NUTRIENT SENSING IN THE ALIMENTARY TRACT

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NUTRIENT SENSING IN THE ALIMENTARY TRACT

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ABSTRACT

Background Metabolic syndrome is a complex disorder that increases the risk of cardiovascular diseases. Metabolic syndrome has an unknown aetiology, but shifting dietary patterns such as increased consumption of industry-processed refined sugars have been implicated in the pathogenesis of the syndrome. Distributed along the length of the alimentary tract are macronutrient sensing mechanisms that play a key role in regulating feeding behaviour and energy balance and control intestinal nutrient-transport capacity and the release of peptide hormones. Recent studies have suggested that the sweet-taste receptor, T1R2/3, senses small-intestinal glucose levels and co-ordinates transport capacity and GLP1 release; however, the precise role of T1R2/3 in intestinal sugar sensing remains controversial. Studies have also suggested that macronutrient sensing is attenuated in diet-related metabolic disease; however, the mechanisms by which this occurs are not known.

Aims We hypothesised that T1R2/3 expressed in enterocytes regulates intestinal sugar-transport capacity. We also hypothesised that macronutrient sensors demonstrate a diurnal rhythm that is controlled by clock genes and that in obesity/diabetes the gene-expression levels of such sensors are diminished.

Methods To test our hypotheses we studied the expression levels of intestinal sugar sensors in nocturnal feeding rodents, rodents chronically fed a high-sucrose diet and Caco-2 cells — an enterocyte model.

Results T1R2/3 gene expression was highest in the tongue, absent from the stomach and detected at low levels in the small intestine. SGLT3, a novel sugar sensor, was not

expressed in the tongue but was expressed in the stomach and small intestine. A clear diurnal rhythm of clock genes CRY2 and BMAL1 was found in the tongue, stomach and small intestine. A diurnal rhythm for T1R2, T1R3 and the novel sugar sensor SGLT3 was detected in the stomach and small intestine, but not the tongue. SGLT3 expression, but not T1R2/3, was significantly decreased in mice fed a high-sucrose diet. SGLT3 and T1R3, but not T1R2, were detected in Caco-2 cells; however, we found no evidence of a functional sweet-taste receptor.

Conclusion Taken together, these data suggest a novel interaction between intestinal clock genes and sugar-sensor mechanisms. Disturbances in clock gene/nutrient sensing interactions may be important in the development of diet-related diseases. T1R2/3 regulation of sugar transport most likely occurs via an enteroendocrine cell/enterocyte interaction.

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Patrick O'Brien

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
ANOVA	Analysis of variance
Arntl	Aryl-hydrocarbon receptor nuclear translocator-like protein
bHLH	Basic helix-loop-helix
Bmal	Brain and muscle ARNT-Like
BSA	Bovine serum albumin
CB	Cytochalasin B
CCG	Clock-controlled gene
CCK	Cholecystokinin
CD	Cluster of differentiation
cDNA	Complementary DNA
CO	Corn oil
Cq	Cycle of quantification
Cry	Cryptochrome
Cyp	Cytochrome
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DPPIV	Dipeptyl-peptidase IV
DTT	Dithiothreitol
Duo	Duodenum
EEC	Enteroendocrine cell
ENaC	Epithelial sodium channel
ErbB	Epidermal growth factor receptor
FBS	Fetal bovine serum
FEO	Food-entrainable oscillator
FFAR	Free fatty-acid receptor
Fig.	Figure
FRX	Farnesoid X receptor
GIP	Gastric inhibitory peptide
GLP	Glucagon-like peptide
GLUT	Glucose transporter
GO	Gene onthology
Glu	Glucose
GPCR	G-protein coupled receptor
HFCS	High fructose corn syrup
HSD	High-sucrose diet
HT	Hydroxytryptamine
Ile	Ileum
iNOS	Inducible nitric oxide synthase
Jej	Jejunum

LCM	Light condensed milk
LEO	Light-entrainable oscillator
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
ND	not detected
NEFA	Non-esterified fatty acids
NHANES	National Health and Nutrition Examination Survey
NO	Nitric oxide
ns	not significant
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pen	Penicillin
Per	Period circadian protein homolog
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PT	Phloretin
PVDF	Polyvinylidene difluoride
PYY	Peptide tyrosine tyrosine
PZ	Phloridzin
RAR	Retinoic acid receptor
RE	Response element
RNA	Ribonucleic acid
RPM	Rounds per minute
ROR	RAR-related orphan receptor
RT	Reverse transcriptase
RXR	Retinoid X receptor
SCN	Suprachiasmatic nucleus
SD	Standard deviation
SDS	Sodium-dodecyl-sulphate
SEM	Standard error mean
SLC	Solute carrier family
SGLT	Sodium-glucose linked transporter
SNP	Single nucleotide polymorphism
SSB	Sugar sweetened beverages
Strep	Streptomycin
TBS	Tris-buffered saline
TBS-T	TBS-Tween
TNF	Tumour necrosis factor
TRP	Transient receptor potential ion channel
UPL	Universal probe library
USDA	US Department of Agriculture
VIP	Vasoactive intestinal peptide

CHAPTER 1

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

1.1 Metabolic syndrome & the modern diet

Metabolic syndrome is a major medical and economic challenge of modern society and is a disorder associated with a combination of metabolic abnormalities including obesity, hypertension, dyslipidaemia and insulin resistance and linked to cardiovascular diseases (Flegal et al., 2007). Increased dietary intake of carbohydrates and fat, dysregulated energy homeostasis, central obesity and reduced insulin sensitivity contribute to the metabolic derangements (Després & Lemieux, 2006). Why there has been such a continuous rise since the last century and the exact pathophysiological aetiology of metabolic syndrome are not clear. Obesity is one of the major risk factors for metabolic syndrome and has drastically increased from 13.3% in 1960-62 to 34.7% in 2005-08 based on data from the National Health and Nutrition Examination Survey (Fig. 1) (NHANES, 2012).

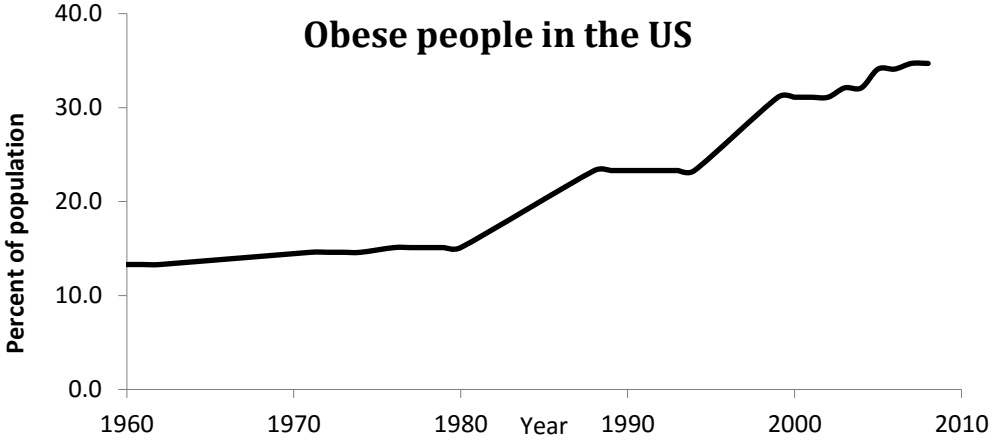

Fig. 1 Increase in the percentage of people classed as obese (BMI ≥ 30.0) since the 1960s in the USA. Data from: (NHANES, 2012).

The higher prevalence in obesity can only be explained by an increased energy intake and/or a reduced expenditure of energy (Prentice & Jebb, 1995). Indeed, daily

energy consumption has increased by approximately 425 kcal per capita since the 1970s in the US as estimated by the US Department of Agriculture (Fig. 2) (USDA, 2011). This dataset is based on food disappearance (corrected for waste), but other studies estimating daily food consumption using alternative methods produce very similar results (Swinburn et al., 2009; Duffey & Popkin, 2011)

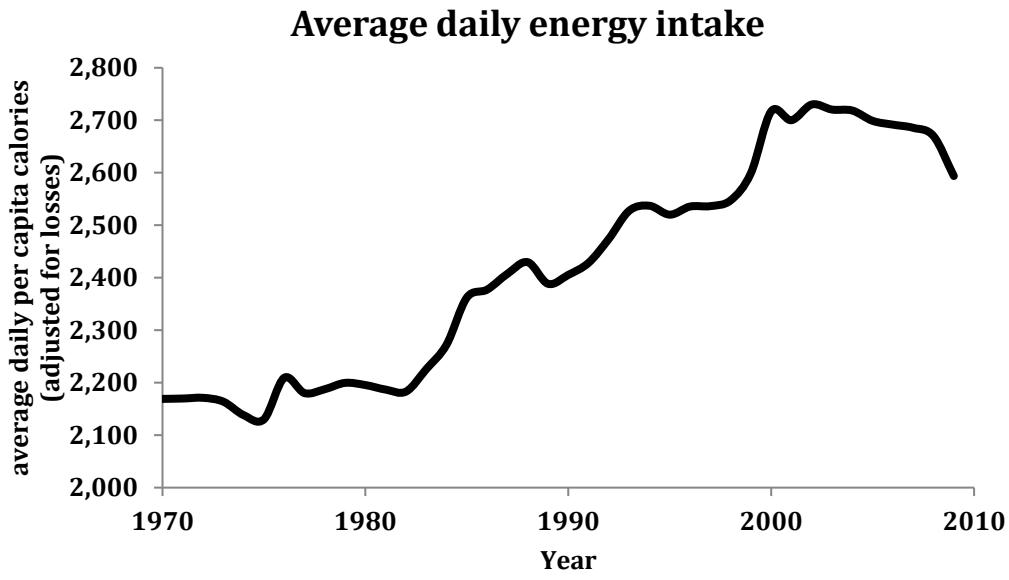

Fig. 2 Daily per capita calorie consumption since the 1970s based on food disappearance corrected for waste loss, based on data from the US Department of Agriculture.

When the total energy intake is broken down into macronutrients it appears that the calories from protein have been stable over the last 40 years (Fig. 3). Although it seems that percent calories from fat decreased slightly, absolute amounts in grams have been stable. The energy surplus can hence be accounted for by a higher consumption of carbohydrates. On average the percentage of kcal from carbohydrates increased from 43.9% to 48.5% between 1971 and 2008 (Fig. 3).

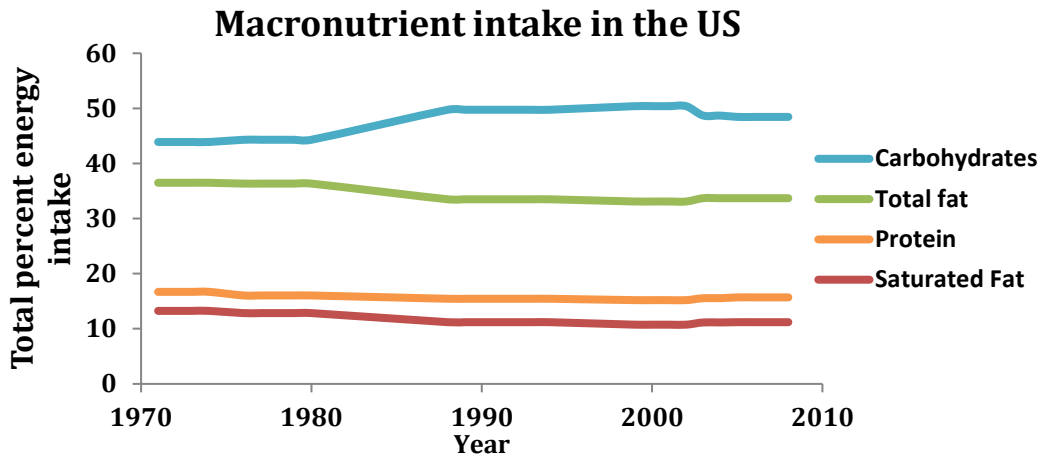

Fig. 3 Percentage of protein, fat and carbohydrates consumed daily in the US. Data are based on food disappearance corrected for waste loss supplied by the US Department of Agriculture (USDA, 2011).

Carbohydrates are sourced from grains, fruit and vegetables in the form of simple sugars and starches or are added to foods as sugars. When the 'carbohydrates' group is broken down into its sources, it can be seen that not only the consumption of flour and cereal products in general, but also of caloric sweeteners, has increased (Fig. 4).

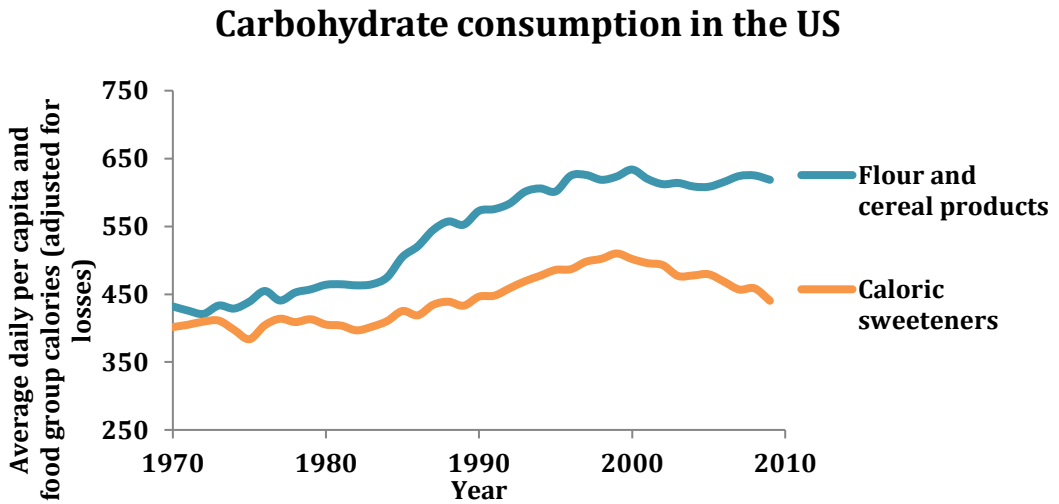

Fig. 4 Average daily calories obtained from flour and cereal products and caloric sweeteners. Together both account for ~40% of our daily energy intake. Data are based on food disappearance corrected for waste loss supplied by the US Department of Agriculture (USDA, 2011).

Although corn is the major grain grown in the US, but most is fed to animals, hence the bulk of consumed carbohydrates is produced from grains, and especially wheat (USDA, 2011).

The 'caloric sweeteners' can be broken down again and it demonstrates that refined sugar has been produced less from sugar cane and beet since the 1980s, but more from corn in the form of 'High Fructose Corn Syrup' (HFCS), dextrose and glucose (Fig. 5). HFCS gained a remarkable share of 50% over the years in the USDA data set. Natural sweeteners such as honey and edible syrups have not changed (Fig. 5).

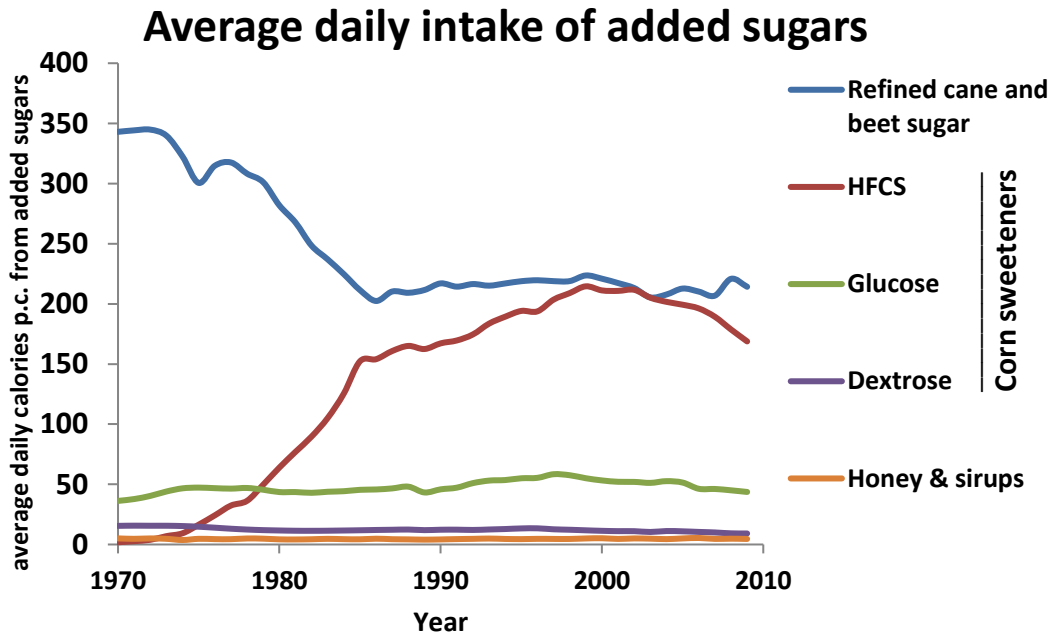

Fig. 5 Average daily calories obtained from caloric sweeteners broken down in its sources. Data are based on food disappearance corrected for waste loss on data by the US Department of Agriculture (USDA, 2011).

Other US based studies estimate that 15% and 21.4% of energy intake for adults and adolescents respectively are 'added sugars' (Marriott et al., 2009; Welsh et al., 2011). In Britain mean energy intake of 'non-milk extrinsic sugars' is currently 12% among adults and 15% among children (Bates et al., 2011). Consumption of high

amounts of added sugars has been hypothesised as a contributing factor to the rising rates of metabolic syndrome observed over the past few decades (Kaur, 2014).

Although the USDA's database gives a lot of information on the food use of the basic primary products (e.g. flour, eggs, potatoes etc.), little data exists on the processing and final product form. A study on beverage consumptions shows a move away from whole milk to either low-fat milk or sugar-sweetened beverages (SSB) (Popkin, 2010). A doubling in the consumption of soda and fruit drinks (and alcohol) between 1977 and 2006 was found, which indicates that more processed, highly palatable and rewarding foods, rich in mono- and di-saccharides and sweeteners, are being consumed (Popkin, 2010). SSBs are the main source of added sugars (Malik et al., 2010), and associations of SSB consumption and weight gain, type 2 diabetes and cardiovascular disease have been found in several studies (Tordoff & Alleva, 1990; Berkey et al., 2004; Schulze et al., 2004; Bes-Rastrollo et al., 2006).

Highly palatable, calorie dense food are highly rewarded in the hypothalamic and hindbrain nuclei, which is at the core of the inability to limit excessive food consumption, uncontrolled weight gain and the onset of metabolic syndrome (Cordain et al., 2005; Lenoir et al., 2007; Volkow et al., 2011). From an evolutionary perspective this behaviour is advantageous as it ensures that nutrients are consumed when available and energy is stored as fat to increase survival in times of scarcity. However, in our modern world where food is widely available and designed to be highly rewarding, this adaptation has become a problem.

1.2 Metabolism & the gut-brain axis

The brain centrally coordinates energy intake and expenditure by directly sensing nutrient levels (glucose, amino acids and fatty acids) and by integrating messages from all peripheral tissues including adipose, liver, muscle, pancreas and gastrointestinal tissues (Badman & Flier, 2005; Guyenet & Schwartz, 2012). The hypothalamus is able to regulate food intake, physical activity, basal metabolic rate and endocrine systems by integrating the long-term energy status via leptin from adipose tissue and insulin via the pancreas and also recent nutrient intake via signals from the gut.

The alimentary tract is responsible for the digestion and absorption of foods and assimilation of nutrients. There are anticipatory physiological responses to feeding, the cephalic phase responses, that set in motion an array of digestive and signalling cascades (neuronal & endocrine). This preparatory adaptation not only increases the efficiency of digestion, absorption and metabolisation of nutrients but also directly and indirectly regulates meal size and duration of eating.

1.2.1 Gut peptides

Pre-digested food from the mouth is transported via the oesophagus to the gastrointestinal tract. The stomach and intestine are the main sites of digestion and nutrient uptake and a highly developed organ with autonomous nervous and immune systems. The gut delivers messages about the nutritional status to the brain through afferent nerves and the vagus nerve system, but also via endocrine pathways. Throughout the gastrointestinal tract highly specialised endocrine cells are found

between the parenchymal cells secreting more than twenty gut hormones and the gut is considered the 'largest endocrine organ in the body' (Ahlman & Nilsson, 2001).

Ghrelin is a gut hormone produced in the stomach. Levels peak before meal ingestion and fall to a minimum within one hour of eating. It is thought that ghrelin serves as a signal to start eating. It has been suggested the gastric taste cells sense food load in the stomach and suppress ghrelin production which protects from overeating and overfilling of the stomach (Hass et al., 2010). A strong argument for the sensing capabilities is that the degree of ghrelin suppression depends on the type of nutrient consumed, with proteins and carbohydrates being more suppressive than lipids (Foster-Schubert et al., 2008).

The small intestine has a variety of cell types involved in chemosensation and hormone secretion. Intestinal enteroendocrine cells (EECs) appear long and narrow with their apical membrane and microvilli projecting to the lumen. In response to meal ingestion I-cells secrete the gut hormone cholecystokinin (CCK), K-cells glucose-dependent insulinotropic polypeptide (GIP) and L-cells the peptides glucagon-like peptide-1 and 2 (GLP1 & 2) and peptide tyrosine tyrosine (PYY) (Young, 2011b). These gut hormones have been found to be important in regulating food intake and functions in whole body metabolism. While ghrelin stimulates food intake, CCK from the small intestine inhibits eating, slows gastric emptying, mediates intestinal motility and stimulates pancreatic and gall-bladder secretions. In response to intraluminal chyme, plasma CCK levels rise and peak within minutes of eating, remain elevated throughout feeding and decline to baseline levels by the time the meal is terminated (Liddle et al., 1985).

PYY and GLP1 levels rise immediately after food intake, but peak after meal ingestion (Wen et al., 1995). Both gut peptides also inhibit eating, although GLP1 degrades very rapidly due to the activity of dipeptyl-peptidase IV (DPPIV). PYY and GLP1 decrease gastric motility, but also slow pancreatic secretions into the small intestine, which delays digestion and slows nutrient absorption, maintaining satiety. Furthermore, GLP1 increases insulin secretion, but inhibits glucagon production in the pancreas, facilitating the disposal of ingested nutrients and the lowering of blood glucose levels (Ahrén et al., 1995). GIP likewise leads to glucose-dependent insulin secretion, but also promotes energy storage in adipose tissue and bone formation. The gut peptides' insulin stimulatory effects are termed 'incretin response' (Drucker, 2006). It has been shown that the incretin response is mediated not only via endocrine pathways, but also via neuronal pathways. Vagotomy blunts the incretin response to fat stimulus (Rocca & Brubaker, 1999), but exact mechanisms are still unclear.

1.2.2 Nutrient sensing in the alimentary tract

The molecular identity of nutrient sensor mechanisms has emerged over the last 15 years (Hoon et al., 1999; Chaudhari & Roper, 2010). The mouth and tongue is the first entity in contact with food where lingual sensors detect the basic tastes sweet, salty, bitter, fat, sour, umami and possibly a few others. Tasting helps in finding not only energy- and nutrient-rich but also toxin-free food sources. Furthermore, it has been suggested that lingual tasting primes the gastrointestinal tract to digestion and nutrient absorption to maximise macro- and micronutrient intake ultimately leading to an increased chance of survival (Butterworth et al., 2011). Mammals have evolved a host of

nutrient sensors that are conserved between species, of a transmembrane nature and specific for each taste quality.

There are three distinct taste cells In the oral cavity, which are nested in onion-like structures in the various taste papillae (Calvo & Egan, 2015). Type-II cells are receptor cells equipped with sensors for sweet, umami and bitter taste. Type-III cells are presynaptic cells, potentially involved in 'sour' tasting, but especially important as a connector for Type-II cells, forming synapses with nerves. Receptor cells activated by sweet, bitter and umami compounds are induced to release adenosine triphosphate (ATP) through pannexin1 (PANX1) hemi-channels. The extracellular ATP excites ATP receptors (P2X, P2Y) on sensory nerve fibres and on Type-III cells which leads to neurotransmitter release and signal transduction to the brain. Type-I cells are potentially involved in 'salty' sensing, but also appear to be involved in terminating synaptic transmission and restricting the spread of transmitters (Chaudhari & Roper, 2010)

Distinct receptors for each taste quality can be found on the aforementioned taste cells (Chaudhari & Roper, 2010). The sweet-taste receptor is made up of a heterodimer of T1R2 and T1R3 and is stimulated by a wide variety of sugars, but also by artificial sweeteners of a chemically varied nature. Umami, the taste of L-glutamate and a few other L-amino acids, is mediated via a heterodimer of T1R1 and T1R3, but metabotropic glutamate receptors have also been implicated (Chaudhari et al., 2000). Bitter chemicals are sensed by a large number of taste sensors in the T2R family. The saltiness of Na^+ is detected by direct permeation of Na^+ ions through membrane ion channels such as the epithelial Na channel (ENaC) to depolarise the membrane (Lin & Finger, 1999). Sour taste mediated by protons excites presynaptic cells, but the

molecular identity of the sensor is not clear (Tomchik & Berg, 2007). Triglycerides can be rapidly hydrolysed in the oral cavity and free fatty acids can be sensed by several sensors, including CD36, GPR40 and GPR120 (Laugerette et al., 2005; Wellendorph et al., 2009).

A landmark finding was that sweet-, umami-, fat- and bitter-taste receptors are not restricted to the tongue, but can also be found throughout the body, including the pancreas, stomach and small intestine (Dyer et al., 2005; Bezençon et al., 2007; Mace, Affleck, et al., 2007). In the stomach mucosal cells for chemosensation and ghrelin production have been found to express T1R3 and αGustducin (Hofer et al., 1996; Hass et al., 2010). Like ghrelin producing cells in the stomach, EECs express a variety of taste receptors and the necessary signalling molecules (reviewed in Janssen & Depoortere, 2013). A proposed model of gastrointestinal nutrient sensor mechanisms is shown in Fig. 6.

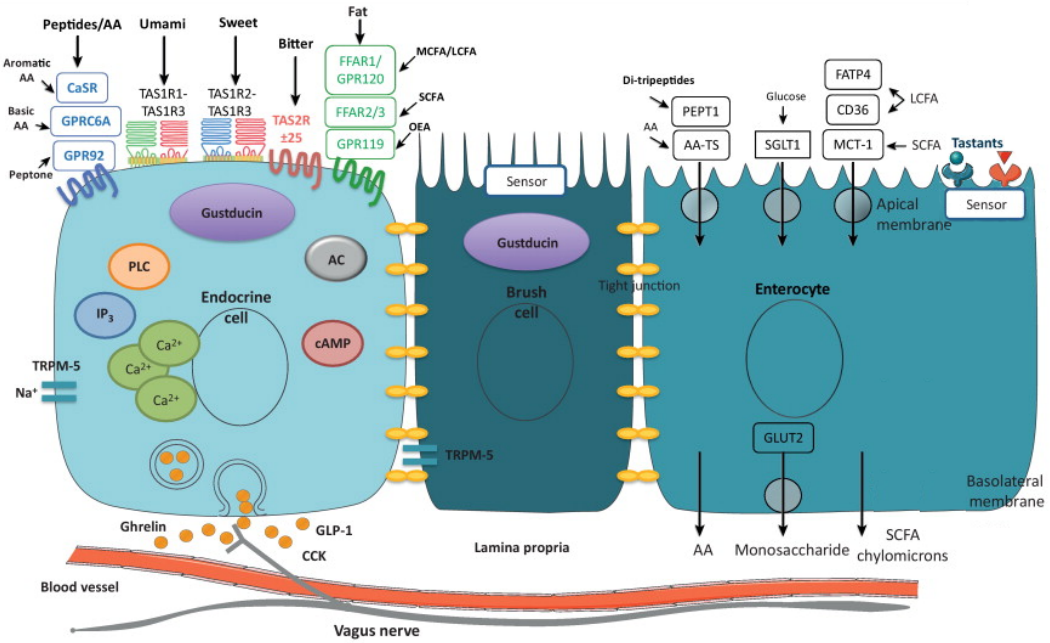

Fig. 6 Model of components involved in gastrointestinal nutrient sensing. Nutrients are sensed by luminal GPCRs or transporters in several cell types in the mucosa (EECs, enterocytes and brush

cells) which in turn can affect each other's expression. Second-messenger release induced by sensors can lead to the release of basolateral gut peptides which can then affect metabolism via the blood-stream or stimulate vagal afferents. Figure adapted from Janssen & Depoortere, 2013.

Luminal nutrients can be sensed by taste receptors and transporters on EECs (Gerspach et al., 2011; Steinert et al., 2011). The luminal nutrient-receptors are connected to the intracellular second-messenger machinery via αGustducin. This can lead to either the release of gut peptides or to membrane depolarisation via basolateral ion channels such as the Transient receptor potential cation channel subfamily M member 5 (TRPM5). This enables the tissue to adapt to the nutrient composition of the chyme and also regulates metabolism in the body. Gut peptides have effects on the brain and metabolic tissues in the periphery (pancreas, adipose tissue, liver) directly via endocrine pathways or indirectly via neuronal pathways.

Recent studies have revealed that the 'classic' gut peptides are also expressed in taste cells on the tongue (reviewed in Dotson *et al*., 2013). Genetic and pharmacological experiments indicate a critical role for lingual gut peptides in the modulation of taste responsiveness and preferences. Mostly auto- or paracrine function have been assigned to these tongue-expressed peptides, as the hormones and their respective receptors are expressed on the same or neighbouring cells such as nerve fibres (Dotson et al., 2013). Yet it has also been shown that GLP1 is secreted into the circulation within minutes of the application of glucose to the tongue (Kokrashvili et al., 2014). This data suggests that the involvement of lingual taste sensing and peptide-hormone release is important for the cephalic phase of digestion and potential feed-forward mechanisms to the gastrointestinal tract in order to prepare the distal digestive tract.

Yet it has also been shown how hormones such as leptin and gastrointestinal gut peptides change the responsiveness of lingual chemosensory cells to taste stimuli (Nakamura et al., 2008). Indeed, it has been suggested that changes in the levels of circulating hormones mediate the changes in taste perception observed after gastric bypass surgery (Hajnal & Kovacs, 2010; Miras & Roux, 2010; Bueter et al., 2011). These findings implicate a feed-backward loop from the gastrointestinal tract up to the oral cavity and brain to regulate food intake.

1.3 Expression of sugar transporters and sensors in the alimentary tract

The goal of this thesis is to study nutrient sensor mechanisms in health and disease. In order to do this it is important to know the spatial expression of such sensors. We therefore analysed the cephalocaudal expression of sugar transporters and nutrient sensors along the alimentary tract of mice.

1.3.1 Expression of sugar transporters in the gastrointestinal tract

We evaluated our system by analysing the gene expression of known hexose transporters (SGLT1, GLUT2 and GLUT5) along the small intestine. 15 mg of small-intestinal tissues from eight six-week-old CD-1 mice were dissected, RNA extracted and cDNA transcribed for subsequent RT-qPCR (methods outlined in 3.4). Exact locations (proximal or distal) is not known and whole tissues including the *serosa*, *muscularis propria*, *submucosa* and the three layers of the *mucosa*. Consistent with previous reports (Yoshikawa et al., 2011), mRNA expression of *slc5a1* (SGLT1), *slc2a2* (GLUT2) and *slc2a5* (GLUT5) is highest in the proximal regions, duodenum and jejunum, but is lower in the ileum (Fig. 7). This matches the higher luminal concentration of sugars in the duodenum and jejunum.

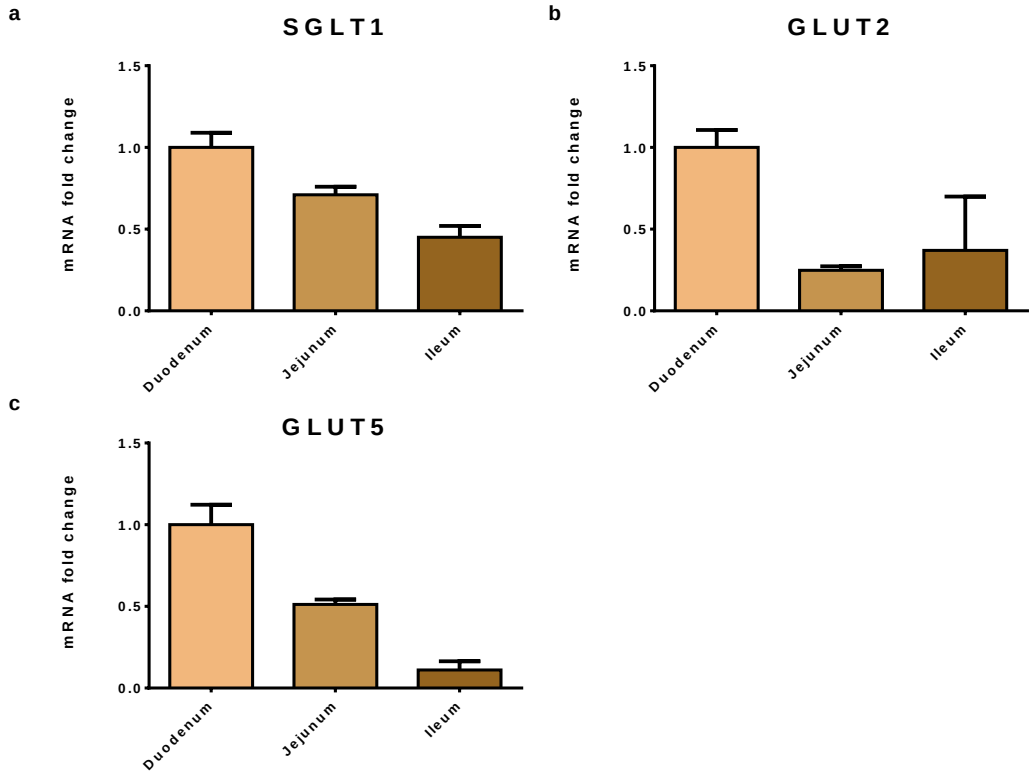

Fig. 7 mRNA expression of hexose transporters in the small intestine of adult mice. Whole duodenal, jejunal or ileal tissue was dissected from six-week old CD-1 mice (n=8) for subsequent quantitative PCR of *slc5a1* (SGLT1), *slc2a2* (GLUT2) and *slc2a5* (GLUT5). Values are normalised against three reference genes and relative quantitation is performed using the ddCT method and expressed relative to duodenum.

1.3.2 Expression of the sweet-taste machinery in the alimentary tract

In addition to the hexose transporters we evaluated the gene expression of nutrient sensors along the murine alimentary tract including tongue, stomach and small intestine. As demonstrated in Fig. 8, the umami- and sweet-taste receptor molecules *tas1r1* (T1R1), *tas1r2 (T1R2)* and *tas1r3* (T1R3) and downstream signalling molecules *gnat3* (αGustducin) and *gnat1* (αTransducin) are all highly expressed in the tongue, where they have been described first (Hoon et al., 1999).

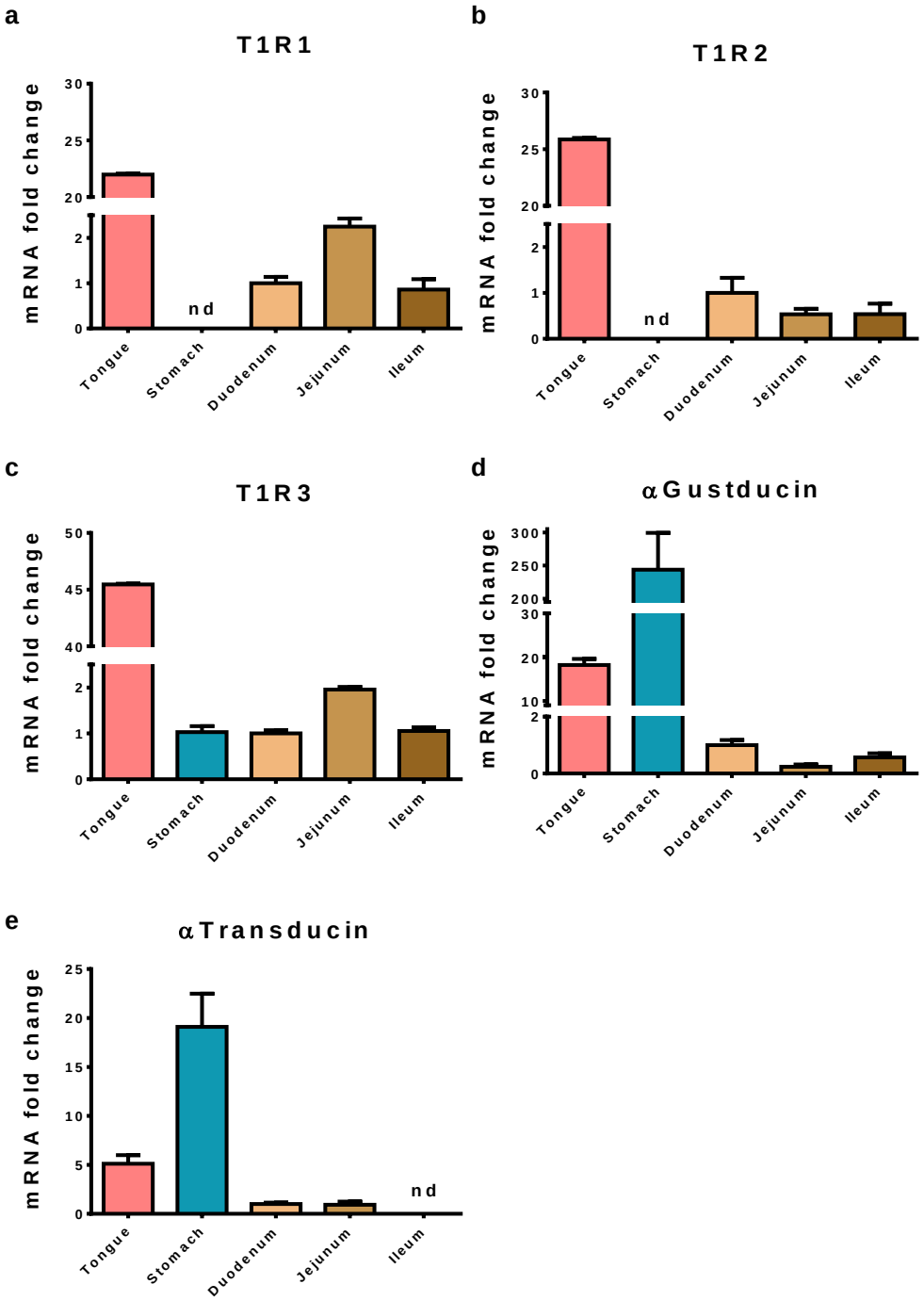

Fig. 8 mRNA expression of nutrient sensor genes in the small intestine of adult mice. Whole tongue, stomach, duodenum, jejunum and ileum was dissected from six-week old CD-1 mice (n=8) for subsequent quantitative PCR of *tas1r1* (T1R1), *tas1r2* (T1R2), *tas1r3* (T1R3), *gnat3* (αGustducin) and *gnat1* (αTransducin). Values are normalised against 3 reference genes and relative quantitation is performed using the ddCT method and expressed relative to duodenum. ND = not detected.

T1R1 and T1R2 are not expressed in the stomach, but T1R3 is. Interestingly, we detected high expression levels of αGustducin and αTransducin in gastric tissues, with ~4x and ~20x higher expression than in lingual extracts. Contrary to data published on humans we were not able to show expression of T1R1 and T1R2 in the stomach, but showed T1R3 mRNA levels (Bezençon et al., 2007). Another study confirms our findings with no expression of T1R2, but expression of T1R3 (Young et al., 2009). This study also dissected the expression between fundus, corpus and pylorus and showed higher expression in fundus and corpus and lower expression in antrum. Further discrepancies appear comparing αGustducin expression in stomach. Bezençon *et al*., 2007 were not able to detect αGustducin in stomach, whereas other studies (Hofer et al., 1996; Young et al., 2009) and our own experiments show gastric expression. αGustducin in the stomach has been suggested to be involved in L-glutamate sensing via mGluR1 (San Gabriel et al., 2007), which has been associated with increased protease secretion necessary for the gastric phase of protein digestion (Vasilevskaia et al., 1993). Similarly, gastric αGustducin is also involved in the control of bitter sensor mediated ghrelin release, resulting in delayed gastric emptying and stimulated food intake (Janssen et al., 2011). Regarding sweet-taste sensation, the expression of T1R2 is rather low/undetectable, thus rendering the formation of a T1R2/3 heterodimer unlikely. T1R3 has been suggested to works on its own as a homodimer to detect sweet compounds (Nelson et al., 2001; Zhao et al., 2003). It is possible that T1R3 could also dimerise with a yet unknown partner or a completely different sensor, as we expect, similar to the other nutrients, a gastric phase of chemosensation necessary for feed-forward mechanisms and signalling to the brain.

T1R1, 2 and 3, αGustducin and αTransducin are expressed in the small intestine, although at ~5 - 45x lower levels than in the tongue (Fig. 8). A similar trend regarding the expression levels from proximal to distal can be observed with higher levels in the duodenum and jejunum than in the ileum (Fig. 8). αTransducin was not even detected in the ileal samples. Taste receptors followed a similar pattern to the hexose transporters, with higher expression in the proximal regions. This suggests that T1R1/3 and T1R2/3 via αGustducin/αTransducin are involved in proximal nutrient detection. This so called 'duodenal brake' is important to inhibit gastric emptying and acid secretion (Rønnestad et al., 2014)

1.3.3 Expression of the novel sugar sensor SGLT3 in the alimentary tract

SGLT3 has been suggest to be a sugar sensor rather than a transporter (Barcelona et al., 2012). SGLT3/*slc5a4* belongs to the same family as SGLT1/*slc5a1*. Despite a 76% genetic identity match of SGLT1 and SGLT3 in humans (NCBI blast), SGLT3 does not transport D-Glu, but results in Na^+- and Phloridzin (PZ)-sensitive depolarisation (Diez-Sampedro et al., 2003). While in humans only one gene codes for SGLT3, a gene-duplication has occurred in mice and rats. Human SGLT3 has 82% mRNA sequence identity with murine SGLT3a and SGLT3b. Murine SGLT3a and SGLT3b have 80% mRNA and 75% amino acid sequence identity (NCBI blast). SGLT3a is similar in its properties to human SGLT3 (high D-Glu affinity, no transport). SGLT3b is more similar to SGLT1, showing D-Glu transport, but at a lower capacity. Yet the exact role of SGLT3(a+b) is not fully understood. A detailed distribution and comparative intensities of expression of the

SGLT3s along the alimentary tract has not been demonstrated and could improve the functional interpretation of these proteins.

We therefore analysed, for the first time, the cephalocaudal axis of gene expression for SGLT3 in the alimentary tract. Our analysis revealed that in the tongue neither *slc5a4a* (SGLT3a) nor *slc5a4b* (SGLT3b) is expressed (Fig. 9), which suggests that it is not involved in sweet-taste perception on the tongue.

In the stomach *slc5a4b* (SGLT3b), but not *slc5a4a* (SGLT3a) is expressed at the mRNA level, although at 20-fold lower levels than in the small intestine where both genes are expressed. SGLT3a follows the pattern of luminal sugar concentrations like the hexose transporters, with higher expression proximally. SGLT3b shows its highest expression in the ileum (Fig. 9).

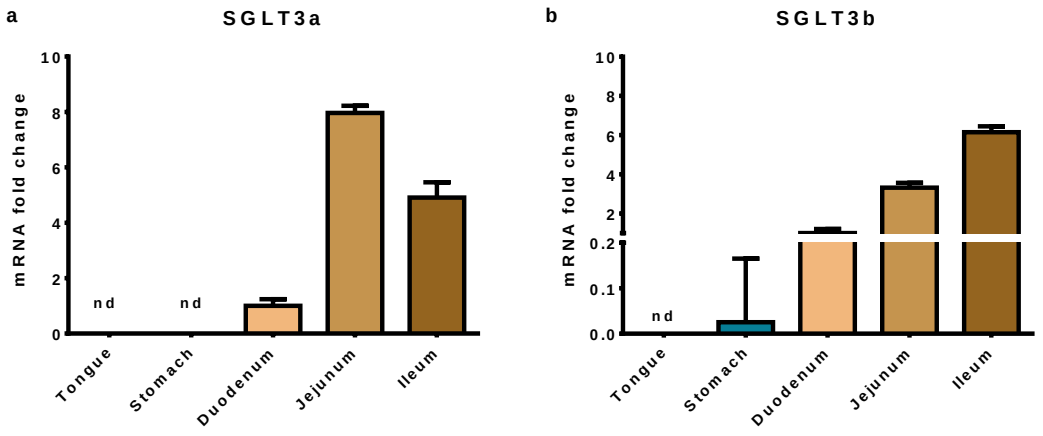

Fig. 9 mRNA expression of SGLT3 in the murine alimentary tract. Whole tongue, stomach, duodenum, jejunum and ileum was dissected from six-week old CD-1 mice (n=8) for subsequent quantitative PCR of *slc5a4a* (SGLT3a), *slc5a4b* (SGLT3b) and *slc2a5* (GLUT5). Values are normalised against 3 reference genes and relative quantitation is performed using the ddCT method and expressed relative to duodenum. ND = not detected.

SGLT3 is expressed in cholinergic neurons of the enteric nervous system and skeletal muscle where it is thought to regulate smooth and skeletal muscle activity (Diez-

Sampedro et al., 2003). Furthermore, SGLT3 has been shown to be expressed in the hypothalamus, spleen, kidney, liver, portal vein and intestinal mucosa (Kong et al., 1993; Gribble et al., 2003a; O'Malley et al., 2006; Kothinti et al., 2012; Delaere et al., 2013). Experimental research has shown that SGLT3 is involved in D-Glu sensing in the hypothalamus (O'Malley et al., 2006), portal vein (Delaere et al., 2013), kidney (Kothinti et al., 2012) and GLP1 secreting L-cells (Gribble et al., 2003a; Lee et al., 2015). Despite these findings, the exact roles of SGLT3a+b are not well established.

Both genes have different expression patterns, which suggests that both genes are regulated differently on the transcriptional/post-transcriptional level. This also suggests different functional roles for both genes/proteins.

SGLT3a has no known transport function, and it has been suggested that it works as a glucose sensor. GLP1 plasma levels rise rapidly after nutrient ingestion and the enteric nervous system has been found to be important for the proximal-distal loop regulating GLP1 release from the L-cells of the distal small intestine (Rocca & Brubaker, 1999). SGLT3a could be involved in assessing luminal or absorbed sugar D-Glu concentrations, strategically expressed in the mucosa or on cholinergic neurons. We speculate that SGLT3a is involved in the proximal-distal loop for the secretion of GLP1.

SGLT3b on the other hand is highly expressed in the distal regions of the small intestine, but also in the stomach. The strategic expression in the stomach might be important for ghrelin release, as well as the proximal-to-distal loop to signal to the ileum. This is important as the ileum is the small-intestinal region with the highest density of GLP1-secreting L-cells (Tolhurst et al., 2009) and GLP1 is central to regulating Insulin secretion and other core metabolic pathways. SGLT3 is potentially involved in

GLP1 secretion as experiments in an L-cell model, GLUTag cells, have shown (Gribble et al., 2003b). Indeed, very recent data using an SGLT3 inhibitor directly links SGLT3 sensing with GLP1 secretion (Lee et al., 2015). We speculate that SGLT3b is a high-affinity D-Glu transporter in the distal small intestine involved in the ileal brake phenomenon. Feedback mechanism like these are important not only to provide information to the central brain regions involved in satiety and feeding behaviour, but also to help digestion and nutrient assimilation by slowing gastrointestinal motility, inducing enzyme, gut peptide secretion and by regulating transport mechanisms. Overstimulation by dietary sugars can make such systems signal resistant, which can then become over/under active and lead to metabolic dysregulations.

1.4 Nutrient sensor mechanisms as targets for metabolic syndrome

Analogues for satiety hormones like GLP1 agonists are already used in the clinic (Tella & Rendell, 2015), but directly targeting nutrient sensors to stimulate/block gut peptide secretion could present an alternative and more holistic approach, as the various chemosensors are present on the many different EECs and would lead to a broader regulation of gut peptide secretion. New targets offer novel approaches for the prevention or treatment of metabolic syndrome. Most progress has been made in the development of free fatty-acid receptor (FFAR) agonists to treat type 2 diabetes. Targeting FFAR2 led to increased GLP1 and PYY levels, which resulted in a reduction of non-alcoholic fatty-liver disease (Chambers et al., 2015). The sweet-taste receptor, specifically T1R3, has also been studied as a target to control body weight and a patent has been submitted to protect the use of this method (Shirazi-Beechey & Iserentant, 2009). Data of marmoset monkeys on an obesogenic diet for 11 weeks treated with the T1R3 inhibitor Lactisole are presented in this patent. While the control animals became obese and doubled their body weight, animals with Lactisole treatment were able to stay on a level comparable to lean controls. A more recent human study was able to show that Lactisole, a T1R3 antagonist, blocked GLP1 and PYY secretion successfully (Steinert et al., 2011).

Despite recent advances, major questions remain regarding the role of nutrient sensors in the regulation of intestinal function in health and disease.

Luminal sugar levels regulate sugar-transport capacities in enterocytes (Ferraris & Diamond, 1989). Yet precise mechanisms of how sugar transporters are regulated in

the short and long term are less understood. It has been suggested that sugar is directly sensed by T1R2/3 expressed on enterocytes to regulate sugar-transport capacities in the short term (Kellett et al., 2008). Yet it has also been suggested that neighbouring EECs regulate sugar transport in enterocytes via paracrine effects of secreted gut peptides (Margolskee et al., 2007). Exact mechanisms and pathways are not clearly established.

The role of nutrient sensing mechanisms in metabolic diseases is an active area of research. Evidence in humans and rodents suggests dysregulation off EECs in metabolic syndrome. It has been shown that obesity and type 2 diabetes can blunt the incretin response (Nauck et al., 1986). In addition, gustatory and ghrelin-producing cells were found to be more numerous in obese patients (Widmayer et al., 2012). Similar findings were found in an obese high-fat-fed mouse model, where the numbers of GPR120 expressing brush cells as well as gastrin- and ghrelin-producing cells were increased (Widmayer et al., 2015). Yet reports of dysregulations of nutrient sensors by dietary factors, especially a high-sucrose diet, are scarce and not well studied (Young et al., 2009; Widmayer et al., 2012; Widmayer et al., 2015).

Disruptions in clock genes have been linked to metabolic disease, but exact mechanisms are unclear. Intestinal processes are regulated not only in response to luminal concentrations, but mammals have developed anticipatory, circadian mechanisms to optimise energy and nutrient uptake. The sugar transporter SGLT1 is regulated in a diurnal fashion and is under the control of clock genes (Corpe & Burant, 1996; Balakrishnan et al., 2012). Furthermore, GLP1 has been shown very recently to display diurnal rhythmicity (Gil-Lozano et al., 2014). Yet the rhythmicity of sensor mechanisms has not been studied under healthy or diseased conditions.

It is therefore important to better understand how nutrient sensors influence transport activity, how their expression is spatially and temporally regulated and how they are affected in metabolic diseases. This will pave the way to finding new targets to prevent or treat metabolic syndrome.

1.5 Aims, hypotheses & rationale

Aims of the study

- *Specific aim 1:* Study the short-term regulation of sugar transport via the sweet-taste receptor T1R2/3 in enterocytes in an *in vitro* model.
- *Specific aim 2:* Evaluate diurnal rhythmicity of nutrient sensor expression along the murine alimentary tract.
- *Specific aim 3*: Examine nutrient sensor gene expression under diabetic conditions.

Hypotheses

- The sweet-taste receptor T1R2/3 is expressed on enterocytes and is able to directly influence transport capacities of the cell.
- Mice show diurnal rhythms of gastrointestinal nutrient sensors anticipatory to nocturnal feeding.
- A chronic diet high in sucrose leads to type 2 diabetes mellitus and attenuates nutrient sensor and gut peptide gene-expression levels in the small intestine.

Rationale

We used an *in vitro* enterocyte model, Caco-2 cells, a human colon cancer cell line that appears enterocyte-like upon culture. These cells express the hexose transporters SGLT1, GLUT2 and GLUT5 (Mahraoui et al., 1994), but also the sweet-taste receptor T1R2/T1R3 (Le Gall et al., 2007).

We used mouse models for *in vivo* studies, as mice have the same conserved core clock machinery found in all mammals (Reppert & Weaver, 2001) and have also been widely used for researching gastrointestinal function, as mice express all the common nutrient transporters and sensors found in humans.

To study gastrointestinal dysregulations in type 2 diabetes we fed mice for six weeks on a test diet of chow plus free access to light condensed milk, to emulate a diet rich in added sugars to induce metabolic syndrome.

CHAPTER 2

SHORT-TERM REGULATION OF SUGAR TRANSPORT IN AN *IN VITRO* MODEL OF ENTEROCYTES — CACO-2/TC7 CELLS

2 SHORT-TERM REGULATION OF SUGAR TRANSPORT IN AN *IN VITRO* MODEL OF ENTEROCYTES — CACO-2/TC7 CELLS

2.1 Summary

Background The gastrointestinal tract plays a key role in regulating nutrient metabolism and energy balance; the response of the gastrointestinal tract to ingested carbohydrates is therefore an active area of research. The molecular mechanisms by which dietary carbohydrates regulate intestinal sugar transport are controversial. Two models currently exist both involving the sweet-taste receptors T1R2/3: an indirect model, whereby T1R2/3 expressed on mucosal EECs is activated by luminal carbohydrates, resulting in the release of gut peptides, which in turn regulate the enterocytes, and a direct model, whereby T1R2/3 is expressed on the enterocyte and directly regulates enterocyte function.

Aims We hypothesised that the sweet-taste receptor T1R2/3 is expressed on enterocytes and directly regulates sugar-transport capacities in enterocytes upon stimulation by sugars and artificial sweeteners.

Methods To study the direct model we used the Caco-2/TC7 cell line, a well-established *in vitro* model of the human enterocyte. Uptake of D-Glu and Fru into Caco-2/TC7 cells was assessed using radiolabelled sugars and expression of the intestinal sugar transporters and sweet-taste receptors was determined by RT-PCR. Regulation of sugar transport by T1R2/3 was assessed using agonists in the form of mono- and disaccharides, artificial sweeteners or the inhibitor Lactisole.

Results Carrier-mediated D-Glu and Fru uptake by SGLT1 and GLUTs was detected in Caco-2/TC7 cells. Increasing concentrations of D-Glu or HFCS in the media resulted in a

4-fold increase in D-Glu uptake, but was not blocked by Lactisole or enhanced by artificial sweeteners. RT-PCR revealed only T1R3 (but not T1R2) expression in Caco-2/TC7 cells. Fru or sucrose had no effect on D-Glu or Fru uptake.

Conclusions Our *in vitro* data suggest that the sweet-taste receptor is not responsible for the detected direct regulation of intestinal D-Glu/Fru transport. Potentially another sensor is involved in sugar sensing, but control by neighbouring EECs cannot be excluded. Furthermore, the Caco-2/TC7 cell model may not be an appropriate model to study direct regulation of sugar transport by T1R2/3.

2.2 Background

The gastrointestinal tract is the site of nutrient digestion and uptake. Nutrient assimilation is not a passive process; mechanisms exist that tightly regulate transport capacities to match luminal nutrient load. Chronic/long-term mechanisms, as well as acute/short-term mechanisms exist to control nutrient uptake following their ingestion. The breakdown of macronutrients, including carbohydrates, is facilitated by several enzymes that are secreted from the exocrine pancreas and liver or are present on the microvillus brush border surface (e.g. lactase, Sucrase-Isomaltase) of the enterocytes. Once carbohydrates are hydrolysed into monosaccharides they can be transported across the enterocyte into the portal circulation.

Dietary D-Glu is transported across the apical membrane of the enterocyte by the Sodium/glucose co-transporter SGLT1 and Fru is transported by the facilitative transporter GLUT5. D-Glu and Fru then exit the enterocyte via basolateral GLUT2 into the bloodstream. SGLT1 has a high affinity, but a low capacity for D-Glu, saturating at a concentration of 30–50 mM (Debnam & Levin, 1975) and can be inhibited by Phloridzin (Donhoffer, 1935). Yet the intestinal transport capacity is linear up to luminal D-Glu concentration of several hundred millimolar (Holdsworth & Dawson, 1964). These observations indicate the presence of a second transport component described as Phloridzin-insensitive and 'diffusive' or 'passive', as it is linear and non-saturable (Debnam & Levin, 1975). This secondary component accounted for 75% of D-Glucose absorption and was hypothesised to be mediated through a paracellular flow mechanism (Madara & Pappenheimer, 1987; Pappenheimer & Reiss, 1987). It was observed that high amounts of water were absorbed and it was proposed that SGLT1

activity leads to the contraction of the perijunctional actomyosin ring (Turner et al., 1997). This contraction then dilated the intercellular tight junctions, facilitating the osmotically driven flow of D-Glu through the paracellular solvent drag. This theory was disproved in several studies (Lane *et al.*, 1999; Ferraris & Diamond, 1997; Schwartz *et al*., 1995) who showed that paracellular D-Glu flow accounted only for 2–7% over a 2 h period in dogs. Furthermore, it had already been shown by Debnam & Levin in 1975 that the facilitative component did not rely on SGLT1 activity, as D-Glu was still readily absorbed after blocking active transport by Phloridzin.

A large body of research has been conducted since, leading to a new dogma for the facilitative component: GLUT2 insertion into the apical membrane. Functional studies revealed that D-Glu transport in normal rat jejunum was mediated mainly by SGLT1 at low luminal concentrations (~10 mM D-Glu), but that GLUT2 was responsible for up to 75% of D-Glu transport if the concentrations was raised to 100 mM (see Fig. 10) (Kellett & Helliwell 2000).

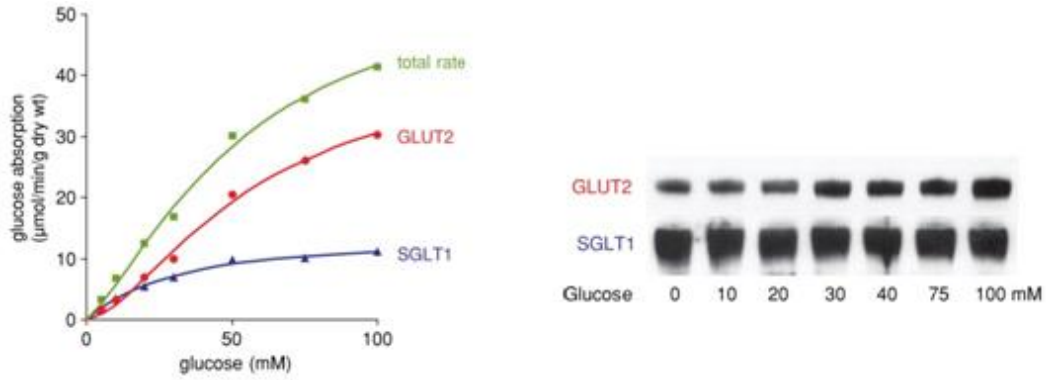

Fig. 10 Acute short-term D-Glu absorption in the perfused rat jejunum. Left panel: D-Glucose transport was dissected into two components, a Phloretin-sensitive GLUT2 component and a Phloretin-insensitive SGLT1-mediated component. Right panel: protein levels of apical SGLT1 and GLUT2 showing that GLUT2 insertion increases with the D-Glucose concentration while SGLT1 stays stable. (Figure reproduced from Kellett *et al.,* 2008).

The transient insertion of GLUT2 into the apical membrane is very rapid ($t_{1/2}$ ~3.5 min) and correlated with an activation of protein kinase C (PKC) isoform βII (Helliwell et al., 2000). Several studies have since confirmed the apical location of GLUT2 (Au et al., 2002; Affleck et al., 2003; Gouyon et al., 2003; Grefner et al., 2006; Tobin et al., 2008).

In 2007 the Kellett group proposed in two papers that apical GLUT2 is reliant on intracellular Ca^{2+} levels mediated by the L-type channel $Ca_v1.3$ (Mace, Morgan, et al., 2007; Morgan et al., 2007), since PKC βII activity is Ca^{2+} dependent (Hug & Sarre, 1993), Ca^{2+} is necessary for cytoskeletal rearrangements (Madara & Pappenheimer, 1987; Turner, 2000) and D-Glu itself is depolarising.

The Kellett model appears to be the established model for intestinal sugar transport (summarised in Fig. 11), but remains controversial.

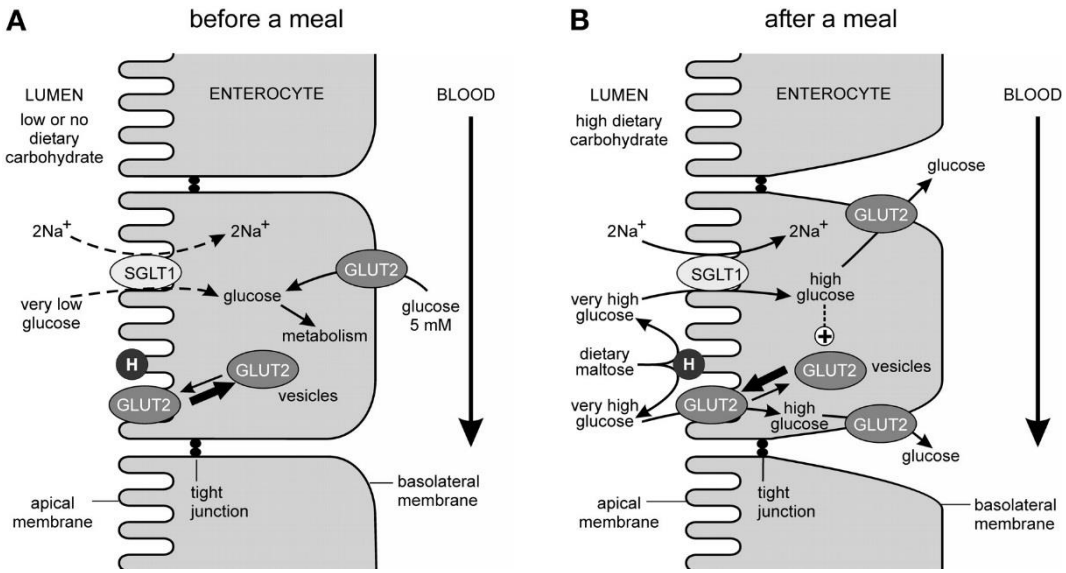

Fig. 11 Proposed mechanisms of monosaccharide absorption in the small intestine. A: SGLT1 transports D-Glu of levels of up to 30 mM. SGLT1 is an active transporter driven by a Na^+ gradient created by the basolateral Na^+/K^+ ATPase. There is low or no apical GLUT2. **B**: After a meal, when higher concentrations of luminal D-Glu are present, GLUT2 is rapidly inserted into the apical membrane as mediated by the depolarising effect of Na^+, which in turn activates the calcium transporter Cav1.3. $Ca2^+$ ions are necessary for the cytoskeletal rearrangements and PKCβII activity is necessary for the insertion of GLUT2 into the apical membrane. (Figure reproduced from Kellett & Brot-Laroche, 2005).

An SGLT1 knockout model revealed that GLUT2 insertion is SGLT1 dependent (Gorboulev et al., 2012). Yet this same study led to a discussion that GLUT2 is responsible for less than 10% of D-Glu transport in the control mice. Consistent with this observation, it was found in a study of human glucose-galactose malabsorption that this is due to SGLT1 mutations, resulting in a non-functional protein (Martín et al., 1996).

Another set of studies also challenged the Kellett model (Stümpel et al., 2001; Santer et al., 2003). GLUT2 knockout mice and GLUT2-deficient patients show no signs of glucose-galactose malabsorption. It was suggested that D-Glu exit from the enterocyte into the plasma appeared by phosphorylation, transfer into the endoplasmatic reticulum and vesicular exocytosis from the basolateral membrane. For how much transport capacity this alternative mechanism accounts is not clear, but it was estimated to be approximately 15% (Kellett et al., 2008).

A refined role of apical GLUT2 has been suggested recently (Naftalin, 2014). GLUT2 could act as an apical osmoregulator to protect the cell from damage from cell-volume change upon increased intracellular D-Glu rather than as a D-Glu transporter, but this view has been highly contested (see review reports of publication for a detailed discussion).

As explained previously, Ca^{2+} ions play an important role in the regulation of apical GLUT2 trafficking, but it has been argued that a second signal is necessary for mass D-Glu absorption. A landmark finding was that taste receptors for bitter (T2R family), umami (T1R1/3) and sweet (T1R2/3) and the necessary downstream signal-transduction machinery, including αGustducin, first described in lingual taste buds, can also be found throughout the gut (reviewed in Iwatsuki & Uneyama, 2012).

Kellett and colleagues suggested that the sweet-taste-receptor machinery can be found in enterocytes (Mace, Affleck, et al., 2007). Sugars and artificial sweeteners bind to the sweet-taste-receptor heterodimer T1R2/3 directly on the enterocyte and connect to the sugar transporters via αGustducin and/or αTransducin and the second-messenger molecules PKCβII and Phospholipase βII (Kellett et al., 2008). This enables the enterocyte to directly and immediately regulate hexose-transport capacities upon luminal nutrient load.

High levels of the 'taste machinery' proteins were also located in small-intestinal EECs like GLP1-secreting L-cells. It has been suggested that EECs also regulate nutrient-transport capacities in enterocytes in an indirect, paracrine fashion (Margolskee et al., 2007). Indeed, GLP2 has been shown to upregulate SGLT1 in enterocytes and to rapidly lead to apical GLUT2 insertion (Cheeseman, 1997; Au et al., 2002). αGustducin knockout mice show diminished GLP1 levels and when chronically fed a high-carbohydrate diet cannot up-regulate SGLT1 expression in enterocytes (Jang et al., 2007). These observations indicate the importance of the indirect regulatory function of EECs for the long-term regulation of sugar transport in enterocytes (Margolskee et al., 2007).

The indirect, chronic EEC-mediated mechanism and the direct, short-term enterocyte-mediated mechanism are not mutually exclusive and could exist in parallel to regulate transport capacity. Despite the inconsistencies, the evidence suggests an involvement of the sweet-taste receptor T1R2/3 in the regulation of sugar transporters. This makes it an attractive target for the treatment of several metabolic pathologies by enabling the stimulation, and also the downregulation, of sugar-transport capacities.

An increase in nutrient uptake could help people with Fru malabsorption. Both directly activating the sweet-taste receptor on enterocytes and indirectly activating them via EECs to release GLP1/2 to upregulate GLUT5 expression or lead to apical GLUT2 insertion in enterocytes are plausible scenarios to increase Fru absorption.

Blocking of Fru absorption could help in the onset of non-alcoholic fatty-liver disease (NAFLD). Decreasing/retarding sugar uptake could reduce the postprandial glycaemic load and reduce the risk of developing metabolic syndrome. NAFLD has been associated with excess Fru consumption, leading to higher hepatic lipogenesis rates, which in turn can lead to an increased lipid burden (Moore et al., 2014). Besides dietary interventions, regulating the rate of Fru absorption to slow the postprandial Fru burden on the liver, as well as stimulating gut peptide secretion, could be beneficial with high Fru loads.

In diabetes, the postprandial blood glucose levels increase rapidly, with homeostatic measures failing to clear the glucose to normal levels fast enough or at all (Laakso, 1999). It has been suggested that these hyperglycaemic spikes are involved in the onset of cardiovascular complications (Laakso, 1999). Similarly, slowing intestinal sugar absorption could be a potential therapy angle to help control postprandial hyperglycaemia.

2.3 Chapter Overview – Short-term regulation of sugar transport *in vitro*

Aims

Study the role of the sweet-taste receptor T1R2/3 in the regulation of intestinal sugar transport by dietary carbohydrates and artificial sweeteners.

Hypothesis

The sweet-taste receptor T1R2/3 is expressed on enterocytes and directly regulates sugar-transport capacities in enterocytes upon stimulation by sugars and artificial sweeteners.

Objectives

- Establish the Caco-2/TC7 cell line and radioactive sugar-uptake assay to study the regulation of sugar uptake
- Study the short-term regulation of sugar transport by dietary sugars in Caco-2/TC7 cells
- Evaluate the role of the sweet-taste machinery in the short-term regulation of sugar transport

2.4 Materials & Methods

2.4.1 Cell culture

Caco-2/TC7 cells were grown in high-D-Glucose (25 mM) Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Dorset/UK), containing 10% fetal bovine serum (FBS; Sigma-Aldrich), 50 units Penicillin and 50 µg Streptomycin (Pen/Strep; Sigma-Aldrich), 0.1 mM MEM non-essential amino acids and additional 2 mM L-Glutamine (Sigma-Aldrich). The cells were grown in T25 flasks, split 1:20 once a week at ~80% confluence and seeded for experimentation at a density of 10,000 cells/cm^2 in 24 multiwell dishes (NUNC, Roskilde/DK). Medium was changed every other day until confluent and daily after reaching confluence. For experiments cell passages 38–50 were used.

2.4.2 Radio-labelled sugar-uptake assay

Assay conditions varied according to the experimental question and any modifications to the following standard protocol are indicated in figure legends/descriptions. Kreb's buffer (KBS) necessary for the protocol contained 30 mM HEPES, 130 mM NaCl, 4 mM KH2PO4, 1 mM MgSO4 and 1 mM CaCl2, and the pH was adjusted to 7.4. For Na^+-free KBS, NaCl was replaced with choline chloride.

Cells were removed from the incubator, washed once with KBS and then preincubated in KBS optionally containing the inhibitors 500 µM PZ, 500 µM Phloretin (PT) or 10 µM Cytochalasin B (CB; Sigma-Aldrich), or D-Glu or Fru (Sigma-Aldrich), for 15 min or 3 h respectively. Preincubation medium was then removed for uptake, but 3 h preincubation experiments had another wash with KBS included. Sugar uptake was measured by exposing cells to 10 mM D-Glu (Perkin-Elmer, Cambridge/UK) or Fru

(American radiolabelled chemicals, St Louis, MO, US) containing 0.1 µCi/ml 14C radio-labelled sugar molecules. Absolute uptake was corrected for simple diffusion by measuring ^{14}C L-Glucose (Perkin-Elmer) diffusion. Cells were lysed with 500 µl 0.2% SDS in dH20 for 60 min at 37°C. 200 µl of cell lysate were added to 5 ml of Ecoscint A scintillation fluid (National Diagnostics, Hessle/UK) and radioactivity counts were measured on a liquid-scintillation counter (LS 6500, Beckman-Coulter, High Wycombe/UK). The amount of substrate in the lysate was calculated using the specific activity of the uptake media.

2.4.3 RNA isolation

Cells on 24-well plates were washed with ice-cold PBS and then lysed with 400 µl cold TRIzol® reagent (Life Technologies, USA). RNA was then extracted, using a modified protocol based on Chomczynski & Sacchi (1987). 80 µl Chloroform and 96 µl RNase-free water were added to the cell lysate, and after vortexing thoroughly for 15 s incubated for 10 min on the bench. Tubes were then centrifuged at 13,000 RPM at 4°C for 10 min. The aqueous phase was transferred to a new tube and RNA was precipitated with ice-cold isopropanol in -20°C freezer for 30 min and then centrifuged 13,000 RPM at 4°C for 30 min. The resulting pellet was washed twice in 75% ethanol and centrifuged for 5 min at 13,000 RPM at 4°C. The ethanol was taken off and let sit on the bench to evaporate and was then redissolved in 30 µl water. The RNA concentration and purity were measured on a Nanodrop 2000 (Thermo Scientific, UK).

2.4.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

1 µg of RNA was digested using a RQ1 RNase-free DNase kit (Promega, UK) to get rid of any genomic contamination by incubating at 37°C for 30 min in a PTC-200 Peltier Thermal Cycler (MJ Research, Canada), as indicated by the manufacturer's protocol. Reverse transcription was performed using a High-Capacity cDNA reverse transcription kit (AppliedBiosystems, US) according to the manufacturer's manual. The resulting cDNA was then diluted 1:10 for further transcriptomic analysis.

PCRs were run with the FastStart Universal Probe Master according to the manufacturer's protocol on a PTC 200 Peltier Thermal Cycler (MT Research) with the following set up (see Table 1 & Table 2). All Primers were obtained from IDT (Leuven, Belgium) and reconstituted with ultrapure water. For PCR assays, primers (Table 3) were diluted to a stock concentration of 10 µM and used at a final concentration of 900 nM.

Table 1 Reaction setup for PCR

Reagent	Volume (µL)
FastStart Universal Probe Master (ROX)	10
Forward primer (900 nM)	1.8
Reverse primer (900 nM)	1.8
RNA-free water	4.4
cDNA (1:10 dilution)	2
Total Reaction Volume	20

Table 2 PCR protocol

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	95	95	60	4
Time (min)	10	0:15	1	∞
Cycles	1	40		1

Table 3 Primer sequences used for gene-expression analyses

Gene	Forward primer: 5'-3'	Reverse primer: 5'-3'	Amplicon
slc2a1	ggttgtgccatactcatgacc	cagataggacatccagggtagc	66 bp
slc2a2	gcatgtgccacactcacac	aaaaccagggtcccagtga	89 bp
slc2a3	gccctgaaagtcccagattt	ttcatctcctggatgtcttgg	115 bp
slc2a5	tccatttggagggtttatcg	aacagcaaggcccctttt	78 bp
slc5a1	ctggcaggccgaagtatg	ccacttccaatgttactagcaaag	68 bp
Slc5a4	aagctgctgcccatgttc	cgcattcagaaggtaccacac	94 bp
tas1r2	tgaagggcattgttcacctt	tgtagcctatcaccttcacttcat	91 bp
tas1R3	ttcagtgcaacgcctcag	cacgtggaaggtcaggttg	89 bp
gnat3	agcgagatgcaagaaccgta	cattcttatggatgatcttcatttgt	96 bp
nos2	gaccagtacgtttggcaatg	tttcagcatgaagagcgattt	73 bp

2.4.5 Agarose gel electrophoresis

PCR products were analysed on a 2% agarose gel made up of UltraPure Agarose (Life Technologies, Paisley/UK), Tris-Borate-EDTA buffer (45 mM Tris-borate/1 mM EDTA) and 5 µg Ethidium Bromide (Alfa Aesar, Heyshamm/UK). 4 µl of Blue/Orange 6X Loading Dye (Promega, Southampton/UK) were added to each PCR sample and run on the gel together with 12 µl DNA Ladder 100bp (Promega) at 120 V for 60 min. Gels were then visualised on a SynGene Genius Bioimaging system using GeneSnap, version 6.00.26 (Synaptics, Cambridge/UK).

2.4.6 Statistics

Data analysis and statistics were carried out either in Excel included in Microsoft Office Pro 2007, Version 12.0.6661.5000 SP3 MSO or in Graphpad Prism, Version 6.01. Data are given as arithmetic mean + standard deviation (SD). Significances were tested using the inbuilt two-sided T-Test of Excel, and ANOVA was tested in Prism with a Tukey post-hoc test with Bonferroni correction. A P-value of 0.05 or less was considered statistically significant.

2.5 Results

2.5.1 D-Glu & Fru uptake is carrier-mediated and linear in Caco-2/TC7 cells

Caco-2 cells are an established and widely used model for the study of transepithelial transport (Delie & Rubas, 1997). A substantial amount of time has gone into the optimisation of growth, differentiation and assay protocols, to use the Caco-2 cells as a model of enterocytes (data not shown). We decided to use the Caco-2/TC7 sub-clone with the protocols outlined in 2.4. This sub-clone has a higher expression of sugar transporters and hence higher sugar-uptake capacities than the parental strain (Chantret et al., 1994). All the following experiments in this chapter use cells grown and differentiated for 21 ±2 days in growth medium containing D-Glu at 25 mM. Sugar uptake assays are performed in 24-well dishes with cells attached to the bottom.

To establish our cell system and test our radio-labelled uptake assay we incubated cells in 10 mM D-Glu or L-Glu uptake media for a time course ranging from 1-30 min. Uptake of L-Glu, a measure for simple diffusion as it cannot be absorbed by any known transporter, is at a very low rate and stays below 20 nMoles at 30 min (Fig. 12).

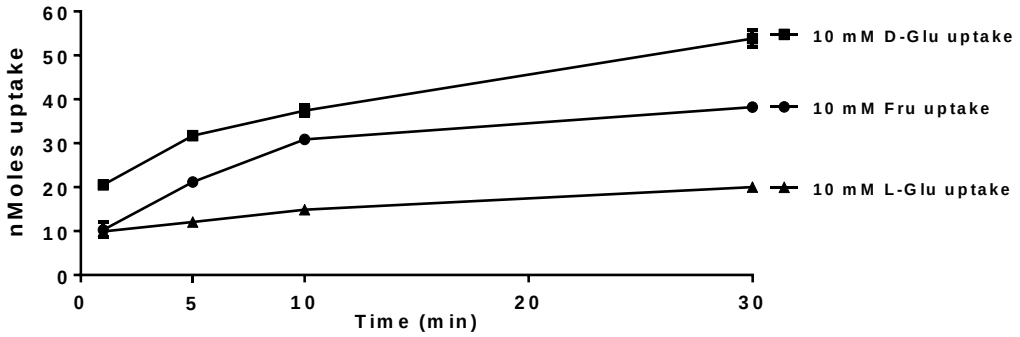

Fig. 12 Carrier-mediated C^{14} 10 mM D-Glu/Fru uptake over time in Caco-2/TC7 cells. Differentiated Caco-2/TC7 cells were incubated in 10 mM C14 D-Glu/Fru/L-Glu uptake media for 1, 5, 10 or 30 min and cellular uptake was measured. Data are expressed as nMoles uptake/well ± SD of n=4 per condition.

D-Glu and Fru uptake is readily detected above the simple diffusion 'baseline' set by L-Glu, indicating carrier-mediated uptake. Furthermore, uptake levels appear linear between 1 and 10 min, but then lag towards 30 min. We therefore used 10 min for further uptake experiments.

2.5.2 D-Glu transport is regulated by D-Glu in Caco-2/TC7 cells

To establish if our *in vitro* model is able to regulate sugar uptake upon nutrient availability, we incubated differentiated Caco-2/TC7 cells in KBS with concentrations of D-Glu between 2.5 and 75 mM for 3 h to mimic the physiologic situation and measured D-Glu-uptake capacity. D-Glu uptake is significantly more up-regulated the higher the concentration of D-Glu is in the preincubation media (Fig. 13). Uptake increased by 73% and 309% between 2.5 and 25 mM and 2.5 and 75 mM, respectively.

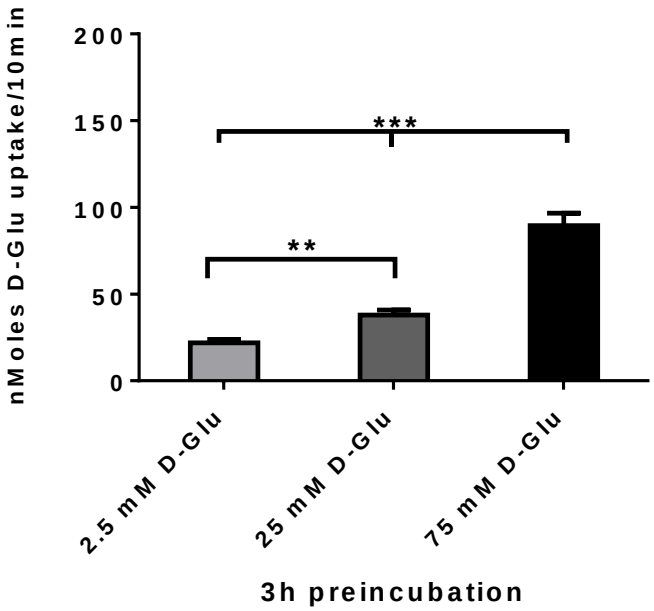

Fig. 13 Carrier-mediated C^{14} D-Glu uptake in Caco-2/TC7 cells after 3 h D-Glu preincubation. Differentiated Caco-2/TC7 cells were incubated for 3 h in KBS including D-Glu at the indicated concentrations. Osmolarity was adjusted for by adding Mannitol. D-Glu uptake was measured with radio-labelled D-Glu. Uptake is corrected for simple diffusion of L-Glu. Data are expressed as nMoles uptake/well/10min ± SD of n=4 per condition. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

2.5.3 Dissection of transporters responsible for higher D-Glu transport

Previous studies have shown that SGLT1, GLUT2 and GLUT5 are the main hexose-transporters in enterocytes in humans and rodents (Ferraris, 2001). To identify which hexose transporter genes are present and could contribute to the observed upregulation seen in Fig. 13, we performed RT-PCR on differentiated Caco-2/TC7 cells.

Gene transcripts for *slc5a1/*SGLT1, *slc2a2/*GLUT2 and *slc2a5/*GLUT5, transporters for D-Glu, D-Glu+Fru or Fru, respectively, are expressed as expected in enterocytes (Fig. 14). In addition, the D-Glu transporters *slc2a1/*GLUT1 and *slc2a3/*GLUT3 are highly expressed in Caco-2/TC7 cells (Fig. 14).

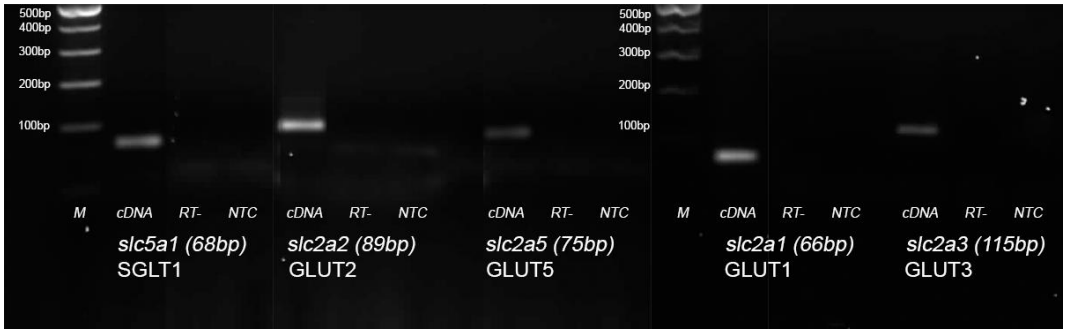

Fig. 14 Gene expression of hexose transporters in Caco-2/TC7 cells. Gene expression for SGLT1 (*slc5a1*), GLUT2 (*slc2a2*), GLUT5 (*slc2a5*), GLUT1 (*slc2a1*) and GLUT3 (*slc2A3*) at 21 days of culture of Caco-2/TC7 cells. Shown are the marker ladder (M), one representative sample (cDNA), a reverse transcriptase negative (RT-) and non-template control (NTC) for each gene.

To determine if the up-regulation in D-Glu transport seen in Fig. 13 is mediated by SGLT1 and/or GLUT2, we measured uptake of D-Glu in the presence of inhibitors. Phloridzin (PZ) is known to inhibit only SGLT1 (Helliwell & Kellett, 2002), whereas Phloretin (PT) only inhibits GLUT2 (Kwon et al., 2007). Cytochalasin B (CB) inhibits GLUT 1-4, 7, 8. Inconsistent with previous publications (Zheng et al., 2011), we were not able to detect inhibition by any of the inhibitors (PZ, PT, CB) at 25 mM D-Glu preincubation concentration (Fig. 15). At 75 mM D-Glu neither PZ nor CB decreased uptake, but PT was

able to inhibit transport by ~33%. This would indicate an involvement of GLUT2 in the upregulation of D-Glu transport. As PZ does not inhibit transport at 25 mM and as PT, but not CB inhbits transport at 75 mM possible reasons for this observation are discussed later and further confirmation is required.

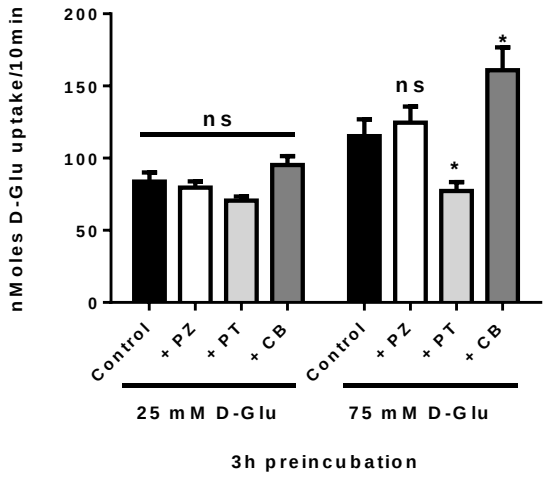

Fig. 15 Carrier-mediated D-Glu uptake in Caco-2/TC7 cells. Differentiated Caco-2/TC7 cells were incubated for 3 h in KBS including D-Glu at the indicated concentrations. Osmolarity was adjusted for by adding Mannitol. Uptake was measured in the presence of inhibitors of sugar transporters; Phloridzin (PZ) & Phloretin (PT) at 500 µM, Cytochalasin B at 10 µM. The control has DMSO added at a final concentration 0.1%. Uptake is corrected for simple diffusion of L-Glucose. Data are expressed as nMoles uptake/well/10min ± SD of n=4 per condition. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

GLUT2 is a known D-Glu, but also Fru transporter. To determine if the upregulation of D-Glu was caused by GLUT2 we repeated the experiment, but measured Fru uptake after D-Glu preincubation. Although Fru uptake in Caco-2/TC7 cells is lower than D-Glu uptake, an acute and statistically significant upregulation can be detected (Fig. 16a). Comparing the 2.5 mM with 75 mM preincubation concentration, Fru uptake is ~81% higher.

We added PT and CB to the uptake media to dissect the transport mechanism. As seen in Fig. 16b, at 25 mM preincubation concentration Fru uptake is inhibited by PT

by ~60%, but not by CB, which indicates GLUT2 activity. At the higher concentration of 75 mM of D-Glu, PT inhibits Fru uptake by ~43% and CB by ~55%, indicating involvement of GLUT2, but not GLUT5.

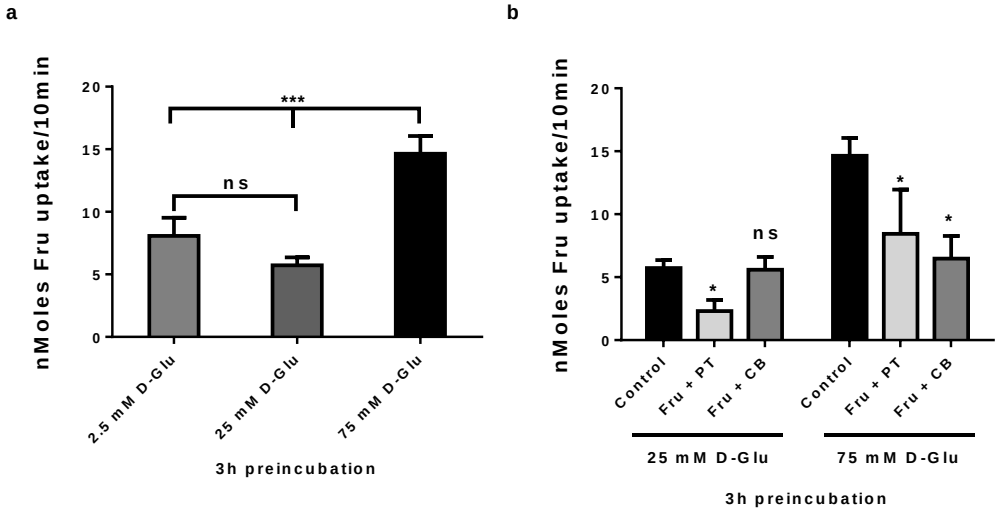

Fig. 16 Carrier-mediated C^{14} Fru uptake in Caco-2/TC7 cells after 3 h D-Glu preincubation. Differentiated Caco-2/TC7 cells were incubated for 3 h in KBS including D-Glu at the indicated concentrations. Osmolarity was adjusted for by adding Mannitol. **a:** Fru uptake was measured with radio-labelled Fru. **b:** Uptake was measured in the presence of inhibitors of sugar transporters; Phloretin (PT) at 500 µM, Cytochalasin B at 10 µM. The control has DMSO added at a final concentration 0.1%. Uptake is corrected for simple diffusion of L-Glucose. Data are expressed as nMoles uptake/well/10min ± SD of n=4 per condition. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

Taken together, this suggests that Caco-2/TC7 cells are able to respond to short-term stimulation (3 h), enabling them to upregulate D-Glu- and Fru-uptake capacities. Yet a dissection of which transporters are responsible for the increased uptake was not fully achieved.

2.5.4 The sweet-taste receptor T1R2/3 does not regulate sugar transport in Caco-2/TC7 cells

It has been described that the rodent intestine stimulates GLUT2 expression/apical translocation via the sweet-taste receptor T1R2/3 (Mace, Affleck, et al., 2007). To determine if the sweet-taste receptor T1R2/3 is expressed on a transcription level in Caco-2/TC7 cells, we extracted RNA from differentiated cells for subsequent RT-PCR. As seen in Fig. 17, only *tas1r3* (T1R3) is expressed, but not *tas1r2* (T1R2) or the downstream signalling molecule αGustducin (*gnat3*).

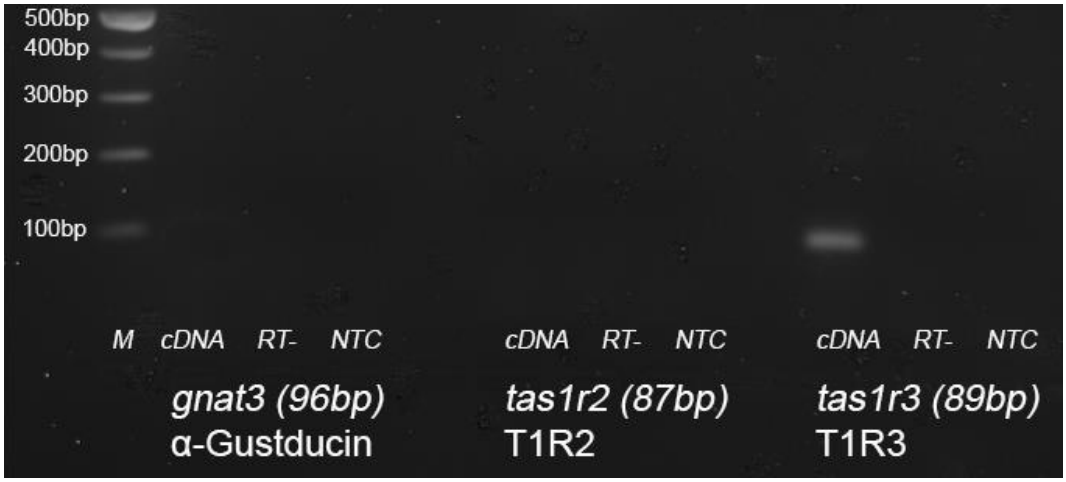

Fig. 17 Gene expression of the sweet-taste machinery in Caco-2/TC7 cells. Gene expression of αGustducin (gnat3), T1R2 (tas1r2) and T1R3 (tas1r3) at 21 days of culture of Caco-2/TC7 cells. Shown are the Ladder (M), one representative sample (cDNA), a reverse transcriptase negative (RT-) and non-template control (NTC) for each gene.

Knockout mouse models have shown that T1R2 or T1R3 on their own can still sense natural sugars at higher molarities, but double knockouts completely lose functional sweet sensing (Zhao et al., 2003). Furthermore, HEK-293 cells transfected with only T1R3 also showed responses to high molarities of sucrose (Zhao et al., 2003). To test if T1R3 can be functional on its own in Caco-2/TC7 cells, we added the T1R3 inhibitor Lactisole to preincubation media and measured D-Glu uptake. As demonstrated in Fig. 18a, Lactisole does not inhibit the acute upregulation of D-Glu.

Furthermore, we added sweet-taste-receptor agonists in the form of the artificial sweeteners AcesulfameK and Sucralose during the preincubation in the presence/absence of Lactisole. Neither is D-Glu uptake enhanced by the added artificial sweeteners, nor is transport activity inhibited by the added Lactisole (see Fig. 18b), indicating no functional sweet-taste receptor in Caco-2/TC7 cells.

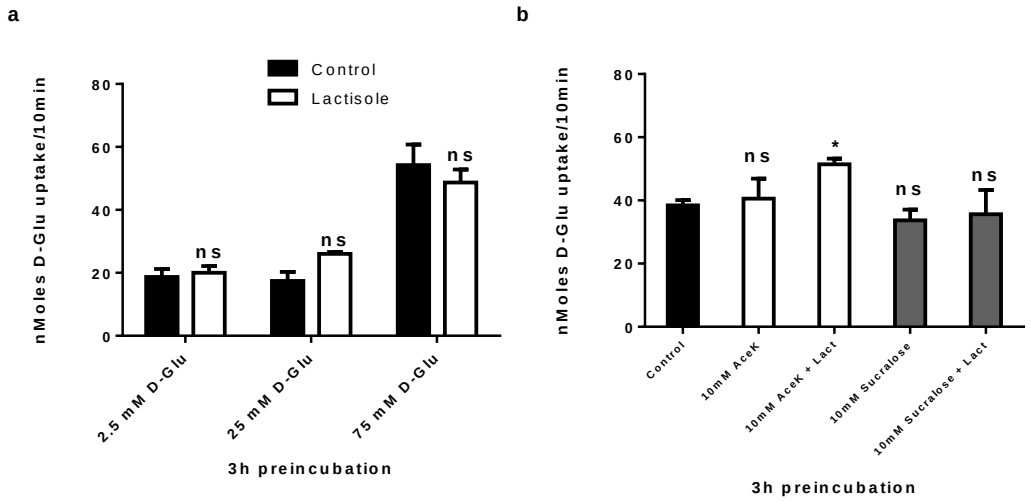

Fig. 18 Regulation of D-Glu uptake by the sweet-taste receptor T1R2/3. Caco-2/TC7 were seeded at a density to reach confluence at day 7 and grown for a total of 21 days to differentiate. **a:** Cells were incubated for 3 h in KBS including D-Glu at the indicated concentrations in the presence of Lactisole at 500 µM. The control contains the same amount of solvent (ddH20). **b**: Cells were incubated with 75mM D-Glu in the presence of 10 mM AcesulfameK K (AceK) or Sucralose with the addition Lactisole at 500 µM. Osmolarity was adjusted for by adding Mannitol. D-Glu uptake was measured. Uptake is corrected for simple diffusion of L-Glucose. Data are expressed as nMoles uptake/well/10min ± SD of n=4 per condition. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

2.5.5 HFCS, but not sucrose or fructose, stimulates sugar transport in Caco-2/TC7 cells

Dietary Fru is able to regulate Fru-transporter activity (Corpe & Burant, 1996). In alignment with the previous experiments, we sought to study if the sweet-taste receptor is involved in the regulation of Fru transport. As the main forms of Fru in our diet are sucrose and HFCS, and to a smaller degree free Fru, we preincubated cells with increasing concentrations of sucrose, a 1:1 mix of D-Glu+Fru or Fru for 3 h and subsequently measured D-Glu and Fru uptake. It has been suggested that HFCS stimulates sugar absorption to a higher degree than sucrose as it skips the rate-limiting cleavage of sucrose by brush-border-bound sucrose-isomaltase.

Cells did not react to the higher concentration of sucrose with increased D-Glu or Fru uptake (Fig. 19a+b). After preincubating cells for 3 h in HFCS D-Glu uptake, but not Fru uptake, increased significantly (Fig. 19c+d). The upregulation is likely due to the free D-Glu molecules, comparing it with sucrose. Adding Lactisole, we saw that the upregulation in D-Glu uptake after HFCS preincubation was not mediated by the sweet-taste receptor (Fig. 19c), confirming our data from section 2.5.4.

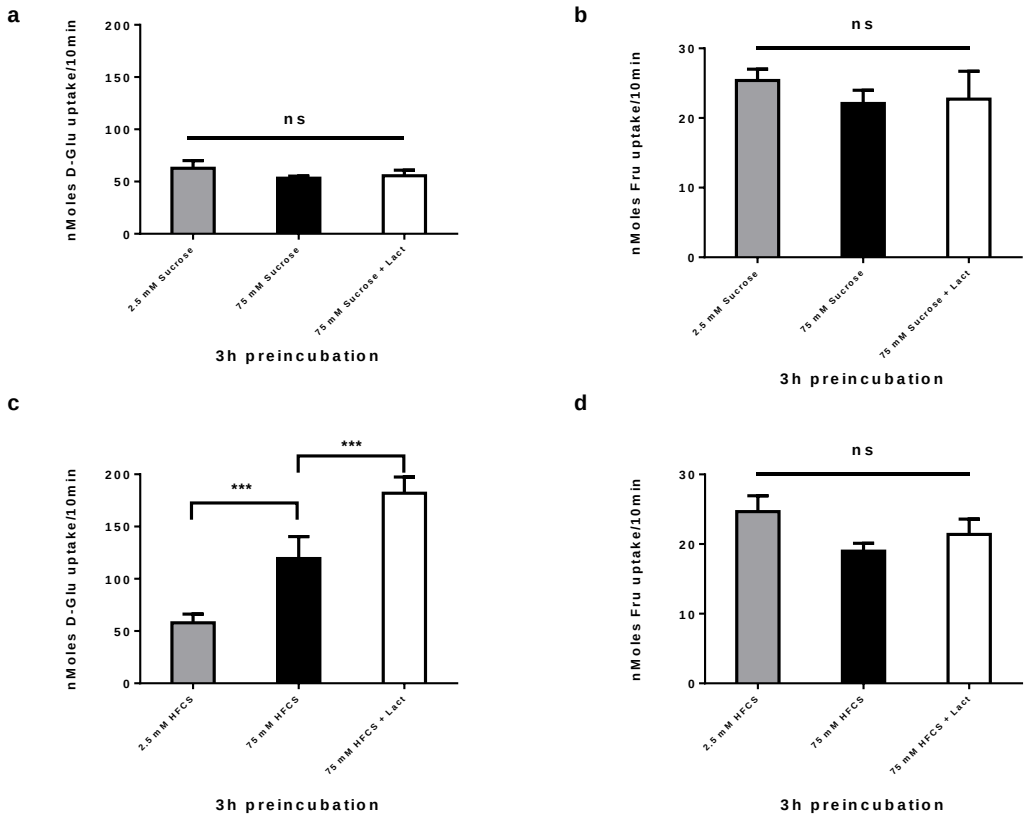

Fig. 19 Stimulation of D-Glu/Fru transport with sucrose and HFCS in Caco-2/TC7. Differentiated cells were incubated for 3 h in KBS including sucrose (**a, b**) or HFCS (**c, d**) at the indicated concentrations. Osmolarity was adjusted for by adding Mannitol. D-Glu uptake (**a, c**) or Fru uptake (**b, d**) was measured. Lactisole at 500 µM was added to block the sweet-taste receptor. Uptake is corrected for simple diffusion of L-Glucose. Data are expressed as nMoles uptake/well/10min ± SD of n=4 per condition. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

Preincubation with Fru did not increase D-Glu- or Fru-transport capacity (Fig. 20a+b).

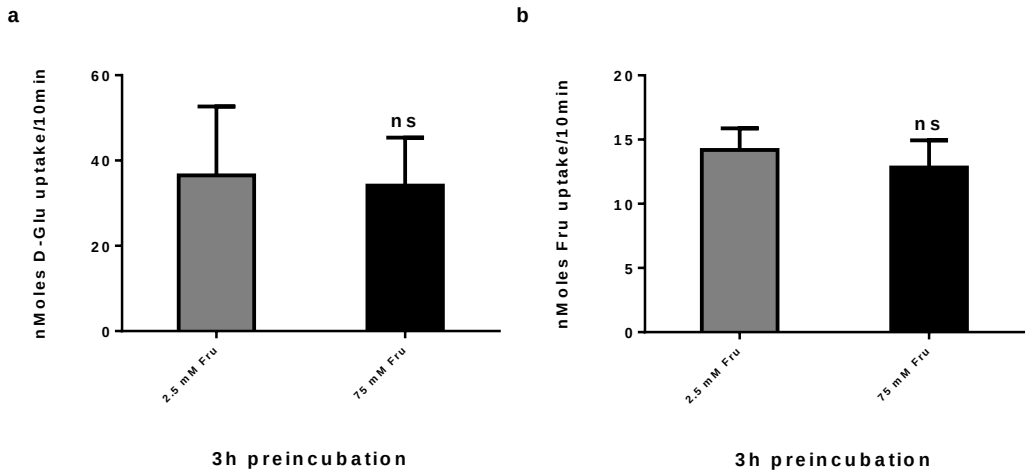

Fig. 20 Stimulation of D-Glu/Fru transport by Fru in Caco-2/TC7 cells. Differentiated cells were incubated for 3 h in KBS including Fru at the indicated concentrations. Osmolarity was adjusted for by adding Mannitol. D-Glu uptake (**a**) or Fru uptake (**b**) was measured. Uptake is corrected for simple diffusion of L-Glucose. Data are expressed as nMoles uptake/well/10min ± SD of n=4 per condition. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

2.6 Discussion

The gastrointestinal tract is the first barrier in contact with nutrients and is a complex endocrine organ integrated into whole body metabolism. It has evolved intricate mechanisms to acutely and chronically regulate its transport capacities upon postprandial nutrient load. Since the late 1990s a new concept has been that the gut senses foods to induce an appropriate response (Raybould, 1998). The molecular mechanisms by which sugar transport is regulated are not well studied, and controversial. Two models exist, both using the sweet-taste receptor to regulate hexose-transport capacities: a direct model where the sweet-taste receptor directly regulates sugar transport capacities on enterocytes and an indirect model where EECs sense nutrients and regulate transport function in neighbouring enterocytes.

We used the intestinal Caco-2/TC7 cell model to identify the role of the sweet-taste receptor T1R2/3 in the regulation of intestinal sugar transport by dietary carbohydrates. We found an acute response of D-Glu transporters after stimulating cells with increasing concentrations of D-Glu, which was potentially mediated via GLUT2 but was independent of the sweet-taste receptor.

We readily detected sugar uptake using our cell model and uptake assay and also detected upregulated D-Glu and Fru transport upon 3 h short-term stimulation with D-Glu. This indicates that monosaccharide load can be sensed and transport regulated. Applying gene-expression techniques, we detected expression of the small-intestinal sugar transporters SGLT1, GLUT2 and GLUT5, as expected. Using inhibitors to dissect transport pathways at the basal level of 25 mM D-Glu preincubation concentration we were not able to establish which transporters are responsible for the observed

upregulation. In contrast, at 75 mM D-Glu we detected PT sensitivity, indicating GLUT2 activity. This observation is in alignment with the direct model of regulation.

To study the existence and functionality of the sweet-taste receptor in Caco-2/TC7 cells, we analysed expression of *tas1r2, tas1r3* and *gnat3* at the mRNA level. We were only able to detect transcripts of *tas1r3,* indicating a functional sweet-taste receptor cannot be existent in Caco-2/TC7 cells. Adding artificial sweeteners or Lactisole in preincubation media to stimulate/block the sweet-taste receptor, we saw no significant enhancement or inhibition of transport, indicating that D-Glu sensing in Caco-2/TC7 cells may not be mediated via the sweet-taste receptor.

Since Caco-2/TC7 cells only express *tas1r3,* and it has been shown that *tas1r2* knockout mice can still sense sugars, although not artificial sweeteners (Zhao et al., 2003), we expanded our work to study and compare the sensing of sucrose, HFCS and Fru in Caco-2/TC7 cells. Incubating cells with increasing concentrations of sucrose or Fru showed no up-regulation in transport of D-Glu or Fru. In contrast, HFCS preincubation showed upregulated D-Glu uptake, which is likely due to the free D-Glu available in HFCS to stimulate transport activity, which is in support of our previous data.

The lack of a functional sweet-taste receptor and the sensing specificity for D-Glu but not for Fru, suggest another sensing mechanism. In fact, it has been suggested that a novel sugar sensor, is involved in intestinal nutrient sensing with high specificity for D-Glu (Diez-Sampedro et al., 2003). First described as expressed in intestinal cholinergic neurons and smooth muscle, it has also been shown to be expressed in the intestinal mucosa of rats (Freeman et al., 2006). We speculate that SGLT3 is potentially involved in D-Glu sensing on the enterocyte, and more research is needed.

Since Caco-2/TC7 cells only express T1R3 on a transcription level and we did not see functionality regarding D-Glu sensing, we expanded our work to Fru and sucrose. We were not able to detect transport activation with increasing concentrations of sucrose or free Fru. Either sucrose is not sensed or Sucrase-Isomaltase is rate-limiting the availability of mono-saccharides, and hence transporter expression or transporter translocation to the apical membrane does not need to be upregulated. Le Gall *et al*. reported in 2007 that Caco-2/TC7 cells can react to Fru incubation with increased SGLT1, GLUT2 and GLUT5 mRNA expression and that insulin is able reduce GLUT2 trafficking to the brush boarder (Tobin et al., 2008). The observed SGLT1 and GLUT5 mRNA upregulation was blockable with Lactisole. The increased mRNA levels may not carry over into increased uptake levels, although this is in strong contrast to animal studies, as it has been shown that Fru regulates GLUT5 transport capacity (Corpe et al., 1996).

Our findings are in contrast to previous literature and models (Le Gall et al., 2007; Zheng et al., 2011; Zheng & Sarr, 2013). The Brot-Laroche lab shows functional sugar sensing in Caco-2/TC7 cells and upregulated sugar-transporter transcripts for SGLT1 and GLUT5 upon stimulation, which is blockable by Lactisole. They also show T1R2 and T1R3 protein expression using immunohistochemistry, but only show RT-PCR data for *gnat3* and *tas1r3*, but not *tas1r2* (Le Gall et al., 2007). We question why a PCR for *tas1r2* has not been carried out/included in the publication. Further confirmation of the immunohistochemistry data is needed, as the specificity of the antibodies used is not shown. Furthermore, Le Gall's study starved differentiated Caco-2/TC7 cells for 2 days prior to performing experiments. Not only is this unphysiological, but from our experience it is very detrimental to cell health/survival; 48 h starvation resulted in mono-layer detachment and cell death for us.

Michael Sarr's group used very similar protocols to us, but utilised the parental Caco-2 cell strain and they were able to show dissection of transport pathways with PZ and PT (Zheng et al., 2011). We therefore acquired the parental Caco-2 strain and repeated our Caco-2/TC7 experiments, but achieved very similar results to the Caco-2/TC7 results (data not shown). Zheng *et al.* also studied the stimulation of D-Glu transport by artificial sweeteners and confirmed our results (insensitivity to T1R3 agonists). Yet they were able to demonstrate that with only a 5 min uptake stimulation of carrier-mediated D-Glu uptake with AcesulfameK was blockable with CB or a PLCβII inhibitor (Zheng & Sarr, 2013). The activation of the sweet-taste receptor was able to increase D-Glu uptake at concentrations higher than 25 mM although no statistical analysis was performed to support their data.

2.7 Limitations of the study

Despite the interesting findings from our study, several limitations appear. The variable findings in our inhibitor experiments to dissect transport pathways and the lack of sweet-taste-receptor mediation could be due to our cell line. As Caco-2 cells are a colonic adenocarcinoma cell line that spontaneously differentiates to an enterocyte-like phenotype, certain cancer-like characteristics cannot be removed during their spontaneous differentiation and we show high expression of GLUT1 and GLUT3.

Furthermore, it is very common for cell lines to evolve and change phenotypic properties upon long-term passaging or wrong culture conditions, which has been extensively written about regarding Caco-2 cells (Sambuy et al., 2005). Our Caco-2/TC7 clone was obtained many years ago directly from the Brot-Laroche lab and has since been handled by many different researchers and students in the lab. It stands to reason

that there has been a change in the cell's functional characteristics. We also obtained a parental Caco-2 cell line from another lab and a complete fresh stock from ATCC/Sigma in order to compare sub-clones with each other and to the literature, and saw very similar results to the ones demonstrated. It would be of interest to repeat our experiments in other enterocyte *in vitro* models such as RIE-1 or IEC-6 cells.

It cannot be excluded that assay conditions were not optimal regarding the experimental questions, especially regarding inhibitor studies to dissect transport pathways. Yet we used the same assay conditions for SGLT1 or GLUT2 expressing oocytes to study inhibition by PZ, PT and CB which indicates that our sugar uptake conditions are correct (data not shown). The high expression of GLUT1 and GLUT3 may overshadow the uptake by SGLT1, GLUT2 and GLUT5 and hence render the cells insensitive to the inhibitors.

Taken together, it leads us to question the suitability of Caco-2 cell lines as an *in vitro* model to study intestinal sugar sensing in the regulation of sugar transport.

2.8 Conclusion

In conclusion, our *in vitro* data are in line with human studies showing that only natural sugars, but not artificial sweeteners can lead to small-intestinal functional responses, such as slowed gastric emptying, enhanced sugar transport, the release of incretins (Fujita et al., 2009; Ma et al., 2009; Ma et al., 2010). Our data suggest that the observed acute upregulation of D-Glu uptake after D-Glu or HFCS preincubation is not mediated via the sweet-taste receptor, but potentially via another sensor such as SGLT3 or directly by the sugar transporters.

Based on our findings we support the direct model in line with Kellett, Brot-Laroche and Sarr via another sensing mechanism, but only for D-Glu and HFCS. For Fru and sucrose we suggest an indirect model of transport regulation mediated via EECs. Further research (e.g. in EEC cell models) and other, more physiological enterocyte models are needed to elucidate exact mechanisms.

CHAPTER 3

DIURNAL REGULATION OF NUTRIENT SENSORS ALONG THE ALIMENTARY TRACT

3 DIURNAL REGULATION OF NUTRIENT SENSORS ALONG THE ALIMENTARY TRACT

3.1 Summary

Background Distributed along the length of the gastrointestinal tract are macronutrient sensing mechanisms that regulate intestinal nutrient transport capacity and the release of peptides that influence gastrointestinal function and energy balance. Recent data has shown a circadian rhythm of GLP1 secretion. However, there have been no reports showing clock gene regulated expression of macronutrient sensors.

Aims We hypothesised that macronutrient sensors and gut peptides display a diurnal rhythm anticipatory to nocturnal feeding in the alimentary tract that is controlled by clock genes.

Methods To test our hypothesis we studied the expression levels of clock genes, nutrient sensors and gut peptides in the alimentary tract of nocturnal feeding rodents. We applied gene expression profiling techniques to further study clock-controlled genes in the murine tongue.

Results The novel sugar sensor SGLT3a showed clear rhythmicity in the small intestine and SGLT3b demonstrated a clear rhythm in the stomach, whereas T1R1, T1R2 and T1R3 displayed a much more limited rhythm. The tongue showed robust clock gene activity, but no rhythmicity in the studied nutrient sensors. Microarray analysis on the tongue revealed a high regulation of bitter sensors, enzymes and gut peptides, and also of genes involved in immunity and signal transduction.

Conclusions Taken together, this data suggests a novel interaction between clock genes and nutrient sensor mechanisms in the alimentary tract that is possibly involved in the

food-entrainable part of the mammalian clock. Disturbances in clock gene/nutrient sensing interactions may be important in the development of diet-related diseases.

3.2 Background

3.2.1 Circadian clock

Most living organisms on earth depend upon the energy harnessed from sunlight. The daily pattern of night and day caused by the earth's rotation around its own axis has influenced the evolution of life forms and species. Circadian adaptions can be found in all branches of the tree of life, as they provide advantages for organisms to anticipate environmental oscillations. This is most obvious in plants and the daily change of leaf movements of the Tamarind tree was described as early as 400 BC (Bretzl, 1903).

In animals, the circadian system regulates daily rhythms of behaviour and physiology, enabling organisms to anticipate periodic alterations in the environment and adjust necessary adaptive mechanisms. The most obvious of these adaptions is sleep/wake cycles. Animals can be categorised according to their highest activity levels, which usually means food-search behaviour; these groups are described as during the day (diurnal), during the night (nocturnal), in the twilight (crepuscular), in the morning twilight (matutinal) or in the evening (vespertine). Different processes in the body need to be active during certain times of the day, and others shut off so as not to waste energy/resources. Such behaviour enables optimal reproduction and energy utilisation (Panda, Hogenesch, et al., 2002).

The behavioural and physiological adaptations are governed by a highly developed, conserved and complex circadian machinery in animals. Light signals on the retina are sent to the suprachiasmatic nucleus (SCN) in the brain via the retinohypothalamic tract. The SCN is termed the 'master clock', as it synchronises the 24

h light-dark cycle and entrains the body with necessary phenotypic adaptions. Peripheral clocks are also found in all tissues and are governed by the SCN. The central and peripheral clocks are established on a molecular level. The canonical mechanism is a transcriptional–translational feedback loop that drives a ~24 h expression pattern of the core clock machinery. CLOCK and BMAL1 proteins, members of the basic helix-loop-helix (bHLH) family, dimerise and bind E-Box elements of target genes to enhance transcription. Two target gene groups are *period (per1-3)* and *cryptochrome (cry1, cry2),* which heterodimerise, translocate into the nucleus and promote histone deacetylase binding, which represses CLOCK:BMAL1 expression. This completes the negative feedback loop and establishes an oscillating pattern (see Fig. 21a).

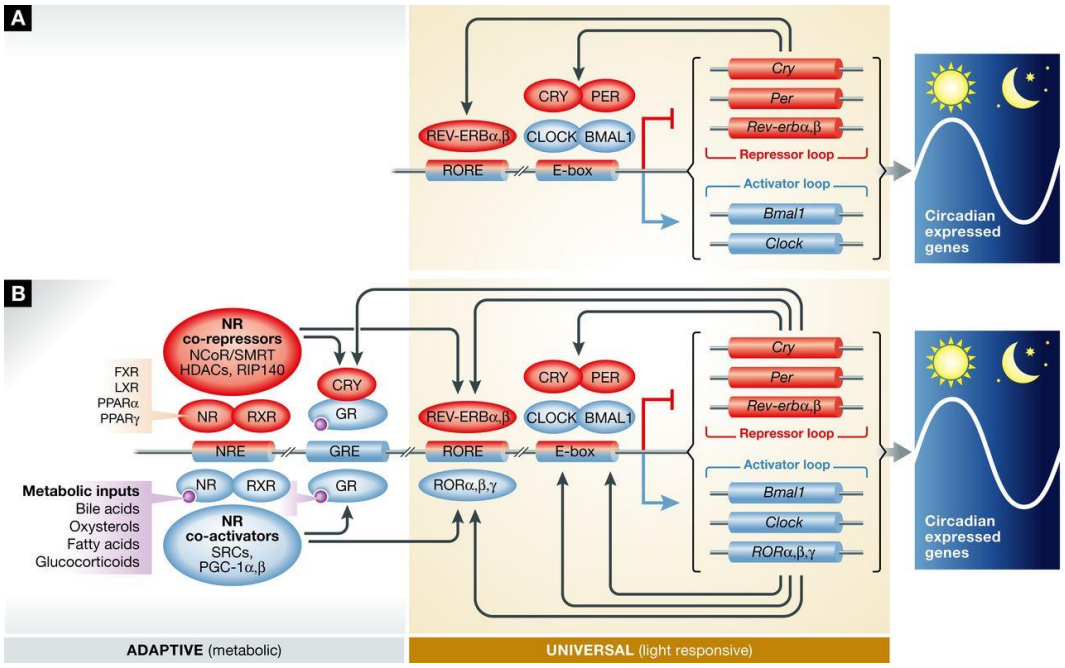

Fig. 21 Mechanism of the molecular clock. A: The canonical, core mechanisms governed by CLOCK, BMAL1, CRY and PER inducing a transcriptional–translational negative feedback loop. **B**: The emerging model of the cellular clock mechanism with a variety of transcription and nuclear factors regulating and fine tuning the cellular circadian rhythm. (Figure reproduced from ZHAO *et al.*, 2014).

Over the years, knockout, mutation and experimental studies have revealed a more complex and fine-tuned model (Fig. 21b). A host of nuclear factors such as the retinoic acid-related orphan nuclear-receptor family, including REV-ERB and RXR, bind response elements (RE) in promotors of core clock genes to stimulate/repress transcription. Nuclear receptors have a variety of dimerisation partners such as Peroxisome Proliferator-activated receptors (PPAR), which in turn can be bound by a multitude of ligands and metabolites (e.g. fatty acids, Vitamin D, retinoic acid; Fig. 21b). Not only do clock genes and nuclear factors regulate each other, but it has been suggested that ~10% of cellular gene transcripts are diurnally regulated (Panda *et al.*, 2002). These genes are termed clock-controlled genes (CCGs).

3.2.2 Circadian clock and metabolic disease

In today's society meal timing and patterns have changed, and this is most evident in shift workers. It has been suggested that these changes increase the risk for metabolic diseases (Pan et al., 2011). Evidence is accumulating on how perturbations of clock systems and sleep constitute risk factors for diseases (e.g. cancer) and metabolic disorders such as obesity, diabetes mellitus and cardiovascular disease (Zelinski et al., 2014). Polymorphisms in circadian regulatory genes have been linked to obesity and type 2 diabetes (Scott et al., 2008). Disruption of *arntl1*/BMAL1 gene function caused an abnormal metabolic profile with hyperleptinemia, glucose intolerance and dyslipidaemia (Rudic et al., 2004). *Clock/*CLOCK mutant mice not only have disrupted sleep and feeding patterns, but are hyperphagic and develop obesity (Turek et al., 2005). Similarly, disruption of sleep and sleep patterns such as in shift workers have been shown to

disturb circadian rhythm and increase the risk of metabolic syndrome (Lund et al., 2001; Pan et al., 2011). Sleep–wake cycles can become disturbed and light exposure and eating during the night pose time-inappropriate cues that disrupt clock mechanisms in the SCN, in the periphery and on a cellular level. Altered circadian rhythmicity could be affecting the body through both disrupted metabolism and consequent behavioural modification, producing a vicious cycle of altered cellular machinery, activity levels and adverse dietary choices, leading to a disease state.

Maintaining circadian rhythms protects the body from metabolic diseases (Nohara et al., 2015). Behavioural, environmental and dietary measures are at the core of maintaining and stabilising these rhythms, but targeting clock genes and CCGs with pharmacological agents is also an emerging possibility (Challet, 2013). Small molecules, dubbed 'clock-amplitude-enhancing small molecules', have been identified and are able to target core clock genes. These molecules have been shown to have stabilising and beneficial properties on sleep and metabolism (Chen et al., 2012; Chen et al., 2013).

The central clock is synchronised and reset every day by light on the retina, termed 'Light-entrainable oscillator' (LEO), but food and feeding regimens have been also shown to synchronise a 'food-entrainable oscillator' (FEO). The FEO increases 'food-anticipatory activity' before mealtime, which raises general activity levels and food-finding patterns. The FEO also prepares the body for feeding, including body temperature and corticosterone and growth-hormone levels, as well as intestinal motility (Krieger, 1974; Moberg et al., 1975; Comperatore & Stephan, 1987).

The FEO is thought to be synchronised by an array of neuronal and humoral signals from the gastrointestinal tract, including gut peptides and also blood-glucose and

insulin levels (reviewed in Carneiro & Araujo, 2009). Yet the exact molecular identity of the sensors and the precise mechanisms responsible for FEO entrainment are not clear. It has been shown that a host of intestinal processes display diurnal rhythmicity. Nutrient transporters such as hexose and peptide transporters on enterocytes display circadian rhythmicity anticipatory to feeding (Corpe & Burant, 1996; Pan et al., 2002). GLP1 has been demonstrated to have a circadian pattern of expression only very recently (Gil-Lozano et al., 2014). Insulin is secreted from the pancreas in a circadian fashion (Jarrett et al., 1972; Boden et al., 1996). A GLP1-receptor agonist, Liraglutide, has been shown to restore the rhythmicity of clock genes and insulin secretion in pancreatic β-cells of diabetic mice (Wang et al., 2015). The regulation of GLP1 secretion is taste-receptor-dependent (Gerspach et al., 2011; Steinert et al., 2011). Furthermore, sweet-taste recognition in humans displays diurnal rhythmicity (Nakamura et al., 2008). Yet there are no reports in the literature demonstrating a diurnal pattern of expression for the umami- and sweet-taste receptors or the novel D-Glu sensor SGLT3 in the alimentary tract. Their role as sensors implicate their ability to mediate food entrainment cues into the peripheral and central clock. Since insulin and GLP1 secretion display circadian rhythms, we hypothesised that nutrient sensors in the alimentary tract also display diurnal rhythmicity anticipatory to feeding.

Better understanding of circadian rhythmicity of chemosensors in the gastrointestinal tract could help to optimise meal timing and also to avoid metabolic problems observed in shift workers.

3.3 Chapter Overview – Diurnal expression of nutrient sensors along the alimentary tract

Aims

Evaluate the diurnal expression of nutrient sensors in the murine alimentary tract.

Hypothesis

Mice show diurnal rhythms of nutrient sensors in the alimentary tract anticipatory to nocturnal feeding, which are regulated by the mammalian clock machinery.

Objectives

- Evaluate diurnal rhythms of nutrient sensor gene expression in the alimentary tract of mice
- Study the promotors of nutrient sensors for circadian E-Box elements
- Assess the expression profiles in the tongue for diurnally regulated genes

3.4 Materials & Methods

3.4.1 Animal *ex vivo* studies

Sixteen juvenile male CD-1 mice were kept for six weeks under standard conditions with free access to standard chow and water. A standard 12-hour dark/light cycle, with the start of the light cycle at 6am and the start of the dark cycle at 6pm, was under automatic regulation. The mice were randomly divided into two groups (7am and 7pm) before being euthanised by cervical dislocation whilst under halothane anaesthesia. 7am mice (n=8) were euthanised between 6 and 7am, whilst 7pm mice (n=8) were euthanised between 6 and 7pm. Tissues were dissected into tubes and immediately snap-frozen in liquid nitrogen for further RNA extraction.

Due to the small tissue size available from the CD-1 mouse samples, more tissue was dissected from C57BL/6 mice. Fifteen eleven-week-old male mice were housed with a 12 h light/dark cycle. They were randomly assigned into two groups (7am: n = 8, 7pm: n=7). As before, mice were anaesthetised and euthanised and tissues were dissected into tubes and immediately snap-frozen in liquid nitrogen for further protein extraction.

Transcriptomic findings found in the CD-1 tissues were confirmed in C57BL/6 tissues to justify the proteomic analysis.

3.4.2 RNA extraction

1 ml RNAlater®-ICE solution (Life Technologies, USA) was added to tissues and tubes were transferred to a -20°C freezer to penetrate the tissues overnight. 15 mg of tissue were then weighed out into a 2 ml tube with a 5 mm steel bead (Qiagen, Netherlands) while kept on dry ice. 1 ml of Qiazol (Qiagen) was then added to

homogenise the tissues in a Qiagen TissueLyser for 3x30 s at 30 Hz. RNA was then extracted using an RNeasy Lipid Tissue Mini Procedure (Qiagen, Netherlands), following the manufacturer's protocol.

3.4.3 Microarray analysis

400 ng of RNA was pooled together from each group (7am/7pm) and integrity was confirmed by analysing it on an Agilent Bioanalyzer chip. RNA was then labelled with a GeneChip® 3' IVT Express Kit (Affymetrix, USA) and analysed using a GeneChip MouseGenome 430A 2.0 Array following the manufacturer's instructions. Data was analysed using the GeneGo software (MetaCore™, version 6.19 build 65960 - Thomson Reuters).

3.4.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

1 µg of RNA was digested as indicated in 2.4.4. The RNA was then reverse transcribed with a High capacity RNA-to-cDNA kit (Applied Biosystems, USA) according to the manufacturer's protocol in a PTC-200 Peltier Thermal cycler. The resulting cDNA was diluted 1:10 in water for further analysis.

Primers were designed by using Roche's online ProbeFinder Software, ver. 2.50. The human genome was selected to design PCR assays over exon-exon borders for transcriptome specificity (Table 4). All primers were obtained from IDT (Leuven, Belgium) and reconstituted with ultrapure water to a stock concentration of 10 µM.

cDNA was amplified using a FastStart Universal Probe Master (Rox) (Roche, Switzerland) mastermix with primers (see Table 4) at a concentration of 900 nM each, unless otherwise indicated. 2 µl of diluted cDNA were added, to achieve a total volume

of 20 µl. This amounts to ~10 ng cDNA per reaction. PCR was performed with the following protocol on a PTC-200 thermocycler: 10 min at 95°C for polymerase activation, 40 cycles of 95°C annealing for 15 s and 1 min at 60°C for amplification. PCR products were visualised as outlined in 2.4.5.

Table 4 Primer sequences and probes used in RT-PCR & real-time PCR

Gene	Forward primer: 5'-3'	Reverse primer: 5'-3'	Amplicon bp	Probe #
18s	gcaattattccccatgaacg	gggacttaatcaacgcaagc	68	48
hprt1	tcctcctcagaccgctttt	cctggttcatcatcgctaatc	90	95
hmbs	tccctgaaggatgtgcctac	aagggttttcccgtttgc	73	79
slc5a1	ctggcaggccgaagtatg	ttccaatgttactggcaaagag	65	49
slc2a2	gggccatcaacatgatcttc	aatcatcccggttaggaaca	86	70
slc2a5	agagcaacgatggaggaaaa	ccagagcaaggaccaatgtc	64	94
slc5a4a	aaacccattcccgatgttc	tcgattctttcctccttactgttc	71	107
slc5a4b	ccgattcctgatgttcacct	atccgctcctctgtgttgtt	65	67
gcg	cacgcccttcaagacacag	gtcctcatgcgcttctgtc	69	33
cck	tgatttccccatccaaagc	gcttctgcagggactaccg	103	9
gip	caggtaggaggagaagacctcat	cctagattgtgtcccctagcc	77	79
ghrl	ccagaggacagaggacaagc	catcgaagggagcattgaac	70	17
arntl1	tacagtggccctttgcatct	cccaaattcccacatctga	72	47
cry2	ccgcctgtgggacttgta	ctccattcggtcaaacctg	130	49
gnat3	tccaaagaactggagaagaagc	tttcccagattcacctgctc	93	88
gnat1	agagctggagaagaagctgaaa	tagtgctcttcccggattca	95	89
tas1r1	gggcctgataacactgacca	tgctcgcctcatagctgac	85	74

3.4.5 Real-time PCR

Quantitative PCR was performed using the same master mix as in 3.4.4, but the total volume was changed to 25 µl including 2.5 µl cDNA. Furthermore, a Universal probe library (UPL) fluorescent probe was added (see Table 4) and real-time PCR was performed on an AB 7000 qPCR cycler (Applied Biosystems, USA).

For relative quantification the ddCT method was performed using the reference genes *18s*, *hprt1* and *hmbs* for normalisation.

3.4.6 QuantiFast® *tas1r2* & *tas1r3* real-time PCR

As we were not successful in quantifying expression of *tas1r2* and *tas1r3* with normal UPL assays, we used QuantiFast® Probe Assay kits (Qiagen) with predesigned primers and probes - *tas1r2 (*Cat. No: QF00096929*) and tas1r3* (Cat. No: QF00030016) - and followed the manufacturer's manual. PCRs were performed on an AB 7000 qPCR cycler (Applied Biosystems, USA).

3.4.7 Protein extraction

15 mg of tissue was dissected on dry ice and added to a 2 ml tube with a 5 mm steel bead (Qiagen, Netherlands). 400 µl ice-cold protein-extraction buffer (1mM fresh DTT, 50mM Tris-HCl pH 7.4, 250mM Mannitol, 100mM NaCl, 1mM EDTA, 1mM EGTA, 10% glycerol) containing 1x protease-inhibitor cocktail (Sigma-Aldrich P8340, Dorset/UK) was added to the tubes and immediately disrupted in a Qiagen TissueLyser for 2x60s at 30 Hz and then kept on ice. Then 44 µl extraction buffer containing 10% Triton-X 100 was added for a final concentration of 1% of Triton-X 100. Samples were then incubated on a rotating spinner for 30min at 4°C and then centrifuged at 13,000 RPM for 10 min at 4°C. The protein-containing supernatant was transferred into a new tube.

3.4.8 Bicinchoninic acid (BCA) assay

Protein content was quantified using a Pierce™ BCA Protein assay kit. Bovine serum albumin (Sigma Aldrich A9647, Dorset/UK) was made up at a concentration of 2 mg/ml stock and diluted to 1, 0.5, 0.125 mg/ml to serve as standards. Protein samples were diluted 1:10 in water. 25 µl of standards and diluted samples were added to a 96-well plate. Reagent A was mixed 50:1 with reagent B and then 200 µl was added to each

well. The plate was mixed for 30s on a plate mixer and then incubated for 30 min at 37°C. Absorbance was measured at 562 nm on a BIO-TEK Synergy HT-1 spectrophotometer. Concentrations of unknowns were calculated from a standard curve drawn in Microsoft Excel.

3.4.9 Western blotting

Protein lysates were made up at a final concentration of 5 µg/µl, including 4x NuPAGE® LDS sample buffer (Life Technologies) and 10x sample-reducing agent (Life Technologies). Samples were then heated for 5 min at 95°C and 20 µg were loaded onto a 4-12% gradient NuPAGE Bis-Tris Protein gels. Proteins and a Amersham ECL High-Range Rainbow Molecular Weight Marker (GE Healthcare, Little Chalfont/UK) were separated for 55 min at 200 V in an XCell sure-lock tank with 1x NuPAGE® MOPS SDS running buffer (Life Technologies). The Western transfer was then performed using a Bio-Rad TurboBlot by sandwiching the gel between two filter papers soaked in cold Transfer buffer (25mM Tris-base, 75 mM Glycine, 10% Methanol, 0.1% SDS) and a 0.45 µm Immobilon-P PVDF membrane (Merck Millipore, Cork/IRL). Proteins were transferred for 90 min at 20V with a 100 mA cut-off to avoid overheating and drying out of membrane.

PVDF membranes were then blocked in 5% milk powder in TBS+Tween 0.1% (TBST) for 1 h. Blots were then rinsed 3x and washed for 3x 5 min in TBST. Primary antibodies were prepared at a concentration of 1:2000 in TBST with 0.05% Sodium Azide and blots were incubated over-night at 4°C. Antibodies were from Santa Cruz Biotechnology, Heidelberg/DE: βActin sc-130656, GLUT2 sc-31826, SGLT3 sc-134521.

The next day antibodies were removed and blots were rinsed 3x and washed for 3x 5 min in TBST and blocked for 10 min. Anti-rabbit secondary antibody (sc-2004) was made up in blocking buffer at a final concentration of 1:2000, to be incubated for 1 h at room temperature while rocking. Blots were rinsed 3x and washed for 3x 10 min in TBST to then be visualised. Blots were rid of any excess wash buffer and placed on an acetate sheet and 800 µl ECL Prime (GE Healthcare, Little Chalfont/UK) was added according to the manufacturer's protocol and they were then incubated for 3 min while rocking by hand. Luminescence generated by antibodies was visualised for 1-20 min using a SynGene G:BOX with the GeneSnap software, version 7.12 (Synaptics, Cambridge/UK).

3.4.10 *In silico* promotor analysis

To fetch the upstream sequence for a specific gene, we used the genome browser at UCSC (Kent et al., 2002). The mouse or human genome and the latest assembly (mouse: Dec. 2011 (GRCm38/mm10), human: Feb. 2009 (GRCh37/hg19)) were selected. The gene-accession number was pasted in the identifier field and 'sequence' chosen for output format before clicking on 'get output'. On the next page 'genomic' was chosen and on the final page 5000bp was entered in the 'Promotor/Upstream' field to retrieve the sequence in text format. Promotor regions are automatically selected according to the orientation on the genome (sense or antisense). E-Box elements were then retrieved manually in Microsoft Word.

3.4.11 Statistics

Data analysis and statistics were performed using Excel included in Microsoft Office Pro 2007, Version 12.0.6661.5000 SP3 MSO. Data are given as arithmetic mean + standard deviation (SD). Significances were tested using the inbuilt two-sided T-Test of Excel. A P-value of 0.05 or less was considered statistically significant.

3.5 Results

3.5.1 Diurnal rhythm of sugar transporters in the gastrointestinal tract

Diurnal rhythms of nutrient transporters have been described previously in rats (Corpe & Burant, 1996) and mice (Fatima et al., 2009). To confirm these findings and to have a baseline for our further experiments we analysed the diurnal rhythmicity of hexose transporters SGLT1, GLUT2 and GLUT5 in the small intestine of mice comparing the mRNA expression at 7am and 7pm. As seen in Fig. 22, a clear diurnal pattern with significant and high expression anticipatory to nocturnal feeding can be observed for all three transporters in all small-intestinal regions. Upregulations range from at least ~2- to 4-fold at 7pm, which is consistent with the literature.

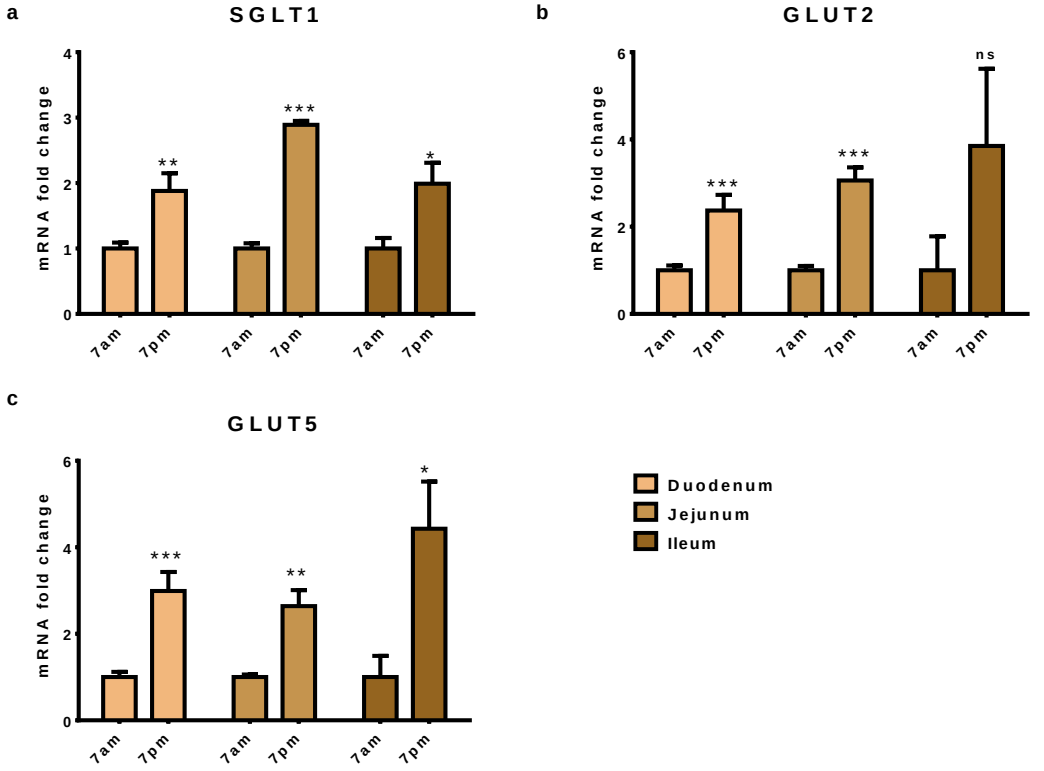

Fig. 22 Diurnal mRNA expression of hexose transporters in the murine small intestine. Whole duodenum, jejunum and ileum was dissected from six-week-old CD-1 mice at 7am or 7pm (n=8 per group) for subsequent quantitative PCR of *slc5a1* (SGLT1), *slc2a2* (GLUT2) and *slc2a5* (GLUT5). Values were normalised against 3 reference genes and relative quantitation was performed using the ddCT method and are expressed relative to 7am. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

In order to confirm the mRNA findings on a proteomic level, we extracted total protein and performed Western blot analyses to relatively quantify the expression of GLUT2 normalised against βActin. GLUT2 protein levels follow the same pattern as we saw for the mRNA (Fig. 23). It appears that the rhythm is less pronounced with the jejunum and ileum, showing only a ~50% increase at 7pm (Fig. 23b+c).

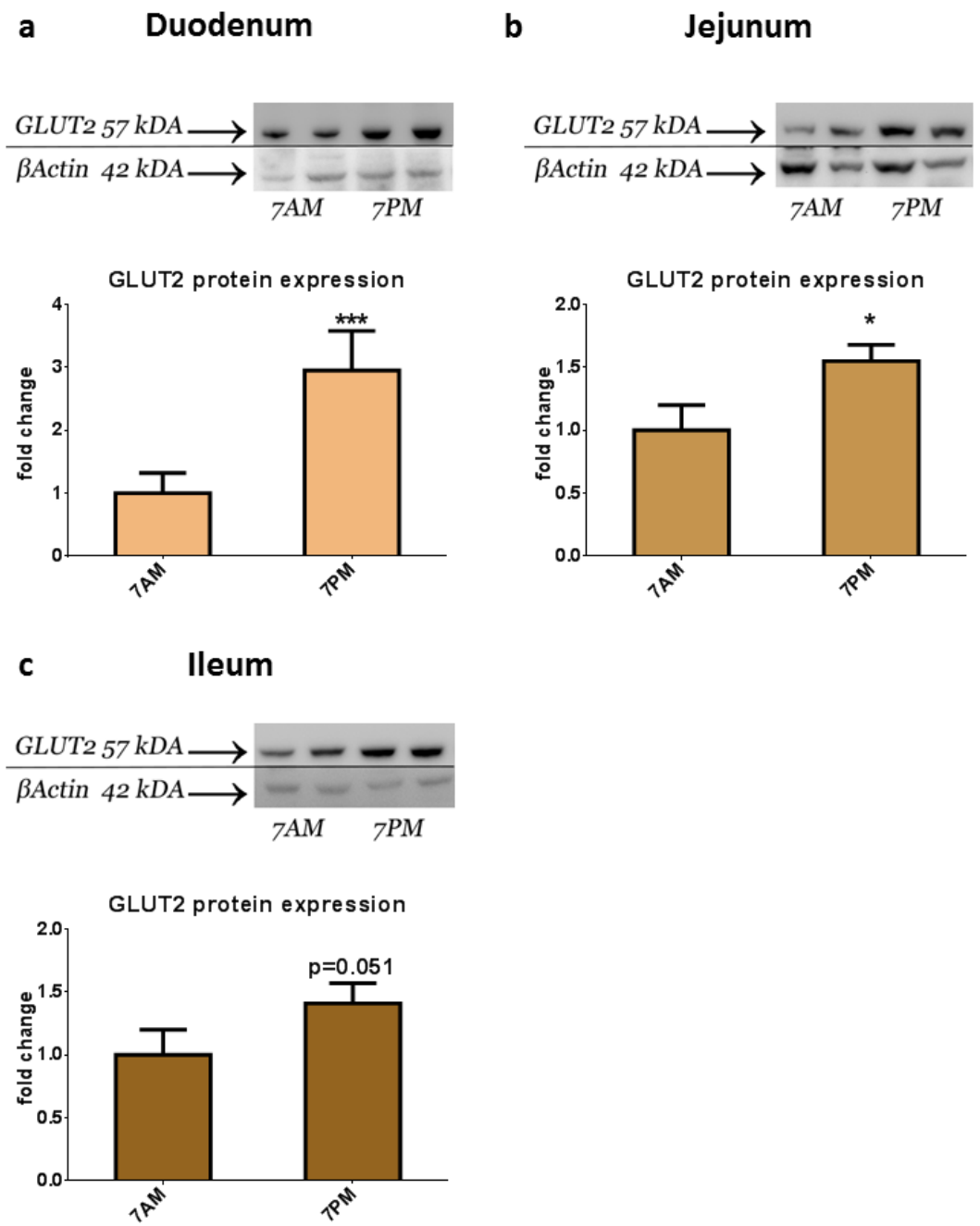

Fig. 23 Diurnal protein expression of GLUT2 in the murine small intestine. Whole duodenum, jejunum and ileum was dissected from 10 week old C57BL/6 mice (n=8 per group) for subsequent protein extraction and Western blots of βActin and GLUT2. Representative blots are shown. Blots were quantified and normalised against βActin and are expressed relative to 7am. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

3.5.2 Diurnal rhythms of taste sensors in the gastrointestinal tract

With a clear diurnal expression pattern of hexose transporters anticipatory to feeding, we hypothesised that nutrient sensors like the sweet-taste receptor also display such a rhythm along the alimentary tract.

In the tongue, T1R1 shows a significant but small (31%) upregulation at 7pm, whereas T1R2, T1R3, αGustducin and αTransducin do not show any rhythmicity (see Fig. 24a). In the stomach only αGustducin shows a rhythm, with a two-fold induction that does not reach significance ($p=0.08$). In the small intestine, T1R1 shows a trend of induction in the duodenum ($p=0.09$) and the ileum ($p=0.19$). The sweet-taste receptor molecules T1R2 and T1R3 both show a pattern in the proximal regions (Fig. 24b+c). T1R2 shows ≤2-fold upregulations in the duodenum and jejunum (Fig. 24b). T1R3, on the other hand, only demonstrates a significant upregulation in the duodenum, with 38% higher mRNA-expression levels. αGustducin shows trends in all tissues, with a two-fold induction in the stomach just short of reaching statistical significance ($p=0.08$). αTransducin, on the other hand, only shows an induction of 53% in the duodenum, not reaching significance ($p=0.08$).

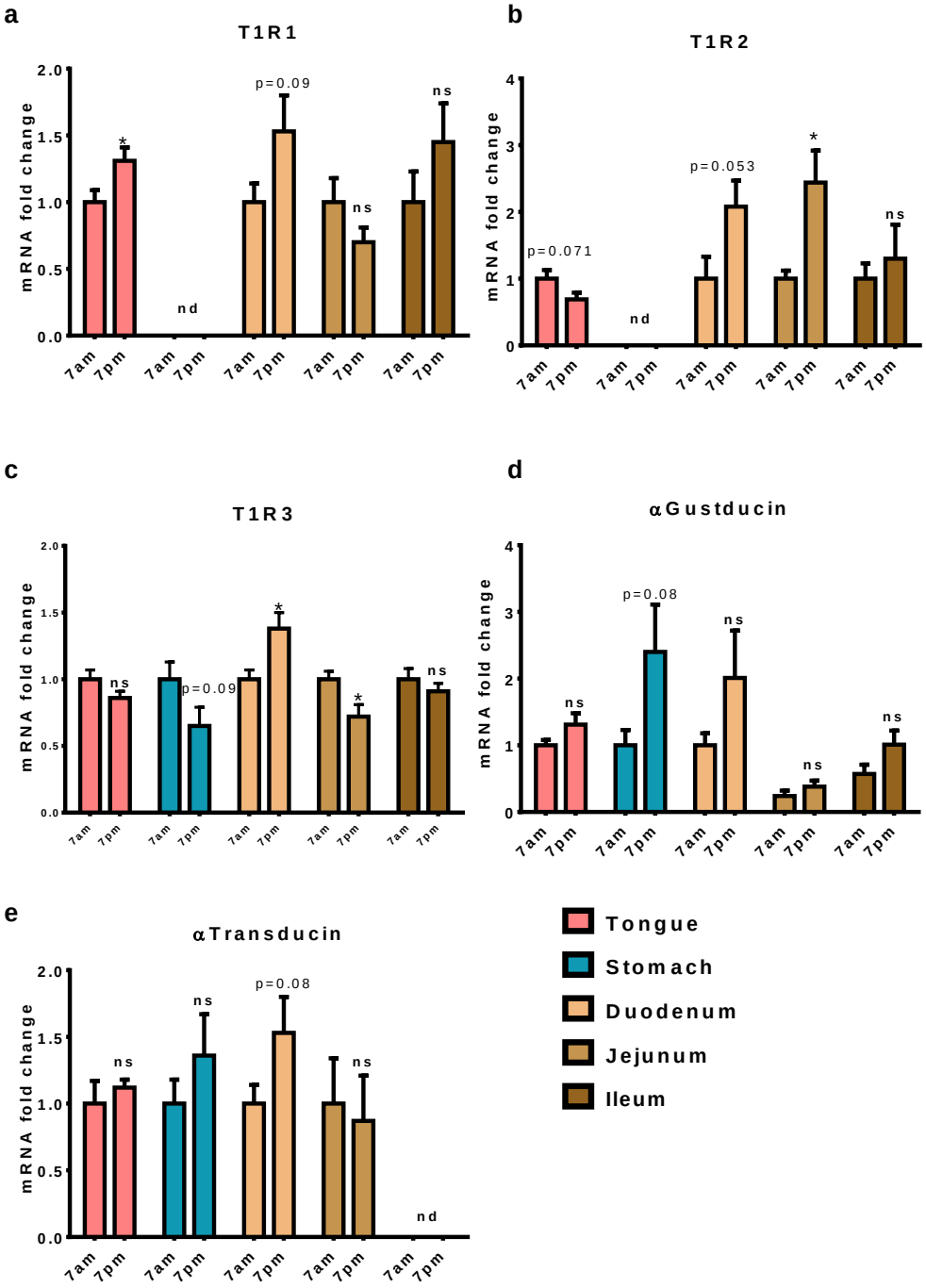

Fig. 24 Diurnal mRNA expression of nutrient sensors in the murine alimentary tract. Whole tongue, stomach, duodenum, jejunum and ileum was dissected from six-week-old CD-1 mice at 7am or 7pm (n=8 per group) for subsequent quantitative PCR of *tas1r1* (T1R1), *tas1r2* (T1R2), *tas1r3* (T1R3), *gnat3* (αGustducin) and *gnat1* (αTransducin). Values were normalised against 3 reference genes and relative quantitation was performed using the ddCT method and are expressed relative to 7am. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

Compared to the more established nutrient sensors, a clearer diurnal expression pattern appears for the novel sugar sensor SGLT3. SGLT3a shows a 6-fold induction in the duodenum and 2.5-fold induction in the jejunum and a 3-fold induction (ns) in the ileum (Fig. 25a). SGLT3b on the other hand shows a five-fold induction only in the stomach, but no rhythm in the small intestine. This clearly demonstrates that SGLT3a and SGLT3b have specific roles in the gastrointestinal tract that are different from each other.

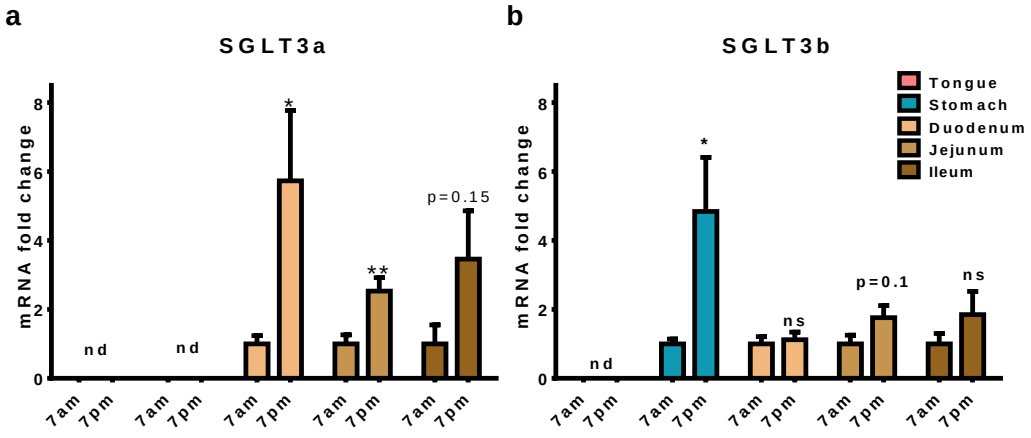

Fig. 25 Diurnal mRNA expression of SGLT3+b in the murine alimentary tract. Whole tongue, stomach, duodenum, jejunum and ileum was dissected from six-week old CD-1 mice at 7am or 7pm (n=8 per group) for subsequent quantitative PCR of *slc5a4a* (SGLT3a) and *slc5a4b* (SGLT3b). Values were normalised against 3 reference genes and relative quantitation performed using the ddCT method and are expressed relative to duodenum at 7am. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

We applied Western blotting to confirm the transcriptomic findings on the proteome level. As we demonstrate in Fig. 26, no rhythm for SGLT3 can be detected by comparing the protein expression at 7am vs 7pm.

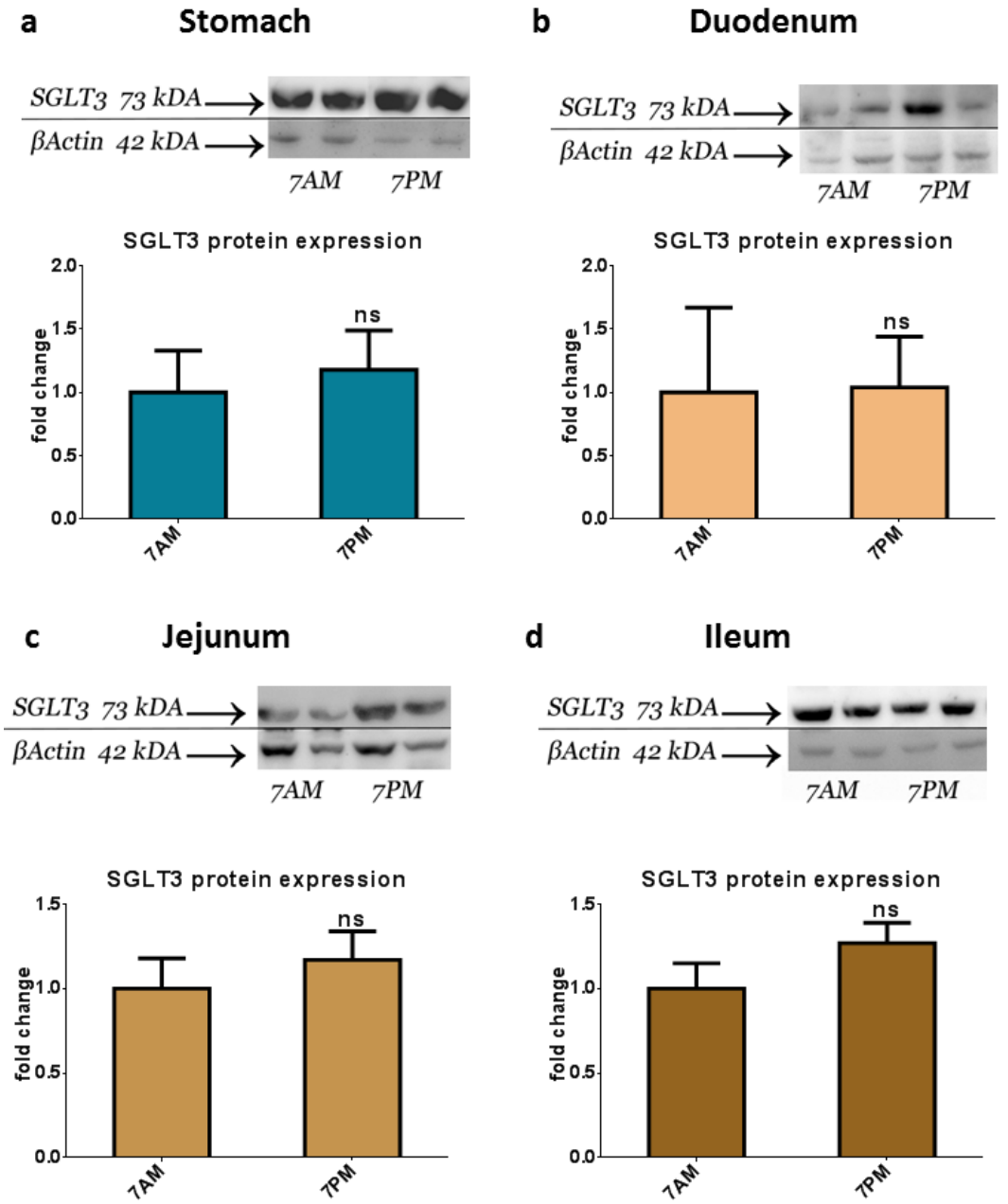

Fig. 26 Diurnal protein expression of SGLT3 in the murine small intestine. Whole stomach, duodenum, jejunum and ileum was dissected from 11-week-old C57BL/6 mice (n=8 per group) for subsequent protein extraction and Western blots of βActin and SGLT3. Representative blots are shown. Blots were quantified and normalised against βActin and are expressed relative to 7am. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

3.5.3 Diurnal rhythms of gut peptides in the gastrointestinal tract

The sweet-taste receptor has been shown to be involved in the sensing of nutrients and subsequent incretin secretion from enteroendocrine cells (Steinert et al., 2011). Only very recently has a circadian rhythm of GLP1 been described on the plasma protein and transcript level (Gil-Lozano et al., 2014). We therefore studied the diurnal expression pattern of the gut peptides CCK, GIP, Preproglucagon (GLP1+2) and ghrelin to see if there is a correlation of the observed nutrient sensor diurnal rhythms with incretin-expression levels. As demonstrated in Fig. 27, none of the studied genes shows a diurnal rhythm. Only the *gcg* gene that gets translated and cleaved to GLP1 and GLP2 in the intestine shows a significant 43% induction in the jejunum and a non-significant induction of 85% in the duodenum.

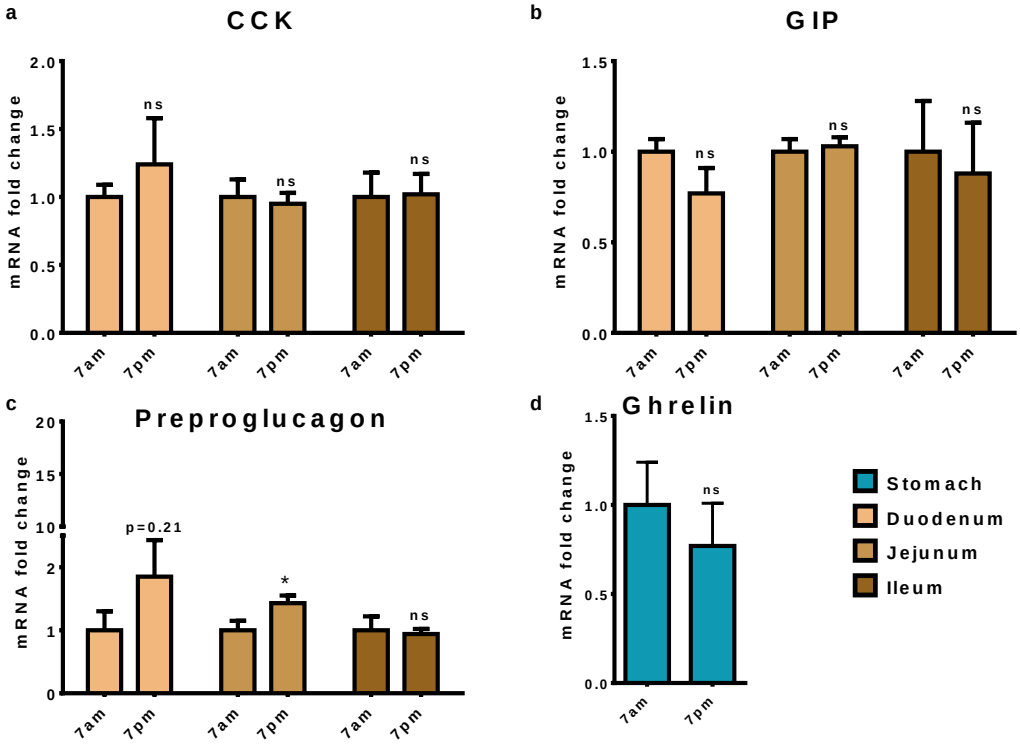

Fig. 27 Diurnal mRNA expression of gut peptides in the murine alimentary tract. Whole tongue, stomach, duodenum, jejunum and ileum was dissected from six-week-old CD-1 mice at 7am or 7pm (n=8 per group) for subsequent quantitative PCR of slc5a4a (SGLT3a) and slc5a4b (SGLT3b). Values were normalised against 3 reference genes and relative quantitation was performed using the ddCT method and expressed relative to 7am. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

3.5.4 Diurnal rhythm of clock genes in the gastrointestinal tract

A marked rhythm in the mRNA levels of two canonical clock genes, *arntl1* (BMAL1) and *cry2* (CRY2), was found, oscillating in opposing phases, as expected (Fig. 28). *Cry2* is induced at 7pm by 50-400% in all tissues, with the stomach as an exception. *Arntl1/*BMAL1, in contrast, is ~7-fold downregulated at 7pm. This suggests the existence of a functional circadian clock in all studied tissues, and that genes including nutrient sensors could be under its control.

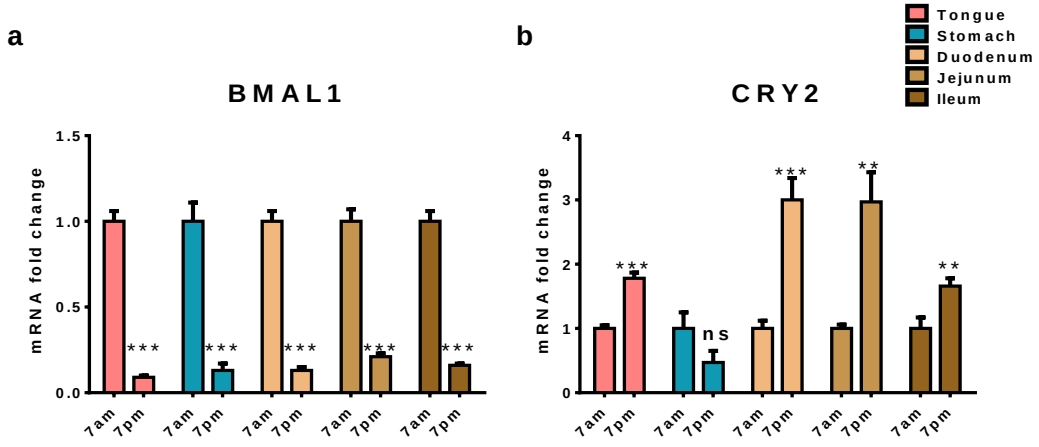

Fig. 28 Diurnal mRNA expression of clock genes in the murine alimentary tract. Whole tongue, stomach, duodenum, jejunum and ileum was dissected from six-week-old CD-1 mice (n=8 per group) for subsequent quantitative PCR of *artnl1* (BMAL1) and *cry2* (CRY2). Values were normalised against 3 reference genes and relative quantitation was performed using the ddCT method and are expressed relative to 7am. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

3.5.5 *In silico* promotor analysis of sugar transporters and nutrient sensors

Clock genes can regulate gene expression by binding to the promotor, within the transcript or downstream of it. Clock genes regulate target genes by binding to E-Box *cis*-regulatory enhancer sequences. It has been suggested that clock genes bind not only the canonical E-Box sequence used by all basic Helix-Loop-Helix transcription factors but also an extended E-Box sequence of the form (G/T)G(A/G)ACACGTGACCC. We demonstrated diurnal rhythmicity of expression for hexose transporters and nutrient sensors, including the sweet-taste receptor and the putative D-Glu sensor SGLT3. We therefore hypothesised that our genes of interest also contain such extended E-Box elements.

We analysed *in silico* 10,000 bp upstream of the transcription-initiation site of hexose transporters, clock genes and the aforementioned nutrient sensors. We found the canonical E-Box sequence **CACGTG** in all genes apart from *cry2, gip* and *gcg* (Fig. 29). Analysing these sequences for the extended E-Box sequence, we found a near perfect fit for *arntl1/*BMAL1 and *slc2a2/*GLUT2 (matches highlighted in bold). *Slc5a1*/SGLT1, *tas1r1*/T1R1, *tas1r2*/T1R2, *tas1r3*/T1R3, *slc5a4a*/SGLT3a, *slc5a4b*/SGLT3b, ghrl/ghrelin, *cck*/cck and *pyy*/PYY have at least two nucleotide matches. No E-Box was found for *cry2*/CRY2*, gip*/GIP or *gcg*/Preproglucagon in the 10,000 bp upstream of the first exon. Several intronic E-Boxes were found for *cry2*. Directly beside each other are two E-Boxes, with one of them having a high match to the extended circadian E-Box sequence; 15,115bp and 15,168bp downstream of the first exon the sequences **TG**TG-**CACGTG**-**A**G**CC** and CT**G**C-**CACGTG**-C**C**AG were found.

E-Box elements in 10,000 bp upstream region of genes

	-7	-6	-5	-4	Consensus	+4	+5	+6	+7
Gene name	**G/T**	**G**	**A/G**	**A**	**CACGTG**	**A**	**C**	**C**	**C**
arntl1	**T**	**G**	**G**	**A**	**CACGTG**	C	**C**	**C**	G
cry2					**X**				
slc5a1	**G**	C	C	T	**CACGTG**	G	G	A	**C**
slc2a2	**T**	**G**	**G**	**A**	**CACGTG**	**A**	G	**C**	**C**
tas1r1	C	C	**G**	C	**CACGTG**	C	G	**C**	G
tas1r2	C	A	T	T	**CACGTG**	G	**C**	**C**	**C**
tas1r3	C	A	**G**	**A**	**CACGTG**	C	T	**C**	**C**
slc5a4a	C	C	**A**	**A**	**CACGTG**	T	**C**	T	T
slc5a4b	**G**	A	**G**	C	**CACGTG**	T	T	**C**	T
ghrl	**T**	A	C	**A**	**CACGTG**	G	**A**	**A**	**A**
gip					**X**				
cck	**T**	**G**	**A**	G	**CACGTG**	T	**C**	**C**	T
gcg					**X**				
pyy	**T**	C	C	T	**CACGTG**	T	G	**C**	T

Fig. 29 *In silico* promotor analysis of hexose transporters and nutrient sensor genes for sequences of diurnal E-Box elements. 10,000bp upstream of each translation start were used to find E-Box sequences and matching extended consensus sequences are marked in bold.

These observations suggest that our genes of interest could be potentially under the control of clock genes. Directed mutagenesis to study these sequences would be needed to evaluate our findings.

3.5.6 Gene-expression profiling of tongue tissue at 7am vs 7pm

3.5.6.1 Analysis of the clock machinery

In the tongue, we were not able to detect diurnal rhythms for the sweet-taste receptor T1R2/3 and only a very small induction for the umami-taste receptor T1R1 (see Fig. 24). Yet we showed strong clock activity in the tongue (see Fig. 28). DNA synthesis and mitotic activity have been shown to be regulated in a diurnal fashion in the tongue (García et al., 2001). Furthermore, glucocorticoids, which display strong diurnal rhythms, co-localise with *tas1r3* in type-II taste cells and are involved in food entrainment (Peek et al., 2012; Parker et al., 2014). Together, this data suggests that genes involved in taste preference could be diurnally regulated. We therefore used pooled RNA from 8 mice per group and compared the expression patterns on an Affymetrix microarray.

Looking specifically for the clock-machinery genes and CCGs, we first analysed the array for clock genes and important nuclear factors that have gained an important role in the emerging model of circadian rhythm as outlined in 3.2.1. Nuclear factors, which are controlled by clock genes and oscillate in a circadian fashion, can affect gene-transcriptional control. Despite only using pooled samples rather than individual samples to save costs, we confirmed our qPCR findings presented earlier, with the microarray with *cry2* being upregulated and *arntl1* being highly downregulated (Fig. 30). Furthermore, strong rhythms exist for the clock genes *clock*, *per1* and *per3* as hypothesised. The nuclear factors presented indicate diurnal rhythmicity with a downregulation of RAR-α and high upregulation of Rev-erbβ and RXR-β (Fig. 30). This further confirms the diurnal rhythmicity in our tissue samples and suggests the existence of CCGs.

Clock gene & nuclear factor regulation in mouse tongue

Protein	Gene name	Fold change 7am vs 7pm
CLOCK	*clock*	**-1.52**
PER1	*per1*	**1.92**
PER2	*per2*	1.24
PER3	*per3*	**8.05**
CRY1	*cry1*	-1.00
CRY2	*cry2*	**1.79**
BMAL1	*arntl1*	**-11.03**
BMAL2	*arntl2*	1.42
Rev-erbα	*nr1d1*	1.23
Rev-erbβ	*nr1d2*	**3.53**
RAR-α	*rara*	**-1.74**
RAR-β	*rarb*	-1.21
RAR-γ	*rarg*	1.35
RXR-α	*rxra*	1.05
RXR-β	*rxrb*	**2.50**
RXR-γ	*rxrg*	-1.23

Fig. 30 Fold changes of clock genes and nuclear factors in the murine tongue. RNA was pooled from 8 mice per group and analysed on an Affymetrix microarray chip. mRNA expression was compared between 7am and 7pm and fold changes are given.

3.5.6.2 Analysis of lingual taste receptors

The sweet-taste receptor did not show a diurnal rhythm, yet we detected high clock-gene activity. We therefore manually extracted taste- and nutrient sensing genes from the microarray, including sweet-, fat-, umami-, sour- and bitter-taste genes, as well as signalling molecules needed for the downstream signal transduction. As expected, no regulation of the sweet-taste receptor was detected (Fig. 31). A 6.5-fold and 1.39-fold induction was noted for the genes of αTransducin and TRPM5 respectively. TRPM5 is an ion channel that is important for the creation of action potentials and shows a 39% induction at 7pm.

Diurnal regulation of nutrient sensors in mouse tongue

	Protein	Gene name	Fold change 7am vs 7pm
Sweet	T1R2	*tas1r2*	**-1.52**
	T1R3	*tas1r3*	1.21
	SGLT3a	*slc5a4a*	1.17
	SGLT3b	*slc5a4b*	1.01
Fat	CD36/FAT	*cd36*	1.24
	FFAR2	*ffar2*	**-1.74**
	GPR120	*gpr120*	NA
Umami	mGlu4r	*grm4*	NA
	T1R1	*tas1r1*	**6.01**
Sour	TRPP3	*pkd2l1*	NA
	ENaCα	*scnn1a*	**-1.56**
	ENaCβ	*scnn1b*	**-1.48**
	ENaCγ	*scnn1c*	1.13
Bitter	T2R105	*tas2r105*	**2.41**
	T2R108	*tas2r108*	**-1.88**
	T2R119	*tas2r119*	**-3.89**
Signalling	αGustducin	*gnat3*	NA
	αTransducing	*gnat1*	**6.52**
	TRPM5	*trpm5*	**1.39**
	Phospholipaseβ2	*plc*β2	1.06

Fig. 31 Fold changes of diurnally regulated taste sensors and necessary signalling molecules. RNA was pooled from 8 mice per group and analysed on an Affymetrix microarray chip. mRNA expression was compared between 7am and 7pm and fold changes are given. NA= not available in microarray chip used.

The fat receptor *cd36* shows a slight increase, whereas *ffar2* is downregulated by 74% (Fig. 31). The umami-taste receptor mGlu4r was not available in the microarray, but *tas1r1* shows a 6-fold induction, although we confirmed this finding with qPCR with only a slight induction of 31% (p=0.04, Fig. 31).

It has been suggested that ion channels are involved in sour/H^+ sensing. The TRPM5-related transporter *pkd2l1* was not available in the array, but the epithelial sodium-channel ENaC subunits ENaCα+β show a 50% downregulation (Fig. 31).

Most striking is the regulation of the T2R family, sensors of bitter tastants. The *tas2r* family consists of at least 30 members (Meyerhof, 2005), but in the applied microarray only three genes belonging to this family were available, namely *tas2r105, tas2r108* and *tas2r119*. *tas2r105* is 41% upregulated, whereas *tas2r108* and *tas2r119* are downregulated by 88% and 289% respectively (Fig. 31).

We used the GeneGo® 'bitter taste' map function to expand the network of genes involved in bitter sensing in Taste II taste cells (Fig. 32).

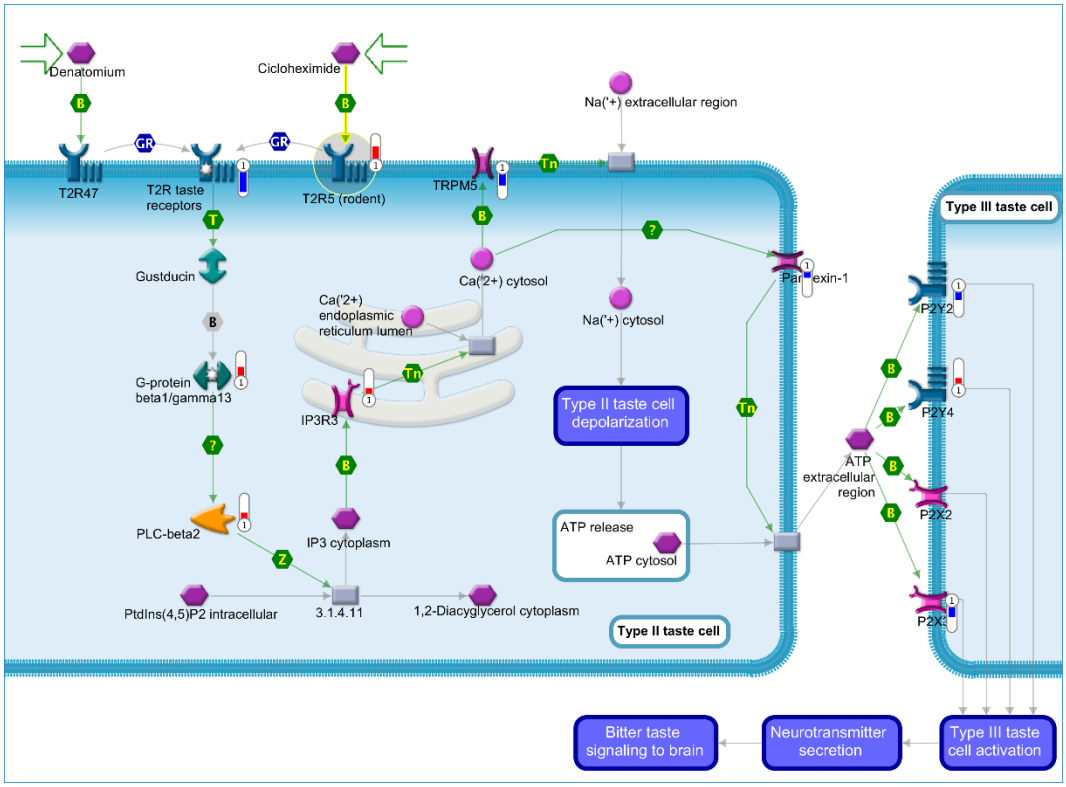

Fig. 32 Bitter-taste map generated by GeneGo®. Shown are a Type-II taste cell for bitter sensing and the connection to a Type-III taste cell connecting the taste cell with nerve fibres. Demonstrated are intracellular and membrane-bound receptors and signalling molecules needed, as well as their interactions with each other. Thermometers indicate intensity of up/downregulation.

Shown is a Type-II taste cell that tastes sweet, umami and bitter and how it connects to a Type-III, taste cell which acts as a connector between Type-II cells and

nerve fibres and ultimately to the brain. Of note is the second messenger machinery of PLCβII and IP3 and the ion channel TRPM5 involved in taste-signal transduction, which is regulated in our model.

Furthermore, of interest is the connection between the Taste-II and Taste-III cells. Pannexin1 is located on the Type-II cell membrane and is an ion and small-molecule channel (e.g. ATP or cAMP) that connects the intracellular with extracellular space between cells. Pannexin1 is downregulated, comparing 7am with 7pm. On the membrane of the Type-III membrane we find the purinergic signalling-molecule family P2X and P2Y (Fig. 32). These large receptor families are activated by nucleotides such as ATP, ADP, AMP, UDP and UMP and connect to downstream signals via various G-Proteins (Abbracchio et al., 2006). In our array we saw down- or upregulation for *p2ry1* (+1.33), *p2ry2* (-1.38), *p2ry12* (+1.40), *p2ry14* (-1.18). For the P2X family we were able to detect two-fold downregulation for *p2rx1* and *p2rx2,* whereas *p2rx7* was three-fold downregulated.

3.5.6.3 Analysis of lingual gut peptides

Recent findings have shown that many of the known gut peptides such as GLP1, ghrelin and CCK can also be detected in lingual taste buds (Herness et al., 2002; Gröschl et al., 2005; Shin et al., 2008). Oral PYY has now been found to be involved in reducing food intake (Acosta et al., 2011). As some of the nutrient receptors are regulated in this study, we hypothesised that this also affects lingual peptide-hormone levels. Indeed, the expression of *gcg, pyy, ghrl* and *cck* was downregulated, whereas *gip* expression was upregulated (Fig. 33).

Diurnal regulation of gut peptides in mouse tongue

Protein	Gene name	Fold change 7am vs 7pm
Preproglucagon	*gcg*	-3.23
GIP	*gip*	2.12
PYY	*pyy*	-1.90
Ghrelin	*ghrl*	-2.65
CCK	*cck*	-1.61

Fig. 33 Fold changes of diurnally regulated gut peptides in the murine tongue. RNA was pooled from 8 mice per group and analysed on an Affymetrix microarray chip. mRNA expression was compared between 7am and 7pm and fold changes are given.

3.5.6.4 Circadian-rhythm network map

Using the automated 'circadian rhythm' function in GeneGo, we were able to visualise a network map of clock genes and CCGs (Fig. 34). The interaction between clock genes (e.g. CRY1+2, PER1-3, CLOCK, BMAL1) and nuclear factors (e.g. Rev-erbβ and ROR-α) is clearly visible in the nucleus. Also shown are the targets and affected genes of the clock machinery, especially in the cytosol and plasmalemma.

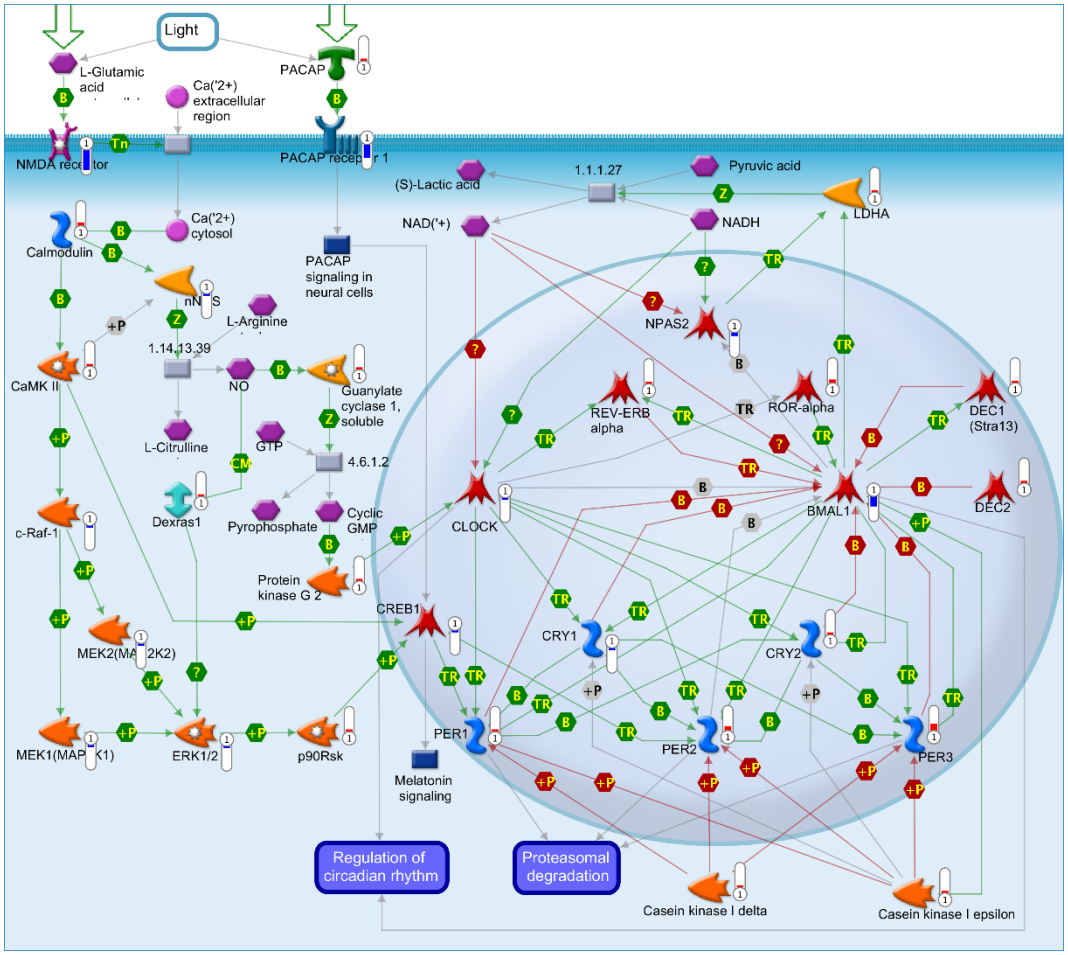

Fig. 34 Network map built with the 'Circadian rhythm' function in the GeneGo software. Shown are nuclear, intracellular and membrane-bound clock genes and clock-affected genes, as well as their interactions with each other. Thermometers indicate intensity of up/downregulation.

Of interest is the regulation of PACAP and its membrane-bound receptor. PACAP has been found to stimulate enterochromaffin-like cells in the intestine and CCK

secretion in the L-cells (Chang et al., 1996). We found a 64% upregulation for PACAP and 2-fold downregulation for the PACAP receptor (see membrane in Fig. 34).

Also notable is the high downregulation of the NMDA receptor (top left corner, Fig. 34). It has been suggested that the NMDA receptor is an alternative L-glutamate receptor for umami taste (Lin & Kinnamon, 1999). In the map, all NMDA receptors are combined as 'NMDA receptor'. Several genes were identified in this group and extracted from the microarray, including *grin1, grin2a, grin2b* and *grin2c,* with downregulations of 23-fold, 0.4-fold, 3-fold or 5-fold respectively.

3.5.6.5 Top 10 diurnally regulated genes

To further study the phenotype we manually extracted the top 10 regulated genes (enrichment analysis of the dataset can be seen in appendix I). The top 10 upregulated genes either are involved in immunity (*dock2, h2-k1, vcam1, hivep3*) or adrenergic-nerve signalling (*ankrd1, adrb3*) or are transcriptional regulators (*dbp, tead2, per3*), and range with upregulations between 7- and 22-fold (Fig. 35). All of the downregulated genes are enzymes (proteases, carbohydrases, lipases), and have very high downregulations between 300- and 64,000-fold.

Top ±10 diurnally regulated genes in the tongue

Protein	Gene name	Fold change 7am vs 7pm
Ankyrin repeat domain-containing protein 1	*ankrd1*	**+22.02**
D site albumin promoter binding protein	*dbp*	**+17.76**
Histocompatibility 2, D region locus 1	*h2-k1*	**+16.38**
Dedicator of cyto-kinesis 2	*dock2*	**+10.98**
Vascular cell adhesion molecule 1	*vcam1*	**+9.96**
HIV type I enhancer binding protein 3	*hivep3*	**+9.06**
TEA domain family member 2	*tead2*	**+8.39**
Period homolog 3	*per3*	**+8.05**
chloride channel 2	*clcn2*	**+7.69**
adrenergic receptor, beta 3	*adrb3*	**+7.12**
Phsopholipase A2	*pla2g1b*	**-326**
Carboxypeptidase B1 (tissue)	*cpb1*	**-358**
Carboxypeptidase A1	*cpa1*	**-389**
Chymotrypsinogen B1	*ctrb1*	**-534**
Pancreatic lipase-related protein 2	*Pnliprp2*	**-640**
Kallikrein 1	*klk1*	**-738**
Amylase 2, pancreatic	*amy2*	**-1022**
Trypsin5	*try4*	**-4038**
Colipase, pancreatic	*clps*	**-8520**
Elastase 3, pancreatic	*ela3*	**-64529**

Fig. 35 Fold changes of the top ±10 diurnally regulated genes in the murine tongue. RNA was pooled from 8 mice per group and analysed on an Affymetrix microarray chip. mRNA expression was compared between 7am and 7pm and fold changes are given and sorted by fold change.

3.6 Discussion

The alimentary tract is the first barrier in contact with nutrients. It has evolved intricate mechanisms to sense nutrients and react to nutrient load. Yet the body not only reacts to nutrients upon stimulation but is also able to prepare anticipatory to feeding to optimise nutrient uptake. This is governed by central and peripheral clock machinery. Our modern diet and eating behaviour have changed since the Palaeolithic, and this could implicate a mismatch of physiologic mechanisms, leading to the increasing prevalence of metabolic diseases. Circadian rhythmicity of hexose transporters was demonstrated many years ago (Corpe & Burant, 1996), but a rhythm for GLP1 has only been shown very recently (Gil-Lozano et al., 2014). GLP1 secretion from L-cells is controlled by the sweet-taste receptor and its downstream signalling machinery, including αGustducin (Jang et al., 2007). We therefore hypothesised that nutrient sensors like the sweet-taste receptor also show diurnal rhythmicity anticipatory to feeding in the small intestine, but also in the tongue and stomach, and regulate gut peptide secretion. To test this hypothesis, we used wild-type mice fed a normal chow diet and compared the gene and protein expression at 7am and 7pm.

The sugar transporters SGLT1, GLUT2 and GLUT5 and the clock genes CRY2 and BMAL1 demonstrated a clear diurnal pattern of expression at the mRNA level, and we also confirmed this on a proteomic level for GLUT2. We were not able to show diurnal expression for the sweet-taste receptor T1R2/3 and only a very small induction for the umami-taste receptor T1R1. For the first time a diurnal rhythm of expression was seen for the putative sugar sensors SGLT3a and SGLT3b. The gut peptides GLP1, CCK, GIP and ghrelin did not display diurnal rhythmicity in the gastrointestinal tract, but showed

rhythmicity in the tongue. Clock genes showed a strong rhythm and we were able to identify E-Box elements necessary for transcriptional regulation in gene promotors of most genes of interest. Gene expression profiling using a microarray on tongue RNA revealed bitter taste receptors, especially, to be highly regulated as were new processes including adrenergic signalling and immunity.

3.6.1 Sugar transporters, clock genes, nutrient sensors & gut peptides

The distinct circadian rhythmicity of nutrient transporters (Corpe & Burant, 1996), insulin and GLP1 secretion (Boden et al., 1996; Gil-Lozano et al., 2014) led us to study temporal changes of expression for nutrient sensors, gut peptides and clock gene, and also to study promotor sequences of genes of interest *in silico*. Despite the clear rhythms of clock genes *cry2* and *arntl1* in all tissues, the sweet- and umami-taste receptors displayed very little diurnal regulation in the tongue and gut. SGLT3a and SGLT3b, on the other hand, showed a clear pattern. SGLT3a, which does not transport sugar has hence been suggested that it is a sugar sensor (Barcelona, 2012), displayed a marked upregulation of expression at 7pm (compared to 7am) in the duodenum and jejunum. SGLT3b, a high-affinity D-Glu transporter, was significantly upregulated in the stomach at 7pm compared to 7am. This is the first evidence that SGLT3a and SGLT3b exhibit a diurnal rhythm of expression in the gastrointestinal tract. These findings add evidence to the theory that SGLT3a and SGLT3b are two distinct proteins with different roles.

SGLT1 expression is under the control of clock genes (Balakrishnan et al., 2010). SGLT1 has high gene- and protein-sequence similarity with SGLT3a and SGLT3b. Not only

did our *in silico* promotor study find circadian E-Box elements for genes that have been shown to have a clear circadian rhythm (e.g. GLUT2), but we were also able to find these E-Box elements in the clock gene *arntl1*, nutrient sensors including the sweet-taste receptor T1R2/3 and SGLT3 and the gut peptides ghrelin, CCK and PYY. Experimental evidence has shown that clock genes directly regulate the expression of the oligopeptide transporter PEPT1 and the Na^+/H^+ exchanger NHE3 (Rohman et al., 2005; Saito et al., 2008).

Our data suggest that the genes analysed in our study can potentially be under clock-gene control via circadian E-Boxes. Further exploration and functional promotor studies are needed to establish exact transcriptional control mechanisms by clock genes. Many new sequencing technologies like 'nascent-Seq' have been developed to allow direct and accurate measurement of gene activity and post-transcriptional modifications which could be potentially be used for further research (Kojima & Green, 2015). Also, many more mechanisms besides E-Boxes exist to control circadian expression. SGLT1 has been shown to be regulated by PER1 in an E-Box-independent fashion (possibly by acting on other clock genes that bind to non-E-Box elements in the promotor) (Balakrishnan et al., 2012). Furthermore, micro RNAs (miRNAs) and long non-coding RNAs (lncRNAs) have been found to be important regulators of mRNA survival and subsequent translation of clock genes (Lee & Bartolomei, 2013; Du et al., 2014). Lee and Bartolomei suggest that up to 30% of CCGs undergo miRNA-mediated regulation. An array of post-translational modifications have also been implicated in the regulation of CCG protein expression (Kojima & Green, 2015).

SGLT3 was duplicated to SGLT3a and SGLT3b in rodents and hence has high similarity with SGLT3a and SGLT3b. Yet our cephalocaudal data suggests that the gene expression of SGLT3a and SGLT3b is regulated differently and highly specific to accommodate two different roles in the gastrointestinal tract, as discussed earlier. Our work on the diurnal rhythmicity of both genes further suggests two different functions in the gastrointestinal tract and are interesting in the context of metabolism and its dysregulations. In today's society meal timing and patterns have changed (most evident in shift workers) and such changes have been suggested to increase the risk for metabolic diseases (Pan et al., 2011). Polymorphisms in circadian regulatory genes have been linked to obesity and type 2 diabetes (Scott et al., 2008).

We demonstrated that SGLT3b is highly upregulated anticipatory to feeding in the evening. An interesting study found, using pyloric cuffs to prevent gastric emptying, that a post-gastric (i.e. intestinal) sensor is necessary for glucose-induced gastric ghrelin suppression (Williams et al., 2013). Yet their study protocol takes many hours and it stands to reason that the researchers performed their experiments during the day. Based on our findings, we suggest that the study by Williams *et al.* did not find a sensor because it had not yet been expressed. This is interesting as it could be implicated in dysregulated feeding behaviour. SGLT3 has been shown to be important for glucose sensing in the portal vein. Strategically expressed on neurons in the wall of the vein, it can relay messages about the nutrition status to the hypothalamus to suppress appetite (Mithieux, 2014). It could be speculated that gastric SGLT3b has a similar role, but if food is ingested during times of the day when expression is low (i.e. humans in the night, rodents during the day), satiety signals to the brain are not as strong which can lead to

overeating and over time induce obesity. It would be interesting to repeat the study by Williams *et al.* in the evening time to test this.

SGLT3b is also higher expressed in the distal small intestine, which suggests it could be involved in the ileal-brake phenomenon (Spiller et al., 1984; Maljaars et al., 2008). It has been shown that incretin responses are blunted in diabetic/obese patients (Nauck et al., 1986; Lindqvist et al., 2005) and GLP1 has been demonstrated to have a circadian pattern of expression only recently (Gil-Lozano et al., 2014). The regulation of GLP1 secretion is taste-receptor-dependent and has been linked to SGLT3 (Lee et al., 2015). If clock mechanisms are disturbed it is also possible that the diurnal expression of SGLT3b is disturbed. When D-Glu arrives in the ileum, but is less sensed by SGLT3b, the incretin response could be attenuated. The incretin response is important for insulin secretion and satiety signalling to the brain, as well as decreasing gastric motility and emptying. A blunted incretin response due to decreased SGLT3b mediated sensing, hence, could be a factor leading to the metabolic dysregulations seen in groups such as shift workers.

In contrast to SGLT3b, SGLT3a displayed high diurnal regulation in the proximal small intestine, which is also where luminal sugar concentrations are highest. Luminal D-Glu induces functional feedback via vagal nerve and gut peptide pathways (Young, 2011a), including the regulation of gastric emptying, transport function and food intake (Raybould & Hölzer, 1992; Horowitz et al., 1993; Pilichiewicz, 2007). It can be speculated that either optimal eating times exist when SGLT3a is highly expressed or that if clock mechanisms become disturbed and hence SGLT3a's diurnal rhythmicity becomes blunted, intestinal feedback mechanisms upon luminal stimulation are also blunted. This

in turn could then affect intestinal motility, insulin secretion and satiety and lead to metabolic disease.

The rhythms in the central and peripheral clocks are partly entrained by the FEO, which in turn are guided by intraluminal nutrient-concentrations and gut peptide levels (Balakrishnan et al., 2008; Pan & Hussain, 2009). As outlined above, we speculate that SGLT3a+b play major roles in the detection of gastrointestinal sugar concentrations crucial for the entrainment of clock systems, but are also implicated in metabolism and feeding. It would be of interest to study if the expression and diurnal rhythmicity of SGLT3 is dysregulated in metabolic disease.

Furthermore, humans only have one form of SGLT3. Rodents are adapted to a diet high in carbohydrates which could explain the gene duplication. Potentially this means that humans are maladapted to a diet based on carbohydrates (Last & Wilson, 2006). Further research on SGLT3 in humans is needed.

3.6.2 Gene expression profiling in the tongue

In the tongue we demonstrated strong clock-gene activity, but no diurnal rhythmicity for the sweet- and umami-taste receptors. Yet the high activity of clock genes suggests the presence of CCGs. We therefore used microarrays for gene-expression profiling to identify diurnally controlled genes related to nutrient sensing.

Dissecting the different nutrient sensor groups, we were able to identify the bitter-taste sensor group to be highly regulated. The T2R group consists of at least 40 receptors. Bitter tasting provides an important protective function, as toxic foods are generally bitter and the ability to detect these compounds at low thresholds gave an

evolutionary advantage (Callaway, 2012). We speculate that an anticipatory expression of such bitter-taste chemosensors ensures that potential toxic foods are readily detected when the animal starts eating. A broader study approach of more T2R genes will help understand better why certain bitter sensors are upregulated and some downregulated anticipatory to feeding.

The cephalic response to nutrient intake is gaining more importance as a player in metabolism, including postprandial glycaemia (Ahrén & Holst, 2001; Zafra et al., 2006; Power & Schulkin, 2008). Gut peptides have been shown to be expressed in the tongue to mediate local and systemic effects. We therefore evaluated the diurnal regulation of gut peptide expression in the tongue. We were able to identify strong diurnal rhythms for *gcg, gip, pyy, ghrl* and *cck.* This highlights the potential importance of gut peptides outside of the gastrointestinal tract and potential involvement in feed-forward mechanisms from the oral cavity to the distal alimentary tract. These anticipatory mechanisms are potentially involved in preparing the alimentary and especially the gastrointestinal tract for incoming food to enable optimal nutrient assimilation. However, whilst such a pattern would be conducive to maximising nutrient intake in times of scarcity it could mean a potential 'maladaptation' to today's food availability.

Using the microarray, we were able to identify several interesting groups of genes with diurnal rhythmicity:

The NMDA receptor group was highly downregulated. The NMDA receptor is an L-glutamate receptor and ion-channel protein that is found in nerve cells. Activated by a ligand like L-glutamate, it leads to the influx of positively charged ions. In an EEC model it has been shown that the NMDA receptor is involved in gut peptide secretion

(Fukunaga et al., 2010). In the tongue, it has been suggested that the NMDA receptor acts as an ionotropic umami-taste receptor beside the classic metabotropic receptors T1R1/3 and mGlu4R (Lin & Kinnamon, 1999). Our data suggests that the NMDA receptor could potentially be involved in lingual gut peptide secretion, which is important for feed-forward mechanisms.

We were also able to identify genes involved in paracellular signal transmission. We identified pannexin1, which is a channel for second messengers like cAMP, and receptors for such nucleotides (p2x and p2y family) on neighbouring cells. This could be important for the signal transduction from taste cells to nerve cells with Taste-III cells lying in-between (Yoshie, 2009). These results need to be confirmed with transcriptomic and proteomic techniques and *in situ* experiments will help identify exact location of expression.

Manually selecting the top 10 up- and downregulated genes opened up new avenues for further research. The top 10 upregulated genes were either adrenergic nerve-signalling (*ankrd1, adrb3)*, immune processes (*dock2, h2-k1, vcam1, hivep3*) or transcription factors (*dbp, tead2, per3)*. *dbp* and *per3* are actually clock genes, and are highly involved in circadian rhythmicity (Stratmann et al., 2012).

The regulation of the adrenergic nerve-signalling mechanisms is interesting in combination with our findings of the NMDA receptor and transcellular signalling of pannexin1. Even if taste receptors are not regulated diurnally, but downstream signalling pathways are, the signals translated to the brain could potentially display rhythmicity. Indeed, studies have shown diurnal rhythmicity for sweet preference (Nakamura et al., 2008). Better understanding of the mechanisms underlying these

observations are important for better understanding the cephalic phase of digestion with respect to appetite, satiety and obesity.

A recent study also revealed how adrenergic nerve-signalling is important for signal circadian recruitment of immune cells to peripheral tissues, including skeletal muscle (Scheiermann et al., 2012). *vcam1,* especially, was pointed out in this study as this 'vascular cell-adhesion molecule-1' is necessary for leukocyte arrest on endothelial cells and subsequent extravasation into tissue. Based on this study and our results, we suggest that leukocyte infiltration mediated via adrenergic signals also takes place in the tongue. This could potentially help fend off infectious agents, as more contact with bacteria and viruses is made during times of high activity such as food finding and eating.

The top 10 downregulated genes are all enzymes (proteases, carbohydrases, lipases). Most of them (*try4, pla2g1b, cpb1, cpa1, ctrb1, clps, and ela3)* are highly specific to the pancreas. Oral enzymes such as amylase and lipase are important for the cephalic response of insulin secretion (Zafra et al., 2006). It would be expected to measure higher expression anticipatory to feeding rather than downregulation, as we saw in our experiment. This would help start digestion in the oral cavity and make it possible to sense exact nutrients in the food and prepare the rest of the digestive tract accordingly. As we used crude tongue tissue including muscle, epithelium, nerves and blood vessels, exact localisation of where these genes are expressed would help to shed light on the exact mechanism. Co-localisation with taste papillae would be interesting and requires further research.

The automatic 'circadian map' function in GeneGo™ enabled us to analyse new genes involved with clock genes. PACAP and its receptors were identified. PACAP has high similarity to the vasoactive intestinal peptide (VIP) and was 64% upregulated. PACAP binds to the VIP and PACAP receptor and stimulates adenylate-cyclase function. VIP has been found to be expressed in type-II taste cells in the tongue, and to be co-localised with αGustducin. Type-II cells also express the sweet-, bitter- and umami-taste receptors, which need αGustducin for signal transduction. VIP and PACAP share similar functions regarding metabolism and gastrointestinal function and VIP knockout mice can substitute the loss of VIP with PACAP (Winzell & Ahrén, 2007). One of PACAP's effects is to stimulate enterochromaffin-like cells in the intestine, and it has been shown to stimulate CCK secretion in the L-cell model, STC-1 (Chang et al., 1996). The high similarity to VIP and PACAP's involvement in intestinal nutrient sensing suggest further research on its role in the tongue. We speculate a potential involvement of PACAP in the cephalic phase similar to that of the gut peptides as discussed earlier.

3.7 Limitations of the study

Despite the vast amount of novel findings, several limitations appear in our study. We obtained our stomach samples frozen in tubes from a collaborator, and only dissected ~15 mg of material from the whole tissue block. We were not able to determine from which region the extracted RNA came. This is important, as the stomach has different regions with distinct functions, cell populations and mRNA expression-profiles (Young et al., 2009).

We were able to show matching diurnal rhythms for GLUT2 at the mRNA and protein levels. Unfortunately, we were not able to show diurnal rhythmicity for the sweet-taste receptor at the mRNA level or confirm our mRNA findings for SGLT3 on the protein level. A recent paper on circadian rhythms in the liver was able to show that transcriptomic rhythms do not always match proteomic rhythms and also showed how rhythms in the proteome do not necessarily stem from circadian regulated transcripts (Mauvoisin et al., 2014). This can be explained by the vast amount of transcriptional and post-transcriptional, as well as the various post-translational mechanisms, that alter mRNA and protein stability as discussed earlier. Furthermore, protein expression tends to slightly lag behind mRNA expression as seen for hexose transporters (Houghton et al., 2006). Generally, diurnal rhythms of the proteins also seem to be less clear, due to the sensitivity limitations of Western blot assays. Again, more time points will be needed to confirm our findings. In addition, proteins were extracted from tissues from other animals due to lack of tissue material. Proteins were taken from C57BL/6 inbred mice instead of the CD-1 outbred strain used for gene expression. We did run test qPCRs on those tissues to ensure diurnal rhythmicity, but many studies have shown differences in

study outcomes when comparing mouse strains (e.g. Barone *et al*., 1993). It cannot be excluded that differences between these two animal strains exist on a protein level, despite the same gene-expression results.

Furthermore, we unfortunately only had two time points available for our study. Many other studies show at least four time points, which make it possible to see a clearer circadian rhythm. Our two time points might be exactly when the oscillation is at the same level, and we therefore might have missed the diurnal rhythm. Moreover, even though the clock genes showed a clear rhythm other studies with many more time points show that maximum peaks for clock genes are just a few hours into the light and dark phases and not at the beginning, like in our study (Lamia et al., 2008; Takahashi et al., 2013). As the clock genes are generally higher induced than clock-controlled genes, this could explain why we see rhythms for clock genes, but not for the umami- and sweet-taste receptors. A repeat of the study with more time points is needed.

We were able show a slight diurnal rhythm for *gcg* in the jejunum. Gil-Lozano *et al.*, 2014 who published evidence for a circadian rhythm of GLP1 only in late 2014, despite many other studies having failed to do so, state that identical nutrient composition and caloric loads need to be ensured, as otherwise the rhythm gets obscured. Furthermore, the paper also shows *gcg* gene expression and shows peaks later at night during the dark phase. Our two time points, 7am and 7pm, would have barely shown a rhythm in their study. Again, more time points would have been useful, and blood GLP1 levels would have been instrumental. Later and more time points would also be beneficial for the other gut peptides (GIP, CCK, ghrelin), as we also suspect a diurnal rhythm.

3.8 Conclusions

This study was able to confirm findings of diurnal rhythms of expression of intestinal sugar transporters and clock genes shown from previous studies. However, we demonstrated for the first time that the novel sugar sensor SGLT3 displays diurnal rhythmicity in the stomach and small intestine tract at the mRNA, but not protein level. We suspect that the novel sugar sensor SGLT3 is expressed in a circadian, oscillatory pattern at the mRNA and protein levels and is under transcriptional regulation by clock genes, which are themselves entrainable by food within the intestinal lumen. SGLT3 may therefore play an important role in the FEO mechanism. The full function of SGLT3a+b is currently not clear, but the findings of this study suggest a role in the regulation of gastrointestinal function, such as sugar uptake and gut hormone secretion, as well the control of food intake by the brain. We also showed diurnal rhythmicity of expression of lingual gut peptides potentially involved in feed-forward mechanisms to the gut.

Our findings may be implicated in metabolic diseases such as diabetes and obesity. Better understanding of the underlying physiology will help optimise meal-timing and find new targets for dietary intervention and pharmaceutical treatment in metabolic disease.

CHAPTER 4

EFFECTS OF METABOLIC SYNDROME ON NUTRIENT-SENSOR EXPRESSION LEVELS IN THE ALIMENTARY TRACT AND INTESTINAL FUNCTION

4 EFFECTS OF METABOLIC SYNDROME ON NUTRIENT-SENSOR EXPRESSION LEVELS IN THE ALIMENTARY TRACT AND INTESTINAL FUNCTION

4.1 Summary

Background The prevalence of metabolic syndrome is increasing at an alarming rate. Yet there is a lack of understanding of the role of the alimentary tract in the development of metabolic syndrome. We hypothesised that macronutrient sensors and gut peptides are dysregulated in metabolic syndrome.

Aims: The aim of the study is to identify changes in gene expression affecting the function of the alimentary tract in a mouse model of metabolic syndrome induced by a chronic high-sucrose diet (HSD).

Methods To test our hypotheses, we studied gene-expression levels in a rodent model of metabolic syndrome. Mice had free access to chow, but HSD mice were given *ad libitum* access to sugar-rich condensed milk for six weeks. We applied gene-expression profiling techniques to further study genes in the murine affected by the high-sucrose feeding.

Results Mice were hyperphagic, had higher weight and became diabetic with high fasting-glucose in response to the chronic HSD. SGLT3a and SGLT3b were downregulated in the small intestine. Jejunal CCK and gastric ghrelin expression was increased. Furthermore, the microarray revealed a high regulation of inducible nitric oxide synthase (iNOS) in the tongue and small intestine, and also a high induction of inflammatory processes.

Conclusions Taken together, these data demonstrate a dysregulation of gastrointestinal nutrient-sensor and gut peptide expression and an inflammatory state in metabolic syndrome, suggesting such disturbances may be important in the development of diet-related diseases.

4.2 Background

The aetiology and pathophysiology of metabolic syndrome are a vast and complex interplay between genetic and environmental factors (Kaur, 2014). Yet the underlying issue and cause is the overconsumption of food and reduced activity levels, causing obesity. The hyperplasia and hypertrophy of fat cells leads to increased production of cytokines including TNFα, which leads to tissue inflammation and a cascade of consequences including macrophage infiltration (Xu et al., 2003). The local inflammatory state leads to systemic chronic inflammation, which ultimately drives insulin resistance (Hotamisligil, 1999). The effects metabolic syndrome, obesity and type 2 diabetes can have on tissues are extensive. Furthermore, what is causative to the phenotype or a consequence of another dysregulated system in the body can be hard to dissect. The influences and mechanisms in adipose, muscle, liver, brain and pancreatic tissues are well studied. The role in the aetiology of metabolic syndrome, but also the consequences on the alimentary tract, are less studied, but are becoming clearer. The sugar transporters GLUT2 and GLUT5 have been shown to be increased in the small intestine of diabetic rats (Corpe et al., 1996). GLUT2 has also been shown to be increased in intestinal brush-border membranes of obese patients (Ait-Omar et al., 2011). This observation has been explained to be potentially due to the loss of insulin-mediated GLUT2 internalisation (Tobin et al., 2008). This evidence suggests that the small intestine can contribute to postprandial hyperglycaemia, which is implicated in cardiovascular disease and type 2 diabetes.

Furthermore, it has been shown that the incretin response via GLP1 is blunted in type 2 diabetes (Nauck et al., 1986). GLP1 is important for inhibiting gastrointestinal

secretion and motility, especially gastric emptying (Wettergren et al., 1993; Nauck et al., 1997). The physiological role of GLP1 is hence the control of the delivery of digestive chyme to the proximal intestine and insulin secretion stimulated by insulinotropic nutrients mediated via incretins (Layer et al., 1995). Not only does GLP1 exert its effects via endocrine pathways, but the nervous system seems to be highly involved. Several observations speak for this. First of all, GLP1 is degraded rapidly within minutes due to the high activity of DPPIV, which may only allow action locally in the gut. Furthermore, intraportal infusion of GLP1 caused higher impulse activity in vagal nerve-trunks (Nishizawa et al., 1996). These impulses might be transmitted to the pancreas and also nucleus of the solitary tract and hypothalamus, linking the gut with the brain (Nakabayashi et al., 1996; Nakagawa et al., 2004). GLP1 inhibits appetite via these routes or directly in the brain, and it has been shown that in obesity GLP1 secretion is highly reduced after meal ingestion compared to lean controls (Ranganath et al., 1996). The loss of appetite-inhibition might then start a vicious circle, aggravating the uncontrolled weight gain.

Ghrelin, a hunger hormone produced and secreted from the stomach, has also been shown to be dysregulated in metabolic syndrome. The exact mechanisms for its regulation are not clear, but it has been suggested that a post-gastric sensor is needed to decrease its release upon eating (Williams et al., 2013). Duodenal αGustducin and the fat receptor GPR120 have been shown to be involved (Janssen et al., 2012; Gong et al., 2014). Studying the stomachs of obese patients revealed that gustatory cells, positive for TRPM5, were twice as numerous in stomachs of obese patients (Widmayer et al., 2012). The authors also found that neighbouring ghrelin-producing cells were also more

abundant. Similarly, in a high-fat-fed mouse model it was found that the fat receptor GPR120 was highly increased in the stomach, as were ghrelin- and gastrin-producing cells (Widmayer et al., 2015). Despite these observations in rodents, it has been observed that in obese humans ghrelin blood levels are actually lower (Tschop et al., 2001). Widmayer *et al.* suggest though that the higher number of ghrelin-producing cells is a compensatory response to the higher number of gustatory cells, as long-chain fatty acids have been shown to inhibit ghrelin production via GPR120 (Lu et al., 2012; Gong et al., 2014). Young *et al.* (2009) showed no change of TRPM5, T1R2 and T1R3 expression in duodena of type 2 diabetic patients, but demonstrated an inverse correlation to blood-glucose concentrations, although results were not significant. GLP1 release from intestinal L-cells is sweet-taste-receptor dependent (Gerspach et al., 2011).

Furthermore, iNOS has been shown to be enriched in gastric tufted cells, where it has been suggested to be an alternative to cellular PLCβ2 signalling, as well as stimulating interstitial nerve endings and neighbouring EECs, blood vessels and epithelial cells (Hofer et al., 1996). The sweet-taste receptor is also expressed in the pancreas, where dysregulated inducible NO synthase (iNOS) has been shown to play a role in the impairment of βcell function, ultimately leading to defective insulin secretion (Qader et al., 2003; Hegyi & Zoltán Rakonczay, 2011). NO released by lingual taste buds has been shown to act as a paracrine signalling-molecule that results in the depolarisation of taste cells, which leads to a nerve signal (Kretz et al., 1998; Lindemann, 2001; Nakamura et al., 2005).

As outlined earlier, it has been suggested that added sugars from SSBs are one of the factors responsible for the epidemic of metabolic syndrome. A recent study in

mice has shown how a diet high in sucrose increased total caloric intake, enhanced liver triglyceride accumulation and increased intestinal GLUT2, GLUT5 and CCK mRNA expression levels (Ritze et al., 2014). Hexose-transporter expression is potentially under the control of gut peptides from neighbouring EECs (Margolskee et al., 2007).

Based on these observations, we hypothesised that nutrient-sensor, gut-peptide and iNOS expression is dysregulated in the alimentary tract of mice with metabolic syndrome. We therefore used a mouse model chronically fed with a diet high in sucrose to induce metabolic derangements.

4.3 Chapter Overview – Effects of metabolic syndrome on nutrient-sensor expression levels in the alimentary tract and intestinal function

Aims

Identify the effects of high-sucrose feeding on the metabolic phenotype and small-intestinal gene expression.

Hypothesis

A chronic diet high in sucrose leads to type 2 diabetes mellitus and changes nutrient-sensor and gut-peptide expression levels in the small intestine.

Objectives

- Characterise the phenotype and metabolic profile of chronically high-sucrose-fed mice
- Study nutrient-sensor, gut-peptide and signalling gene-expression in the alimentary tract of high-sucrose-fed mice
- Evaluate the gene-expression profile via microarrays in the small intestine
- Evaluate the effects of chronic sucrose exposure in an *in vitro* enterocyte model, Caco-2/TC7 cells

4.4 Materials & Methods

4.4.1 Laboratory animals & study protocol

Sixteen C57BL/6 juvenile male mice (28 days of age) were obtained from Charles River, UK, and were randomly assigned to two groups (n = 8); either a high-sucrose (HSD) or a control diet. Mice were weighing 16.5 ±0.59 g and 16.5 ±0.39 for the control group and the HSD group, respectively. The animals were individually housed in cages with wood-chip bedding (Utemp1284 cages, Techniplast, UK; Aspen-wood chips, B and K Ltd) under controlled temperature (20–22˚C) and humidity conditions (45–65%) with a 12 h light/dark cycle.

All animals were acclimatised for one week with free access to the control diet (2% corn oil pellets) and water. After the acclimatisation period, the control mice were continued on the control diet and the HSD mice were given free access to light condensed milk (LCM) (Carnation milk, Nestlé, UK) in addition to the control diet (AIN93G) for a period of six weeks (see Table 5 for diet composition). As mice completely switch to LCM only, 1% corn oil was added to LCM to make the fat-to-carbohydrate ratio similar to the control diet. This diet is considered a low-fat diet, as fat content is below 4.5%.

Throughout the intervention, body weights, chow intake and LCM consumption were recorded daily. Animal husbandry was carried out by Prof Victor Preedy and Dr Peiying Sim and tissue isolation by Dr Christopher Corpe.

Table 5 Macronutrient composition of control pellet chow and LCM

	2% corn oil chow		LCM+ 1% corn oil	
	g / 100 g	**kcal/100 g**	**g/100 g**	**kcal/100 g**
Total carbohydrate	68	255	59.5	223.1
• **Starch**	58	217.5	0	0
• **Sucrose**	10	37.5	47.3	177.4
• **Lactose**	0	0	12.2	45.7
Fat	2	18	1.2	10.8
Protein	20	80	9.3	37.2
Total	**100**	**353**	**100**	**271.1**

4.4.2 Serum biomarker analysis

After the six-week dietary intervention, all animals were fasted overnight to minimise the variation in the time between the last food intake and blood and tissue collection. All animals were sacrificed via decapitation. Blood samples were collected and whole blood was processed to obtain serum. Serum glucose, insulin, cholesterol and triglycerides were analysed using Siemens Healthcare Diagnostics Ltd kits, following the manufacturer's instructions. Non-esterified fatty acids (NEFA) were analysed using a kit by WAKO Chemicals. Serum analysis was carried out by Prof Victor Preedy and Dr Peiying Sim.

4.4.3 Transcriptomic analysis

RNA extraction, cDNA synthesis, qPCR and Microarray analysis were performed as described in section 3.4. Statistical analysis of qPCR data was performed as described in section 3.4.5.

4.4.4 Caco-2 experiments

Caco-2/TC7 cells were grown as outlined in section 2.4.1. After 18 days of culture in 25 mM D-Glu, cells were split into two groups (n = 6/group). One group was switched to 25 mM sucrose, whereas the other group was continued in 25 mM D-Glu. Media was glucose-free DMEM equivalent to 'growth media' including FBS, MEM, Pen/Strep as outlined earlier, but 25 mM D-Glu or sucrose was added. After three days of culture in sucrose or D-Glu, media was either collected for nitric oxide measurements (see 4.4.5) or RNA harvested as outlined for further transcriptomic evaluation using qPCR (see 2.4.3).

4.4.5 Nitric-oxide detection

Cell media was harvested at day 21 from Caco-2/TC7 cells as outlined in section 4.4.4. Media (0.5 ml) was diluted 1:3 in reaction buffer and filtered using Millipore 10,000 MWCO filters (Fisher Scientific, Leicestershire, UK) by centrifugation for 5 min with 14,000 × g on an Eppendorf 5417R centrifuge. Filtrate was harvested and nitric oxide determined using a 'Nitric Oxide (total), detection kit' following the supplied protocol (Enzo Lifesciences, Exeter/UK).

4.5 Results

4.5.1 High-sucrose diet: Food intake & calorie consumption

Mice had free access to either the control diet or control pellet chow with free access to LCM (HSD). The control diet consisted of pellet chow including 2% corn oil and the HSD group had access to the control pellet chow with free access to LCM including 1% corn oil. Food and calorie intake was calculated daily over six weeks of the experiment, including the acclimatisation week. As seen in Fig. 36, mice completely neglected pellet chow when LCM was offered. The higher palatability of LCM led to increased consumption of food per day than in the control group with a daily average of 3.15±0.07 g/day vs 4.88±0.06 g/day for the control group and HSD group, respectively.

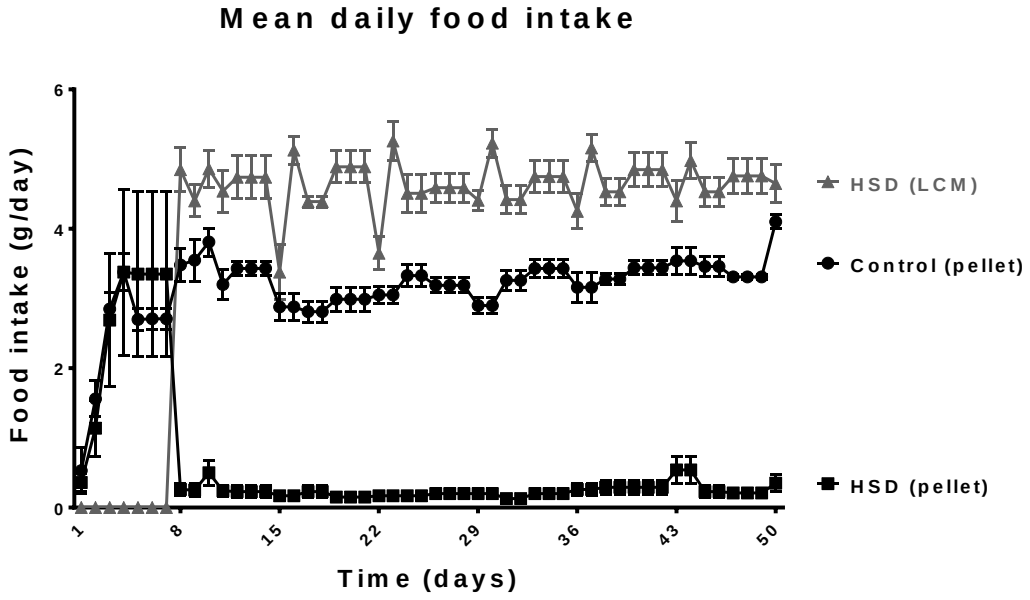

Fig. 36 Mean daily food intake (g·day−1) in C57BL/6J mice during prolonged HSD or control feeding. Mice at 4 weeks of age were caged singly, acclimatised on the control diet for 1 week and then weaned on either the control diet or the HSD. Daily consumption of pellet chow and additional LCM in the HSD group were measured. Data presented are means ± SEM (n = 8).

The increased food intake results in higher energy intake over the course of the study (Fig. 37a). Mean daily intake was of 11.56±0.15 vs 13.42±0.16 kcal/day ($p \leq 0.001$) for the control group or HSD group respectively (Fig. 37b). The higher energy consumption led to increased body weights over the six week experiment. Control mice reached 20.6 ±0.66 g body weight, whereas the HSD group had 22% higher body weight with 25.2 ±0.42 g (Fig. 37c).

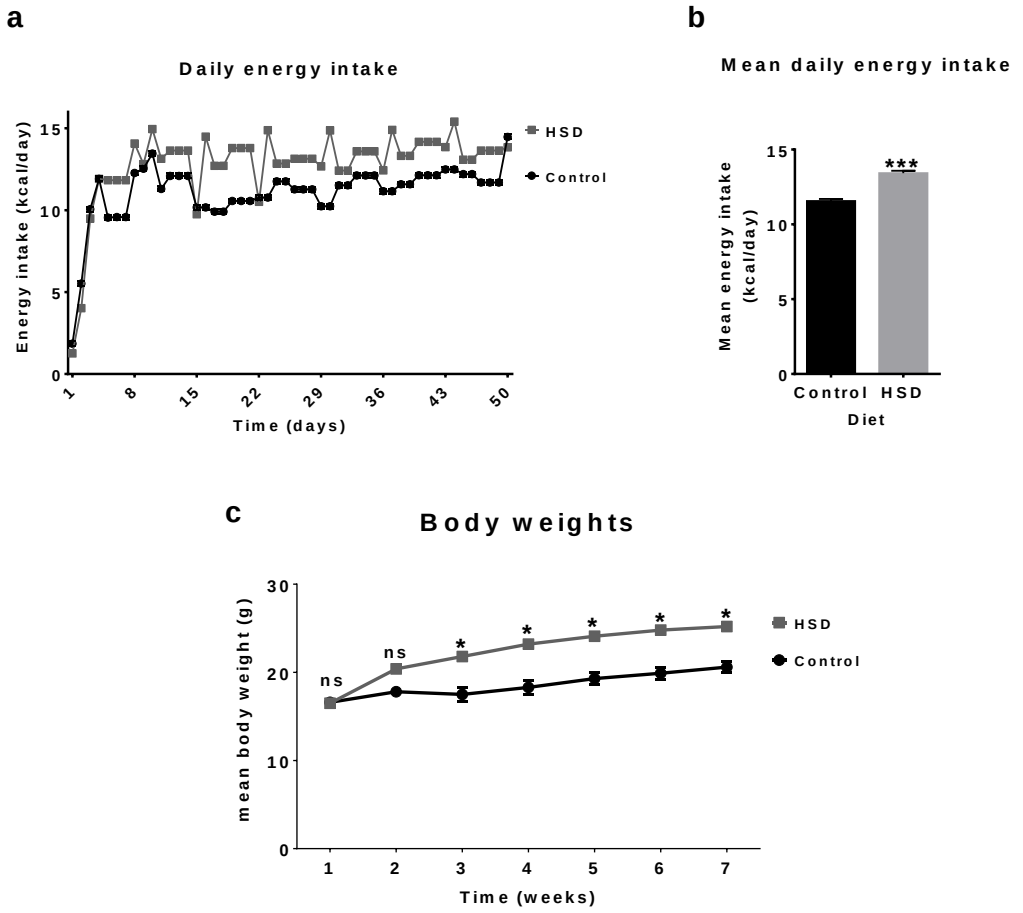

Fig. 37 Energy intake and body weights in C57BL/6J mice during prolonged control or HSD feeding. Mice at 4 weeks of age were caged singly, acclimatised on the control diet for 1 week and weaned on either the control diet or the HSD. Daily energy intake was calculated based on dietary composition (**a**). Mean daily energy intake was calculated over the six-week dietary intervention (**b**). Body weights were measured weekly and means are given (**c**). Data presented are means ± SEM (n = 8). Statistical significance was established with a Student's t-test or one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

4.5.2 High-sucrose diet: Macronutrient intake

The high preference and consumption of LCM changed the macronutrient intake in both groups. Mice in the HSD group had significantly higher intake of carbohydrates, especially sucrose and lactose, but significantly lower intake of protein (Fig. 38). Fat consumption was below 1% of total energy intake and no change was observed between control and HSD mice. HSD mice derived 62% of their total caloric intake from sucrose, compared to only 11% in the control group ($P < 0.001$).

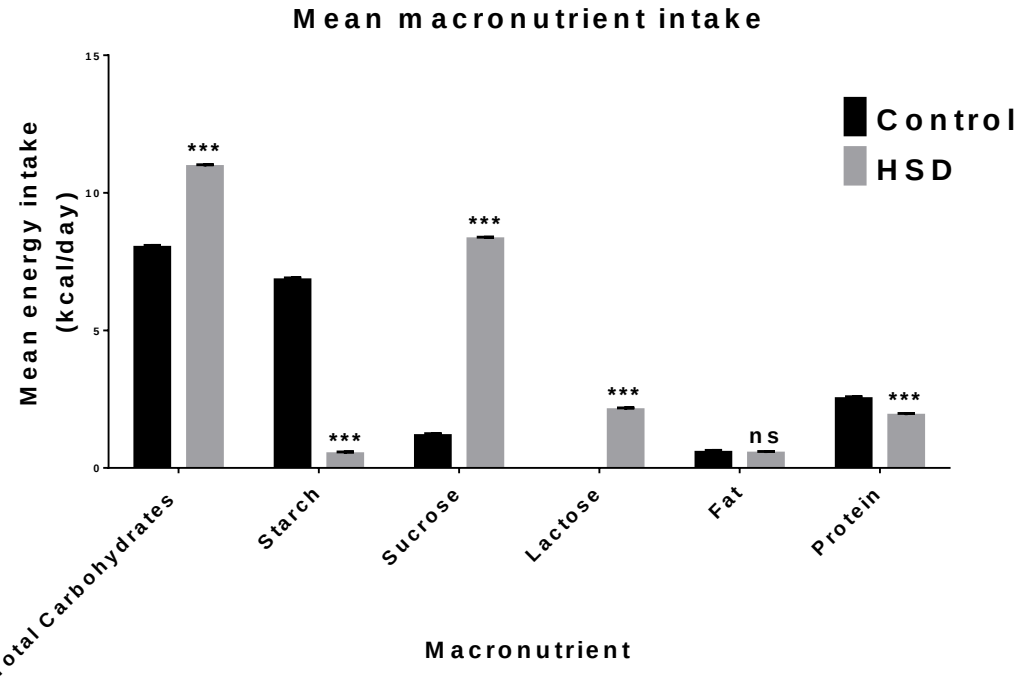

Fig. 38 Macronutrient intake of C57BL/6J mice during prolonged HSD or control feeding. Macronutrients were calculated from the mean daily food intake (pellet and LCM) based on the composition information outlined in methods section 4.4.1. Data are presented as means ± SEM (n = 8). Statistical significance was tested with a Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

4.5.3 High-sucrose diet: Blood analysis

Not only does the high intake of carbohydrates, especially the simple disaccharides lactose and sucrose, led to higher body weights, but biochemical analysis displayed mean fasting glucose levels of 11.89 mMol/L in HSD mice compared to 5.23 mMol/L for control mice (Fig. 39). HSD mice were not hyperinsulinemic, but had increased mean serum cholesterol levels (2.08 vs 1.82 mMol/L, p=0.06 HSD vs control). Serum triglyceride and non-esterified fatty acids (NEFA) were decreased between groups. Serum triglycerides levels decreased from 0.71 to 0.48 mMol/L ($P < 0.05$) and NEFA levels from 1.50 to 0.85 mMol/L ($P < 0.05$) in HSD mice.

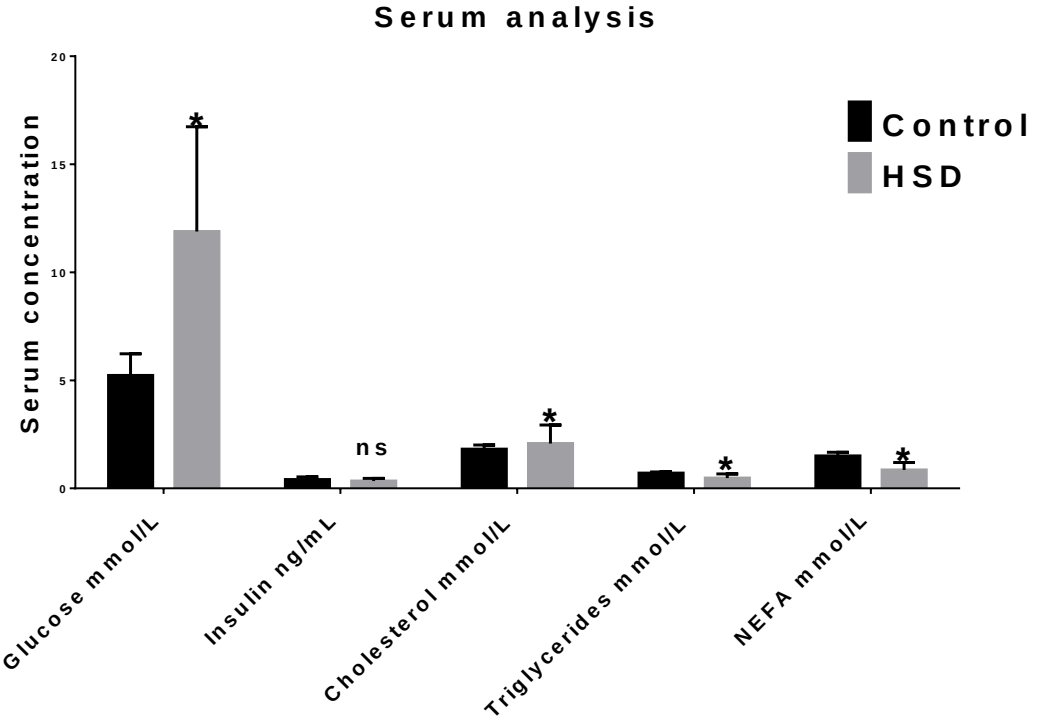

Fig. 39 Blood serum analysis for metabolic biomarkers of C57BL/6J mice during prolonged HSD or control feeding. Levels were measured from blood serum as outlined in methods section 4.4.2 and data are presented as means ± SEM (n = 8). Statistical significance was tested with a Student's t-test. *P < 0.05, ns = not significant.

4.5.4 Nutrient-sensor, gut-peptide and iNOS expression in the alimentary tract of mice on a high-sucrose diet

We used microarrays to study gene-expression profiles in the small intestine after six weeks of high-sucrose feeding. We manually extracted taste- and nutrient-sensing genes including fat-, umami-, sour- and bitter-taste genes, as well as downstream signalling molecules, from the microarray. Only very little change for sweet, umami, sour and bitter receptors, as well as αGustducin and αTransducin, was found (see Fig. 40).

Regulated nutrient sensors in the small intestine of HSD fed mice

	Protein	Gene name	Fold change Ctrl vs HSD		
			Duo	Jej	Ile
Sweet	T1R2	*tas1r2*	1.15	-1.03	1.12
	T1R3	*tas1r3*	1.13	1.07	-1.05
	SGLT3a	*slc5a4a*	**-2.61**	**-1.73**	**1.19**
	SGLT3b	*slc5a4b*	**-4.23**	**-3.0**	**-1.13**
Fat	CD36/FAT	*cd36*	**-4.62**	**-3.12**	**-1.41**
	FFAR2	*ffar2*	1.02	1.04	1.09
	GPR120	*gpr120*	-1.10	-1.21	-1.13
Umami	mGluR4	*grm4*	-1.03	-1.13	-1.01
	T1R1	*tas1r1*	1.11	-1.07	-1.15
Sour	TRPP3	*pkd2l1*	1.15	1.00	-1.13
	ENaCα	*scnn1a*	-1.09	-1.15	-1.06
	ENaCβ	*scnn1b*	1.00	-1.31	-1.07
	ENaCγ	*scnn1c*	NA	NA	NA
Bitter	T2R102 – T2R143	*tas2r102 – tas2r143*	-1.43 – 1.33	-1.59 – 1.22	-1.27 – 1.06
		36 genes			
Signalling	αGustducin	*gnat3*	1.28	1.31	1.29
	αTransducing	*gnat1*	1.19	-1.44	-1.74
	TRPM5	*trpm5*	**1.66**	1.20	1.14
	Phospholipaseβ2	*plcb2*	**1.52**	1.18	-1.04

Fig. 40 Fold changes of regulated nutrient-sensor genes in the small intestine of HSD-fed mice. RNA was pooled from 8 mice per group and analysed on an Affymetrix microarray chip. mRNA expression was compared between Ctrl and HSD mice and fold changes are given.

CD36 was downregulated in all three tissues studied, but the highest regulation was in the proximal regions (-4.62x in the duodenum and -3.12x in the jejunum) compared to a downregulation of only -1.41 in the ileum. This cephalocaudal pattern corresponded to the signalling genes *trpm5* and *plcb2*, which were only upregulated in the proximal regions of HSD-fed mice (Fig. 40).

Interestingly, we also found high regulation of the SGLT3 sensor/transporter genes with the same pattern cranial to caudal. SGLT3a and SGLT3b were downregulated -2.61- and -4.23-fold in the duodenum, but only -1.73- and -3-fold in the jejunum and were barely regulated in the ileum (Fig. 40).

We were able to confirm the microarray findings via qPCR. While T1R2 showed no regulation in all tissues, T1R3 showed downregulation by 28% ($P<0.05$) and 27% ($P=0.08$) in the jejunum and ileum (Fig. 41a+b). SGLT3a and SGLT3b were both downregulated in the duodenum and jejunum, as indicated by the microarray (Fig. 41c+d). Furthermore, the fat receptor CD36 was highly downregulated in both the duodenum (-80%) and the jejunum (-64%), but was not regulated in the tongue, stomach or ileum (Fig. 41e).

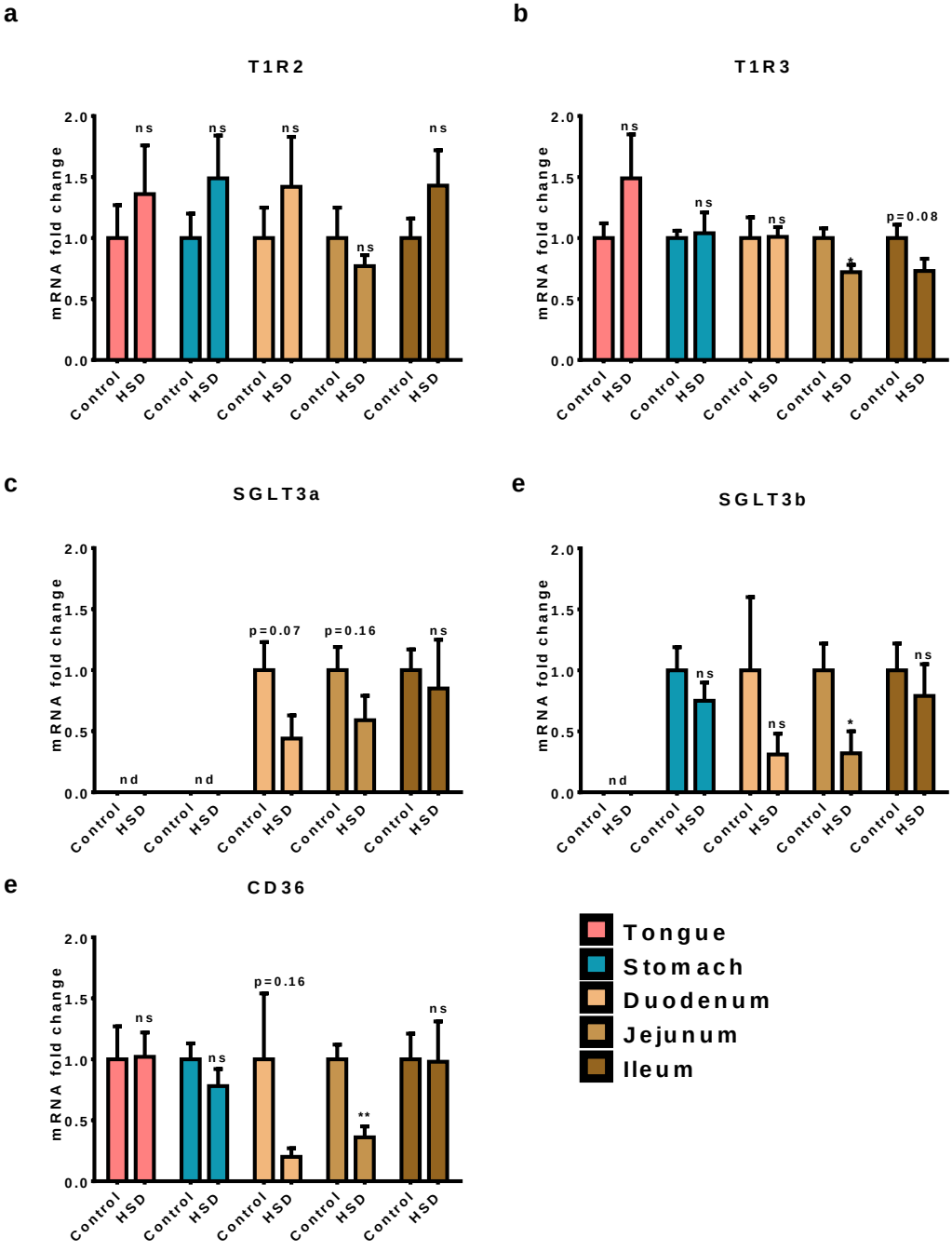

Fig. 41 Gene-expression change of nutrient receptors in the alimentary tract of mice fed a HSD. Whole tongue, stomach, duodenum, jejunum and ileum were dissected from C57BL/6 fed either the HSD or the control diet. RNA was extracted for subsequent quantitative PCR of the sweet-taste receptor subunits **a)** *tas1r2* (T1R2) **b)** *tas1r3* (T1R3), **c)** *slc5a4a* (SGLT3a), **d)** *slc5a4b* (SGLT3b) and **e)** *cd36b* (CD36). Values are normalised against 3 reference genes, relative quantitation was performed using the ddCT method and expressed relative to the control diet. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant, nd = not detected.

As nutrient sensor can regulate gut peptide secretion, we analysed the gene expression of CCK, ghrelin and preproglucagon. CCK expression was significantly upregulated by 65% in the jejunum of HSD mice (Fig. 42a). Ghrelin showed a 4-fold induction in the stomach (Fig. 42b), but did not reach statistical significance (p=0.13). GLP1+2 was induced in the ileum by 65% (p=0.055) (Fig. 42c).

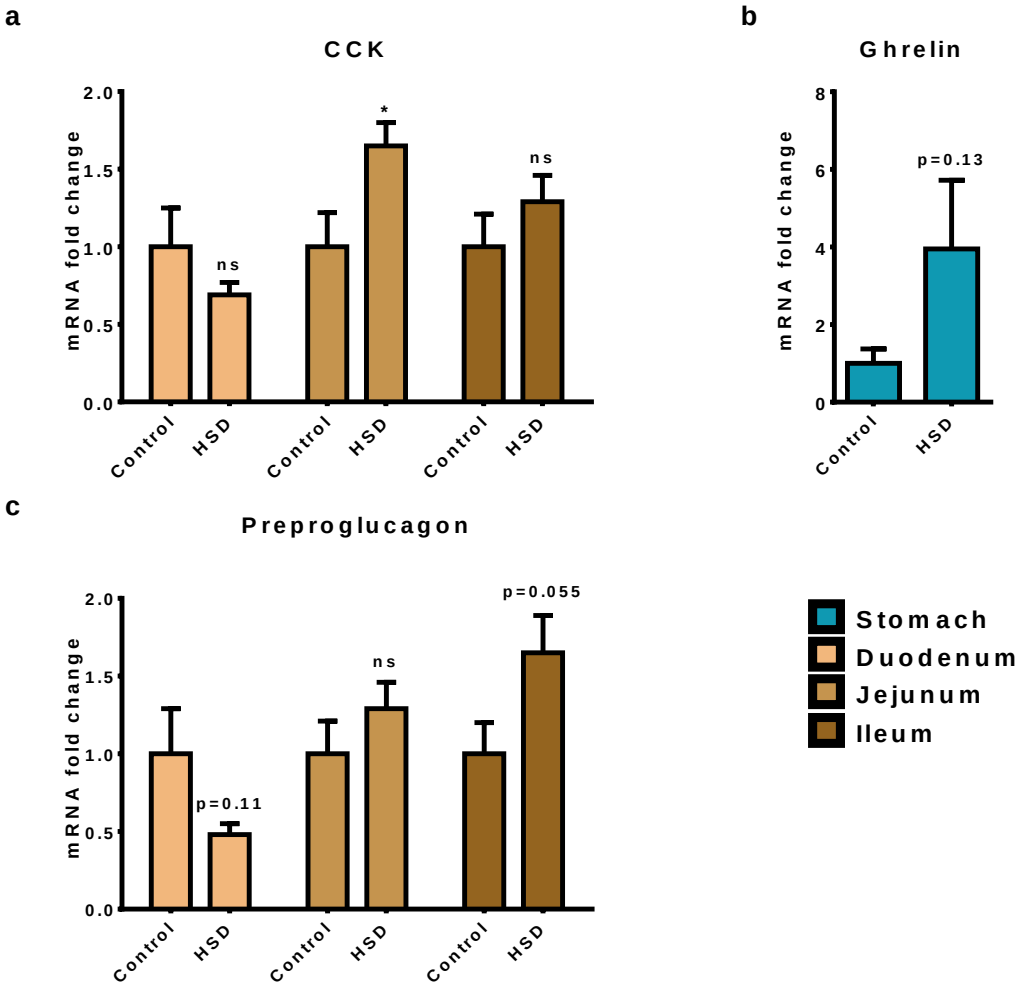

Fig. 42 Gene-expression change of gut peptides in the gastrointestinal tract of mice fed a HSD. Whole stomach, duodenum, jejunum and ileum were dissected from C57BL/6 fed either the HSD or the control diet. RNA was extracted for subsequent quantitative PCR of the gut peptides **a**) *cck* (CCK) **b**) *ghrl* (ghrelin) and **c**) *gcg* (preproglucagon). Values are normalised against 3 reference genes, relative quantitation was performed using the ddCT method and expressed relative to the control diet. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

Our array data showed an upregulation in the duodenum. As NO signalling has been implicated in pancreatic dysfunction, lingual taste-signal transduction and gastric tufted cell signalling, we hypothesised that iNOS could also be an important signalling molecule in EECs and that the expression of *nos2* is also upregulated along the alimentary tract of HSD-fed mice. Indeed, *nos2* was highly induced in the tongue and small intestine of HSD mice compared to control animals (Fig. 43). *nos2* was upregulated 7-fold in the tongue, 34-fold in the duodenum, 6-fold in the jejunum and 4-fold in the ileum, but not in the stomach (Fig. 43).

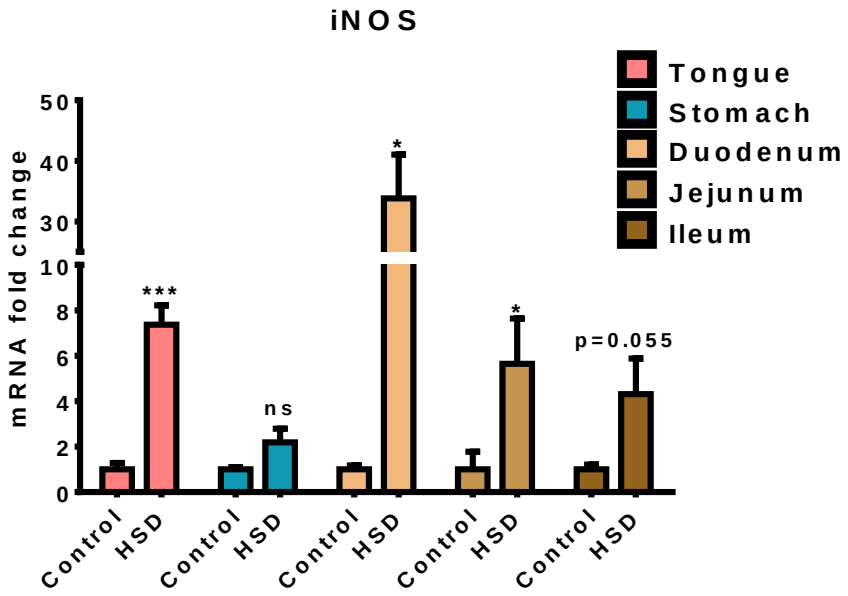

Fig. 43 Gene-expression change of iNOS/*nos2* in the alimentary tract of mice fed a HSD. Whole tongue, stomach, duodenum, jejunum and ileum were dissected from C57BL/6 fed either the HSD or the control diet. RNA was extracted for subsequent quantitative PCR of *nos2* (iNOS). Values are normalised against 3 reference genes, relative quantitation was performed using the ddCT method and expressed relative to the control diet. *P < 0.05, **P < 0.01, ***P < 0.001, ns = not significant.

4.5.5 Gene-expression profiling of the small intestine of high-sucrose fed mice

4.5.5.1 Enrichment analysis

We further used the microarray data to compare the expression profiles of the duodenum, jejunum and ileum, which revealed 81 common and 297 similar genes affected by the dietary intervention. Yet 408, 369 and 403 genes were uniquely regulated in the duodenum, jejunum and ileum respectively (Fig. 44).

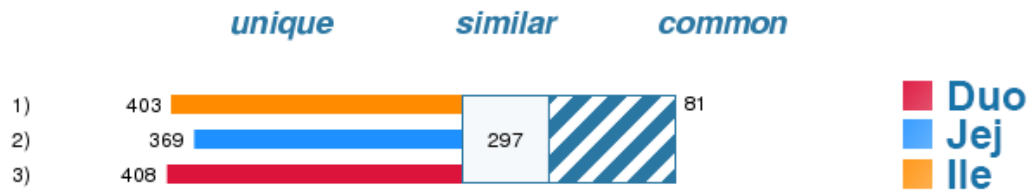

Fig. 44 Unique, similar and common genes regulated in the HSD model between the duodenum, jejunum and ileum.

Enrichment analysis revealed that common pathway maps, GO processes and networks were related to either cell cycle (mitosis, replication, transcription), immune response (antiviral, B-cell receptor, interferon signalling) or regulation of metabolism (progesterone signalling, retinol metabolism, bile-acid regulation) (see appendix II).

4.5.5.2 Top 10 regulated genes

We manually extracted the top 10 up- and downregulated genes in all three tissues. On the one hand it confirmed the high regulation of immune-system genes (*reg2, fut2, reg3d, susd2, anxa10, nos2*), but it also revealed novel genes to be regulated by the diet. Genes regulated in the duodenum were involved in Golgi glycosylation (*mgat4c, b3galt5, fut2),* NADPH/ROS metabolism (*duoxa2, cubn*), Cytochrome superfamily (*cyp2c38, cyp3a44, cybrd1*), gluconeogenesis (*pck1*), Calcium channel (*trpbv),* lactate/acetate *(slc5a12*) or Vitamin C (*slc23a1)* absorption and neurotransmission (*nos2)* (Fig. 45). Especially of interest is the 4-fold downregulation of the novel sugar sensor *slc5a4b*/SGLT3b and the fat sensor *cd36*/CD36 (Fig. 45).

Top ±10 regulated genes in the Duodenum

Protein	Gene name	Fold change Ctrl vs HSD
Regenerating islet-derived 2	*reg2*	**+14.33**
Dual oxidase maturation factor 2	*duoxa2*	**+9.51**
Inducible nitric oxide synthase	*nos2*	**+4.74**
Acetylglucosaminyltransferase	*mgat4c*	**+4.50**
Galactosyltransferase 5	*b3galt5*	**+4.44**
Fucosyltransferase 2	*fut2*	**+4.08**
Regenerating islet-derived 3	*reg3d*	**+4.00**
Annexin A10	*anxa10*	**+3.97**
Cytochrome b reductase	*cybrd1*	**+3.94**
Resistin like beta	*retnlb*	**+3.85**
Sushi domain containing 2	*susd2*	**-3.60**
Solute carrier family 23, member 1	*slc23a1*	**-4.02**
Cytochrome P450, family 3, subfamily a	*cyp3a44*	**-4.03**
Solute carrier family 5, member 4b	*slc5a4b*	**-4.23**
Transient receptor potential cation channel	*trpv6*	**-4.56**
CD36 Antigen/FAT	*cd36*	**-4.62**
Cytochrome P450, family 2, subfamily C	*cyp2c38*	**-5.00**
Phosphoenolpyruvate carboxykinase 1	*pck1*	**-5.82**
Cubilin (intrinsic factor-cobalamin receptor)	*cubn*	**-7.35**
Solute carrier family 5, member 12	*slc5a12*	**-33.87**

Fig. 45 Fold changes of the top ±10 HSD regulated genes in the murine duodenum. RNA was pooled from 8 mice per group and analysed on an Affymetrix microarray chip. mRNA expression was compared between Ctrl and HSD and fold changes are given.

An overlap of certain genes between the duodenum and the jejunum was noticed (*duoxa2, b3galt5 susd2*) (Fig. 46), and a majority of genes, mainly upregulated, were also involved in immune function (*ifi44, ms4a1, ccl20, bank1, sell, h20-ob, susd2, plb1)* or in the Cytochrome superfamily (*cyp2c38, cyp4a31, cyp4a10*). Highly regulated was the gene encoding phospholipase B1 *plb1,* with an 87-fold downregulation. Two genes were involved in ketone/lipid metabolism (*oxct1, ces1*), *nt5e* is a nucleotidase converting extracellular nucleotides to membrane-permeable nucleosides, *bpm9* is involved in cell maturation, especially of cholinergic neurons and *ddah1* is involved in generating nitric oxide by regulating cellular concentrations of methylarginines, which in turn inhibits nitric oxide synthase activity (Fig. 46).

Top ±10 regulated genes in the Jejunum

Protein	Gene name	Fold change Ctrl vs HSD
Interferon-induced protein 44	*ifi44*	**+5.75**
Membrane-spanning 4-domains	*ms4a1*	**+5.12**
Chemokine ligand 20	*ccl20*	**+4.66**
Dual oxidase maturation factor 2	*duoxa2*	**+4.23**
B-cell scaffold protein	*bank1*	**+4.10**
Selectin, lymphocyte	*sell*	**+3.73**
Histocompatibility 2, O region, beta locus	*h2-ob*	**+3.41**
3-oxoacid CoA transferase	*oxct1*	**+3.30**
Histocompatibility 2, class II E beta 2	*h2-eb2*	**+3.24**
Galactosyltransferase 5	*b3galt5*	**+3.11**
5' Nucleotidase, ecto	*nt5e*	**-3.98**
Dimethylarginine aminohydrolase	*ddah1*	**-4.07**
Carboxylesterase 1	*ces1*	**-4.10**
Cytochrome P450, family 2, c, 38	*cyp2c38*	**-4.71**
Phsophoenolpyruvate carboxykinase 1	*pck1*	**-4.88**
Cytochrome P450, family 4, a, 31	*cyp4a31*	**-5.45**
Cytochrome P450, family 4, a, 10	*cyp4a10*	**-5.51**
Sushi domain containing 2	*susd2*	**-5.72**
Bone morphogenetic protein 9	*bmp9*	**-6.59**
Phospholipase B1	*plb1*	**-86.97**

Fig. 46 Fold changes of the top ±10 HSD regulated genes in the murine jejunum. RNA was pooled from 8 mice per group and analysed on an Affymetrix microarray chip. mRNA expression was compared between Ctrl and HSD and fold changes are given.

In the ileum, too, a high induction of immune genes was noticed, especially interferon-signalling genes (*ifi44, ifi1, herc5, ddx60)* and genes belonging to the gasdermin family (*gsdmc4, gsdmc3, and gsdmc2)* were downregulated, whereas B-Lymphocyte genes *cd19* and *ms4a1* were down (Fig. 47). Also regulated were genes involved in lipid metabolism (*adipoq*, *lpl, scd1),* imino-acid transport (slc6a20b), histone acetylation (*hist1h2ab*) and a carbonic anhydrase (*car5b*) (Fig. 47).

Top ±10 regulated genes in the Ileum

Protein	Gene name	Fold change Ctrl vs HSD
Gasdermin-C4	*gsdmc4*	**+6.36**
Solute carrier family 6, member 14	*slc6a14*	**+5.85**
Gasdermin-C3	*gsdmc3*	**+5.76**
Gasdermin-C2	*gsdmc2*	**+3.64**
interferon-induced protein 1	*ifi1*	**+2.93**
Interferon-induced protein 44	*ifi44*	**+2.75**
Potassium inwardly-rectifying channel, J, 13	*kcnj13*	**+2.75**
DEAD box polypeptide 60	*ddx60*	**+2.67**
Hect domain & RLD 5 isoform 1	*herc5*	**+2.61**
Histone cluster 1, H2ab	*hist1h2ab*	**+2.48**
Adiponectin, C1Q	*adipoq*	**-3.21**
Lipoprotein lipase	*lpl*	**-3.21**
Stearoyl-coenzyme A desaturase 1	*scd1*	**-3.49**
Solute carrier family 6, member 20b	*slc6a20b*	**-3.83**
Carbonic anhydrase 5b	*car5b*	**-3.85**
B-lymphocyte antigen CD19	*cd19*	**-4.15**
Cytochrome P450, family 4, a, 10	*cyp4a10*	**-4.99**
B-lymphocyte antigen CD20	*ms4a1*	**-5.44**
Cytochrome P450, family 4, a, 31	*cyp4a31*	**-6.69**
Cytochrome P450, family 2, e, 1	*cyp2e1*	**-6.89**

Fig. 47 Fold changes of the top ±10 HSD regulated genes in the murine ileum. RNA was pooled from 8 mice per group and analysed on an Affymetrix microarray chip. mRNA expression was compared between Ctrl and HSD and fold changes are given.

4.5.6 Effects of chronic sucrose exposure in an enterocyte *in vitro* model, Caco-2/TC7 cells

Our gene-expression studies in high-sucrose fed animals revealed a downregulation of *slc5a4*/SGLT3 in the small intestine. Furthermore, we detected an induction of *nos2* in the tongue and small intestine and a downregulation of *ddah1* in the small intestine, both involved in NO production. Yet we do not know if the sucrose is causative for the gene down-regulation, in which cell-types these genes are downregulated and if the higher expression of iNOS leads to higher NO levels. We hypothesised that the higher activity of iNOS in enterocytes leads to NO generation and in turn downregulation of *slc5a4* and *cd36.*

We therefore used Caco-2/TC7 cells as a model for intestinal epithelium. To test if Caco-2/TC7 cells express SGLT3, we performed RT-qPCR for SGLT1 (*slc5a1*), SGLT3 (*slc5a4*) and CD36 (*cd36*) in differentiating Caco-2/TC7 cells at day 5, 10 and 21. As expected, SGLT1 expression is increased during the differentiation of the Caco-2/TC7 cells and 73x higher at day 21 compared to day 5 (Fig. 48a). SGLT3 shows a similar, pattern with mRNA expression increasing 29-fold on day 21 (Fig. 48b). CD36 was not expressed (data not shown).

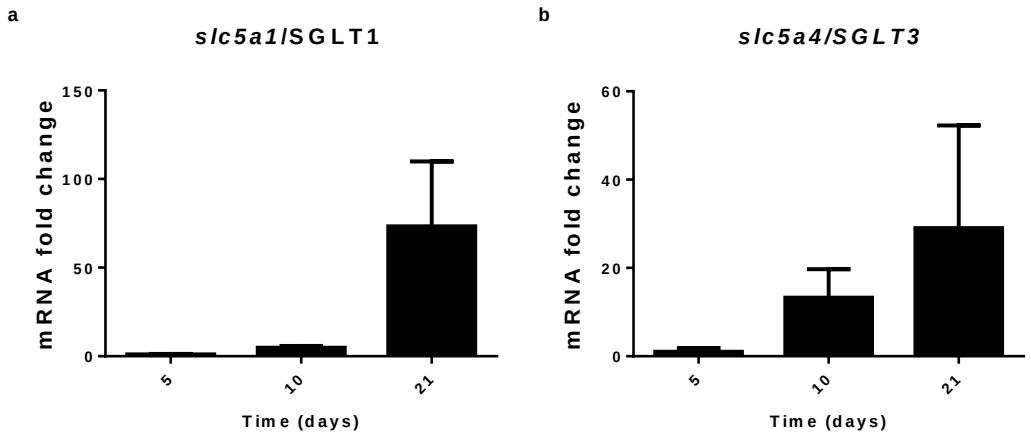

Fig. 48 SGLT1 and SGLT3 mRNA expression time-course in Caco-2/TC7 cells. RT-qPCR was performed on 6 samples on day 5, 10 or 21 and normalised against βActin expression. Values are expressed relative to day 5.

62% of the energy intake in the HSD group is from sucrose. We therefore stimulated differentiated Caco-2/TC7 cells for three days with 25 mM sucrose or D-Glu to compare expression levels of SGLT1, SGLT3 and iNOS and also measured NO produced by the cells. Sucrose stimulation did not increase gene expression for *slc5a1*/SGLT1, *slc5a4*/SGLT3 or *nos2*/iNOS (Fig. 49a). Nitric oxide in cell media was indeed increased by 169%, but did not reach significance (p = 0.09, Fig. 49b).

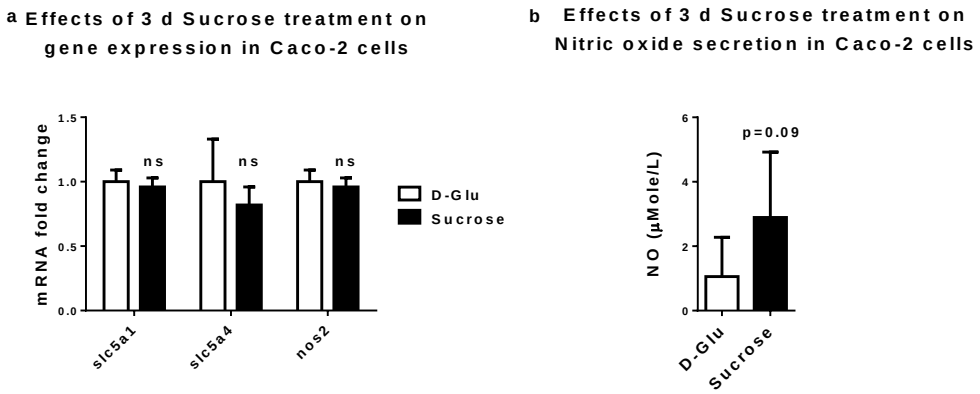

Fig. 49 Effect of 3-day sucrose feeding of Caco-2 cells on gene expression and nitric oxide secretion. Differentiated, 18-day-old Caco-2/TC7 cells were maintained in either 25 mM D-Glu (white bars) or sucrose (dark bars). After 3 days cells were harvested either for RNA extraction and RT-qPCR (**a**) or nitric oxide measurements from media (**b**). ns = not significant.

4.6 Discussion

At the heart of metabolic syndrome, obesity and type 2 diabetes lies the overconsumption of foods. Carbohydrate intake in the form of added sugars in foods and SSB has significantly increased in recent decades, and it has been suggested that this increase is one of the factors responsible for the obesity epidemic. The gastrointestinal tract has been established as an important endocrine organ and regulator of metabolism and its role in the pathophysiology of metabolic syndrome is gaining more importance. Only few studies have examined whether gastrointestinal nutrient sensing and gut peptides are dysregulated in metabolic syndrome and mainly focus on the stomach (Young et al., 2009; Widmayer et al., 2012; Ritze et al., 2014; Widmayer et al., 2015). We therefore aimed to evaluate how a diabetogenic diet high in added sugars changes the function of the alimentary tract with special focus on nutrient-sensor expression levels.

4.6.1 Metabolic observations, nutrient sensing & gut peptides

To induce metabolic syndrome in our study we used fat-reduced condensed milk, which combines several things: 1) a preferred concentration of sugar and sweetness, 2) high energy density, 3) a soft and preferred texture and 4) constant accessibility. The high reward and palatability led to increased food intake, which in turn led to higher energy intake and the slow accumulation of body weight as demonstrated. Metabolic profiling on fasting blood revealed that HSD mice were diabetic, characterised by fasting hyperglycaemia and hypercholesterolemia. Our study adds to the pool of studies associating high sugar intake with effects on metabolic health.

The macronutrient analysis of the diet revealed that the HSD group not only has higher intake of carbohydrates but also shows a switch from starch to the simple, refined sugars sucrose and lactose. Many studies in rodents have been conducted comparing complex and simple carbohydrates on body-fat accumulation. Sucrose-rich pellet chow can induce body-fat gain in some contexts when compared to starch (Reiser & Hallfrisch, 1977; Hallfrisch et al., 1979; Toida et al., 1996). In some cases, though, sugar actually leads to similar or less body-weight increases than starch (Gutman et al., 1987; Surwit et al., 1995; Black et al., 1998; Cresci et al., 1999). A potential reason for the varied results is the concentration of sugars in the test diets, as too low or too high a concentration has been shown to change the taste preference and a 'sweet spot' at ~25% sugar in water has been established (Richter & Campbell, 1940). Adding sugar to drinking water is actually another very effective way to fatten animals. Offering animals sweet sugar rich liquids consistently causes body-fat accumulation (Kanarek & Orthen-Gambill, 1982; Young et al., 2009).

4.6.2 Nutrient-sensor & gut-peptide expression

The metabolic observations led us to hypothesise that the metabolic dysregulations were due to disturbances in the small intestine, especially of nutrient-sensing mechanisms and gut-peptide secretion. The analysis of the nutrient-sensor expression levels revealed some novel and interesting findings. Although T1R2 and T1R3 were not regulated in any of the tissues studied, strong downregulations were found for CD36 and SGLT3a+b in the intestine. All three sensors were more downregulated in the duodenum and jejunum than in the ileum. This is interesting, as the proximal intestine is

where luminal nutrient concentrations are highest. Furthermore, CCK expression was increased in the jejunum and ghrelin was highly induced in the stomach, which is in line with other studies (Ritze et al., 2014; Widmayer et al., 2015).

These adaptions could be part of a response mechanism adjusting the gastrointestinal system to the constant energy and sugar surplus. It is plausible that such a downregulation of SGLT3a+b expression in the small intestine might be due to desensitisation of the cells as a consequence of a sustained access to sucrose and its breakdown products. Yet the fact that SGLT3, expressed in the enteric nervous system, is downregulated could have several effects. Meal-induced ghrelin suppression relies on a small-intestinal nutrient sensor. Indeed, we measured higher expression of ghrelin in the stomach in HSD mice. This could be a potential explanation for the overeating of the animals. Furthermore, the enteric nervous system is involved in the incretin response. It is possible that the pancreas is less stimulated to secrete insulin in our model, which could explain the high fasting-glucose levels measured. Lastly, gastrointestinal nerves are crucial in the translation of signals from the intestine in terms of control of food intake. Downregulated SGLT3 could mean fewer signals to satiety centres in the brain, which could explain the demonstrated hyperphagia. Interestingly, SGLT3 has been suggested as a portal vein glucose sensor important for the crosstalk between the periphery and the hypothalamus to regulate meal size and duration (Delaere et al., 2013; Mithieux, 2014).

The upregulation of CCK is interesting and could be explained by the fact that it stimulates pancreatic insulin secretion, which is important for controlling the hyperglycaemia observed in the HSD animals. GLP1 did not show a change in gene

expression, but GLP1 with its short half-life could be more important for the short-term regulation of postprandial blood glucose, whereas CCK is more important in the long term.

4.6.3 Nitric oxide

NO is a widely used molecule in the body, including the alimentary tract (Lane & Gross, 1999; Keklikoglu et al., 2008) and is involved in feeding, taste-cell and EEC signalling, but has also been implicated in pancreatic dysfunction. We hypothesised that NO is involved in lingual and gastrointestinal chemosensory cells as a signalling-molecule and therefore analysed the expression of *nos2* in the alimentary tract of HSD mice.

We measured a strong induction of *nos2* expression and identified *ddah1* expression to be downregulated in the tongue. *Nos2* produces NO, whereas *ddha1* regulates cellular concentrations of methylarginines, which in turn inhibits NOS activity. Taken together, these observations could lead to higher production of NO. As the condensed milk used in the HSD is highly palatable and sweet, this could potentially mean that taste reception and signalling to the brain in the HSD model is increased via NO in taste papillae and hence could be involved in the observed hyperphagia.

nos2 was also highly induced in the small intestine, especially in the duodenum. Since SGLT3 levels were decreased, the high *nos2* expression could be explained as a compensatory mechanism to overcome the decreased SGLT3 mediated signalling to the brain. Furthermore, an interesting study found that CCK secretion is iNOS/NO dependent (West et al., 2003). We speculate that the higher expression of *cck* in our model is caused by an upregulation of *nos2*. It would therefore be interesting to study

sucrose-dependent gut-peptide secretion in EEC *in vitro* models to measure direct effects and establish whether iNOS/NO is part of the signalling cascade, in order to confirm a functional link between our CCK and iNOS gene-expression data.

We emulated the HSD model *in vitro* by chronically feeding Caco-2/TC7 cells with sucrose, in order to study the effects on *slc5a4* and *nos2* expression as well as NO production. It has been suggested that enterocytes directly sense nutrients to regulate cellular gene expression such as sugar transporters. We hypothesised that the high luminal sucrose levels led to NO mediated downregulation of SGLT3 expression directly in enterocytes. Although sucrose feeding did not change gene levels for iNOS or SGLT3 in Caco-2/TC7 cells, NO levels were increased although not significant. This suggests sucrose is not an agonist for SGLT3 or SGLT3's role *in vivo* is not on enterocytes. Furthermore, we speculate, also in regard to our *in vivo* findings regarding CCK, that the measured NO produced in enterocytes is used as a paracrine signal for gut peptide secretion from neighbouring EECs. These findings suggest further research to identify the target of NO, and also the exact process it is involved in.

Yet NO is also involved in many processes, such as smooth-muscle cell-relaxation, that can lead to vasodilation. A potential function in the intestine of our HSD model could be to directly relax the smooth muscle cells in the intestinal wall. This in turn reduces motility and increases transfer times of the chyme ultimately enabling greater nutrient absorption. A pattern favourable in times of scarcity maximising nutrient intake, this could mean a potential maladaptation to the *ad libitum* food availability in the HSD model.

Another role of NO is in the immune system. Not only can NO lead to vasodilation to slow blood flow to enable easier tissue infiltration by immune cells, but phagocytes are also equipped with iNOS for various different functions (e.g. T-cell maturation, B-cell proliferation, induction of apoptosis) (Bogdan, 2001). Gene-expression profiles generated using microarrays revealed several interesting observations. Enrichment analysis and top 10 gene evaluation for GO processes and pathways revealed the highest genes regulated were involved either in cell cycle, metabolic regulation or immunity. The high regulation of immune-function genes is in line with the systemic and chronic inflammatory state observed in metabolic syndrome. Furthermore, it has been shown how a high-fat diet induces inflammatory proteins in the small intestine of mice (Ding et al., 2010). This was due to diet-induced dysbiosis of the gut flora and it has been suggested that intestinal inflammation then leads to epithelial hyperpermeability and endotoxemia, which induces systemic inflammation leading to the onset of insulin resistance (Moreira et al., 2012). Interestingly, the intestinal component of this cascade is present and measurable before the onset of obesity or diabetes (Ding et al., 2010). A link between local intestinal inflammation and insulin-resistance of enterocytes has also been established (Monteiro-Sepulveda et al., 2015). Insulin internalises apical GLUT2 in healthy, but not diabetic mice, which can lead to uncontrolled energy absorption and metabolic disorders (Tobin et al., 2008). This puts the gastrointestinal tract at the centre of metabolic deregulations and not just a tissue affected by systemic inflammation.

The local inflammation we found may also explain the high regulation of cell cycle/replication-related genes. As we have crude intestinal tissue, this could mean

several cell types. Intestinal stem cells could be induced to proliferate to counteract the epithelial damage and immune cells multiply to defend it, are both plausible explanations.

We analysed macrophage markers in the microarray, but did not find any change of expression (data not shown). This suggests that the location of the upregulated *nos2* is not in macrophages, but could indeed be in the endothelium, enterocytes or EECs, as discussed above. It would be of interest to study the effects of inflammation on nutrient sensors in *in vitro* models for these cell types.

Interestingly, it has been shown that CCK can inhibit inflammation via the vagus nerve upon nutrient stimulation (Luyer et al., 2005). CCK has also been shown to play a role in gastric mucosal defence systems after damage from irritants (West et al., 2003). CCK could hence be upregulated in our model to counteract any damage caused by a dysregulated microbiota.

4.6.4 Metabolic regulation

Furthermore, high regulation of genes involved in 'metabolic regulation' was detected, including bile-acid-regulating genes. A link between the microbiome, bile acids and metabolism has been established. Bile acids are highly metabolised by gut bacteria and have been shown to regulate intestinal homeostasis (Jones et al., 2014; Brandsma & Houben, 2015). They are recognised as ligands in a wide range of metabolic processes including the Farnesoid X receptor (FXR) and also the bile-acid-responsive G protein-coupled receptor 5 (TGR5) on L-cells, where it is involved in GLP1 secretion (De Aguiar Vallim et al., 2013).

Manual extraction of the top 10 regulated genes revealed another set of genes regulated in the HSD model. A similar picture regarding the high regulation of immunity genes was found, especially the interferon and gasdermin family. Furthermore, several members of the cytochrome P450 family were downregulated, notably *cyp4a10* and *cyp4a31*. Involved in fatty-acid oxidation and eicosanoid formation, *cyp4a10* has been shown to be downregulated after fasting (Van den Bosch et al., 2007). Several other genes involved in lipid metabolism were also regulated. Since the fat intake in both dietary groups was below 1%, but unchanged between groups, this poses the questions of why these genes are dysregulated and how they are involved in metabolism. Studies in hepatocytes and skeletal muscles have shown how high amounts of added sugars can increase glycolytic flux, which directly reduces fatty-acid oxidation (Coyle et al., 1997; Cox et al., 2012). We therefore suggest that as the added sugars are digested and assimilated by the gastrointestinal tract intestinal glycolytic flux increases, which in the long term leads to a suppression in genes necessary for fatty-acid oxidation. Potentially this phenomenon is also involved in the measured downregulation of CD36 gene expression in the HSD-fed mice.

In the ileum of HSD-fed mice *slc6a14* was highly regulated, with a 6-fold induction. This solute carrier was not regulated in the proximal regions. *Slc6a14* is interesting, as it encodes an alanine transporter, which potentially regulates tryptophan availability for serotonin synthesis. A very strong association of obesity with a single nucleotide polymorphism (SNP) in *slc6a14* has been shown in a Finnish cohort (Suviolahti et al., 2003; Cheung & Mao, 2012). Interestingly, serotonin or 5-hydroxytryptamine (5-HT) has been found to be involved in gastric emptying by

targeting 5-HT_3 receptors on duodenal vagal efferents (Raybould et al., 2003). Furthermore, 5-HT antagonists have been shown to reduce the anorexigenic effect of carbohydrates in the intestine (Savastano et al., 2005). 5-HT is highly expressed in αGustducin- and GLP1-positive cells (Sutherland et al., 2007) and vagal afferents have been reported to have increased responsiveness to 5-HT in an inflammatory state (Coldwell et al., 2007). 5-HT is potentially a co-signal to GLP1 initiating satiation after carbohydrate ingestion (Sutherland, 2008). There could be a potential link between *slc6a14*-expressing EECs and SGLT3-expressing neurons. If *slc6a14* expression and hence 5-HT production is higher in EECs, vagal afferents are more stimulated, which could reduce SGLT3 expression as detected in our model. Further research is needed to elucidate links and exact mechanisms.

4.7 Limitations of the study

Our results indicate a clear dysregulation of the chemosensory pathways and gut peptides. Yet these observations are just associations and we do not know if dysregulated nutrient sensors/gut peptides are causative for the phenotype; this requires further research. Pair-feeding would enable us to study the effects of the dietary composition independent of the calories consumed (Ellacott et al., 2010). Furthermore, a shorter feeding experiment would allow us to study if the observed changes are already present before the onset of type 2 diabetes

It would also be of high interest to measure blood gut peptide levels and determine exact locations/cell populations of affected genes, as we used crude tissue

samples for our analysis. Furthermore, it would valuable to confirm transcriptomic findings on a proteomic level.

In our study crude intestinal tissue was used. Using mucosal scrapings or *in situ* experiments would have been highly beneficial in order to identify locations of gene changes such as enterocytes, EECs, neurons or smooth muscle cells.

Furthermore, it has been shown that shifting circadian rhythms in mice induces gastrointestinal, metabolic and immune alterations that are influenced by the core clock machinery and ghrelin (Laermans *et al*., 2014). It would be of interest to study how the HSD affects the core clock machinery and the diurnal rhythmicity of the studied sensors.

The intestinal tract is highly studied regarding its microbiota and how diet can affect the microbial colonies and even lead to disease (Ramakrishna, 2013). Microbiotic profiling with genetic techniques would help to determine exact subpopulations and possibly to identify a switch to a pathogenic profile.

4.8 Conclusions

In conclusion, our findings suggest an association between high-sucrose feeding and dysregulation of nutrient sensors and induction of gut peptide and iNOS expression.

Many more systems including inflammation, bile-acid secretion and serotonin signalling, processes highly involved in metabolism, were identified to be affected by the sugar-rich diet. More in-depth research will be needed to fully understand the processes involved and could eventually lead to a better understanding of the physiology and potential targets for therapy of metabolic syndrome.

CHAPTER 5

GENERAL CONCLUSION & FUTURE STUDIES

5 GENERAL CONCLUSION & FUTURE STUDIES

5.1 Conclusion

Nutrient sensors in the alimentary tract, in particular sugar sensors, have been implicated in the regulation of feeding and metabolism. Disruptions of such systems are hence potentially involved in the pathology of metabolic diseases. The purpose of this thesis was to study the short-term regulation of sugar transport by the sweet-taste receptor using an *in vitro* enterocyte model, as well as to evaluate nutrient-sensing mechanisms in the long-term under healthy and diseased conditions.

We were able to demonstrate that in the short term the enterocyte *in vitro* model, Caco-2/TC7 cells, was able to respond to changes in external sugar substrate concentrations (D-Glu or HFCS) with higher transport capacities for D-Glu and Fru. Cells were not responsive to higher Fru or sucrose concentrations. Yet we showed that the displayed upregulation of transport was not mediated via the sweet-taste receptor, as artificial sweeteners or an inhibitor did not change transport activity. We speculated that another sensor could be responsible for our observations and the long-term studies revealed a dietary regulation of SGLT3. We therefore chronically exposed Caco-2/TC7 cells to sucrose, but did not measure any regulation of SGLT3 gene expression. We confirmed that SGLT3 is not involved by using parental Caco-2 cells, which do not express SGLT3, but demonstrated similar results regarding the D-Glu mediated short-term stimulation of D-Glu and Fru transport by D-Glu as outlined in chapter 2 (data not shown). In conclusion regarding Caco-2/TC7 cells, neither the sweet-taste receptor nor SGLT3 seem to be involved in regulating transport activities upon external sugar load and suspect another sensor/sensing mechanism to be involved. Cellular mechanisms

such as increased vesicle transport or higher amounts of cellular ATP after sugar influx could also lead to transactivation of hexose transporters. Indeed Na^+/K^+-ATPase activity has been shown to be increased in enterocytes of diabetic rats which also had increased brush-border GLUT2 and GLUT5 expression (Corpe et al., 1996). Taken together, these findings are especially interesting in regard to the direct model of sugar sensing as it would not require a specified membrane-bound sensor.

The long-term, high-sucrose feeding of mice revealed that it not only leads to type 2 diabetes, but that SGLT3 was highly downregulated, which suggests SGLT3 is under dietary regulation. Luminal nutrient loads change throughout the day and are synchronised to gastrointestinal nutrient absorption and gut peptide secretory mechanisms. SGLT3 also displayed diurnal rhythmicity supporting the fact that it is under dietary control.

SGLT3 is expressed in various tissues involved in metabolism including the hypothalamus, kidney, liver, intestinal mucosa and enteric nervous system. Studies have shown how SGLT3 is involved in nutrient sensing related mechanisms such as GLP1 secretion and portal vein sensing, but that it is highly specific to D-Glu (Diez-Sampedro et al., 2003). It is likely that in the small intestine SGLT3 is either involved in EEC nutrient sensing or has a intraepithelial function (e.g. on cholinergic neurons) where sucrose has been broken down to D-Glu and Fru and then absorbed.

Taken together, the dietary regulation of SGLT3 suggests that it might be important in diet-related disease. This recommends future studies to determine exact location and function of the protein and if the dysregulated SGLT3 expression is causal or consequential to metabolic disease.

5.2 Future studies

As SGLT3 was dysregulated in metabolic syndrome and displayed diurnal rhythmicity, several questions opened up demanding further research. First, it needs to be demonstrated that SGLT3 has a clear circadian oscillatory expression-pattern at the mRNA and protein levels and is under the transcriptional control of clock genes. Once this is established it would be of interest to study if the circadian rhythmicity of SGLT3 is attenuated in metabolic syndrome and if this is causative for the metabolic disturbances. Using more time-points might also reveal diurnal rhythms for the sweet-taste receptor and other studied genes. Furthermore, it is not clear in which cell type the regulation of SGLT3 occurs and *in situ* expression studies would allow to determine if the changes indeed appear in cholinergic neurons or in mucosal cells such as EECs or enterocytes. As we also showed higher iNOS expression it would be of strong interest to identify in which cells this occurs and which mechanism the potential higher amounts of NO are involved in. As SGLT3 has been implicated in portal vein sensing (Delaere et al., 2013), it would be interesting to also study if these mechanisms are disturbed in the HSD model. Furthermore, it would be of high interest to use *in vitro* models of EECs to study if sugars and artificial sweeteners regulate EECs function in the short and long term.

APPENDIX I: ENRICHMENT ANALYSIS OF THE MICROARRAY ON TONGUE RNA AT 7AM VS 7PM PRESENTED IN CHAPTER 3

We applied the GeneGo MetaCore™ software package and identified the top 10 regulated pathways in the tongues of mice at 7am vs 7pm with an enrichment analysis (Fig. 50). The strongest pathways enriched in our experiment are cytoskeletal arrangement, cell adhesion and cell differentiation/ development mediated via Wnt signalling, but also immunity processes.

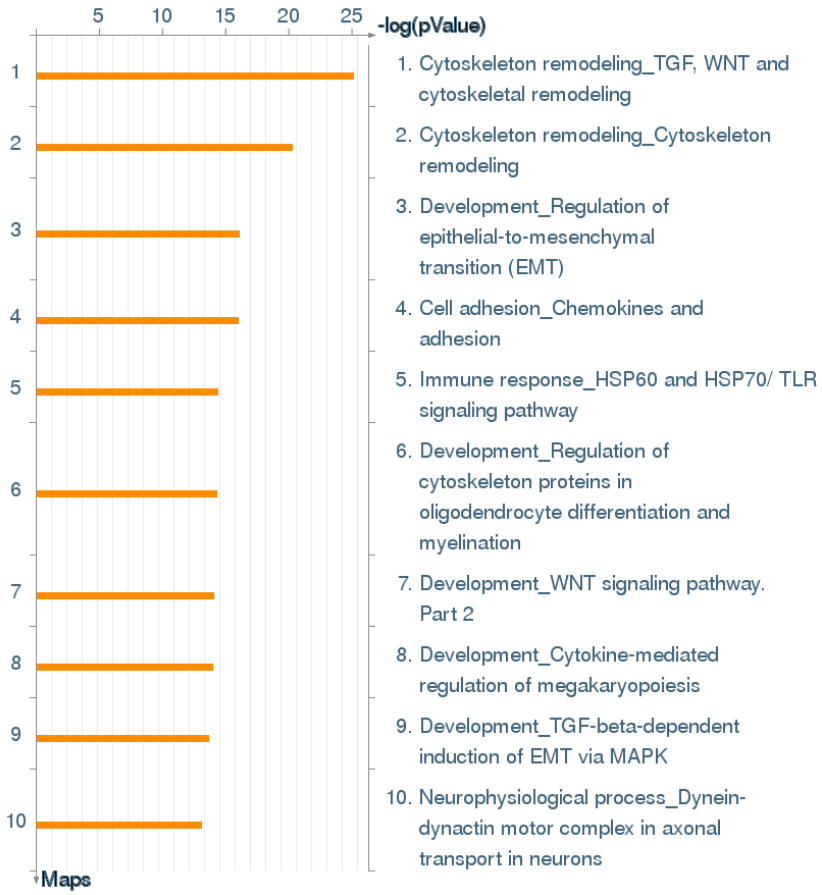

Fig. 50 Microarray data analysis for top 10 diurnally regulated pathways in the tongue. Enrichment analysis for pathway maps was performed in the GeneGo® software.

We enriched our data set for gene onthology (GO) processes (Fig. 51a) and process networks (Fig. 51b) and confirmed that mainly cellular organisation, development and signal transduction are regulated in the tongue, but also inflammatory networks.

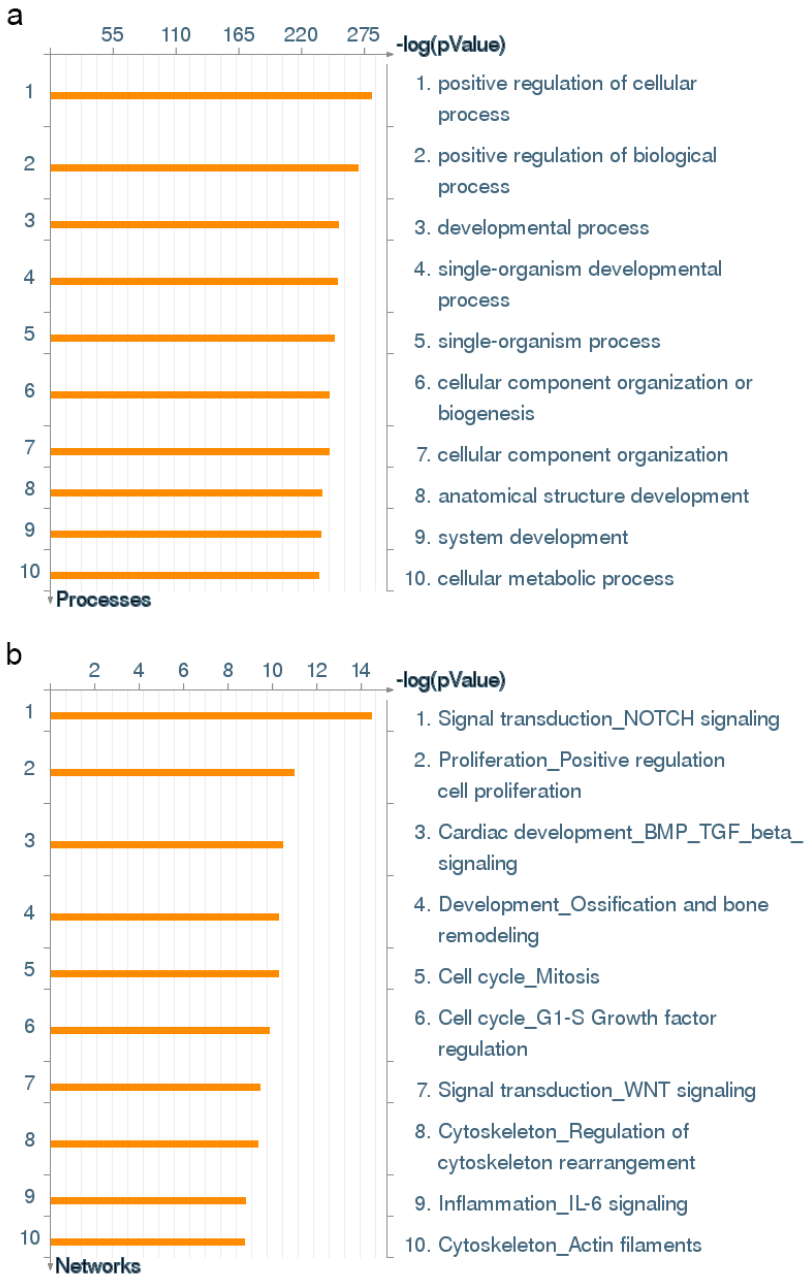

Fig. 51 Microarray analysis for top 10 Gene ontology and process networks. Fold changes were enriched for GO processes **(a)** or process networks **(b)** in the GeneGo® software package.

APPENDIX II: ENRICHMENT ANALYSIS OF THE MICROARRAY ON THE SMALL INTESTINE OF HSD MICE PRESENTED IN CHAPTER 4

We applied the GeneGo MetaCore™ software package to enrich expression profiles for pathways, networks and processes regulated in the duo, jej and ile of HSD mice. The strongest common pathway maps, GO processes and networks regulated in our experiment were related to either cell cycle (mitosis, replication, transcription), immune response (antiviral, B-cell receptor, interferon signalling) or regulation of metabolism (progesterone signalling, retinol metabolism, bile acid regulation) (see Fig. 52, Fig. 53 and Fig. 54)

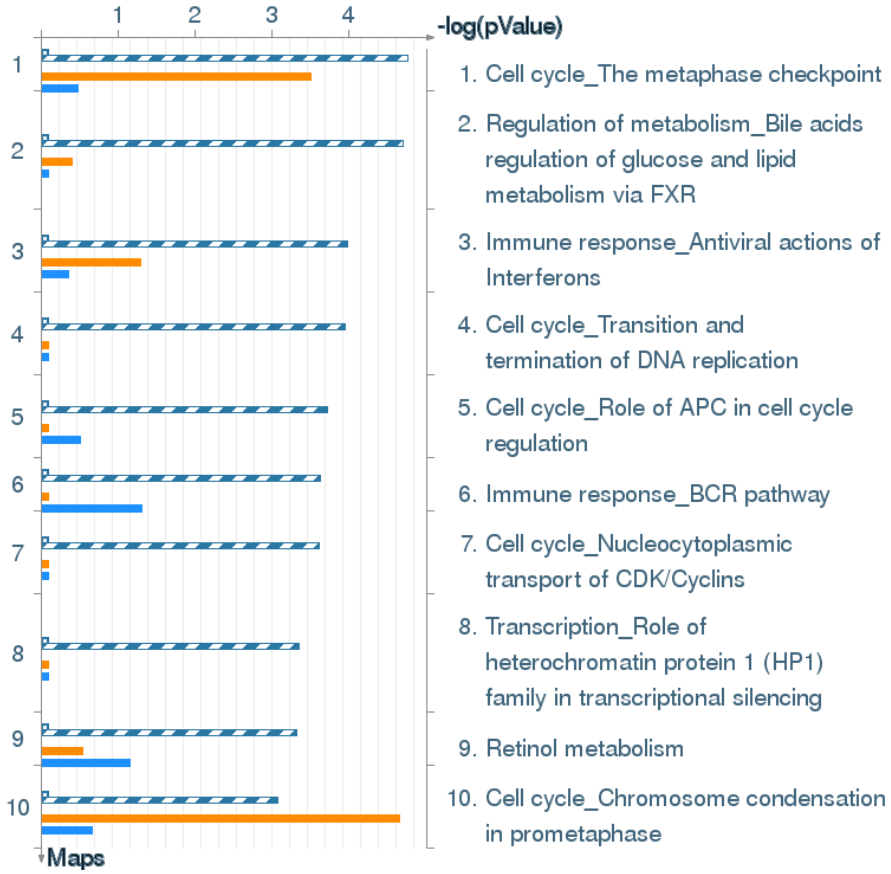

Fig. 52 Microarray data analysis for the top 10 regulated pathways in the small intestines of HSD mice. Common pathways between the Duo, Jej and Ile are displayed as blue/white hatched bars. Significances for individual tissues compared to the Duo are displayed as blue for the Jej and yellow for the Ile. Enrichment analysis for pathway maps in the GeneGo® software.

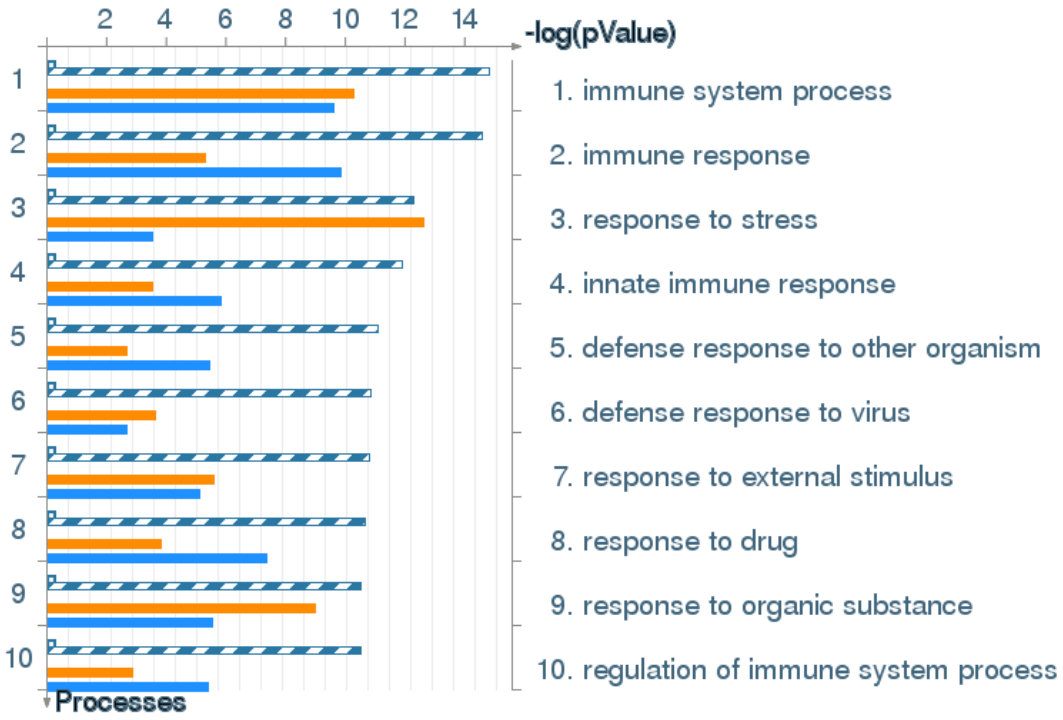

Fig. 53 Microarray data analysis for the top 10 regulated GO processes in the small intestines of HSD mice. Common GO processes between the Duo, Jej and Ile are displayed as blue/white hatched bars. Significances for individual tissues compared to the Duo are displayed as blue for the Jej and yellow for the Ile. Enrichment analysis for GO processes in the GeneGo® software.

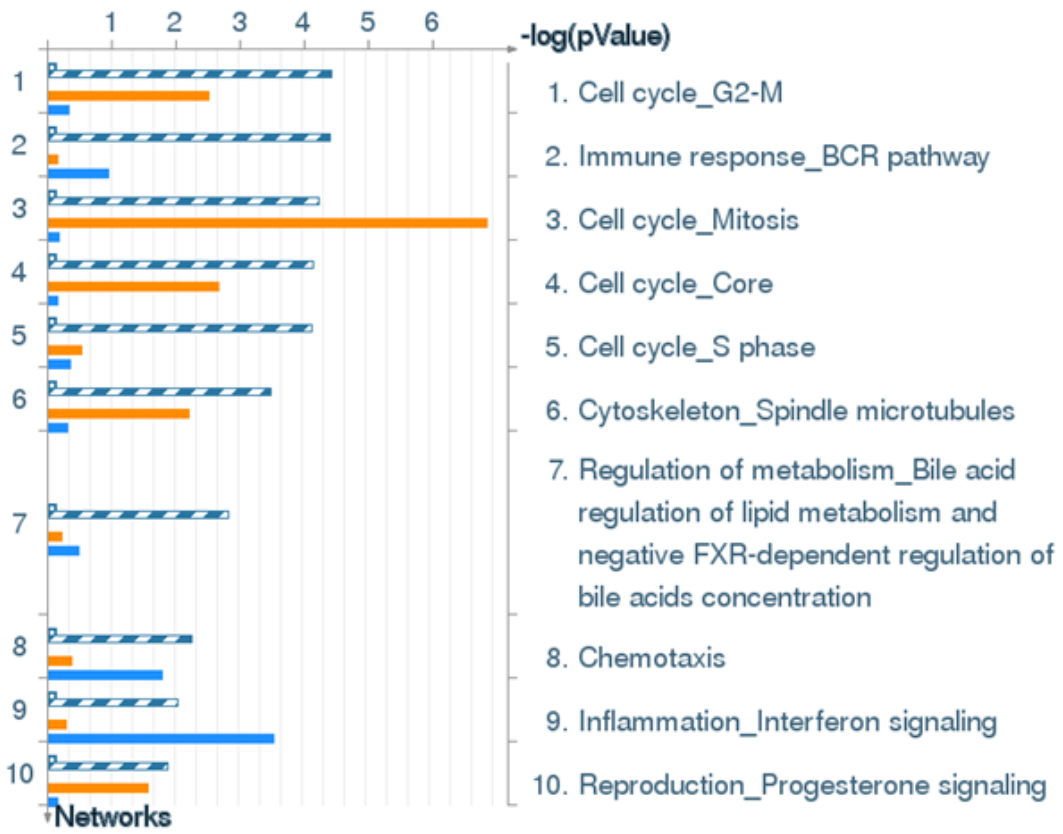

Fig. 54 Microarray data analysis for the top 10 regulated networks in the small intestines of HSD mice. Common networks between the Duo, Jej and Ile are displayed as blue/white hatched bars. Significances for individual tissues compared to the Duo are displayed as blue for the Jej and yellow for the Ile. Enrichment analysis for GO processes in the GeneGo® software.

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