

Effects of Fluctuating Glucose Levels on Neuronal Cells In Vitro

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Abstract There is increasing evidence for glucose fluctuation playing a role in the damaging effects of diabetes on various organs, including the brain. We aimed to study the effects of glycaemic variation (GV) upon mitochondrial activity using an in vitro human neuronal model. The metabolic disturbance of GV in neuronal cells, was mimicked via exposure of neuroblastoma cells SH-SY5Y to constant glucose or fluctuating (i.e. 6 h cycles) for 24 and 48 h. Mitochondrial dehydrogenase activity was determined via MTT assay. Cell mitochondrial activity (MTT) was moderately decreased in constant high glucose, but markedly decreased following 24 and 48 h of cyclical glucose fluctuations. Glucose transport determined via 2-deoxy-D-[1-¹⁴C] glucose uptake was regulated in an exaggerated manner in response to glucose variance, accompanied by modest changes in GLUT 1 mRNA abundance. Osmotic components of these glucose effects were investigated in the presence of the osmotic-mimics mannitol and L-glucose. Both treatments showed that fluctuating osmolality did not result in a significant change in mitochondrial activity and had no effects on ¹⁴Cglucose uptake, suggesting that adverse effects on mitochondrial function were specifically related to metabolically active glucose fluctuations. Apoptosis gene expression showed that both intrinsic and extrinsic apoptotic pathways were modulated by glucose variance, with two major response clusters corresponding to (i) glucose stress-modulated genes, (ii) glucose mediated osmotic stress-modulated

genes. Gene clustering analysis by STRING showed that most of the glucose stress-modulated genes were components of the intrinsic/mitochondrial apoptotic pathway including Bcl-2, Caspases and apoptosis executors. On the other hand the glucose mediated osmotic stress-modulated genes were mostly within the extrinsic apoptotic pathway, including TNF receptor and their ligands and adaptors/activators/initiators of apoptosis. Fluctuating glucose levels have a greater adverse effect on neuronal cell energy regulation mechanisms than either sustained high or low glucose levels.

Keywords Glucose and glycaemic fluctuation · Diabetes · Neurons · Mitochondrial activity

Introduction

There has been recent debate as to the metabolic significance of glycaemic variation (GV) in determining long term outcomes in diabetes [9, 55]. Whilst GV appears to be a determinant of micro- and macro-vascular outcomes in type 2 diabetes, the putative pathophysiologic role of GV in type 1 diabetes remains unclear [34]. This uncertainty is in part due to studies using varying metrics of GV and with differing glycaemic datasets, rendering comparative analysis problematic [4, 9]. In the context of paediatric type 1 diabetes, clinical microvascular pathology is relatively rare in patients with good metabolic control [27, 32]. On the other hand there is an emerging literature highlighting the relative frequency of neurocognitive and psychological pathology in this younger population [10, 35]. This is particularly concerning given that neurodevelopment is arguably the pre-eminent developmental task of childhood and adolescence.

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A constant supply of glucose is critical for normal cerebral metabolism. Children, in particular, have high cerebral energy needs associated with brain growth, ontogeny and “neural pruning” during development and are more sensitive than adults to glucose deprivation [51]. A number of *in vitro*, *in vivo* and clinical research studies have identified the effects of both hypoglycaemia and hyperglycaemia on neural cell activity, survival and function [11, 16, 20, 50]. Developmental psychologists have further highlighted the importance of swings between high and low blood glucose levels in brain development- the “diathesis hypothesis” [49].

A number of *in vitro* studies in human endothelial cells and human olfactory/neuroepithelial cells [19, 40, 43, 46] have indicated that intermittent high glucose has greater cellular damaging effects than constant high glucose [12, 19, 40, 43, 46, 53]. However the cellular and molecular mechanisms involved have not previously been investigated in human neuronal cells. Since glucose is the primary fuel for neurons, and mitochondria play a primary role in the cell’s energy regulation, we chose to focus on mitochondrial function in response to ambient glucose fluctuations.

The purpose of this study then was to examine the effects of fluctuation between high and low ambient

glucose levels upon neuronal viability and mitochondrial function in an *in vitro* model of human neuronal cells.

Materials and Methods

Cell Cultures and Treatments

The Neuronal-type SH-SY5Y human neuroblastoma cell line [26, 48], derived from metastatic neuroblastoma site in a four year-old female [5], was seeded in 24-well plates [3×10^4 cell/well/ml] and cultured for 16 h in 5 mM glucose (normal glucose) complete 10 % FCS (Trace Biosciences, Castle Hill, NSW) DMEM (GIBCO, #11966, Invitrogen, Mulgrave, Victoria 3170, Australia) prior to switch to differentiation treatment with 50 μ M retinoic acid (RA; Sigma Chemical Co., St. Louis, MO, USA) for 48 h. The SH-SY5Y neuroblastoma cells are widely used *in vitro* as model systems for human neuronal cells [1, 14, 30].

Neuronal differentiation by RA of SH-SY5Y at 48 h (prior to treatments, see below) was confirmed by change in cell morphology (NF150kD; Chemicon-Millipore, Temecula, CA, USA) and expression of the neuronal marker Neuro-D6 gene [23] as shown in Fig. 1a. After 48 h retinoic acid was

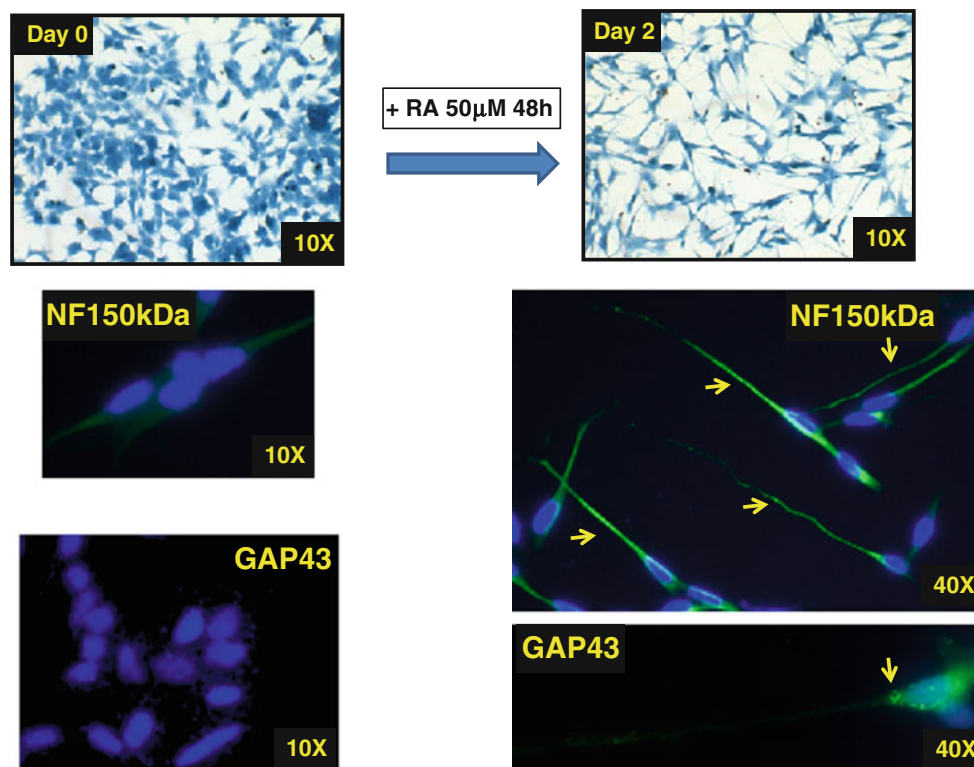


Fig. 1 Morphology of the neuroblastoma cells SH-SY5Y at day 0 (*top left*) prior neuronal differentiation with RA at 50 μ M for 48 h (*top right*). Typical neuronal morphology is demonstrated by immuno-fluorescence (*arrows*) for the neuronal neurofilament marker

NF150 kDa or GAP43 (Alexa-488 green and nuclear DAPI blue stain; *right panels*). No stain for 150 kDa-NF or GAP43 was detected in undifferentiated SH-SY5Y cells. Magnification was $\times 10$ or $\times 40$ as indicated

decreased to 20 μM and this concentration, required for maintenance of the cell neuronal phenotype, was used for the duration of all experiments and in each of the treatments or controls.

Cell number was determined by NBB assay (not shown) and mitochondrial activity by MTT assay as we previously described [26, 48]. The MTT assay (reduction of tetrazolium salts) is a measure of mitochondrial activity which quantitatively reflects cell viability. The yellow tetrazolium MTT [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. MTT assay evaluates the amount of cellular MTT that mirrors the activity of mitochondrial dehydrogenase, whose production decreases with increasing cellular distress [22, 24, 44, 47].

Once differentiated, cells were subjected to constant low or normal or high D-glucose concentrations and to recurrent glucose variance protocol as described below.

Constant Glucose

cells were cultured in 2.5 mM or 5 mM or 25 mM or 50 mM D-glucose with change to fresh media (+RA at 20 μM), and the same D-glucose concentrations, every 12 h, and repeated for up to 48 h.

Glucose Variance

cells were cultured in 2.5 or 5 mM D-glucose for 6 h (low and normal glucose levels; “low phase”) and then supplemented, without a change of media, with concentrated glucose (1 M D-glucose) to elevate both low phase glucose concentrations up to 25 or 50 mM and cultured for another 6 h (high glucose “high phase”). This was then followed by a change to fresh media (+RA at 20 μM) at 12 h, lowering the glucose levels back to low phase glucose concentration of 2.5 or 5 mM. To minimize cell stress due to media change, fresh treatment media was always incubated at 37 °C and equilibrated with 5 % CO₂ prior to addition to cell cultures. These 6 h cycles with change to fresh media (+RA at 20 μM) every 12 h was repeated for up to 48 h.

Although diabetic patients might experience fluctuations of blood glucose levels in a range comparable to those selected for our in vitro model, glycaemic fluctuation might occur more frequently than every 6 hourly. However during the set up of our in vitro model we consistently observed that media change at intervals of 2–4 h significantly affected mitochondrial activity of cells grown in optimal 5 mM glucose. This was not seen when media was changed at intervals of 6 or 12 h as we have used in these studies.

The described treatment protocol was also performed in the presence of mannitol or L-glucose (instead of D-glucose) to control for the effects of changes in media osmolality versus glucose specific metabolic effects. Appropriate volumes of 1 M Mannitol (MW 180 kDa; osmo-mimic for D-glucose) or 1 M L-glucose (enantiomer of D-glucose; not metabolized) were added to the glucose baselines of 5 mM (normal) or 2.5 mM (low) glucose, to obtain the equivalent osmolality as per 25 mM D-glucose (i.e. 20 or 22.5 mM of mannitol or L-glucose to DMEM, RA at 20 μM with 5 or 2.5 mM D-glucose respectively).

To determine the effect of glucose variance on cell number, the above described treatment protocol was performed in low serum (0.5 or 1.0 % FCS) DMEM/RA 20 μM or serum free (no FCS) DMEM/RA 20 μM (data not shown).

Glucose Transport

2-deoxy-D-[1-¹⁴C]-glucose uptake: A variety of radio-labeled or fluorescence-activated 2-deoxy-D-glucose are routinely used for measurement of glucose uptake in vitro systems [2] [42] [28] and in vivo [31, 37]. In the present studies uptake of the 2-deoxy-D-[1-¹⁴C]-glucose tracer was performed as we previously described [26, 48]. This assay does not provide an absolute measurement of glucose uptake or glycolysis rate, but allows the measurement of the amount of the non metabolized 2-deoxy-D-[1-¹⁴C]-glucose tracer trapped inside the cells relatively to the extracellular levels of glucose. Transport/uptake of glucose and therefore of the 2-deoxy-D-[1-¹⁴C]-glucose tracer is expected to be high in low glucose and low in high glucose when compared to glucose or 2-deoxy-D-[1-¹⁴C]-glucose tracer transport/uptake under optimal culture conditions.

In brief, cells were cultured under the key treatments described above (constant glucose at 2.5 or 5 or 25 mM; variable glucose cycles [low phase/high phase] with glucose at 2.5 or 5 mM been shifted at 25 mM) for 24 h. This was followed by a change to fresh media DMEM/RA 20 μM supplemented with 2-deoxy-D-[1-¹⁴C] glucose (1 $\mu\text{Ci/ml}$) and containing glucose at 2.5 or 5 or 25 mM for those cells treated with constant glucose, while the cells exposed to variable glucose cycles (low phase/high phase) were shifted to low phase in the presence of fresh media supplemented with 2-deoxy-D-[1-¹⁴C] glucose (1 $\mu\text{Ci/ml}$) and containing glucose at 2.5 mM or 5 mM. Under each of the treatments, cells were exposed to 2-deoxy-D-[1-¹⁴C]-glucose for 15 min. Some cultures, after fresh media change without 2-deoxy-D-[1-¹⁴C] glucose, were taken through an additional 6 h culture cycle before shifting glucose from 2.5 or 5 to 25 mM and supplementing with 2-deoxy-D-[1-¹⁴C] glucose (1 $\mu\text{Ci/ml}$). Control wells with

cells exposed to constant glucose were also supplemented with 2-deoxy-D-[1-¹⁴C] glucose. In all wells cells were exposed to 2-deoxy-D-[1-¹⁴C] glucose for 15 min and intra-cellular ¹⁴C-glucose after 15 min was measured by β -counter.

Total RNA Extraction, RT-PCR and RT²-PCR Arrays

RNA Extraction

RA differentiated SH-SY5Y cells were grown and treated as described above and in Results, prior to total RNA extraction using RNeasy Minikit (Qiagen, Valencia, CA, USA) as per manufacturer's instructions. DNase digestion was performed using RNase free DNase kit (Ambion, Austin, Texas).

RT-PCR

All reverse transcription and PCR reactions were performed using GeneAmp PCR Core kit (Applied Biosystems). Each semi-quantitative RT-PCR reaction (20 μ l total volume) contained 150 ng of reverse transcribed cDNA. GAPDH primers were as we previously described [23]. Primers for hGLUT1 were (F) TTTGTGGTGGAGC GAGCAGG and (R) AGCCAAAGATGGCCACGATGC TC and for hGLUT3 (F) TGGAGAAAACCTTGCTGCTGA GAAGG and (R) TGGCAAATATCAGAGCTGGGGTGA.

RT²-PCR and RT²-PCR Arrays

First strand cDNA was obtained for each of the constant glucose and glucose variance cycles treatments as described above by reverse transcription [23]. To verify absence of genomic DNA contamination RT-PCR for GAPDH and negative control (-MuLV reverse transcriptase) was also performed. All steps involved in setting up the reaction plate (PAHS-012A Human Apoptosis, SABioscience Corp, Frederick, MD, USA) were performed as recommended in the RT² PCR array Users' manual. Standard cycle conditions were used as for ABI Prism 7300 Real Time thermal cyclers and data analysis was performed using the 2- $\Delta\Delta$ Ct method with the aid of an analysis template provided by the SuperArray website (<http://www.superarray.com/manuals/pcrarraydataanalysis.xls>) and according to the method by KJ Livak and TD Schmittgen [29]. The reaction was performed using an ABI Prism 7300 Real-time Thermal cycler with fluorescence detection (Applied Biosystems) and data obtained from the 7300 Sequence detection software v 1.2.2. All expression levels were normalised to a set of 5 house-keeping genes (HKG: Beta-2-microglobulin [B2 M]; Hypoxanthine phosphoribosyltransferase 1 [HPTR]; Ribosomal

protein L13a [RPL13A]; Glyceraldehyde-3-phosphate dehydrogenase [GAPDH]; Actin, beta [ACTB]) (see also below). Three independent experimental samples were analysed for each of the described treatments.

Immunofluorescence for Neuronal Differentiation Marker

In order to verify that all treatments were administered to neuronal differentiated cells, SH-SY5Y cells were seeded in 4-well chamber slides (BD FalconTM, Bioscience, Bedford, MA, USA) at 3x10⁴ cell/well/ml and cultured for 16 h in 5 mM glucose complete 10 % FCS-DMEM prior to switch to differentiation treatment with RA at 50 μ M for 48 h. Differentiated cells were then fixed in 4 % paraformaldehyde/PBS at R^oT for 20 min, followed by extensive washes in PBS prior to incubation with 1 % denatured BSA (D-BSA) in PBS for 30 min at R^oT to block potential non-specific binding of the applied antibody. This was followed by a cell membrane permeabilisation step with 0.25 % Triton X-100/1 % D-BSA at R^oT. The cells were then incubated with rabbit-anti neurofilament (150 kDa NF; AB1981;1/200) (Chemicon-Millipore, Temecula, CA, USA), the rabbit-anti GAP43 (1/200; SantaCruz Biotechnology, Santa Cruz, CA), the rabbit-anti GLUT-1 or the rabbit-anti GLUT-3 (1/200; Chemicon-Millipore, Temecula, CA, USA) overnight at 4 °C. Cells were then extensively washed in PBS prior to incubation with the goat-anti-rabbit Alexa-fluoro 488 (1:1,000) applied in 1 % D-BSA/PBS overnight at 4 °C. Cells were then extensively washed in PBS prior to DAPI staining (10 min at 1:2,000). Slide were mounted in an anti-fade medium and examined using a 10 \times -magnification objective by an inverted 1 \times 70 UV microscope (Olympus, Japan). Omission of primary antibody or rabbit IgGs were used as negative controls.

Statistical Analysis

GraphPad PRISM was used to perform t-test for MTT and NBB assays. All experiments were performed at least three times with samples run in quadruplicate and data plotted as mean \pm SEM. The RT²-PCR Array data analysis was performed as we and other have previously described [3, 13, 23, 29, 41, 45], and with the aid of the Data Analysis Web Portal using the 2- $\Delta\Delta$ Ct method and an analysis template provided by the SuperArray website (<http://www.superarray.com/manuals/pcrarraydataanalysis.xls>). Three independent experimental samples for each of the indicated treatments were interrogated by the RT²-PCR Array as above (PAHS-012A Human Apoptosis).

Results

Recurrent Glucose Variance Affects Mitochondrial Activity in Neuronal Cells

In order to mimic the metabolic disturbance of glycaemic variation (GV) in neuronal cells the neuroblastoma cells SH-SY5Y were differentiated with RA to a neuronal phenotype as demonstrated by expression of the 150 kDa neurofilament and growth associated protein-43 (GAP43) (Fig. 1). As shown in Fig. 2a, b following exposure of SH-SY5Y cells for 24 h to constant glucose at 5 mM or 25 mM or 50 mM, mitochondrial activity (MTT), a measure of mitochondrial dehydrogenase activity [22, 24, 44, 47], was moderately but significantly decreased only in very high glucose (Fig. 2a, b 50 mM; $p < 0.05$) when compared to the MTT values of 5 mM (normal; 100 %). In contrast, mitochondrial activity measured by MTT was dramatically reduced following 24 and 48 h of the cyclical glucose fluctuations (Fig. 2a, b, G5–25 mM and G5–50 mM, both $p < 0.01$). Cell number measured by NBB assay at 24 and 48 h was not significantly affected under any of the treatment conditions when compared to control in complete medium (data not shown).

Glucose Transport Is Over-Regulated in Response to Glucose Variance

The above described changes in mitochondrial activity are suggestive of a toxic effect of D-glucose, presumably dependent upon its entry into the cells and subsequent metabolism. Therefore it was important to determine whether the transport of glucose, proportionally measured via 2-deoxy-D-[1- 14 C]-glucose uptake, was affected by glucose variance.

In agreement with our previous studies [48], the 14 C glucose uptake (Fig. 3a) appeared to be enhanced in cells exposed to constant 2.5 mM glucose ($p < 0.001$) while it was decreased in cells exposed to constant high glucose (25 mM; $p < 0.001$) when compared to that measured in optimal 5 mM glucose. The 2-deoxy-D-[1- 14 C]-glucose uptake (Fig. 3a) in cells initially exposed to high glucose (25 mM) and then shifted to lower glucose concentrations (2.5 or 5 mM), appeared to closely match (ns) the uptake determined in constant 2.5 or 5 mM glucose. In contrast, when cells were initially exposed to low glucose concentrations (2.5 or 5 mM) and then shifted to 25 mM glucose, the 2-deoxy-D-[1- 14 C]-glucose uptake was dramatically over-regulated and reduced, such that the uptake was significantly (both $p < 0.001$) lower than that of constant 25 mM. GLUT1 and GLUT3 protein were detected in differentiated SH-SY5Y cells (Fig. 3b) and expression of GLUT1 and GLUT3 mRNA was evaluated by real-time PCR. GLUT1 mRNA levels were found moderately reduced when shifting glucose from 2.5 mM or 5 mM to 25 mM (Fig. 3c; GLUT1), while GLUT3 gene expression, although detected, was not significantly modulated by the applied treatments (not shown).

Mitochondrial Activity is Affected by Glucose Level Variance and Not by Osmotic Variance

A consequence of cyclical glucose levels changes in vitro, designed to mimic glycaemic variance in vivo, is the alteration of the media osmolality and subsequent potential cellular osmotic stress.

The data in Fig. 4a, c, show that the osmotic component (mannitol) does not significantly (ns) contribute to the change in mitochondrial activity when compared to their cognate treatments in Fig. 4b, d (Fig. 4a, 24–48 h G5–25 mM, with $p < 0.001$; Fig. 4d G2.5–5 with $p < 0.01$).

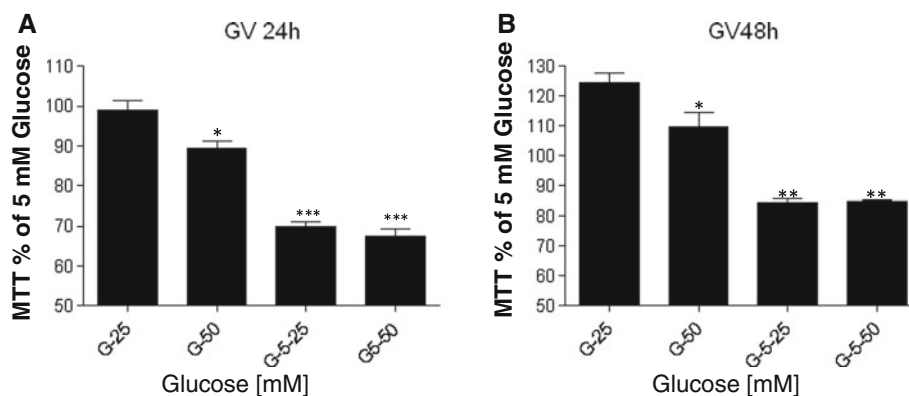


Fig. 2 a–b Recurrent glucose variance affects mitochondrial activity in neuronal cells. **a** Following exposure for 24 and 48 h to constant glucose at 5 or 25 or 50 mM, MTT values are significantly decreased in high 50 mM glucose ($*p < 0.05$) when compared to the MTT

values of 5 mM (normal) glucose. **b** MTT activity at 24 and 48 h is significantly ($***p < 0.001$; $**p < 0.01$) reduced following cyclic glucose fluctuations (G5–25 mM and G5–50 mM)

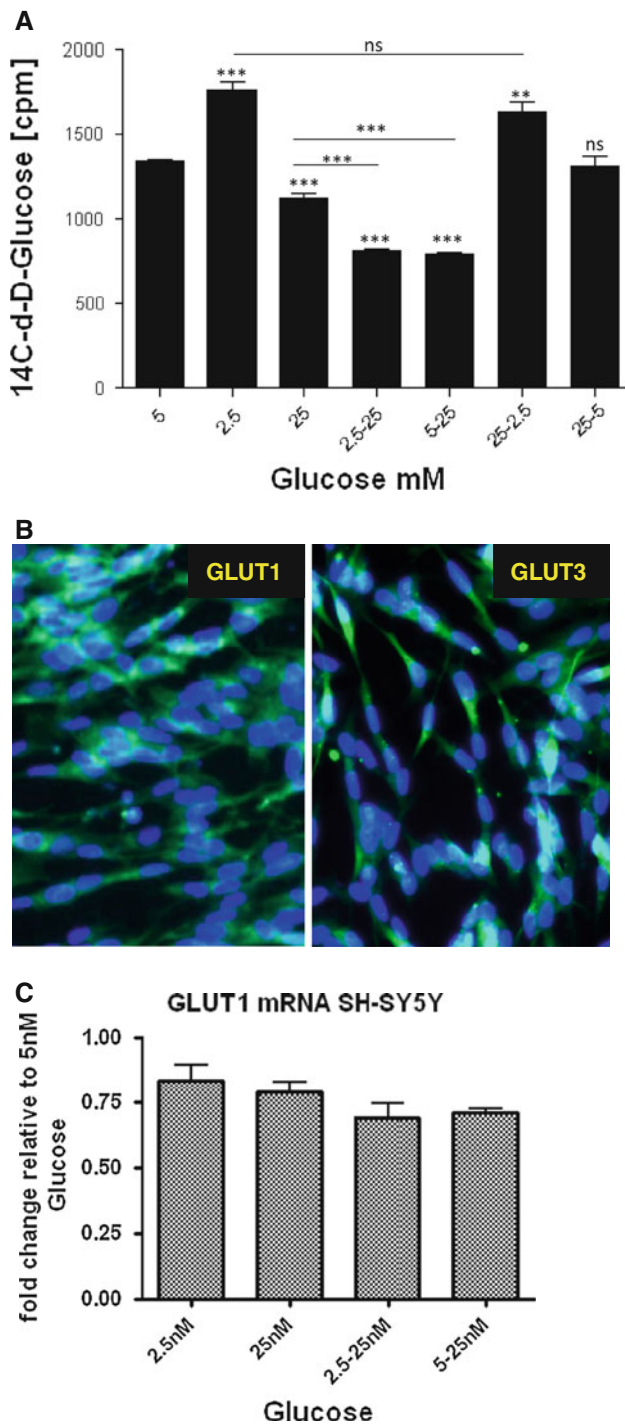


Fig. 3 a–c 2-deoxy-D-[1-¹⁴C]-glucose transport and GLUT1 mRNA levels are over-regulated in response to glucose variance. **a** Cells are exposed to fresh media supplemented with 2-deoxy-D-[1-¹⁴C] glucose (1 μCi/ml) and intra-cellular ¹⁴C-glucose uptake at 15 min is measured by β-counter as described in Materials and Methods. **b** GLUT1 and GLUT3 protein were detected by immunofluorescence (×10 magnification shown) in differentiated SH-SY5Y cells. **(c)** GLUT1 mRNA abundance is reduced when shifting glucose from 2.5 or 5 to 25 mM

The effects of non-metabolisable L-glucose (Fig. 4e) were similar to those of mannitol, suggesting that excursions in glucose (D-glucose) levels are responsible for the reduction in mitochondrial activity seen in Fig. 4f (Fig. 4f, G2.5-5 and G2.5-25, with $p < 0.01$).

Recurrent Cyclical Glucose Variance Results in Modulation of Apoptosis Genes

Although cell number was not affected in the face of a significant reduction in cell mitochondrial activity and an over-regulated glucose uptake, we wished to investigate whether cyclic glucose variance might also result in activation of an apoptotic response, especially involving the “intrinsic” mitochondrial system.

Apoptosis gene expression profile (Fig. 5a–f) was determined for RA differentiated SH-SY5Y cells exposed to constant 2.5 mM or 5 mM or 25 mM glucose or to variable 2.5–25 mM glucose or to 5–25 mM glucose. The 5 mM glucose (normal glucose) and the 2.5 mM glucose (low glucose) were used as comparative baselines for the appropriate treatment. Gene expression was also determined for mannitol at constant 25 mM or at in variable fashion 5–25 mM as described for glucose. Since constant or variable mannitol did not show any significant change in MTT activity, the gene expression profile determined in the presence of the osmotic stressor mannitol (constant or variable) was used to identify which of the glucose regulated genes were indeed genes regulated by glucose acting as an osmotic stressor.

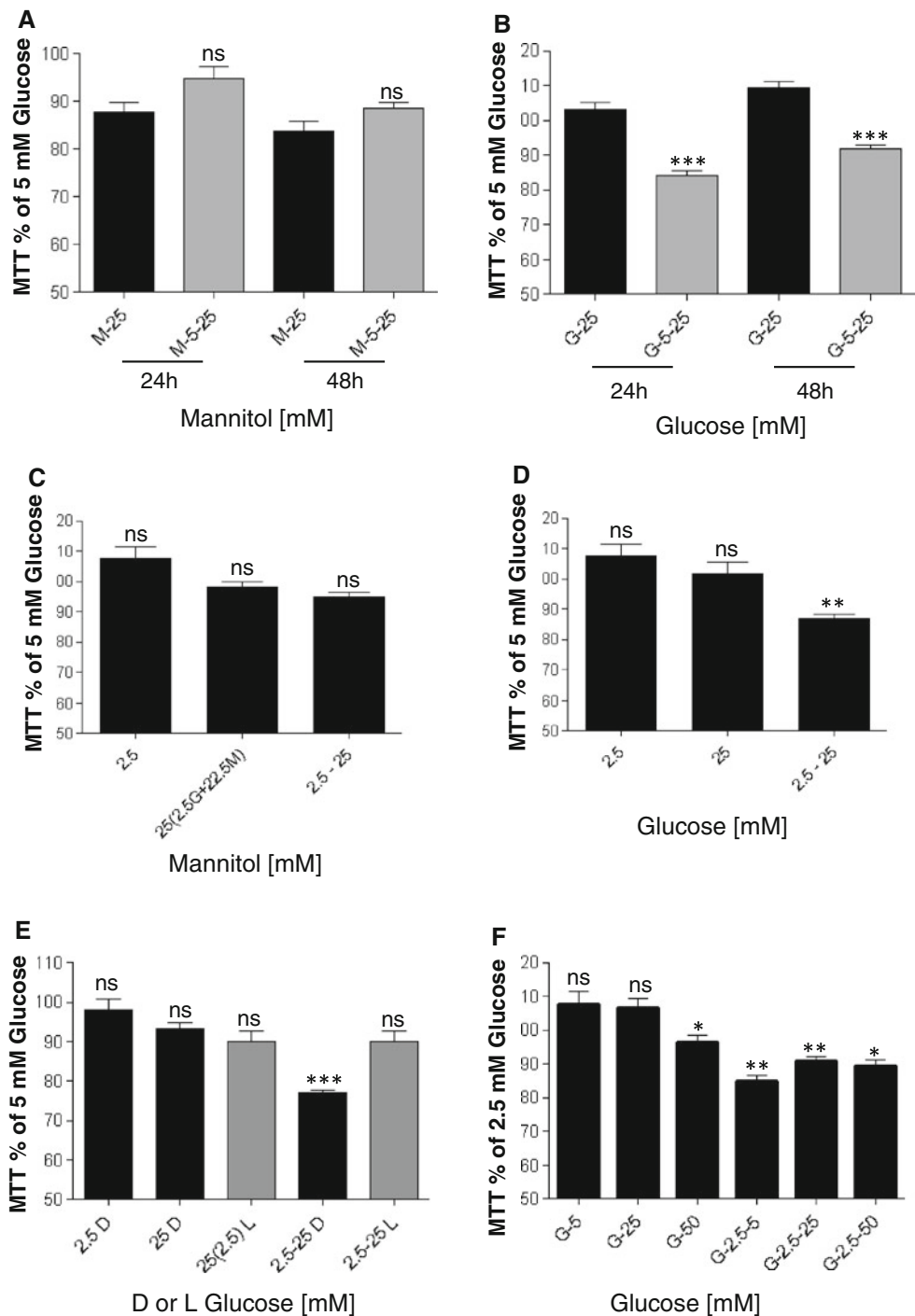
Glucose Stress Modulated Genes [Constant High Glucose]

Up-regulated (at least by +50 %) were BAK1, BCL10, BCLAF1, BFAR, XIAP, BIRC-8, CARD8, FAS, HRK, LTA, CD27, NOL3, RIPK2, TNFSF8 and TP73 compared to their expression at 5 mM (normal) and/or 2.5 mM (low) glucose. Down-regulated (at least by –50 %) were APAF1, BAX, BAG3, BID, NAIP, BNIP2, CARD6, CASP1, CASP10, CASP14, CASP5, CASP7, CD40, CD40LG, CRADD, FASLG, LTBR, TNF, TNFRSF11B, TNFSF10 and CD70 compared to their expression at 5 mM (normal) and/or 2.5 mM (low) glucose.

Glucose Stress Modulated Genes [Variable Normal to High Glucose]

Up-regulated (at least by +50 %) was TNF compared to its expression at 5 mM (normal) glucose. Down-regulated (at least by –50 %) was TP73 compared to its expression at 5 mM (normal) glucose.

Fig. 4 a–f MTT activity is not affected by osmotic variance. To match and mimic the media osmolality fluctuation obtained by variations of glucose (D-glucose) levels (**b, d, f**), the osmo-mimic mannitol [M] (**a, c**) or L-glucose [L] (**e**) are used. The osmotic component does not significantly (ns; mannitol in **a/c** and L-glucose in **e**) contribute to the change in mitochondrial activity. Only excursions in glucose (D-glucose; **b, d** and **f**) levels are responsible for the significant ($p < 0.05$) reduction in MTT activity



Glucose Stress Modulated Genes [Variable Low to High Glucose]

Up-regulated (at least by +50 %) were BCL10, BCLAF1, BFAR, CARD8, FAS and NOL3 compared to their expression at 2.5 mM (low) glucose. Down-regulated (at least by –50 %) were BAX, BCL2, BIRC8, BNIP2, CARD6, CASP1, CASP10, CASP14, CASP5, CASP7, CD40, CD40LG, CIDEA, FASLG, LTA, LTBR, TNF, TNFRSF11B, TNFSF10,

CD70, TNFSF8 and TRAF4 compared to their expression at 2.5 mM (low) glucose.

Osmotic Stress-Modulated Genes with High Glucose Acting as an Osmotic Stressor

Up-regulated (at least by +50 %) was CIDEA compared to its expression at 5 mM (normal) glucose. Down-regulated (at least by –50 %) were CFLAR, GADD45 and PYCARD

compared to their expression at 5 mM (normal) glucose. Down-regulated (at least by -50%) were BCL2L1, BCL2L10, BIRC3, CD27 compared to their expression at 2.5 mM (low) glucose.

Osmotic Stress-Modulated Genes with Glucose Variance Acting as an Osmotic Stressor

Up-regulated (at least by $+50\%$) were BAG1, CIDEA, TNFRSF9 compared to their expression at 5 mM (normal) glucose. Up-regulated (at least by $+50\%$) was BAG1 compared to its expression at 2.5 mM (low) glucose. Down-regulated (at least by -50%) were GADD45 and TRAF2 compared to their expression at 5 mM (normal) glucose. Down-regulated (at least by -50%) were ABL1, BCL2L1, BCL2L10, BIK, BIRC3, CASP8 and CD27 compared to their expression at 2.5 mM (low) glucose.

Osmotic Stress Modulated Genes [Constant High Mannitol]

Up-regulated (at least by $+50\%$) were TNFRSF9 and TRAF3 compared to their expression at 5 mM (normal) glucose. Down-regulated (at least by -50%) were ABL1, BAD, BCL10, BCL2L10, BIRC3, XIAP, CASP8, CIDEB, FADD, GADD45, HRK, PYCARD, TNFRSF10A, CD27, and TRAF2 compared to their expression at 5 mM (normal) glucose.

Osmotic Stress Modulated Genes [Variable Normal Glucose to High Mannitol]

Up-regulated (at least by $+50\%$) were BAG1, CARD6, CIDEA, TNFRSF9 and TRAF3 compared to their expression at 5 mM (normal) glucose. Down-regulated (at least by -50%) were BAG4, BAK1, BCL2L1, BCL2L10, BIK, BIRC3, XIAP, CARD8, CASP2, CFLAR, CIDEB, FADD, GADD45, HRK, TNFRSF21, TP53BP2, TRAF2 and B2 M compared to their expression at 5 mM (normal) glucose.

The gene expression profile data, reported in Fig. 5a–f thus show that glucose variance regulated pro-apoptotic and anti-apoptotic genes in both the intrinsic and extrinsic apoptotic pathways. In contrast to our MTT data, showing no significant effect on mitochondrial activity by the osmotic stressors Mannitol and L-Glucose, the gene expression profile as reported in Fig. 6 (STRING plot analysis), identified two major gene response clusters corresponding to i) glucose mediated metabolic stress-modulated genes, ii) glucose mediated osmotic stress-modulated genes, with high glucose or glucose variance acting as osmotic stressors.

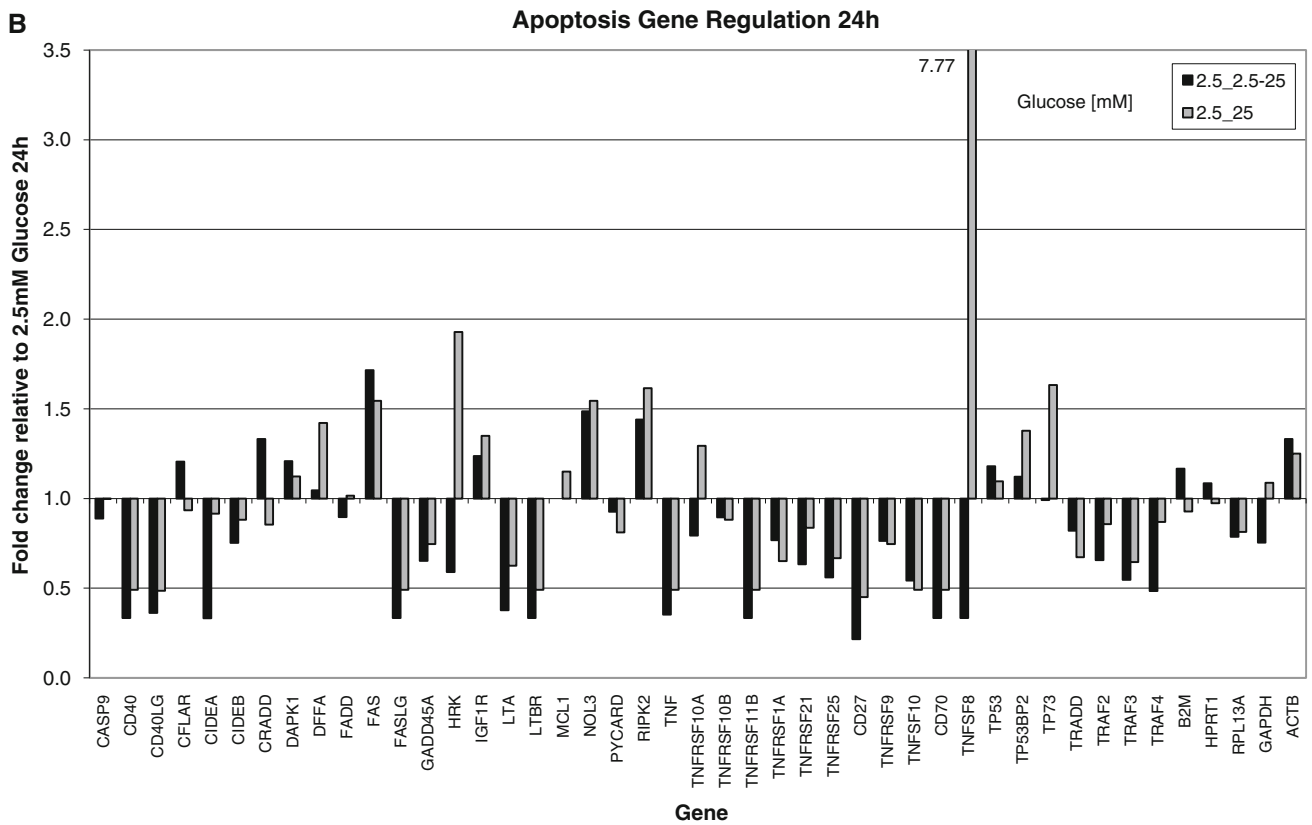
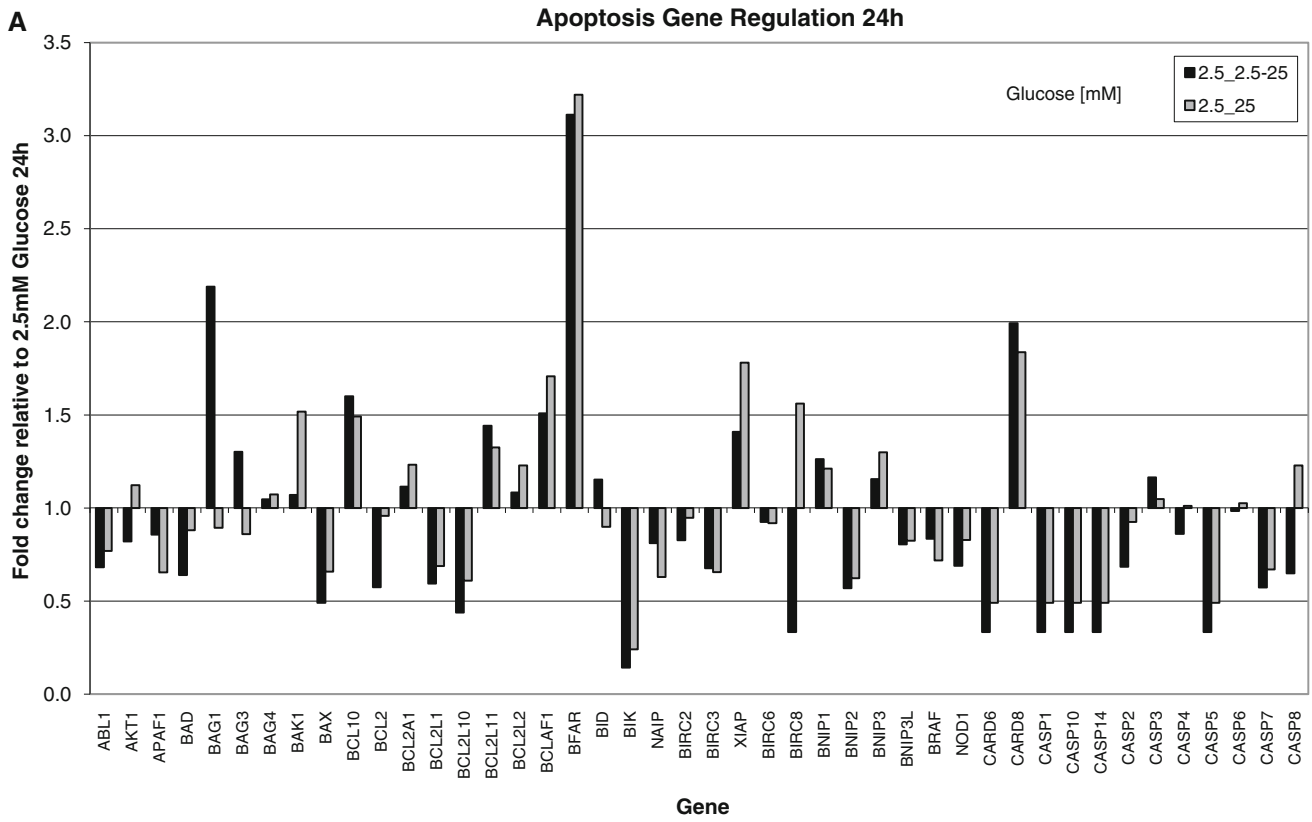
Fig. 5 a–f Sustained glucose variance results in modulation of apoptosis genes. Apoptosis gene expression profile was determined for RA differentiated SH-SY5Y cells exposed to constant 25 mM glucose or to variable 2.5–25 mM glucose (a, b) or 5–25 mM glucose (c, d) and compared to the baseline of 2.5 mM glucose (low glucose; a, b) or to the baseline 5 mM glucose (normal glucose; c, d) as appropriate (Similar analysis was performed in constant (25 mM) or variable (5–25 mM) mannitol (Fig 5 e, f). Regulation of key genes (pro and anti-apoptotic) for both the intrinsic and extrinsic apoptotic pathways is shown

Discussion

This in vitro study is the first to show that recurrent fluctuating glucose levels have a greater adverse effect on mitochondrial activity in neuronal type cells than either sustained high or low glucose levels. These effects appear to be due to a direct effect of glucose mediated mitochondrial stress. Furthermore, these neuronal-like cells respond in an active, but exaggerated manner in response to fluctuating glucose levels, associated with rapid changes in glucose transport. Recurrent variation in glucose levels led to significant gene expression modulation in both intrinsic (mitochondrial) and extrinsic apoptotic pathways.

Neuronal differentiated SH-SY5Y neuroblastoma cells were exposed to recurrent cyclic glucose level changes to replicate in vitro glycaemic variance as seen in paediatric patients with type 1 diabetes. The MTT assay was used to evaluate the activity of mitochondrial dehydrogenase, whose production decreases with increasing cellular distress [22, 24, 44, 47]. Mitochondrial activity, but not cell number, was significantly decreased by recurrent variation in glucose levels. Two potential mechanisms could have reduced mitochondrial activity in the model demonstrated in this study, either direct glucose-mediated oxidative stress [12, 19, 40, 43, 46, 53] within the mitochondria or alternatively an extracellular osmotic stress as shown in other system [38, 39, 57, 58, 60]. In order to elucidate which of these mechanisms was most relevant to the observed effects, the changes in osmolality created by variation in glucose levels were recreated using metabolically inert osmolytes- mannitol and L-glucose. In these experiments only the metabolically active D-glucose showed a significant impact upon mitochondrial activity, while none of the osmolyte agents affected cell number.

The uptake of 2-deoxy-D-[1- 14 C]-glucose, as a measure of glucose transport, was related to the glucose concentration in the media (i.e. high uptake in low glucose), and was independent of variations in media osmolality. These findings suggest that neuronal-like cells are able to sense glucose levels and adjust glucose transport accordingly, however their response to repeated fluctuation in glycaemic exposure is to show a significant further decrease in uptake of glucose (compared to non-fluctuating conditions) in the face of elevated glucose levels. These effects suggest a



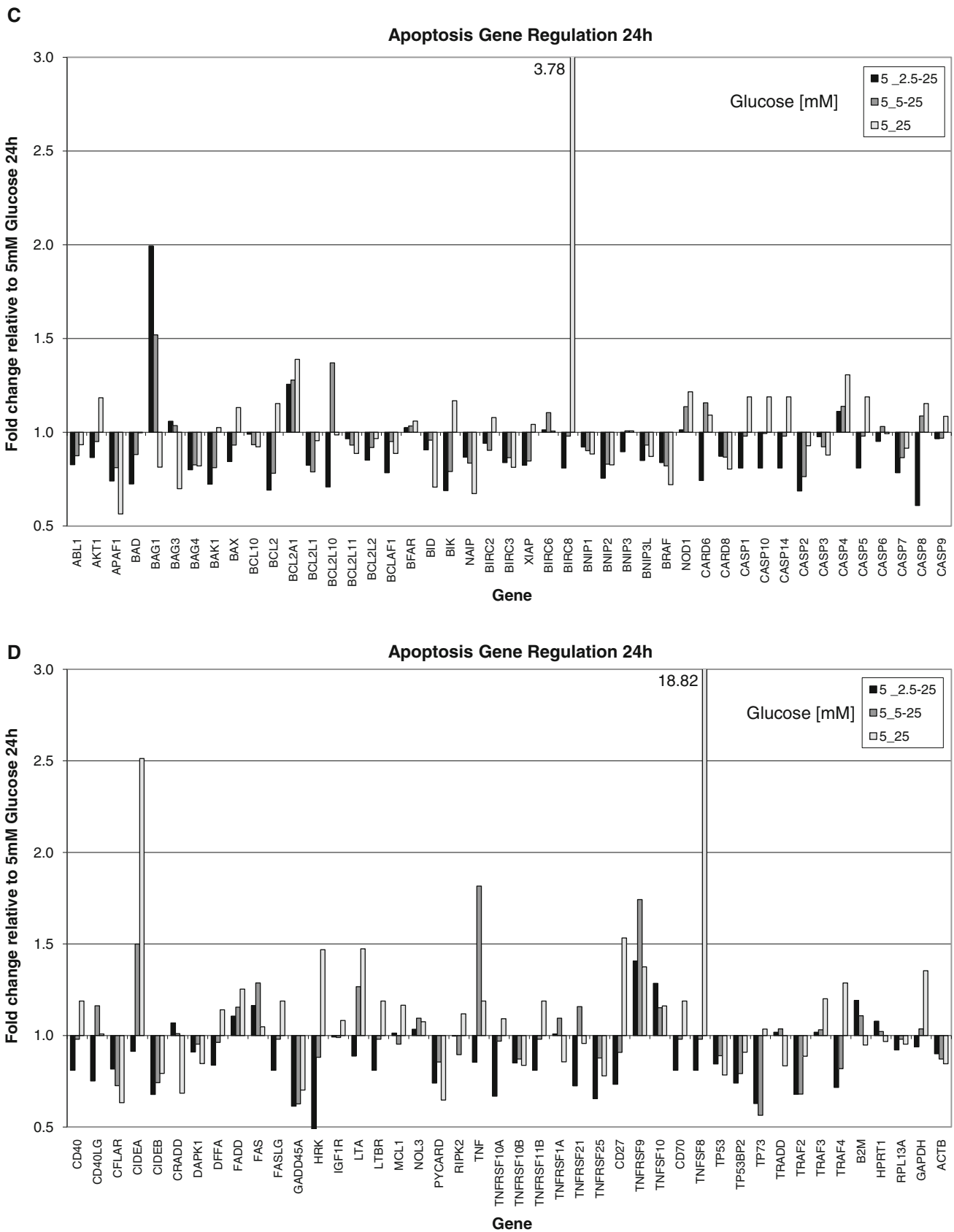


Fig. 5 continued

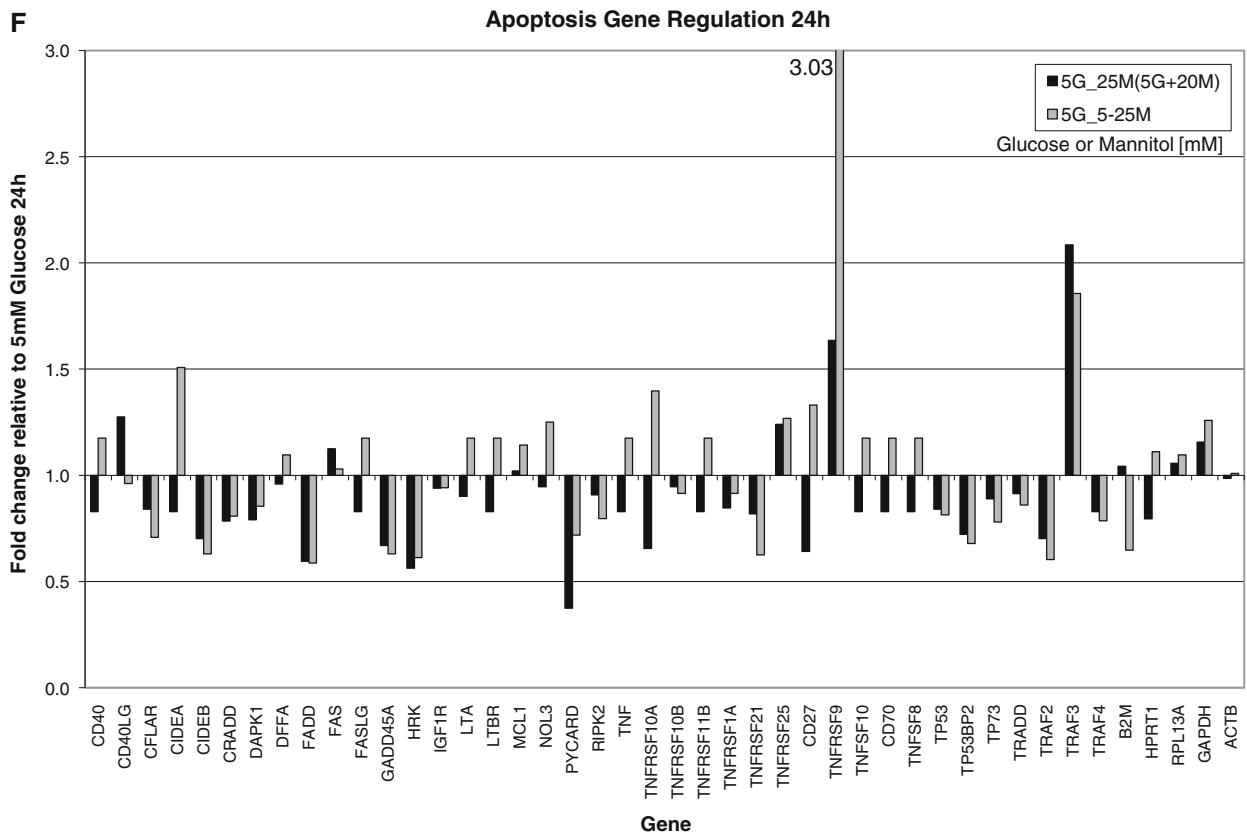
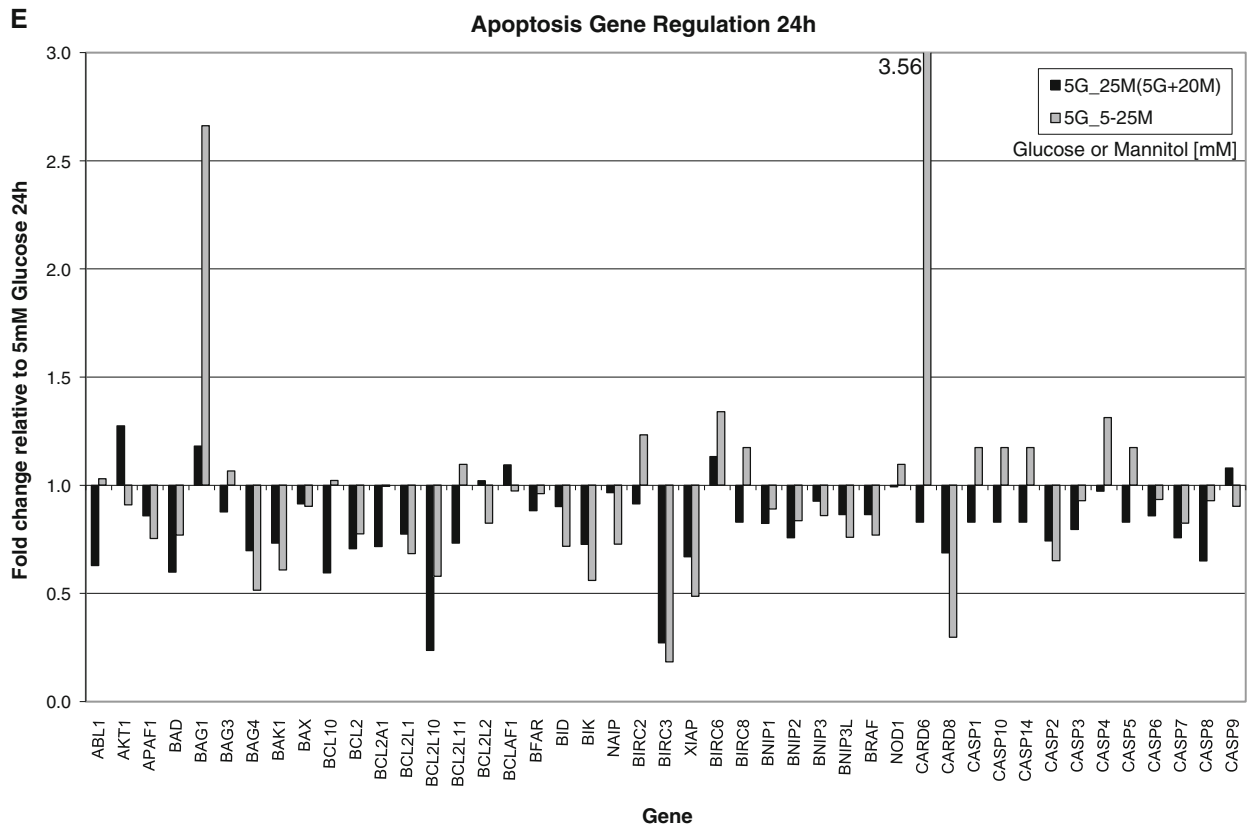


Fig. 5 continued

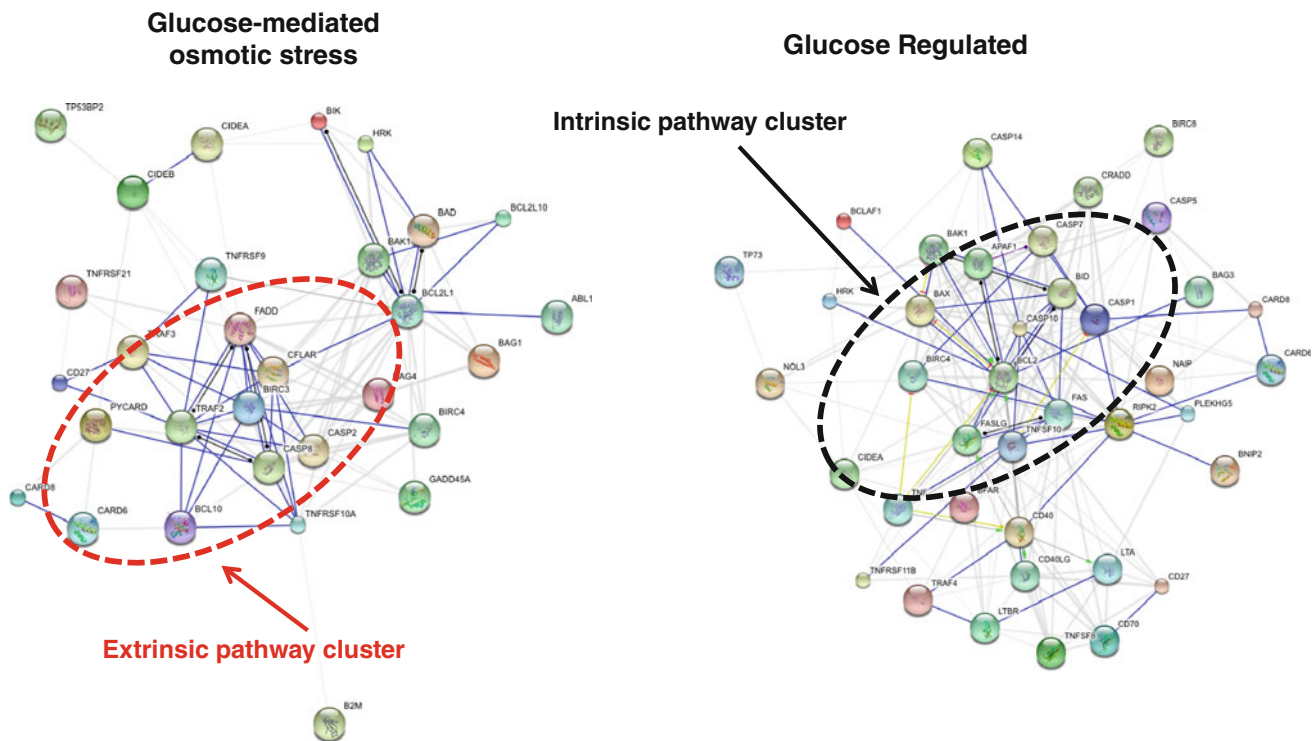


Fig. 6 Sustained glucose variance results in modulation of apoptosis genes. STRING plot (version 8.3; <http://string-db.org>) [56] shows clusters of key genes in both the extrinsic (*left*) and intrinsic (*right*)

regulated by glucose variance or glucose variance mediated-osmotic stress. Pro and anti-apoptotic genes are shown

glucose uptake adjustment to the sudden increase (5–10 fold) in extracellular glucose (i.e. from 5 or 2.5 to 25 mM) in cells that had previously adjusted their glucose transport to a lower extracellular glucose (2.5 or 5 mM). Although GLUT1 mRNA levels were moderately affected by the described treatments, the observed increase or decrease in glucose transport is likely to reflect, in addition to regulation of GLUT1 mRNA, the well-recognised cell surface recruitment or internalization of glucose transporters [6, 17, 18].

The 2-deoxy-D-[1-¹⁴C]-glucose uptake data further suggest that the intracellular levels of glucose would likely have increased to potentially toxic levels very quickly as the extracellular glucose was shifted from low to high (i.e. glucose transport set to high uptake rate in cells exposed to low glucose) and therefore activated an apoptotic response [19, 40, 43, 46]. Recurrence of such events is likely to have a cumulative negative effect on mitochondrial activity and cell survival. We therefore hypothesized that recurrent cyclic glucose variance and the consequent associated changes in glucose uptake/transport, might also activate apoptotic pathways [26, 48, 57]. This was demonstrated by the gene array analysis showing that both the intrinsic (mitochondrial) and extrinsic (death receptor) apoptotic pathways were modulated by recurrent cyclic glucose variance. We were able to determine that both glucose-

mediated metabolic stress and extracellular/osmotic stressors trigger these pathways [21, 48, 57]. However, the most abundant cluster of genes modulated by glucose-mediated metabolic stress predominantly included components of the intrinsic/mitochondrial pathway, whereas the glucose-mediated osmotic stress mostly modulated the extrinsic pathway [21]. In these pathways, both pro-apoptotic and anti-apoptotic genes were significantly regulated. An interesting feature of the apoptotic pathway modulation observed in continuously high or variable glucose was the down-regulation of the core pro-apoptotic genes involved in the intrinsic pathway via either TNF receptor and their ligands, adaptors activators and initiators (i.e. TNFRSF11B, CD70, FASLG, CASP1, CASP5, TRAF4 pro-Caspase 8/10, CRADD, CIDEA, BID) and apoptosis executors (i.e. Caspase 6/7, APAF1, BAX) levels and the up-regulation of the anti-apoptotic XIAP (most potent human inhibitor of apoptosis protein [IAP]), BIRC-8 (inhibitor of neuronal apoptosis/caspase activity) and NOL3 (apoptosis repressor), suggesting, on the balance, of an overall anti-apoptotic response [7, 15, 25, 36, 52, 54, 57, 59]. These modulations appear to oppose/balance those of the up-regulated genes, under the same treatments, which instead initiate/promote apoptosis (i.e. FAS, LTA, HRK, CD27, RIPK2, CARD8, BAK1, BCL10) [8, 21, 33, 54]. It is important to note that, there was a substantial correlation

between the gene expression profile determined in constant high glucose and variable glucose, suggesting that neurotoxicity might be dependent on glucose uptake into the cell.

On the other hand we cannot exclude the possibility that the observed changes in transcription profile are uniquely determined by glucose neurotoxicity, and it is possible that other associated compensatory mechanisms are involved. Nevertheless, the potential for a pro-/anti-apoptotic equilibrium as previously described by Green et al. [21] is consistent with our studies. Our current findings are also in agreement with previous work from our laboratory on glucose regulated genes, examined by subtractive hybridization screen, showing the activation in neuronal cells of both pro- and anti-apoptotic elements in the face of glucose stress [26]. This complex “gene-expression balance” might explain why the cell number was not significantly altered in the presence of high or variable glucose levels while in contrast, mitochondrial activity was decreased.

The effects of osmotic stress upon the extrinsic pathway selectively up-regulated the CIDEA gene (inducer of apoptosis not involving the caspase cascade; insensitive to caspase inhibitors) [59], the pro-apoptotic TNFRSF9 and its interaction protein TRAF3, with the elevation of the neuronal anti-apoptotic BAG1 gene [25, 36, 54]. Also in this case there was a substantial correlation between the gene expression profile determined by osmotic stress in constant high glucose and variable glucose, suggesting that the osmotic component might also contribute to the neurotoxic effects of glucose.

Constant supply of glucose to the brain is critical for normal cerebral metabolism, with both hypoglycaemia and hyperglycaemia affecting activity, survival and function of neural cells [26, 48, 57]. Our data now suggest that varying levels of glucose exposure also triggers cellular and molecular mechanisms that lead to disturbed neuronal metabolism and potential apoptosis. Whilst an in vivo model would theoretically be more informative in terms of neural survival, the concomitant and compounding direct effects of insulin and counter-regulatory hormones upon neural activity render interpretation of individual molecular processes problematic. Functional studies of key regulated genes of the two major clusters within the extrinsic and intrinsic apoptotic pathways would likely provide further insights into the mechanisms of glucose toxicity in neuronal cells. However such studies are outside the scope of this study. Thus, within the obvious limitations of an in vitro model system for human neuronal cells, our studies provide new insights in the cellular and molecular events leading to neuronal dysfunction, degeneration, or loss following cyclical glucose variance. It represents a further step in understanding the disturbed neural biology leading to the recognized patterns of neurocognitive and

psychological pathology associated with type 1 diabetes in childhood and adolescence.

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