing diet were 1.2-fold higher than in littermates on the sugar-free diet (fold change: 1.2 + 0.09 *vs.* 1.0 ± 0.03, $p < 0.05$; Figure 41A) whereas GLUT2 protein abundance again remained unchanged (fold change: 1.0 ± 0.03 and 0.9 ± 0.10; Figure 41B).

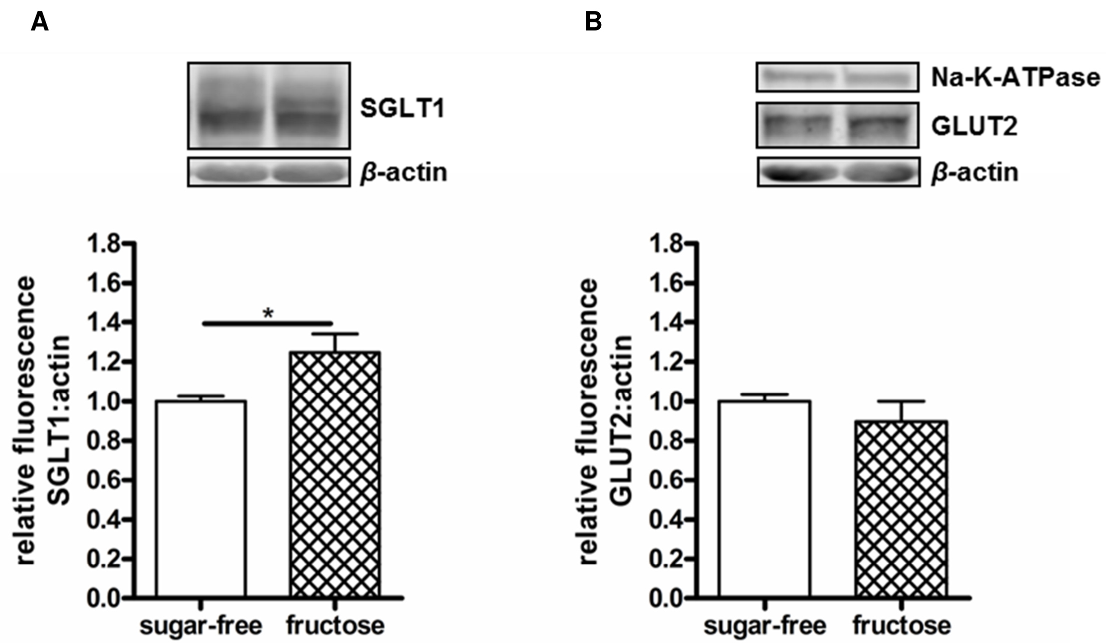


Figure 41: SGLT1 and GLUT2 protein abundance in BBM prepared from *glut5* knockout mice fed the sugar-free (white bars) or the fructose-containing diet (diamond-shaped bars). (A) SGLT1 protein levels following the fructose-glucose gavage. (B) GLUT2 abundance after the fructose-glucose gavage. Values are given in fold change expressed as mean ± SEM. Statistical analysis was performed using unpaired t-test for comparison of SGLT1 expression after fructose-glucose gavage in sugar-free and fructose-fed *glut5* knockout mice. * $p < 0.05$. N = 3 mice per group.

4. Discussion

4.1. Intestinal glucose transport and sensing

Modes and mechanisms by which intestinal sugar absorption occurs have been a subject of controversial scientific discussions for quite a long time. There is even a revival of interest on how and to which extent intestinal carbohydrate absorption is regulated and thereby contributes to the pandemic of obesity and diabetes mellitus type 2 [2]. Conceptually, a two-component system mediating intestinal glucose absorption was established to explain glucose absorption *in vivo* with one component being saturable and a second system with high transport capacity but lacking saturation kinetics – or at least with a very low affinity [42, 119-121, 125]. Whereas the saturable component was identified by expression cloning as SGLT1 by Hediger *et al.* in 1987 [124], the second system is still a mystery. It came back into discussion when Helliwell *et al.* in 2000 proposed that basolateral GLUT2 is recruited from vesicular stores into the apical membrane to mediate bulk absorption of glucose as a uniporter at high luminal glucose concentrations [44]. This translocation of GLUT2 divided and still divides the "scientific world" as shown by contradictory literature and conclusions: Wright *et al.* attribute intestinal glucose absorption solely to SGLT1 [183] and Gorboulev *et al.* conclude from studies in mice that apical GLUT2 plays only a minor role in overall glucose absorption [21] whereas Kellett and co-workers assign the main part of overall glucose absorption to apical GLUT2 [184]. Opposed findings even within one group emphasize the obvious difficulty in proving the presence of apical GLUT2 which also seems to depend on the experimental techniques applied. Thus, GLUT2 in apical membranes was demonstrated by perfusion of the isolated intestine [185] whereas the same authors failed to demonstrate apical GLUT2 in everted gut sleeves of rats [186]. It may well be that environmental factors [137] including diet [109] critically determine a proposed trafficking of GLUT2 to the apical membrane. In order to be able to control the conditions as good as possible we decided to use identical techniques and a highly standardized setting to determine *in vitro* as well as *in vivo* the proteins that control respectively contribute to intestinal glucose and fructose transport with the advantage to have mice available that lack either SGLT1, GLUT2 or GLUT5.

4.1.1. Glucose influx is mediated by SGLT1 whereas GLUT2 provides exit

Intestinal glucose absorption was investigated *in vitro* by using everted gut rings as well as an *in vivo* approach including oral administration of radiolabeled glucose provided by gavage to be able to define the contribution of SGLT1 and GLUT2 at high luminal glucose concentrations in order to prove the hypothesis of a GLUT2 trafficking into the apical membrane as proposed by Kellett *et al.* [44].

Glucose influx into everted gut rings prepared from *sglt1* knockout animals was abolished as was the influx into tissues from wild type mice in the presence of phlorizin. These findings confirm the prime role of SGLT1 in intestinal glucose absorption and are in accordance with the lack of α-MDG uptake into everted gut rings of SGLT1-deficient mice as shown previously by Gorboulev *et al.* [21]. The fact that a glucose gavage – resulting in luminal concentrations exceeding 100 mM – in SGLT1-deficient animals did not reveal any significant glucose uptake into tissues confirms that SGLT1 is the main apical glucose transporter even at high glucose concentrations. However, it was proposed that GLUT2 insertion into the apical membrane is dependent on SGLT1 [187] and hence, in the absence of SGLT1, the incorporation of GLUT2 would not be possible. However, in wild type animals any recruitment of GLUT2 into the apical membrane should have increased glucose uptake and since GLUT2 is in essence non-saturable [44] glucose tracer influx at high luminal glucose levels should have been substantially higher than in the case of mannitol – that was used as an osmotic control – but this was not the case. GLUT2 trafficking was shown to be controlled by PKC βII whose integrity seems to be most crucial [33, 34] for the ability to achieve a GLUT2 recruitment into the apical membrane and which may not be demonstrated *in vitro*. However, Gouyon *et al.* were able to show GLUT2 trafficking using everted intestinal rings and purified brush border membrane vesicles [109] while Scow *et al.* failed to demonstrate this in everted gut sleeves [186].

In contrast to the complete abolition of glucose uptake into everted gut rings there was a remaining 20 % of radiotracer from glucose detectable in intestinal tissues of *sglt1* knockout mice. It is difficult to define the origin of this remaining transport activity. It may well be that it represents adherent radioactivity from the gavage that was not taken up into tissues although this should have been corrected by the use of mannitol tracer but it also could indicate a second system for glucose uptake. By all

means, apical GLUT2 recruitment was shown to depend on SGLT1 [187] and thus it is unlikely that the remaining tracer quantity in our studies is ascribable to GLUT2. Our studies performed *in vitro* using everted gut rings but also *in vivo* applying a glucose gavage containing tracer glucose did not reveal any functional evidence for a role of GLUT2 in apical glucose influx.

Most interestingly, tracer contents in intestinal tissues of animals lacking GLUT2 increased significantly over those obtained in wild type animals. The most likely reason for this is that the lack of GLUT2 markedly reduced the basolateral exit of glucose from the tissue into circulation. This finding, however, contrasts with observations in the same knockout model on the same genetic background from Stümpel *et al.* which in essence failed to demonstrate any difference in portal vein blood glucose in the absence of GLUT2 when 150 mg of glucose were added as a bolus to the perfusate during perfusion of the small intestine *in vivo*. Moreover, by use of S4048, a glucose-6-phosphate translocase inhibitor, the authors concluded that a pathway involving the endoplasmic reticulum and glucose-6-phosphatase rather than GLUT2 critically determines glucose efflux from enterocytes [188]. Our findings in GLUT2-deficient mice with radiotracer glucose provided as an intragastric bolus and analysis of glucose tracer in small intestinal tissue samples 15 minutes thereafter supports the “classical” role of GLUT2 as a basolateral efflux system as indicated in physiology text books.

Significantly diminished quantities of radiotracer in the plasma of *sglt1* and *glut2* knockout mice compared to their respective wild type littermates confirmed at the systemic level the reduced glucose absorption in SGLT1-deficient mice as well as the diminished intestinal efflux of glucose in GLUT2-lacking animals. The rise in blood glucose levels after the glucose gavage was also reduced in these mice. However, both knockout models showed a modest and almost identical increase in blood glucose levels following the gavage. This may be due to glucose coming from the intestine or due to stress-induced glucose release from the liver caused by the gavage per se. Hepatic glucose release has been observed in previous studies in which a water or glucose gavage in *sglt1* knockout animals resulted in comparable blood glucose elevations as to wild type littermates receiving a water gavage (Suppl. Figure 1). Similar blood glucose levels due to hepatic glucose release in SGLT1-

deficient animals were also described by Gorboulev *et al.* [21]. In contrast to our studies, Thorens *et al.* found unaltered blood glucose levels in mice lacking GLUT2 following glucose administration [95]. However, in their study blood glucose was measured after an intraperitoneal glucose injection whereby the intestine is bypassed and a loss of basolateral GLUT2 would be of no consequence.

So far, GLUT2-deficient mice have never been studied to prove putative apical GLUT2 contributing to overall glucose absorption. Only one study assessed the role of apical GLUT2 in fructose absorption and here, fructose uptake was reduced by almost 70 % in brush border membrane vesicles and by more than 60 % in everted gut rings prepared from the small intestines of *glut2* knockout mice. However, this reduction could only be shown when mice were fed a diet rich in fructose prior to the experiments [109]. Using intragastric administration of radiolabeled fructose we found fructose absorption to be comparable in the presence and the absence of GLUT2 suggesting (and as shown later) that GLUT5 is the dominant fructose transporter as described by Barone *et al.* [139].

The majority of studies demonstrating an involvement of GLUT2 in apical glucose uptake has been performed in rats. Due to the lack of proper genetic rat models, inhibitors such as phloretin or cytochalasin B are needed although those are not specific for GLUT2 [48, 110, 189]. Even if the application of phloretin or cytochalasin B resulted in an almost identical inhibition of glucose uptake in some studies [44, 181, 190], any reduction in glucose absorption by these non-specific inhibitors cannot be solely attributed to GLUT2. When we applied phloretin or phlorizin to inhibit glucose influx into everted gut rings of *sglt1* wild type mice we found glucose uptake to be strongly reduced by phloretin and completely abolished by phlorizin. However, this rather reflects the different K_i values of phloretin and phlorizin [48] than inhibition of GLUT2.

Although there are some studies demonstrating GLUT2 in brush border membranes by Western blot analysis [21, 44, 109, 191] only one study used a marker to assess cross-contamination with basolateral membranes [109] and moreover, none of the studies showed any loading control. GLUT2 immunoreactivity in brush border membranes prepared from animals that were not gavaged or after receiving an

intragastric glucose bolus revealed some but almost identical GLUT2 protein levels in apical membrane fractions of *sglt1* wild type and knockout mice but these mainly originated from a cross-contamination with basolateral membranes as demonstrated by considerable amounts of Na-K-ATPase used as a basolateral marker. Although we cannot exclude some GLUT2 protein residing in apical membranes we would have expected to see an increase in the GLUT2 density after the glucose gavage according to the model proposed by Kellett [44, 109] despite the contamination. Furthermore, there also should have been a difference in the GLUT2 density in the brush border membranes isolated from SGLT1-deficient animals [187]. The fact that the density of GLUT2 protein was remained unchanged by the glucose gavage as well as the lack of any difference between GLUT2 protein levels in *sglt1* wild type and knockout mice strongly argues against significant quantities of apical GLUT2 in brush border membranes and its contribution to luminal glucose absorption. It needs to be emphasized that the glucose gavage chosen increased luminal glucose concentrations to approximately 160 mM that should have been more than sufficient to initiate GLUT2 trafficking [44, 109]. Even immunofluorescence staining of tissue sections revealed the prominent basolateral localization of GLUT2 that remained unaffected by the fasting state or glucose-gavage.

Taken together, the studies described here clearly argue against a trafficking of GLUT2 into the apical membrane. Although we confirmed the prime role of SGLT1 in overall glucose absorption, we did not find evidence for increased SGLT1 protein levels after glucose gavage which in turn could enhance glucose absorption as recently reported by Gorboulev *et al.* [21]. This discrepancy might be due to higher amounts of glucose administered and/or the longer duration of the fasting period prior to the intragastric glucose load [21]. Since SGLT1 shows a diurnal rhythmicity [65, 66] it may be critical at what time of day experiments are performed.

4.1.2. Incretin responses are dependent on SGLT1 but not on GLUT2 whereas insulin secretion is impaired in the absence of both transporters.

The incretin hormones GIP and GLP-1 are released from enteroendocrine cells in response to glucose and other nutrients. The incretin effect defines their ability to stimulate glucose-dependent insulin secretion from pancreatic *β*-cells [79-82].

In accordance with recent findings by Gorboulev *et al.* [21] we found that the secretion of GIP and GLP-1 after the glucose gavage was almost completely abolished in SGLT1-deficient mice. Similar findings were reported when phlorizin was used to inhibit SGLT1 by Moriya *et al.* [88]. Here, it was also demonstrated that the mRNA of SGLT1 and GIP is co-localized in the gut whereas this was not the case for the mRNA of preproglucagon, the precursor of GLP-1, which was mainly detected in the distal small intestine. However, studies in human but also porcine and rat intestine revealed a co-localization of enteroendocrine cells secreting both, GIP and GLP-1 [192, 193]. Thus, the intestinal distribution of L-cells in the proximal small intestine likely explains the strongly reduced GLP-1 response when SGLT1 is absent or inhibited. Endocrine cells located in the upper small intestine secreting GLP-1 might also be responsible for the rapid increase in GLP-1 levels following glucose gavage as well as neuroendocrine coupling could stimulate cells in the distal intestine to secrete GLP-1. By administration of *α*-MDG or 3-OMG – which are substrates of SGLT1 that cannot be metabolized – it was demonstrated that the transport per se defined as the co-transport of sodium ions and the substrate activates incretin secretion [88]. These findings clearly confirm that SGLT1 is the prime sensor for a glucose-induced incretin secretion. It leaves the question which role other glucose transporters such as GLUT2 [181] or the sweet taste receptor [194, 195] play in the stimulus-secretion coupling. Sweet receptors were initially proposed to act as sensors in the glucose-dependent incretin release but recent studies in humans consistently showed that this is not the case [88, 196].

In SGLT1-deficient mice insulin secretion in response to the glucose gavage was diminished compared to wild type littermates but this reduction was far less pronounced as in case of the incretin hormones. This was also observed by Gorboulev *et al.* [21] and may be attributed to the lack of incretin secretion following the glucose challenge and the reduced rise in blood glucose levels. Incretin effects are considered to account for ~ 70 % of total insulin secretion [197]. Taken together, we clearly confirmed the role of SGLT1 as a glucose sensor mediating in a function of a “transceptor” all or at least the major effects by which glucose that is ingested or produced during digestion affects GIP- and GLP-1 secretion and in turn insulin release from pancreatic *β*-cells.

In animals lacking GLUT2 GIP levels were slightly reduced whereas GLP-1 levels remained unaffected after the glucose gavage. This contrasts data provided by Cani *et al.* reporting reduced GLP-1 but not GIP levels [198]. This discrepancy may originate from differences in the amount and way of glucose administration as well as the duration of fasting. Furthermore, we quantified active GLP-1 in peripheral plasma whereas Cani *et al.* measured total GLP-1 in hepatoportal vein plasma. Consequently, the GLP-1 levels they quantified were around 10-fold higher than the active GLP-1 levels we measured in systemic blood. Moreover, hepatoportal GLP-1 levels are not necessarily reflected in systemic blood [199]. The finding of either decreased GIP secretion in the present study or diminished GLP-1 response as shown by Cani *et al.* [198] is in contrast to a study in rats by Mace *et al.* that showed both, GIP and GLP-1 to be decreased in the presence of phlorizin and phloretin or cytochalasin B during perfusion of intestinal isolated loops. Using phlorizin to block SGLT1, there was only a partial inhibition of GIP and GLP-1 secretion. However, the authors did not show whether glucose uptake can also be inhibited by phloretin or cytochalasin B alone but only in combination with phlorizin. The subsequent addition of phloretin or cytochalasin B then lowered secretion to basal concentrations. Only by this indirect evidence GLUT2 was ascribed to play a crucial role in the glucose-induced incretin release [181]. However, as mentioned before, phloretin and cytochalasin B inhibit several members of the GLUT family as well as other transporters and for that reason the effects are not GLUT2-specicifc. Moreover, the only partial inhibition of incretin secretion by phlorizin as described by Mace *et al.* [181] contradicts with our findings and those by Gorboulev *et al.* [21] using *sglt1* knockout mice but also with the work in mice applying phlorizin as reported by Moriya *et al.* [88]. One reason for the discrepancies between the studies in mice and rats might be the ratio of glucose to phlorizin which was 5.5:1 [88] and 200:1 [181]. The fact that the ability of phlorizin, which is a competitive inhibitor of SGLT1 [47], to reduce transport decreases with decreasing concentrations respectively increasing glucose concentrations may explain the data obtained in the studies by Mace *et al.* [181] as we also found less inhibition of glucose uptake by phlorizin with a decreasing glucose-to-phlorizin-ratio (Suppl. Figure 2). Other relevant differences relate to the quantification of the incretins but Mace *et al.* did not specify whether active or total GIP and GLP-1 levels were measured. This is of great importance as concentrations strongly vary due to the short half-life of both incretins [200].

Furthermore, Mace *et al.* used a perfusion of isolated intestinal loops during which GIP and GLP-1 secretion was assessed over a prolonged period and was than expressed as AUC [181] which complicates the comparison with other data as does the “serosal secretion samples” instead of blood used in the work by Mace *et al.* [181]. In this respect, all evidence from literature that GLUT2 may act as a sensor for glucose and thereby be involved in incretin secretion is weak and mainly relies on work from one group. In contrast to SGLT1 that was shown to be co-expressed with K- and L-cells [21, 72, 73] there is also no evidence as yet that GLUT2 is expressed in enteroendocrine cells as a prerequisite for a sensor function

Although GLUT2 seems to be of minor – if at all – importance for incretin secretion, its deficiency in mice markedly altered basal insulin levels as well as glucose-induced insulin secretion. A diminished basal insulin level was previously explained by Thorens *et al.* as a consequence of an impaired glucose re-absorption in the kidneys in *glut2* knockout mice whereas in the same paper a normal insulin secretory response in the absence of GLUT2 during hyperglycemic clamps was reported [95]. We found only a tendency towards a lower glucose-induced insulin release compared to *glut2* wild type littermates. The difference in basal insulin concentrations between *glut2* knockout and wild type mice, however, became even more pronounced after the glucose gavage which is in line with findings by Cani *et al.* showing markedly diminished plasma insulin levels in GLUT2-deficient mice compared to wild type animals [198]. The reduced efflux of glucose from the intestinal tissue into blood which we observed after the glucose gavage could well be causative for the decreased insulin response although this was more pronounced as in SGLT1-deficient mice that also showed a reduced blood glucose response. Thus, the lack of GLUT2 may have other and/or further consequences that translate into changes in insulin secretion and insulin levels. Although it was shown that GLUT1-overexpression in *β*-cells in GLUT2-deficient animals restores the first-phase of glucose-stimulated insulin secretion (GSIS) *in vitro*, normalization of insulin secretion *in vivo* was only demonstrated during hyperglycemic clamps [95] whereas in the present study and the work by Cani *et al.* as the only sources insulin responses were assessed after oral glucose administration.

Taken together, the present studies using oral administration of radiolabeled glucose in mice confirm that SGLT1 is the key transporter in the intestine that mediates glucose absorption – even when high glucose concentrations are present in the lumen. Furthermore, the new role of SGLT1 as a “transceptor” was confirmed and extended towards its key role in glucose-induced secretion of the incretin hormones as well as in parts of the insulin secretory response system. Except for its role as a basolateral efflux transporter for hexoses, GLUT2 in our hands does neither play a role in apical glucose uptake nor in incretin secretion.

4.2. Fructose absorption and the role of SGLT1, GLUT2 and GLUT5

In humans, fructose absorption capacity is limited not only in patients suffering from intestinal fructose malabsorption but even in healthy volunteers [159-164] with absorption capacities ranging from less than 5 g to more than 50 g fructose [161]. In most cases of malabsorption, symptoms include abdominal cramps, flatulence and diarrhea [159-164]. Fructose malabsorption is routinely determined based on increased breath hydrogen levels following fructose ingestion [159, 161, 163, 164] with a reduction in hydrogen production and improved symptoms when fructose is ingested together with glucose [159-162]. This improvement of fructose absorption was also demonstrated in perfused, isolated loops of rat intestine but could not be demonstrated in everted gut sleeves or in brush border membrane vesicles [166]. Although the mechanism underlying the enhanced fructose absorption in the presence of glucose has not been defined yet, several hypotheses such as a stimulation of GLUT5 activity via SGLT1 [160], a glucose-dependent fructose co-transport [161], a disaccharidase-related system that jointly transports both, glucose and fructose [166], solvent drag [167, 168] or the GLUT2 recruitment into the apical membrane following high glucose or fructose intake have been proposed [33, 34, 109].

4.2.1. Fructose absorption in the presence of glucose is only enhanced in wild type mice kept in the SPF facility.

In analogy to findings in humans with fructose malabsorption [159-162] we found increased tracer fructose uptake in *sglt1* wild type mice kept in the SPF animal facility when glucose but not when mannitol or *α*-MDG was added to the fructose gavage solution in a 1:1 ratio. Since this phenomenon could not be observed when SGLT1 was absent it seems likely that SGLT1 is involved in this glucose-induced enhancement of fructose transport. Since apical SGLT1 protein levels also increased after the fructose-glucose gavage one might speculate that the increase in SGLT1 protein density leads to an enhanced glucose influx which in turn affects GLUT5 transport activity resulting in an elevation of fructose uptake. Due to lack of a reliable GLUT5 antibody we could not determine apical GLUT5 protein levels so we can only speculate that apical GLUT5 density could also be increased in the presence of fructose and glucose.

Since the improving effect could only be observed with glucose co-administered but not with the non-metabolizable glucose analogue *α*-MDG, the enhancement of fructose absorption obviously involves glucose metabolism and not only SGLT1-mediated transport (Figure 42).

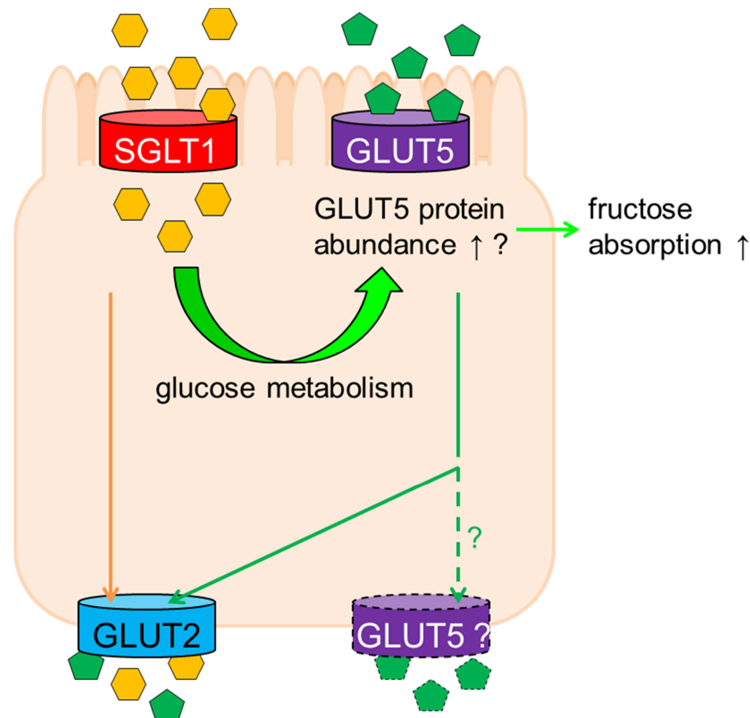


Figure 42: Working model of improved intestinal fructose absorption in the presence of glucose. Glucose (⬡) is transported by SGLT1, fructose (⬠) is taken up by GLUT5. Glucose metabolism enhances GLUT5 protein abundance in the apical membrane which in turn increases fructose absorption. GLUT2 mediates the release of glucose and fructose into the blood stream. Fructose could also exit by another basolateral transporter or efflux system.

GLUT2 protein abundance in brush border membranes was not altered by the addition of glucose to the fructose solution as compared to mannitol in either *sglt1* or *glut2* wild type mice. This is another line of evidence that there is no apical GLUT2 involved in either glucose or fructose absorption as proposed previously [33, 34, 109]. This was supported by similar fructose tracer contents in intestinal tissues of *glut2* wild type mice in the presence of mannitol, *α*-MDG or glucose in the fructose gavage solution and by unchanged fructose absorption rates in the absence of GLUT2. However, a diminished fructose uptake in *glut2* knockout mice was shown by Gouyon *et al.* [109] but it needs to be stressed that the authors observed an impaired fructose absorption in the absence of GLUT2 only when mice were fed a high-

glucose or high-fructose diet whereas feeding a low-carbohydrate diet comparable to the sugar-free diet we used in our study did not lead to reduced fructose uptake in GLUT2-deficient animals [109].

The efflux of fructose across the basolateral membrane is not finally resolved yet. Textbooks refer to GLUT2 as the system that mediates glucose as well as fructose efflux from enterocytes. In humans, evidence for basolateral localization of GLUT5 in the intestine was demonstrated some years ago [146] as were two distinct pathways for the efflux of glucose and fructose in the same *glut2* knockout model as used here in the work by Stümpel *et al.* in which the authors also suggested GLUT5 to be located in the basolateral membrane [188]. Although we obtained various commercial antibodies we were unable to get proper Western blot or immunofluorescence staining with any of the antibodies for probing GLUT5 presence in basolateral membranes.

The finding that GLUT2-deficient mice displayed unaltered fructose-derived tracer levels in intestinal tissues suggests that neither an impaired fructose influx from the lumen nor an altered efflux resulted from the absence of GLUT2. This is essentially in line with the fact that patients suffering from the Fanconi-Bickel-syndrome do not exhibit impaired fructose metabolism [201], indicating that other transporters than GLUT2 may be involved in fructose transport. However, in the present studies lack of changes in fructose tracer levels in intestinal tissues of *glut2* knockout mice also might be due to GLUT5 transporting fructose in a passive manner and thus, uptake was disrupted as soon as the concentration equilibrium was achieved. Furthermore, the fructose amount administered by gavage was less compared to the glucose gavage that previously resulted in glucose accumulation in the absence of GLUT2.

The presence of glucose – when co-administered with fructose in the gavage – failed to increase fructose-derived tracer levels in tissues samples of *glut2* wild type mice in contrast to the finding in *sglt1* wild type animals. This is likely an effect of different housing conditions, i.e. *sglt1* mice are kept in SPF whereas *glut2* animals are housed in a conventional facility. Since both mouse models have the same genetic background we can exclude any genetic difference. When moving *sglt1* wild type and knockout animals from the SPF to the conventional animal facility and repeating the experiment we failed to produce the improving effect. The improving effect of glucose

on fructose absorption therefore is only observed in the SPF but not in the conventional animal facility. Biological and biochemical effects including protein expression and regulatory processes are reported to depend on the hygienic status of the animal facility [202-204] and our studies add another interesting finding to those already described. It is tempting to speculate that differences in the composition of the microbiota may contribute to these controversial findings since it is known that the inflammatory state [205], energy metabolism [206] and sugar transport in particular [207] are affected by the microbiota. The differences found between animal facilities with respect to glucose effects on fructose absorption in *sglt1* wild type mice did not result from altered SGLT1 or GLUT2 protein expression levels but seems likely that the abundance of GLUT5 is affected. Although Swartz *et al.* demonstrated SGLT1 protein levels to be different depending on microbiota composition [207] SGLT1 expression levels in our mice were similar in both animal facilities.

GLUT5 is the main intestinal fructose transporter in the apical membrane [139, 140] and thus, it was not surprising that fructose tracer contents in intestinal tissues of GLUT5-deficient mice were drastically reduced compared to wild type animals. This finding is in line with observations by Barone *et al.* showing significantly diminished fructose uptake into membrane vesicles prepared from *glut5* knockout mice [139]. This drastic reduction of fructose uptake in the absence of GLUT5 was obviously not compensated by apical GLUT2 proposed to mediate fructose uptake [33, 34] or another uniporter that possibly could transport fructose.
A rather surprising finding is that the presence of glucose in the fructose gavage solution did not cause an increase in blood glucose concentrations in GLUT5-deficient mice. This was not due to a reduced SGLT1 protein level (which remained unchanged in the absence of GLUT5) but may originate from a diminished GLUT2 protein abundance which could cause a reduced glucose efflux into circulation similar to the animals that lack GLUT2 (Figure 43). Although GLUT2 mRNA expression in *glut5* wild type and knockout animals was measured in the study by Barone *et al.*, the authors focused on differences caused by diet but not by genotype. In this respect, we need to confirm suggested changes in GLUT2 expression on mRNA level in animals lacking GLUT5 which was not possible due to lack of sufficient tissue samples from *glut5* knockout animals.

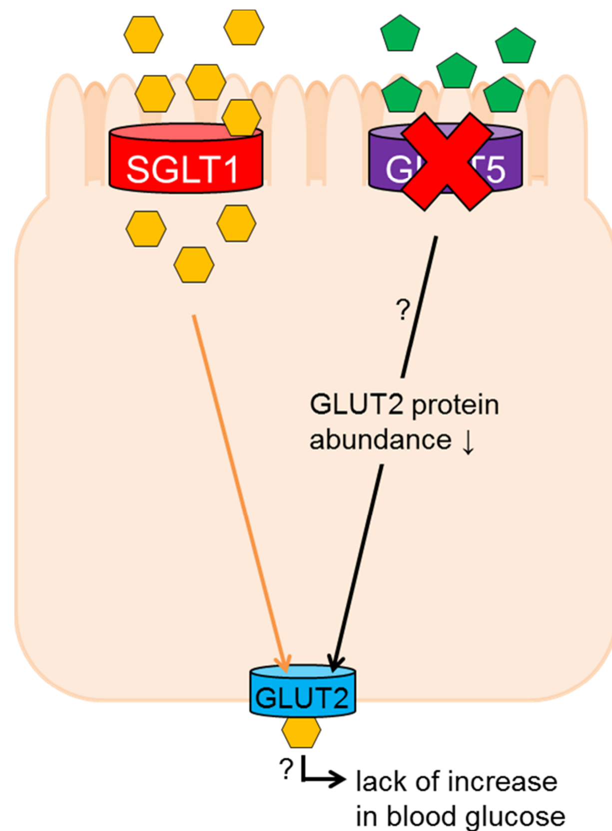


Figure 43: Working model on the effects of GLUT5-deficiency on fructose absorption in the presence of glucose. GLUT2 protein abundance is reduced in the absence of GLUT5 which might result in the lack of increase in blood glucose levels.

4.2.2. Feeding the fructose-containing diet also suggests SGLT1 to be involved in glucose-induced enhancement of fructose absorption.

GLUT5 is known to be regulated by dietary fructose during short-term and long-term exposition [149, 153-156]. Although fructose is not a substrate for SGLT1, diets rich in this monosaccharide were shown to increase the mRNA expression of SGLT1 in *glut5* wild type but not knockout mice [139]. This finding suggests an interaction between SGLT1 and GLUT5 since the absence of GLUT5 seems to affect SGLT1 and *vice versa* as demonstrated here, too, by SGLT1 affecting glucose-induced enhancement of fructose uptake.

In line with the described increased SGLT1 mRNA expression in wild type mice [139], we found one week of feeding a fructose-containing diet to increase SGLT1 protein abundance in wild type mice compared to sugar-free-fed littermates when housed in the conventional animal facility. This enhancement resulted in a significantly stronger rise in blood glucose following the combined fructose-glucose gavage. Furthermore,

feeding the fructose-containing diet led to a significantly diminished fructose uptake in *sglt1* knockout animals compared to wild type littermates but this was not observed when mice were kept in the conventional facility and fed the sugar-free diet. Thus, fructose feeding evoked similar effects – that is reduced tracer contents from fructose in intestinal tissues after the gavage and a lack of rise in blood glucose levels after the combined fructose-glucose gavage in the absence of SGLT – as previously found in *sglt1* wild type and knockout animals kept in the SPF facility and fed the sugar-free diet. SGLT1 thus seems to contribute to a regulation of GLUT5 – either directly or indirectly – that leads to increased intestinal fructose absorption when glucose is administered together with fructose. Housing conditions (or the microbiota) as well as the fructose content of the diet obviously contribute to the regulation of SGLT1 protein expression which in turn affects fructose absorption in the presence of glucose. However, it seems as if not the SGLT1 protein itself but rather its function to transport metabolizable sugars regulates GLUT5 since the increase in fructose absorption was only observed when glucose – which is metabolized – but not when *α*-MDG was co-administered which in turn then could affect GLUT5 function. These findings require more studies on GLUT5 protein expression and its function as well as additional studies using fructose-containing diets in the SPF facility that might reveal more pronounced effects.

Gouyon *et al.* demonstrated that fructose uptake into everted gut rings was greatly increased in *glut2* wild type mice but only moderately enhanced in knockout littermates when animals were fed a fructose-rich diet. Based on the finding of a reduced fructose uptake in the absence of GLUT2 the authors concluded that GLUT2 is involved in fructose absorption [109]. In the present study we found similar fructose tracer contents in intestinal tissues of *glut2* wild type and knockout animals when animals were fed the fructose-containing diet and then tested *in vivo* by gavage. In this respect, our study also did not provide any evidence that GLUT2 could be involved in luminal fructose uptake – even not when a high fructose diet is provided similar to the conclusions drawn by Barone *et al.* assigning GLUT2 only a minor – if any – role in intestinal fructose absorption [139].

Although feeding the fructose-containing diet did not alter tracer levels derived from radiolabeled fructose in intestinal tissues of either *glut2* wild type or knockout animals we found lower fasting blood glucose concentrations in fructose-fed *glut2* knockout

mice compared to wild type littermates as also reported by Gouyon *et al.* [109]. Thorens *et al.* speculated that the loss of GLUT2 in the proximal tubule of the kidney impaired renal glucose re-absorption of glucose resulting in fasting hypoglycemia [95].
Feeding a fructose-rich diet was shown to increase fructose absorption – as demonstrated by higher fructose concentrations in the blood – in the presence but not absence of GLUT5 [139]. In the present study, fructose transport assessed in intestinal tissues of *glut5* knockout mice was also not increased by feeding a fructose-containing diet indicating as well that loss of GLUT5 was not compensated by any other transporter and hence confirming GLUT5 to be the main fructose transporter. In the work by Barone *et al.*, in which feeding a fructose-rich diet to GLUT5-deficient mice did not increase fructose concentrations in the blood, the diet caused a severe malabsorption that was not compensated [139]. GLUT5-deficient mice fed the fructose compared to the sugar-free diet exhibited elevated SGLT1 protein levels as well as an increase in fasting blood concentrations. Despite this, fasting blood glucose was still significantly lower than in wild type animals fed the fructose-containing diet as was the rise in blood glucose levels after the combined fructose-glucose gavage. This could result from reduced GLUT2 protein levels as previously shown in *glut5* knockout animals on the sugar-free diet.

The phenomenon of improved intestinal fructose absorption in the presence of glucose as demonstrated in humans [159-162] could only partly be obtained in the present mouse studies when administering a gavage containing radiolabeled fructose. However, our studies suggest that GLUT5 and SGLT1 (via glucose) are likely to interact to mediate the effect of glucose on enhancing fructose absorption. It should be mentioned here that reduced fructose absorption or malabsorption in humans does not to result from mutations in the *GLUT5* gene including dysfunction or even loss of the protein [138] as it is the case in the GLUT5-deficient mice studied here. Despite various reports suggest that a fructose-rich diet could induce a GLUT2 trafficking to the apical membrane allowing in turn increased luminal fructose uptake [33, 34, 109] we did not find any evidence for a prominent role of GLUT2 in either influx or basolateral efflux of fructose, neither by using the oral fructose administration by gavage nor after feeding animals a fructose-containing diet.

5. Conclusions

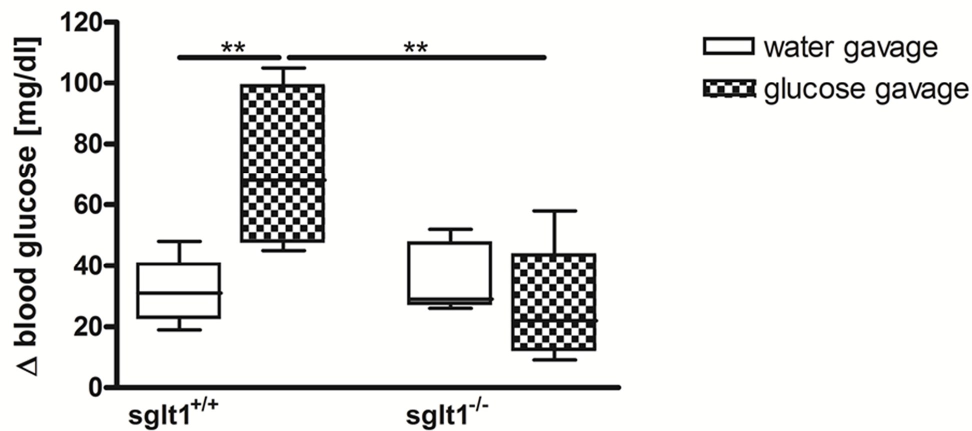
The studies presented here are the first to use intragastric administration of radiolabeled monosaccharides to quantify intestinal sugar absorption in mice that lack the sugar transporters SGLT1, GLUT2 or GLUT5 and thereby define the role of these transporters in the uptake of glucose and fructose.

SGLT1 was confirmed to be the main apical glucose transporter that mediates most – if not all – glucose uptake even at high intraluminal glucose concentrations. A new role of SGLT1 as a “transceptor” was demonstrated and confirmed by the abolition of glucose-induced secretion of GIP and GLP-1 in the absence of SGLT1. In addition, evidence was found for a role of SGLT1 in the increase in fructose absorption when glucose is provided simultaneously to the intestine.

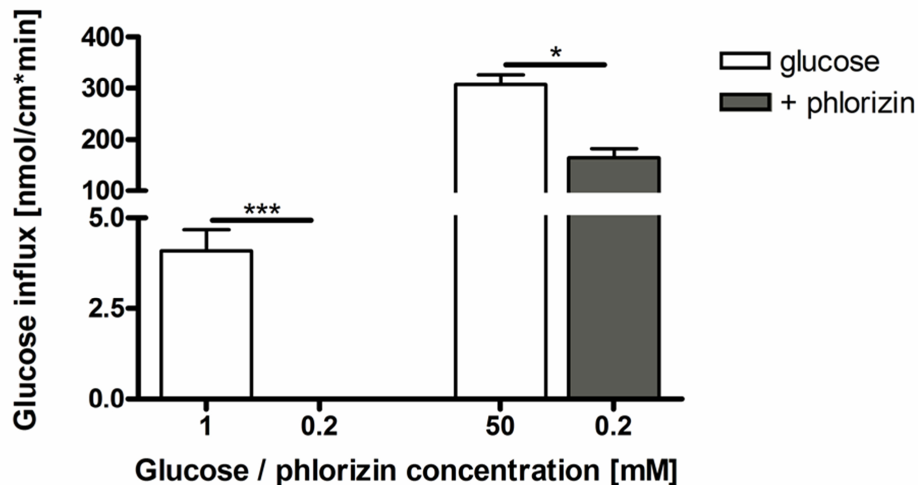
Despite various attempts we were not able to find any evidence that either high luminal glucose concentrations or feeding a fructose-containing diet resulted in a GLUT2 insertion into the apical membrane of enterocytes which in addition to SGLT1 could mediate a significant glucose influx. What we could demonstrate is that GLUT2 mediates most of the glucose efflux from the epithelial cell into circulation. In view of a proposed role of GLUT2 in luminal sensing of glucose we also were not able to confirm this as we found only a tiny change in glucose-induced incretin secretion in animals lacking GLUT2.

GLUT5 was affirmed to be responsible for fructose absorption. When animals were fed a fructose-containing diet as compared to a sugar-free diet no major differences in fructose absorption were found. A significant improvement of fructose uptake in the presence of glucose was only observed in the SPF but not in the conventional animal facility. Moreover, our studies suggest SGLT1 to play a crucial role in this improving effect which requires further studies to assess whether elevated fructose absorption can be attributed to increased levels of GLUT5 and/or its functions as well as on whether GLUT2 shows any impairments when GLUT5 is missing as indicated by lack of blood glucose increase after the combined glucose-fructose gavage.

Supplemental figures



Suppl. Figure 1: Changes in blood glucose levels after water or glucose gavage in *sglt1* wild type and knockout mice. Values are expressed as mean ± SEM. Statistical analyses were performed using 2-way ANOVA with Bonferroni post-tests. ** $p < 0.01$. N = 4-5 mice per group.



Suppl. Figure 2: Inhibition of glucose influx into everted gut rings of wild type mice at different glucose concentrations. Values are expressed as mean ± SEM. Statistical analysis was performed using unpaired t-test with Welch's correction for comparison glucose influx at 1 mM glucose. *** $p < 0.0001$. Unpaired t-test was used to compare glucose influx at 50 mM glucose. * $p < 0.05$. N = 2-4 mice per group.

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List of publications

Peer reviewed pulications

2014 Pia V. Röder, Kerstin E. Geillinger, Tamara S. Zietek, Bernard Thorens, Hermann Koepsell and Hannelore Daniel (2014). **The role of SGLT1 and GLUT2 in intestinal glucose transport and sensing.** PloS One

2013 Kerstin E. Geillinger, Anna P. Kipp, Kristin Schink, Pia V. Röder, Britta Spanier, Hannelore Daniel (2013). **Nrf2 regulates the expression of the peptide transporter PEPT1 in the human carcinoma cell line Caco-2.** Biochim Biophys Acta

Poster presentations

2013 25th European Intestinal Transporter Group (EITG) meeting, Bad Herrenalb, Germany (3rd poster prize)

Pia V. Röder Kerstin E. Geillinger, Tamara S. Zietek and Hannlore Daniel **Is there a GLUT2 trafficking to the apical membrane in mice?**

2012 49. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung e.V. (DGE), Freising, Germany

Nicole Lill, Tilo Wünsch, Alexander Heiseke, Pia Röder, Christine Schulze, Hannelore Daniel **Colonic GLP-1 secretion in response to luminal glucose requires SGLT1.**

Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die bei der promotionsführenden Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der TUM zur Promotionsprüfung vorgelegten Arbeit mit dem Titel

Re-assessing intestinal sugar transport by use of mice lacking
the sodium-dependent glucose transporter SGLT1,
the glucose transporter GLUT2 or the fructose transporter GLUT5

am Lehrstuhl für Ernährungsphysiologie unter der Anleitung und Betreuung durch Frau Prof. Dr. Hannelore Daniel ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 6 und 7 Satz 2 angegebenen Hilfsmittel benutzt habe.

Ich habe keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigungen von Dissertationen sucht oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.

Ich habe die Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt.

Ich habe den angestrebten Doktorgrad **noch nicht** erworben und bin **nicht** in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.

Die öffentlich zugängliche Promotionsordnung der TUM ist mir bekannt, insbesondere habe ich die Bedeutung von § 28 (Nichtigkeit der Promotion) und § 29 (Entzug des Doktorgrades) zur Kenntnis genommen. Ich bin mir der Konsequenzen einer falschen Eidesstattlichen Erklärung bewusst.

Freising, den

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Ausbildung

03/2010 – 11/2013	**Promotionsstudium** Technische Universität München, Freising, Deutschland Wissenschaftszentrum Weihenstephan Lehrstuhl für Ernährungsphysiologie *Re-assessing intestinal sugar transport by use of mice lacking the sodium-dependent glucose transporter SGLT1, the glucose transporter GLUT2 or the fructose transporter GLUT5*
09/2008 – 07/2009	**Masterstudium** Maastricht University, Maastricht, Niederlande Faculty of Health, Medicine and Life Sciences *Predicting activity-induced energy expenditure of five common fitness activities with a tri-axial accelerometer*
10/2005 – 08/2008	**Bachelorstudium** Technische Universität München, Freising, Deutschland Wissenschaftszentrum Weihenstephan Lehrstuhl für Molekulare Ernährungsmedizin *Influence of the fto gene on metabolic parameters*