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Analysis of the glucose-dependent transcriptome in murine hypothalamic cells

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Abstract

We have previously shown that glucose activates CREB-regulated transcriptional co-activator-2 (CRTC-2) in murine, hypothalamic (mHypoA-2/10) cells. Thus, we now analysed the entire glucose-dependent transcriptome of mHypoA-2/10 cells by total RNA-seq. 831 genes were up- and 1390 genes down-regulated by at least 50 %. Signalling pathway analysis revealed activation of the cholesterol biosynthesis pathway by glucose. Accordingly, protein expression of both sterol regulatory element-binding proteins (SREBP) and total cholesterol levels were enhanced by glucose. Analysis of single genes involved in fundamental signalling processes suggested a significant impact of glucose. Thus, we chose ~100 genes and validated the effects of glucose on mRNA levels by qRT-PCR. We identified 15 genes with strong glucose-dependent mRNA expression. Among these genes were *gnai1* to *-3*, *adyc6*, *irs1*, *igfr1*, *hras* and *elk3*. cAMP measurements revealed decreased basal and enhanced noradrenalin-induced cAMP levels at higher glucose concentrations. Serum-response element-dependent reporter assays indicated enhanced basal and insulin-like growth factor-induced activity at higher glucose levels. siRNA against CRTC-2 dampened the effects of glucose on cholesterol synthesis and IRS-1, SREBP-1, SREBP-2 or AC-6 protein expression. These findings could help to understand the functional consequences of physiologically occurring alterations of extracellular glucose concentrations as well as pathologically increased glucose levels.

Introduction

Glucose is a universal source of energy and a key player in diabetes and obesity. Although, plasma glucose levels are kept rather constant at 5.50 mM after fasting, they can reach up to 10 mM after food intake, vary during the day and increase after fasting even to 11 mM in diabetic patients ¹⁻⁴. Brain glucose levels directly correlate with plasma glucose levels, but depending on the specific brain region may be 10 to 15 times lower than plasma levels ^{1,5,6}. Thus, the brain is also exposed to dynamic changes of extracellular glucose concentrations after food uptake and confronted with increased glucose levels in diabetic patients ^{1,7-12}. Furthermore, drugs like heroin induce rapid and significant spikes in brain glucose levels ¹³, further highlighting the dynamics of brain glucose levels.

The hypothalamus is part of the hypothalamic-pituitary-thyroid (HPT) axis, and thus, of prime importance for the central regulation of appetite and energy metabolism ^{14,15}. Dysregulation of the HPT axis has frequently been implicated in severe metabolic disorders including diabetes mellitus (DM) ¹⁶⁻¹⁹. Signalling via tyrosine receptor kinases (RTK) such as the insulin-like growth factor receptor (IGFR) or G protein-coupled receptors (GPCR), such as β -adrenergic receptors, are important elements in the regulation of HPT activity ²⁰⁻²⁵. Effects of altered extracellular glucose levels on signalling induced by both hormones in hypothalamic cells has not been systematically investigated so far.

Besides the universal role of glucose as an energy carrier, glucose-derived ATP is an important

regulator of intracellular signalling. In analogy to pancreatic β -cells, ATP inhibits K_{ATP} channels and thus depolarises hypothalamic neurons²⁶⁻³⁰. Furthermore, ATP dampens AMP-dependent kinase (AMPK) activity in hypothalamic neurons, thereby relieving CRTC-2, from tonic phosphorylation and inhibition by AMPK³¹. AMPK activity inversely correlates with the activity of extracellular-regulated kinases-1/2 (ERK-1/2) in many cell types including hypothalamic cells^{32,33}. Thus, glucose-induced AMPK inhibition leads to ERK-1/2 activation. Because ERK-1/2 are key to the activation of serum response factors (SRF) and ternary complex factors (TCF), increased glucose concentrations may lead to the activation of SRF/TCF-dependent gene expression via inhibition of AMPK. Finally, nuclear translocation of members of the transcription factor family max-like protein X is promoted by glucose-6-phosphate, another product of cytosolic glucose metabolism³⁴. Thus, glucose *per se* has significant potential to serve as a key regulator of gene expression as indicated already in liver cells and pancreatic islets^{35,36}. However, no data about the glucose-dependent transcriptome in hypothalamic cells are available yet.

We have previously studied murine, hypothalamic mHypoA-2/10 cells, and established that physiological changes of extracellular glucose concentrations (0.1 to 2.5 mM) regulate the expression of pro-thyrotropin-releasing hormone via inhibition of AMPK³⁷. In this manuscript, we report the analysis of the entire glucose-dependent transcriptome in mHypoA-2/10 cells. We found that 831 genes were up- and 1390 genes down-regulated by at least 50 %. The cholesterol biosynthesis pathway was most significantly affected on multiple levels, indicating regulatory effects of glucose on hypothalamic lipid synthesis. Further, qRT-PCR analysis of ~100 genes involved in cellular signalling, revealed a list of 15 genes including G proteins (*gnai1*, *gnai2*, *gnai3*), adenylyl cyclase (*adcy6*) and proteins involved in IGF signalling (*irs1*, *igfr1*, *hras* and *elk3*), to be up-regulated by glucose. Thus, we report so far unappreciated effects of extracellular glucose levels on RTK and GPCR signalling in hypothalamic cells. These findings might be of importance to understand the functional consequences of physiologically occurring alterations of extracellular glucose concentrations as well as pathologically increased glucose levels in diabetic individuals.

RESULTS

Cholesterol biosynthesis is the major target of glucose-dependent gene expression. *In vivo* brain glucose concentrations vary between 0.1 to 2.5 mM under physiological conditions^{6,9,26,38-40}. We recently introduced a protocol for mHypoA-2/10 cells, which were initially adapted to low extracellular glucose concentrations (0.1 mM) for 24 h in the absence of fetal bovine serum (FBS)³⁷. Afterwards, glucose was either withdrawn (basal) or increased to 2.5 mM (glucose) for 3 h. Pyruvate (1 mM) was constantly present to ensure equal mitochondrial ATP levels. This protocol led to enhanced activity of a CREB/CRTC2-dependent reporter gene, but did not affect activity of signal transducers and activators of transcription or of nuclear factors-activated in T-cells³⁷.

Here, we took advantage of this protocol and performed total RNA sequencing (RNA-seq) under both conditions in order to determine the glucose-dependent transcriptome in murine hypothalamic cells. The resulting reads per kilobase of transcript per million mapped reads (RPKM) were mapped against the *mus musculus* reference genome (GRCm38.p4 down-loaded from NCBI) and analysed for differential gene expression. When a fold-change $|FC|$ of ≥ 2 in mRNA expression was applied as a cut-off, 641 genes were up- and 766 down-regulated (FDR corrected p-value of ≤ 0.05) by glucose. Thus, in relation to the entire mouse genome (~45,000 genes), ~3 % of the transcriptome was found to be glucose-dependent. When a cut-off for total (glucose-independent) mRNA expression was applied (RPKM of ≥ 1 for at least one condition) 11062 genes remained (Fig. 1). 187 of these genes were up and 480 genes down-regulated by a $|FC|$ of ≥ 2 , and 831 or 1390 by a $|FC|$ of ≥ 1.5 , representing 6.0 or 20 %, respectively, of the transcriptome with a RPKM ≥ 1 (Fig. 1). Hence, total RNA-seq analysis revealed a significant impact of glucose on the transcriptome of hypothalamic cells. Canonical signalling pathway analysis revealed the cholesterol biosynthesis gene network to be most profoundly affected by glucose (positive z-score of 3.6). As shown in figure 2A-I, nine key genes involved in

cholesterol biosynthesis were significantly up-regulated by glucose. SREBP are encoded by the *srebfl* and *sreb2* genes and are key regulators of cholesterol biosynthesis⁴¹. Glucose has been shown to enhance *srebfl* and *sreb2* expression in renal NRK-52E cells, but conversely to inhibit *srebfl* in Schwann cells^{42,43}. RNA-seq data obtained with mHypoA-2/10 cells showed that both *sreb* genes were up-regulated (Fig. 2J&K), portending a major role of glucose as a regulator of cholesterol levels by dynamic regulation of *sreb* expression. In order to substantiate this finding, we next performed Western-blot analysis with specific antibodies against SREBP-1 or -2. As shown in figure 3A, high glucose concentrations enhanced the expression of both SREBP subtypes also on the protein level. It has been shown that the CREB co-activator CRTC-2 regulates SREBP-1 expression in liver cells⁴⁴. We have recently reported that glucose activates CRTC-2 in mHypoA-2/10 cells³⁷. Thus, CRTC-2 activation appeared has a likely mechanism for glucose-induced SREBP expression. We used a siRNA cocktail against CRTC-2 and detected the effects of glucose on SREBP expression by a whole-cell ELISA approach (Fig. 3B). In accordance with the Western-blot data, glucose significantly enhanced SREBP-1 and -2 expression after transfection of a control siRNA. In the presence of the specific CRTC-2 siRNA, effects of glucose on the expression of both SREBP isoforms were almost completely abolished, indicating a major role for CRTC-2 in glucose-induced SREBP expression. To finally validate the effect of glucose on cholesterol synthesis and the role of CRTC-2 in this context, we determined cholesterol levels in mHypoA-2/10 cells after a 24-h treatment with 0.1 or 2.5 mM glucose. Cyclodextrins have a hydrophobic core that is roughly the size of cholesterol. Thus, in the presence of β -cyclodextrin cholesterol is extracted from cells. Glucose-induced cholesterol synthesis should therefore lead to enhanced β -cyclodextrin-promoted cholesterol release into the supernatant of mHypoA-2/10 cells. Indeed, the cholesterol levels in the supernatant of cells transfected with the control siRNA rose from 37.8 ± 6.5 to 58.7 ± 3.9 μ M in the presence of high glucose concentrations (Fig. 3C). This increase was significantly lower (33.4 ± 3.4 to 43.1 ± 5.1 μ M) in the supernatant of cells transfected with the siRNA against CRTC-2 (Fig. 3C). Hence, our data clearly indicate that glucose regulates cholesterol levels of mHypoA-2/10 cells, most likely via CRTC-2-mediated expression of SREBP-1 and -2. In order to investigate whether these effects are restricted to one particular hypothalamic cell line, we took advantage of GT1-7 cells a distinct murine, hypothalamic cell line⁴⁵. Similar to mHypoA-2/10 cells, glucose enhanced β -cyclodextrin-induced cholesterol release in GT1-7 cells (Fig. 3D). Hence, effects of glucose on cholesterol synthesis appeared as a rather common feature among hypothalamic cell line.

Glucose-dependent gene expression alters signalling pathways of RTK and GPCR. After assessing the effects of glucose on entire signalling networks, we subsequently focused on single genes that are involved in intracellular signalling cascades. We found that mRNA levels of monomeric and heterotrimeric G proteins are modulated by glucose as are corresponding signalling molecules such as GTPase-activating protein, GTP exchange factors and transcription factors. Specifically, we found 31 of these genes to be up- and 45 to be down-regulated by glucose (Fig. 4). Further, glucose apparently exerted rather specific effects on certain subtypes within the same gene family. For example, the transcription factor *elk3* but not *elk1* was significantly induced (Fig. 4, middle panel). Similarly, expression of *irs1* but not of *irs2* was enhanced (Fig. 4, lower panel). With regard to α -subunits of heterotrimeric G proteins, *gnai1*, *gnai2*, *gnai3* and *gna11* expression was enhanced, *gnaq* and *gna12* unchanged, while *gna13* expression was reduced by glucose (Fig 4). Next, we aimed at confirming the effects of glucose on the mRNA levels by qRT-PCR. To this end, we created specific primer pairs for 31 up- and for 45 down-regulated genes (Tab. S1). As a control, we also included 23 genes which expression was unchanged. In addition, we also screened the RNA-seq data set for potential reference genes not affected by glucose. We found that mRNA levels of succinate dehydrogenase (*sdha*) and TATA-box binding protein (*tbp*) were glucose-independent (Fig. S1 A&B). Further, *sdha* expression exceeded that of *tbp* by a factor of ~4, thus allowing for controls at different expression levels. We stimulated cells accordingly to the protocol used for the RNA-seq approach and analysed mRNA levels

of *sdha* and *tbp* with different amounts of cDNA by qRT-PCR. Both genes showed almost perfect linear correlation between crossing points (Cp-values) and cDNA amount (Fig. S1 C&D). Furthermore, over the entire range of cDNA concentrations, glucose had no effect on the mRNA expression of either *sdha* or *tbp*. Thus, we generated three independent pairs of mRNAs from cells +/- glucose stimulation and performed qRT-PCR with the abovementioned 99 genes, and analysed their mRNA expression normalized to *sdha* or *tbp*. The heat map in figure 5 combines the glucose-induced changes obtained in the RNA-seq experiment shown in figure 4 with the qRT-PCR data obtained. The entire set of qRT-PCR data is shown in table S2. Some candidates derived from the RNA-seq experiment like *igf2r*, *kras*, *elk4*, *rab7b* and *arhgef19* were insensitive to glucose in the qRT-PCR. Others, like *srf*, *crtc2* and *arhgef39* exhibited opposite effects, when RNA-seq were compared to qRT-PCR data. However, for 65 of the 99 genes analysed, the effect of glucose on mRNA levels observed in RNA-seq was confirmed by the qRT-PCR experiments for at least one reference gene. Of note, the heat map only indicates absolute changes, but does not provide any information about the significance of these changes. Thus, we performed a rigorous statistical analysis based on three parameters: 1) Bonferroni corrected FDR p-values of the RNA-seq data, 2) ANOVA/Sidak's p-values of the qRT-PCR experiment using $\Delta\Delta C_p$ based on *sdha* or *tbp* and 3) two-sample t-test based p-values of the qRT-PCR experiment using $\Delta\Delta C_p$ based on *sdha* or *tbp*. Genes, which glucose-dependent mRNA levels reached statistical significance (p-value of ≤ 0.05) in all three categories and with both reference genes are listed in table 1.

Irs1 appeared as the most profoundly glucose-induced gene (72 ± 26 %). Interestingly, *irs2* expression was unchanged by altered glucose concentrations (Fig. 4 and 5, Tab. S2). Considering the RNA-seq data, it appeared that glucose induced *irs1* expression increased total RPMKs of both *irs* from 9.8 to 11.6 (Fig. 6A). Further, although glucose selectively increased *irs1* mRNA levels by almost 2-fold, the total expression level of *irs2* was still significantly higher, indicating that *irs2* remained the most abundant *irs* subtype upon glucose treatment in mHypoA-2/10 cells (Fig. 6A). At last, RNA-seq data also suggested a tendency of glucose-induced *irs2* depression. Thus, we cultivated mHypoA-2/10 cells for 24 h either with 0.1 or with 2.5 mM glucose and analysed mRNA levels of *irs1* and *irs2* again by qRT-PCR (Fig. 6B). We could confirm a significant increase of *irs1* expression by 48 ± 15 % and observed a significant glucose-induced decrease of *irs2* expression by 12 ± 6 % in the same cells. Thus, glucose induced an increase in total *irs* expression and a shift from *irs2* to *irs1* in hypothalamic cells. Of note, the *igfr1* gene was also induced by glucose (Fig. 4 and 5, Tab. 1), suggesting that glucose modulates IGF signalling by three distinct mechanisms: increase of total *irs* and *igfr1* expression and a changed expression ratio of *irs2* towards *irs1*. In order to validate this hypothesis, we analysed effects of glucose on the protein level of IRS-1 by western-blotting and ELISA (Fig. 6C&D). We observed that glucose-induced expression of the IRS-1 protein after 24 h. Down-regulation of CRT-2 with siRNA blocked glucose-induced IRS-1 expression almost completely (Fig. 6D), indicating that the CREB co-activator is involved. We next validated the consequences of glucose-induced IRS-1 expression on the function of RTK by expressing a luciferase reporter gene containing binding sites for SRF/TCF in mHypoA-2/10 cells. We incubated these cells either with 0.1 or 2.5 mM glucose for 24 h and analysed IGF- or epidermal growth factor (EGF)-induced SRF/TCF-dependent reporter activity⁴⁶. When analysing basal reporter activity, we found an increase of 220 ± 73 % under high-glucose condition (Fig. 6E). This increase could be caused either by glucose-induced expression of *hras* and/or *elk3* shown in table 1 or by the aforementioned glucose-promoted ERK-1/2 activation via AMPK inhibition^{32,33}. Interestingly, IGF further enhanced SRF/TCF activity at high glucose by 170 ± 28 % and only by 115 ± 15 % at low glucose (Fig. 6F). Thus, as expected from our expression analysis, glucose enhanced IGF signalling. Of note, EGF-mediated effects on SRF/TCF activity were glucose independent (Fig. 6F), which is in line with the inconsistent effects of glucose on the expression of EGF receptors or their adapter proteins GAB or GRB (Fig. S2) and the glucose-independent expression of the *sos1* and *sos2* genes (Fig. 4, Tab. S2). Thus, extracellular glucose concentrations exhibited rather selective effects on IGF, but not on EGF signalling. Of note, this also

appeared as a rather common feature of hypothalamic cells, since glucose treatment also affected IGF but not EGF signalling in GT1-7 cells (Fig. 6G).

Our list of glucose-induced genes also includes three α -subunits of the *gnai* family and *adcy6*, suggesting effects of glucose on cAMP signalling. An increase of total *gnai* expression by glucose implies decreased cytosolic cAMP levels. In contrast, increased expression of *adcy6* should result in enhanced cAMP signalling. Of note, *adcy6* is the predominant adenylyl cyclases (AC) in mHypoA-2/10 cells (Fig. 7A). Thus the relative increase of *adcy6* mRNA (44 ± 11 %, Tab. 1), has robust effects on the total amount of *adcy* expression (total *adcy* RPKM increased from 16.9 at basal to 23.6 with glucose). Further, analysis of the glucose-dependent protein level (Fig. 7 B&C) revealed enhanced AC-6 expression at higher glucose concentrations. This, effect was also diminished after transfection of the CRCT-2 siRNA, albeit not completely (Fig. 7C). In order to determine the effects of glucose on cAMP levels, we incubated cells for 24 h either with 0.1 or with 2.5 mM glucose in the presence of [3 H]-adenine and measured basal, noradrenalin (NA)- and forskolin (FSK)-induced cAMP accumulation afterwards. NA activates β_2 - and β_3 -AR in mHypoA-2/10 cells and FSK is a direct activator of AC ⁴⁷. Using sequential chromatography, we first tested whether glucose would affect the conversion of [3 H]-adenine to [3 H]-ATP. Because ATP is the substrate of the reaction catalysed by AC, altered labelling efficiency due to different cytosolic glucose concentrations may theoretically affect the enzymatic reaction independently from AC activity. As shown in figure 7D, [3 H]-ATP levels were not significantly affected by extracellular glucose levels, indicating that the glucose stimulation protocol did not affect labelling of the cells with [3 H]-adenine. When the product of the AC reaction was analysed, slightly decreased basal [3 H]-cAMP levels were detected at high glucose concentrations (Fig. 7E). When NA- and FSK-induced cAMP accumulation were determined, significantly increased x-fold over basal signals were obtained at high glucose concentrations (Fig. 7F). Interestingly, when x-fold over basal signals obtained at 0.1 mM glucose were set to 100 %, high glucose levels increased NA-induced cAMP accumulation by 123 ± 46 % and FSK-promoted signals by 20 ± 28 %, indicating selective effects of glucose on NA-induced signalling (Fig. 7G).

Discussion

Plasma and brain glucose levels vary under physiological and pathological conditions. Glucose has been established as a regulator of gene expression in several tissues, but glucose-dependent gene expression in hypothalamic cells has not yet been analysed in detail ^{36,48-51}. Altered gene expression has been reported in the brain of diabetic rodents, but *in vivo* models can hardly differentiate between the effects of glucose or insulin on gene expression ^{52,53}. Herein, we analysed glucose-dependent gene expression *in vitro*, independent of insulin using a hypothalamic cell line and a four-step strategy: 1) We determined the entire transcriptome of cells at low and high glucose levels by RNA-seq. 2) We selected candidates and re-evaluated their glucose-dependent mRNA levels by qRT-PCR, 3) we monitored protein expression levels by Western-blot and ELISA and 4), we determined second messengers or reporter gene activities at different glucose concentrations.

RNA-seq data indicated that depending on the cut-off of total gene expression and the amount of change, 700 - 2200 genes were glucose-dependent corresponding to around 3 to 7 % of the entire murine genome. Previous studies using either healthy or diabetic human/murine islets or skin cells obtained similar results ⁴⁸⁻⁵¹.

The cholesterol biosynthesis pathway was most prominently induced. Glucose is fundamental for cholesterol synthesis, because its degradation product pyruvate is converted to acetyl CoA which serves as the precursor of cholesterol synthesis. Of note, we kept pyruvate levels constant at 1 mM, such that pyruvate deficiency did not occur under low glucose conditions. Thus, our data show that alterations of cytosolic products of glucose metabolism (most likely ATP) affect the expression of SREBP's and of enzymes involved in the cholesterol synthesis pathway. In line with this notion, glucose enhanced cholesterol levels in two distinct hypothalamic cell lines. Thus, our data indicate a strong correlation between extracellular glucose levels and the cholesterol synthesis pathway in

hypothalamic cells. AMPK is inhibited by ATP and inhibits CRTC-2 activity in hypothalamic cells^{31,37}. Hence, CRTC-2 appeared as a promising candidate for glucose-induced SREBP expression, in particular, when considering that CRTC-2 has been reported to induce SREBP-1 expression in liver cells⁴⁴. In line with these data, down-regulation of CRTC-2 expression by siRNA diminished the effects of glucose on the expression of both SREBP subtypes and on cholesterol synthesis. In addition to the positive effects of glucose on the cholesterol levels in mHypoA-2/10 cells, the insulin induced gene-1 (*insig1*) gene belongs to the 15 glucose-dependent genes revealed herein (Tab. 1). *Insig1* RNA levels were enhanced by 62 ± 23 % at high glucose levels. INSIG-1 is the most profoundly insulin-induced protein in liver cells and functions as an inhibitor of SREBP activity and thus as a negative regulator of cholesterol synthesis^{54,55}. Insulin-independent effects of glucose on *insig1* expression have not yet been shown. Furthermore, induction of *sreb1* and *insig1* in hypothalamic cells by glucose suggests effects on cholesterol synthesis on multiple cellular levels. DM is associated with alterations of the central nervous system including reduction of cholesterol synthesis in the brain^{56,57}. Glucose-induced expression of enzymes involved in cholesterol synthesis indicated by our data and by others appears to contradict this notion, taking into account increased plasma glucose levels in diabetic individuals⁵⁸. However, glucose-induced *insig1* expression may account for reduced cholesterol levels in DM. Reduced glucose sensitivity is also a hallmark in the origin and progression of DM. Thus, although glucose initially induces cholesterol synthesis, this effect might diminish after sustained elevated glucose levels, finally resulting in reduced cholesterol levels. Fukui et al., recently demonstrated in GT1-7 cells, that reduction of cellular cholesterol levels by 30 % reduced IGF-induced ERK-1/2 phosphorylation⁵⁹. These data are in accord with increased IGF-induced SRF/TFC activation at high-glucose concentrations shown in figure 6D. Thus, in addition to the already mentioned effects of glucose on total *irs* and *igf1* expression and the altered *irs2* versus *irs1* expression ratio, glucose might also affect IGF signalling via modulating cholesterol levels.

We also observed depression of *gnai* genes and induction of the *adcy6* gene. To the best of our knowledge, this is the first description of direct modulation of key proteins involved in cAMP production by glucose. Accordingly, when measuring glucose-dependence of cAMP accumulation, we found dual effects of glucose on cAMP signalling. Inhibition of basal cAMP levels could be attributed to enhanced expression of *gnai* genes, whereas increased *adcy6* expression may account for enhanced NA-promoted cAMP signalling. NA-induced cAMP signals were independent from *pertussis toxin*, an inhibitor of all G_i proteins in mHypoA-2/10 cells (data not shown), providing a possible explanation, why enhanced G_i protein expression by glucose did not affect NA-mediated cAMP signalling. Glucose also enhanced cAMP accumulation induced by the direct AC activator FSK, but to a significantly smaller extent compared to NA (Fig. 7F). Interestingly, in contrast to NA, *pertussis toxin* treatment affected FSK-induced cAMP accumulation (data not shown), suggesting that the involvement of G_i proteins in ligand-induced cAMP accumulation contributes to the glucose effect on this process. However, NA is a key activator of the HPT axes, because it induces thyreoliberin release from the hypothalamus via cAMP production^{22,47}. Thus, it is reasonable to assume that glucose-induced enhancement of NA signalling might activate the HPT axis and thus enhance metabolism. Dysregulation of this process in diabetic individuals may provide a new link between DM and altered metabolic activity.

Overall, we discovered so far unappreciated effects of glucose on gene expression in murine hypothalamic cells, affecting cholesterol biosynthesis as well as IGF and NA signalling. Effects of glucose on cholesterol synthesis, SREBP-1 and -2 or IRS-1 expression were completely and on AC-6 expression partially dependent on CRTC-2, suggesting a key role of the CREB co-activator. This is in line with our previous study, showing that glucose enhances CRTC-2 activity by inhibition of AMPK³⁷. However, at this point, we cannot exclude the participation of other factors, such as SRF/TCF or max-like protein X. Detailed promoter analysis of the 15 genes shown in table 1 will be required in order to dissect the exact mechanism leading to glucose-induced gene regulation.

Lastly, we employed an *in vitro* model and rather pronounced changes of glucose concentrations.

Therefore, at this point, we can only speculate to what extent our data can be extrapolated to the *in vivo* situation. Hence, it will be an enlightening task for future studies to validate which of the genes identified in hypothalamic cell models are, indeed, glucose-dependent *in vivo* and whether or not these changes contribute to the pathophysiology of DM.

Experimental procedures

Materials & Methods

Materials - IGF-1, EGF, FSK and noradrenalin were from SigmaAldrich. The SRF/TCF-dependent reporter from Dr. Susanne Mühlich, Walter-Straub-Institute, LMU, München Germany. Oligonucleotids (primer) were from Metabion, Martinsried, Germany. [2,8-³H]-adenine was from PerkinElmer (Boston, USA). The anti-SDHA (ab14715) and the anti-IRS (ab131487) antibodies were from abcam. The anti-SREBP-1 (sc-13551), anti-SREBP-2 (sc-13552) and the anti-ERK1 D2 (sc-1647) antibody was from santa cruz. The anti-ADCY6 (PA5-101284) antibody was from Thermo Fisher scientific.

Cell culture - mHypoA-2/10 (Clu-176) cells were from Cedarlane. GT1-7 cells were kindly provided from Dr. Weiner, University of California, San Francisco, USA). All cells were cultured in DMEM with 10 % FBS, 25 mM glucose, 2 mM L-glutamine and penicillin/streptomycin at 37 °C and 5 % CO₂⁶⁰. mHypoA-2/10 cells stably expressing the *arntl1* or SRF/TCF reporter were previously published²⁰. In order to down-regulate CRCT-2 expression we recently established the following protocol³⁷. A cocktail of three specific siRNAs (sc-45833) or a control siRNA (sc-37007) from Santa Cruz was transfected into mHypoA-2/10 cells via electroporation using the Neon® transfection system from Invitrogen according to the manufacturer's protocol. Briefly, for one pulse 500,000 cells together with 40 nM of the corresponding siRNA were challenged with 1450 V for 30 ms.

RNA-seq - Isolation of total RNA, RNA-seq experiments and gene expression analysis were performed by IMG M Laboratories GmbH, Planegg, Germany. After stimulation of the cells in house, the reaction was stopped by adding RNA stabilizer (RNAlater from LifeTechnologies). Samples were frozen in liquid nitrogen and stored at -80 °C. After transportation on dry ice, lysates were thawed and total RNA extracted using the RNeasy Mini Kit (Qiagen). TruSeq®stranded mRNA HT kit (Illumina, Baltimore, USA) was used for library preparation according to the manufacturer's protocol. Amplified cDNA was purified using a solid phase reversible immobilization (SPRI) paramagnetic bead-based technology (AMPure XP beads, Beckman Coulter) with a bead to DNA ratio of 1 to 1. Quality control of each sample was carried out using 2100 Bioanalyzer DNA 1000 LapChip (Agilent Technologies) and cDNA quantification was determined using Qubit® ds DNA assay kit (Thermo Fisher Scientific). cDNA was denaturated by NaOH and sequencing performed with a 1% PhiX v3 control library spike (Illumina, Baltimore, USA) in a NextSeq® 500 sequencing system (Illumina, Baltimore, USA). Primary image processing was performed on the NextSeq® 500 instrument using Real Time Analysis 2.4.11 Software (RTA). Primary data analysis was performed using the bcl2fastq 2.15.04 software package. The Illumina Sequence Analysis Viewer (SAV) 2.1.8 was used for imaging and evaluation of the sequencing run performance. The CLC Genomics Workbench 9.5.3 (CLC bio, a Qiagen company) was used for in-depth analysis of differential gene expression. Image and signal processing, such as imaging and base calling, was carried out by the Illumina NextSeq® 500 inherited NCS and RTA software packages applying the *FastQ only* processing pipeline. Data obtained were mapped against the *mouse reference genome (GRCm38.p4)* downloaded from NCBI as reference sequence. The expression values were processed to RPKM, a normalized measure of relative abundance of transcripts. It is defined as reads per kilobase of exon model per million mapped reads. For the analysis of differential gene expression the three data sets of low glucose were compared to three data sets of cells kept at high glucose. The p-value of a pairwise comparison was derived from the Wald test. To

control the false positive rate (FDR) Bonferroni corrected p-values were calculated. The method of Benjamini and Hochberg was used to control for the FDR. Functional analysis was performed using the Ingenuity Pathway Analysis (IPA) software, build version: 463341M, content version: 42012434.

Quantitative RT-PCR (qRT-PCR) - Serum-starved mHypoA-2/10 cells were either kept at low or high glucose concentrations as described below. Stimulation was terminated by rapid cooling on ice and total RNA was isolated using the Trizol[®] reagent (Invitrogen, Darmstadt, Germany) according to the manufacturer's instructions. First strand synthesis was carried out with oligo(dT)₁₈ primer using 2 µg of total RNA and the RevertAid[™] H Minus First Strand cDNA Synthesis Kit (Fermentas, Sankt-Leon Roth, Germany). qRT-PCR was done using the LightCycler[®] 480 SybrGreen I Master Mix kappa (Roche, Mannheim, Germany), intron-spanning primer pairs at a final concentration of 1 µM each and 0,08 µl of the first strand synthesis reaction in a LightCycler[®] 480 II (Roche) using the following conditions: initial denaturation for 2 min at 94 °C, 55 cycles of 94 °C for 10 sec, 55 °C for 10 sec and 72 °C for 10 sec. Crossing points (Cp) were determined by the software supplied with the LightCycler[®] 480 and data analysed by the $\Delta\Delta C_p$ method ($2^{-((\text{target gene} - \text{ref. gene})_{\text{high glucose}} - (\text{target gene} - \text{ref. gene})_{\text{low glucose}})}$).

Glucose stimulation - Two distinct protocols were used. For RNA analysis, cells were first starved for 24 h with 0.1 mM glucose in DMEM with 1 mM pyruvate in the absence of FBS. Afterwards one pool of cells was kept at 0 mM and the corresponding pool at 2.5 mM glucose for 3 h. For reporter, cAMP, western blot, whole cell ELISA or cholesterol assays cells were kept for 24 h at 0.1 or 2.5 mM glucose in the absence of FBS.

Reporter assay - Cells were lysed in 100 - 200 µl of lysis buffer (25 mM Tris/HCl pH 7.4, 4 mM EGTA, 8 mM MgCl₂, 1 mM DTT and 1 % Triton-X-100) and a volume of 90 - 180 µl was transferred to white-bottomed, 96-well plates. Luciferase activity was measured after automatically injecting the luciferase substrate (20 - 40 µl) using a FLUOstar[®] Omega plate reader. Total light emission was detected every second for 10 s post injection and average emission between 2 and 10 s was calculated.

Western-blot - Cells were seeded on 6-well plates (~100,000/well), cultured for one day, serum-starved with 0.1 or 2.5 mM glucose for 24 and then lysed in Laemmli buffer. Lysates were subjected to SDS-PAGE and proteins transferred to nitrocellulose by western-blotting. For detection of two distinct proteins on the same blot, blots were separated in two parts, by a horizontal cut at ~53 kDa or ~100 kDa. The upper part was used to analyse SREBP-1, SREBP-2, IRS-1 or AC-6 expression and the lower part to detect ERK-2 or SDHA proteins as a loading control.

Whole-cell ELISA - Whole-cell ELISA assays were performed as described previously with slight modifications^{37,61,62}. Electroporated cells were seeded in 24-well plates (~15,000/well) two days prior to the experiment. The next day, cells were kept at either 0.1 or 2.5 mM glucose for 24 h. Total SREBP-1, SREBP-2, IRS-1 or AC-6 expression was then analysed with the according antibody. After fixation for 15 min with 4 % PFA, cells were permeabilized with ice-cold methanol/acetone (50:50) for 5 min. Next, cells were washed with PBS and then incubated with 1.0 % of BSA in PBS for 15 min at RT to block unspecific binding sites. After an additional washing steps, cells were incubated for 30 min at RT with the corresponding first antibody (1:2,000) in PBS/BSA. As a control, a pool of cells was incubated in PBS/BSA without first antibody. Next, antibodies were removed, cells washed and incubated with the corresponding HRP-conjugated secondary antibody (1:4,000) in PBS/BSA for 30 min at RT. After several washing steps, 200 µl of the 1-Step[™]-Ultra-TMB-ELISA substrate (ThermoScientific, Rockford, USA) was added to the cells and incubated for 15 min at RT. The reaction was stopped by adding 50 µL of 1 M H₂SO₄, 200 µl transferred to 96-well plates with clear bottom and OD₄₅₀ measured in a FLUOstar[®] Omega plate reader. Background values of the control cells (without first antibody) were subtracted.

Cholesterol detection - In order to detect cholesterol levels, the cholesterol ester-glo assay® from Promega (J3190) was used accordingly to the manufacturer's protocol. Dependent on the amount of cholesterol present, cholesterol dehydrogenase produces NADH. This NADH is used by a reductase to convert a non-luminescent reductase substrate into luciferin, which can be detected in the presence of the ultra-glo™ luciferase, ATP and Mg²⁺ based on its bioluminescence. Thus, cholesterol levels directly correlate with the bioluminescence measured. Because glucose might affect NADH or ATP levels in cells, we avoided direct cholesterol measurements in the cells. We have chosen to extract cellular cholesterol with cyclodextrins. In detail, we first cultured cells with 0.1 or 2.5 mM glucose for 24 h in 96-well plates (~10,000 cells per well) and then, extracted cholesterol with 20 mM β -cyclodextrin in 100 μ l DMEM with 0.1 mM glucose and 2 % BSA for 4 h. 20 μ l of these supernatants was used to detect cholesterol levels independently from NADH or ATP levels in the cells. Cholesterol standards were used to normalize luminescence signals to the cholesterol concentration.

cAMP accumulation - To determine agonist-induced cAMP accumulation, 100,000 cells were seeded in 12-well dishes 48 h prior to the experiment and labelled in serum-free DMEM containing 1 μ Ci/ml of [³H]-adenine as described above. Cells were stimulated for 20 min in DMEM containing 1 mM 3-isobutyl-1-methylxanthine along with noradrenalin or FSK. The reaction was terminated by removing the medium and adding ice-cold 5 % trichloroacetic acid to the cells. [³H]-ATP and [³H]-cAMP were then purified by sequential chromatography (dowex-resin/aluminium oxide columns). Radioactivity was measured by scintillation counting using a WinSpectral 1414 (PerkinElmer).

Quantification and Statistical Analysis - Values represent the mean $\pm$ SD of three to six independent experiments. Statistical analysis was performed two-sample student's t-test, one-way or two-way ANOVA followed by Sidak's or Tuckey's post-test using the GraphPad prism software 9.1. One symbol indicates a *p*-value of < 0.05, two of < 0.01 and three of < 0.001.

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Declaration of interest

The authors declare that they have no conflict of interest.

Abbreviations

AMPK, AMP-dependent kinase; β -AR, β -adrenergic receptors; CRTC-2, CREB-regulated transcriptional co-activator-2; DM, diabetes mellitus; EGF, epidermal growth factor; ERK-1/2, extracellular-regulated kinases-1/2; FSK, forskolin; GPCR, G protein-coupled receptors; HPT, hypothalamic-pituitary-thyroid; IGFR, insulin-like growth factor receptor; NA, noradrenalin; TRH, pro-thyrotropin-releasing hormone; RPKM, reads per kilobase of transcript per million mapped reads; RTK, tyrosine receptor kinases; SREBP, sterol regulatory element binding proteins; SRF, serum response factors; TCF, ternary complex factors

Author contributions

AB designed and performed the experiments, analysed the data and wrote the manuscript. LW designed and performed the experiments and analysed the data. DF and SZ performed experiments. TG analysed the data and wrote the manuscript.

Figure legends

Figure 1. Volcano plot of RNA-seq data obtained with mHypoA-2/10 cells at low and high glucose levels. Only genes with RPKM of ≥ 1 for at least one condition (11062 genes) are shown. The number of genes with a fold-change of $|FC| \geq 3$, $|FC| \geq 3$ or $|FC| \geq 1.5$ are given. For each condition, triplicates were analysed as described under experimental procedures.

Figure 2. Glucose induces RNA expression of both *sreb* genes and of key enzymes involved in cholesterol biosynthesis in mHypoA-2/10 cells. Cells were cultured under low and high glucose concentrations and RPKM determined by RNA-seq. For each condition, triplicates were analysed as described under experimental procedures. In (A) data for 3-hydroxy-3-methylglutaryl-CoA synthase 1 (*hmgcs1*), in (B) for 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgcr*), in (C) for mevalonate kinase (*mvk*), in (D) for farnesyl-diphosphate farnesyltransferase-1 (*fdft1*), in (E) for lanosterol 14 α -demethylase (*cyp51*), in (F) for hydroxysteroid 17- β dehydrogenase-7 (*hsd17b7*), in (G) for NAD(P)-dependent steroid dehydrogenase-like (*nsdhl*), in (H) for 24-dehydrocholesterol reductase (*dhcr24*), in (I) for 7-dehydrocholesterol reductase (*dhcr7*), in (J) for the sterol regulatory element-binding protein gene-1 (*sreb1*) and in (K) for the sterol regulatory element-binding protein gene-2 (*sreb2*) are shown. Only genes with at least one RPKM ≥ 1 were considered. Data are expressed as the mean $\pm$ S.D. Asterisks indicate significant differences calculated based on FDR corrected Bonferroni p-values of the entire data set obtained by RNA-seq. Hash marks indicate significant differences based on two-sampled t-tests.

Figure 3. Glucose induced SREBP protein expression and cholesterol synthesis via CRT-2 in mHypoA-2/10 cells. Cells were culture for 24 h with either 0.1 or 2.5 mM glucose. In (A), SREBP-1 or -2 expression was analysed by western-blotting. Detection of ERK-1 served as the loading control. In (B and C), cells were transfected with a control or a specific siRNA against CRT-2. In (B) SREBP-1 or -2 expression was determined by ELISA. In (C) cholesterol levels in the supernatant was detected after incubation of the cells with 20 mM β -cyclodextrin for 4 h. In (D), cholesterol levels were determined in the supernatant of GT1-7 cells after incubation of cells with 20 mM β -cyclodextrin for 4 h. Data of 3 independent experiments performed in quadruplicates are shown as the mean $\pm$ S.D. Asterisks indicate significant differences based on two-way ANOVA followed by Tukey's post-hoc test.

Figure 4. Effects of glucose on mRNA expression of single genes involved in intracellular signalling processes in mHypoA-2/10 cells. Cells were cultured under low and high glucose concentrations and RPKM determined by RNA-seq. RPKM values for 99 genes are given. Genes marked in red were significantly upregulated and those marked blue downregulated by glucose (FDR corrected p-value of ≤ 0.05). Black genes were not significantly affected by glucose. In the lower panel genes with RPKM between 1 and 10, in the middle panel between 10 and 40 and in the upper panel between 40 and 400 are shown.

Figure 5. Heat map of glucose-dependent gene expression in mHypoA-2/10 cells combining RNA-seq and qRT-PCR data in mHypoA-2/10 cells. Cells were cultured under low and high glucose concentrations and RNA levels determined by RNA-seq or qRT-PCR with *sdha* or *tbp* as a reference gene. Redish colours indicate gene induction blueish depression. For qRT-PCR experiments, data of 3 independent experiments performed in triplicates are shown. Data are also provided in Tab. S2.

Figure 6. Glucose enhanced IRS-1 expression via CRT-2 and IGF- but not EGF-induced activation of a SRF/TCF-dependent luciferase reporter gene. Cells were cultured under low and

high glucose concentrations and RPKM determined by RNA-seq in (A) or by qRT-PCR in (B). In (A), for each condition triplicates were analysed for *irs1* or -2 expression. In (B) *irs-1* and -2 expression was analysed with *sdha* as the reference in 3 independent experiments performed in triplicates (mean $\pm$ S.D). Data obtained at 0.1 mM glucose (basal) were set to 1.0. In (A), asterisks indicate significant differences calculated based on FDR corrected Bonferroni p-values of the entire data set obtained by RNA-seq in B), asterisks indicate significant differences based on two-way ANOVA followed by Tukey's post-hoc test. In (C), IRS-1 expression was analysed by western-blotting. Detection of SDHA served as the loading control. In (D), after transfection of a control or a specific siRNA against CRTC-2, IRS-1 expression was determined by ELISA. Data of 3 independent experiments performed in quadruplicates are shown as the mean $\pm$ S.D. Asterisks indicate significant differences based on two-way ANOVA followed by Tukey's post-hoc test. In (E and F), cells were transfected with an SRF/TCF-dependent-reporter gene construct, cultured for 24 either with 0.1 or with 2.5 mM glucose, stimulated or not with IGF or EGF (both 100 nM) for 4 h and luciferase activity determined. In (E) basal reporter activity is shown normalized to the basal obtained at 0.1 mM (100 %). In (F), the corresponding basal value was set to 100 % and GF-induced reporter activation calculated as the percentage of basal. Data of 4 independent experiments performed in triplicates are expressed as the mean $\pm$ S.D. Asterisks indicate significant differences based on two-sampled t-tests (E) or two-way ANOVA followed by Tukey's post-hoc test (F). In (G), GT1-7 cells were transfected with a SRF/TCF-dependent-reporter gene construct, cultured for 24 either with 0.1 or with 2.5 mM glucose, stimulated or not with IGF or EGF (both 100 nM) for 4 h and luciferase activity determined. Data of 4 independent experiments performed in triplicates are expressed as the mean $\pm$ S.D. Asterisks indicate significant differences based on two-way ANOVA followed by Tukey's post-hoc test.

Figure 7. Glucose enhanced AC-6 expression and NA-induced cAMP accumulation in mHypoA-2/10 cells. Cells were cultured under low and high glucose concentrations and RPKM determined by RNA-seq (A). For each condition, triplicates were analysed as described under experimental procedures. Only genes with at least one RPKM $\geq$ 1 were considered. Data are expressed as the mean $\pm$ S.D. Asterisks indicate significant differences calculated based on FDR corrected Bonferroni p-values of the entire data set obtained by RNA-seq. Hash marks indicate significant differences based on two-sampled t-tests. In (B), AC-6 expression was analysed by western-blotting. Detection of ERK-1 served as the loading control. In (C), after transfection of a control or a specific siRNA against CRTC-2, AC-6 expression was determined by ELISA. Data of 3 independent experiments performed in quadruplicates are shown as the mean $\pm$ S.D. Asterisks indicate significant differences based on two-way ANOVA followed by Tukey's post-hoc test. In (D to G), cells were labeled with [3 H]-adenine, cultured for 24 h either with 0.1 or with 2.5 mM glucose, stimulated or not with 10 μ M FSK or noradenalin (NA) for 20 min and [3 H]-cAMP and [3 H]-ATP determined. In (D) basal [3 H]-ATP and in (E) basal [3 H]-cAMP levels are shown. In (F) ligand-induced signals of [3 H]-cAMP signals are shown as x-fold over basal. In (G) ligand-induced signals at 0.1 mM glucose were set to 100 % and ligand-induced signals at 2.5 mM calculated as the percentage. Data of 4 independent experiments performed in triplicates are expressed as the mean $\pm$ S.D. Asterisks indicate significant differences based on two-sampled t-tests (B and D) or two-way ANOVA followed by Tukey's post-hoc test (C).

Table 1. mHypoA-2/10 cells were cultured under low and high glucose concentrations and RNA levels determined by RNA-seq or qRT-PCR with *sdha* or *tbp* as a reference gene. For the RNA-seq data differences were calculated based on FDR corrected Bonferroni p-values. For the qRT-PCR data, $\Delta\Delta\text{Cp}$ using *sdha* or *tbp* were calculated and significant differences determined using two-way ANOVA and Sidak's post-test or two-sampled t-tests. Mean change indicates alterations based on all three data sets.

gene		RNA-seq		qRT-PCR						
name	function	Change [%]	corrected Bon-ferroni	tbp			sdha			Mean change [%]
				Change [%]	ΔΔCp ANOVA Sidak´s	ΔΔCp t-test	Change [%]	ΔΔCp ANOVA Sidak´s	ΔΔCp t-test	
irs1	adaptor protein for IR and IGFR	123	***	41	***	*	52	***	*	72 ± 26
igfr1	IGF receptor	34	***	21	**	***	28	**	***	28 ± 4
adcy6	cAMP production	66	***	28	**	***	37	**	**	44 ± 11
gnai1	inhibition of cAMP production	43	***	15	*	***	30	*	**	29 ± 8
gnai2	inhibition of cAMP production	40	***	24	**	***	39	***	***	35 ± 5
gnai3	inhibition of cAMP production	16	*	17	*	**	30	*	***	21 ± 5
gna11	PLC activation / Ca ²⁺	17	*	24	*	**	30	*	**	24 ± 4
hras	monomeric G protein / MAPK activation	38	***	15	*	***	22	*	**	25 ± 7
elk3	transcriptional activator / SRF/SRE	61	***	40	***	**	50	***	**	50 ± 6
insig1	cholesterolsynthesis inhibitor	108	***	34	***	**	43	***	**	62 ± 23
rap2a	monomeric G protein / MAPK activation	105	***	27	***	**	45	***	**	59 ± 24
tbc1d1	Rab inhibitor	90	***	29	***	**	38	***	**	52 ± 19
s100a10	calcium-binding protein	59	***	51	***	**	72	***	**	61 ± 6
arhgef2	Rho activator	-58	***	-46	***	**	-47	***	***	-50 ± 4
tbc1d31	Rab inhibitor	-61	***	-55	***	***	-53	***	***	-56 ± 2

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

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