

# **Analysis of the transcriptome and DNA methylome in response to acute and recurrent low glucose in human primary astrocytes**

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## ABSTRACT:

**Aims/hypothesis:** Recurrent hypoglycaemia (RH) is a major side-effect of intensive insulin therapy for people with diabetes. Changes in hypoglycaemia sensing by the brain contribute to the development of impaired counterregulatory responses to and awareness of hypoglycaemia. Little is known about the intrinsic changes in human astrocytes in response to acute and recurrent low glucose (RLG) exposure.

**Methods:** Human primary astrocytes (HPA) were exposed to zero, one, three or four bouts of low glucose (0.1 mmol/l) for three hours per day for four days to mimic RH. On the fourth day, DNA and RNA were collected. Differential gene expression and ontology analyses were performed using DESeq2 and GOseq respectively. DNA methylation was assessed using the Infinium MethylationEPIC BeadChip platform.

**Results:** 24 differentially expressed genes (DEGs) were detected (after correction for multiple comparisons). One bout of low glucose exposure had the largest effect on gene expression. Pathway analyses revealed that endoplasmic-reticulum (ER) stress-related genes such as *HSPA5*, *XBP1*, and *MANF*, involved in the unfolded protein response (UPR), were all significantly increased following LG exposure, which was diminished following RLG. There was little correlation between differentially methylated positions and changes in gene expression yet the number of bouts of LG exposure produced distinct methylation signatures.

**Conclusions/interpretation:** These data suggest that exposure of human astrocytes to transient LG triggers activation of genes involved in the UPR linked to endoplasmic reticulum (ER) stress. Following RLG, the activation of UPR related genes was diminished, suggesting attenuated ER stress. This may be mediated by metabolic adaptations to better preserve intracellular and/or ER ATP levels, but this requires further investigation.

**Key words:** astrocytes, diabetes, differential gene expression, DNA methylation, endoplasmic reticulum stress, hypoglycaemia, RNA sequencing, recurrent low glucose, unfolded protein response.

## INTRODUCTION

Iatrogenic hypoglycaemia is a limiting factor to optimal glycaemic control in people with type 1 diabetes (T1DM; [1]). Recurrent hypoglycaemia (RH) leads to an impaired counterregulatory response (CRR) to restore blood glucose levels, at least in part mediated by the central detection of hypoglycaemia.

Astrocytes are glial cells with important roles in regulating neurotransmission, immune support, memory formation, long-term potentiation, and metabolic support to neurons. Astrocytes in the nucleus of the tractus solitarius (NTS) in the hindbrain increase intracellular  $\text{Ca}^{2+}$  levels in response to gluoprivation [2] and astrocytic glucose transporter GLUT2 is required for hypoglycaemia counterregulation [3]. Astrocytic glutamate uptake is impaired following RH [4], contributing to counterregulatory failure, yet little is known about the intrinsic changes within astrocytes, especially human astrocytes, following recurrent low glucose (RLG). In this study, we used both RNA sequencing and an epigenome-wide association study (EWAS) of DNA methylation (DNAm) to examine for the first time, changes to the human astrocyte transcriptome and methylome following acute and recurrent low glucose exposure.

## RESEARCH DESIGN AND METHODS

### **Astrocyte isolation and cell culture.**

HPA cells were isolated from post-mortem sub-ventricular deep white matter following consent from next-of-kin, and with ethical approval from the North and East Devon Research Ethics Committee as previously described [5]. The recurrent low glucose (RLG) model has been previously described (see ESM for details [6]). Samples were split for RNA extraction and DNA extraction, with a total of five and six replicates for RNA sequencing and DNA methylation studies, respectively. Cells were confirmed as mycoplasma free using the MycoAlert kit (Lonza, Slough, UK).

### **RNA sequencing**

Briefly, RNA was extracted and cDNA libraries generated. Sequencing reads were generated using the Illumina HiSeq 2500 and fastq sequence quality was checked using MultiQC before alignment to the human genome (Build GRCh38.p12) using STAR. Mapped reads were counted using the FeatureCounts function of the subread package. Differential gene expression was calculated using DESeq2 [7] using the Likelihood ratio test function to analyse all groups together followed by the Wald-test for pairwise analysis. Genes with a false discovery rate (FDR)  $\leq 0.05$  were considered differentially expressed. Functional gene ontology analysis was performed using GOSep. See ESM for details. Raw RNAseq files are available at GEOLINK.

### **DNA methylation analysis**

DNA was extracted and DNA methylation (DNAm) examined using the Infinium MethylationEPIC BeadChip platform (Illumina Inc.; EPIC). See ESM for details of quality control and normalisation processes. 729727 probes remained after QC processes. The one-way analysis of variance (ANOVA) test was used to test for differentially methylated sites associated across the three groups: LG, aRLG, RLG compared to control. To determine which group was driving the association behind the significant ANOVA results, the *T* statistics for control versus each of the three groups were extracted from the regression model.

## RESULTS

### Low glucose-induced changes in gene expression in human astrocytes

In HPA cells, expression of 1240 genes were significantly ( $p < 0.05$ ) altered in response to glucose variation; 24 of which were significantly differentially expressed (DE) after FDR correction (adjusted  $p < 0.05$ ; Fig. 1A). Volcano plots displaying the pairwise comparisons of each treatment group versus control shows that LG (Fig. 1Ai) produced the largest effect on gene expression, whereas changes induced by aRLG (Aii) and RLG (Aiii) were more modest. LG and RLG shared similar DE patterns (Fig. 1B) and importantly *TXNIP*, regulated by glucose [8], was significantly downregulated in both LG (log2 fold-change -2.16,  $p = 1.09 \times 10^{-5}$ ) and RLG (log2 fold-change -1.46,  $p = 2.91 \times 10^{-3}$ ; Fig. 1C). Of the other DE genes there was a predominance of genes related to endoplasmic reticulum (ER)-stress. X-box binding protein 1 (*XBP1*; log2 fold-change 0.28,  $p = 1.56 \times 10^{-4}$ ; Fig. 1D), heat shock protein family A member 5 (*HSPA5*; log2 fold-change 0.34,  $p = 3.55 \times 10^{-6}$ ; Fig. 1E), and mesencephalic astrocyte-derived neurotrophic factor (*MANF*; log2 fold-change 0.41,  $p = 7.55 \times 10^{-6}$ ; Fig. 1F) showed increased expression following LG exposure which was blunted following RLG. Similarly, mitochondrially encoded NADH:ubiquinone oxidoreductase core, subunit 4 and subunit 4L (*ND4* and *ND4L*) had increased gene expression in acute LG (*ND4* log2 fold-change 0.37,  $p = 3.5 \times 10^{-6}$ ; *ND4L* log2 fold-change 0.47,  $p = 5.75 \times 10^{-7}$ ) and a diminished, but still significant increase following RLG (Fig. 1G,H). Pathway analysis of the DE genes identified seven gene ontology (GO) terms that were significantly altered after correction for multiple comparisons, which were related to the unfolded protein response (UPR) and ER-stress (ESM Table 1).

### LG and RLG produce distinct DNA methylation profiles

Our analyses did not identify any differential methylated positions (DMP) that reached genome-wide significance for DNA methylation association analyses (Fig. 2Ai-iii;  $p < 9.42 \times 10^{-8}$ ; [9]). However, 65 probes reached nominal significance of  $p < 0.0001$ . Hierarchical clustering of these top probes showed four distinct groups that matched with the four experimental conditions suggesting a DNA methylation profile specific to each condition (Fig. 2B). Of the differentially methylated CpG sites, several were related to energy or ion homeostasis. *SLC19A3* (cg07417745,  $p = 5.16 \times 10^{-7}$ ,  $\beta\Delta = 0.23$ ), encoding the thiamine transporter was hypermethylated after LG showing a linear relationship with the number of bouts of LG exposure (Fig. 2C). Similarly, methylation

of the *GRID1* gene, encoding the ionotropic glutamate receptor  $\delta 1$  (cg16777181) was hypermethylated following LG exposure ( $p=1.90E-3$ ,  $\beta\Delta=0.18$ ) and this remained elevated following RLG (Fig. 2D). In contrast, cg1102254 (*NIPA1*,  $p=2.65E-6$ ,  $\beta\Delta= -0.02$ ), cg11692715 (*SLC8B1*;  $p=1.61E-5$ ,  $\beta\Delta= -0.16$ ) and cg22467827 (*CLHC1*,  $p=4.28E-4$ ,  $\beta\Delta= -0.03$ ), which encode a  $Mg^{2+}$  transporter [10], a  $Na^+/Ca^{2+}$  antiporter, and clathrin heavy chain linker domain containing 1 respectively, were hypomethylated following RLG (Fig. 2E-G). The probe cg22467827 (annotated to the gene *CLHC1*) was also differentially expressed (log2 fold-change 0.80,  $p=1.03E-4$ ) in relation to RLG (Fig. 2H). The two datasets (RNAseq and EPIC) were integrated resulting in 28 DE genes that overlapped with 31 differentially methylation positions (Fig. 2I).

## DISCUSSION

The central adaptations in response to RH that may mediate defective CRR require further investigation, with little known about how astrocytes respond or adapt to RH. We sought to examine changes in HPA gene expression and DNA methylation to determine which, if any, pathways were altered by acute and RLG exposure. DE and GO pathway analyses revealed that the major pathway altered by acute LG was the UPR. Protein folding within the ER requires hydrolysis of ATP (for review see [11]) and reductions in ATP content driven by energy stress increases protein misfolding to activate the UPR [12]. ER stress, via ATF6 promotes the production of XBP1 [13], which is spliced by IRE1 $\alpha$ , to produce a potent transcriptional activator, XBP1s that increases HSPA5 [13] and MANF expression [14]). *MANF* is upregulated by UPR to inhibit cell proliferation and prevent ER-stress-related cell death [15, 16]. Interestingly, here expression of *XPB1*, *HSPA5*, and *MANF* were increased following a single bout of LG. Similar ER stress responses have been reported in pericytes [17], cardiac tissue [18], rat primary astrocytes [19] and primary hippocampal neurons [12] in response to LG. Following RLG, the increase in UPR-related gene expression was substantially diminished. Given that energy deficiency increases ER stress, it is plausible that acute LG exposure causes a marked increase in ER stress and that following successive bouts of LG, a concomitant metabolic adaptation, as previously reported [6], better preserves cellular ATP levels, thus attenuating (or delaying) subsequent LG-induced ER stress. In the data presented here, expression levels of *ND4L* and *ND4* following RLG remained elevated above control, suggesting a persistent adaptation. Whether this is mediated by better preservation of intracellular and intra-ER ATP levels remains to be determined.

The EWAS identified 65 DMPs associated with LG/RLG that reached nominal significance, while we did not identify any DMPs that reached the suggested array-wide significance ( $p < 9.42 \times 10^{-8}$ ). Hierarchical clustering revealed distinct patterns of DNA methylation across the four conditions. One of the most significant DMPs (cg07417745) is located in intron 1 of the *SLC19A3* gene, which encodes a thiamine transporter [20], and showed a linear relationship between increased DNA methylation and the number of LG exposures. Interestingly, expression of this gene has previously been found to be modulated by hyperglycaemic-like conditions [21]. Conversely,

methylation of cg11022541 and cg11692715 located within the genes *NIPA1* and *SLC8B1* respectively, decreased following RLG. As these genes encode a  $Mg^{2+}$  transporter [10] and a  $Na^+/Ca^+$  exchanger [22], this may indicate the energetic cost of ion handling within the cell, which requires further investigation. The main limitation in this study was that we were underpowered in the DNA methylation analyses as power analysis indicated we had 50% power to detect a difference of 10% in half of all the sites on the EPIC array. Moreover, the relationship between DNA methylation and gene expression is complex, with the direction of effect dictated by sequence context [23]. Furthermore, the annotation of DNAm sites to genes is purely based on proximity rather than empirical derived data [24], both these factors make inferences between DMPs and gene expression complicated. Despite these challenges we looked for overlapping genes between the datasets and identified 28 that were significantly altered ( $p < 0.05$ ) in both analyses. For example, *CLHC1* gene expression was significantly increased and a DMP (cg22467827) located in intron 1 was hypomethylated. This tentatively suggests that DNA methylation within the first intron may be mediating the upregulation of this gene in the response to LG glucose.

In summary, there are both shared and unique gene expression and DNA methylation profiles in human astrocytes following LG and RLG exposure. A single bout of LG exposure induced expression of genes associated with the UPR linked to ER stress. This response diminished after 4 bouts of LG exposure, suggesting an attenuated stress response. Taken together with previous observations that astrocytes adapt to RLG by increasing reliance on fatty acid oxidation to maintain intracellular ATP levels, activation of the UPR by glucose deprivation may be attenuated following RLG exposure.



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## **Contribution Statement**

A.R.J, E.D. and C.B. conceived the project. P.G.W.P, S.W., A.R.J and E.D. contributed to data acquisition, analyses and writing of the manuscript. J.E.H and N.J.G obtained human post mortem tissue from which astrocytes were isolated, cultured and characterised as the stable human primary astrocyte population thus permitting the investigation of intrinsic changes in human astrocyte responses to be undertaken. C.B. wrote and edited the manuscript and accepts full responsibility for the work and/or conduct of the study, had access to the data and controlled the decision to publish.

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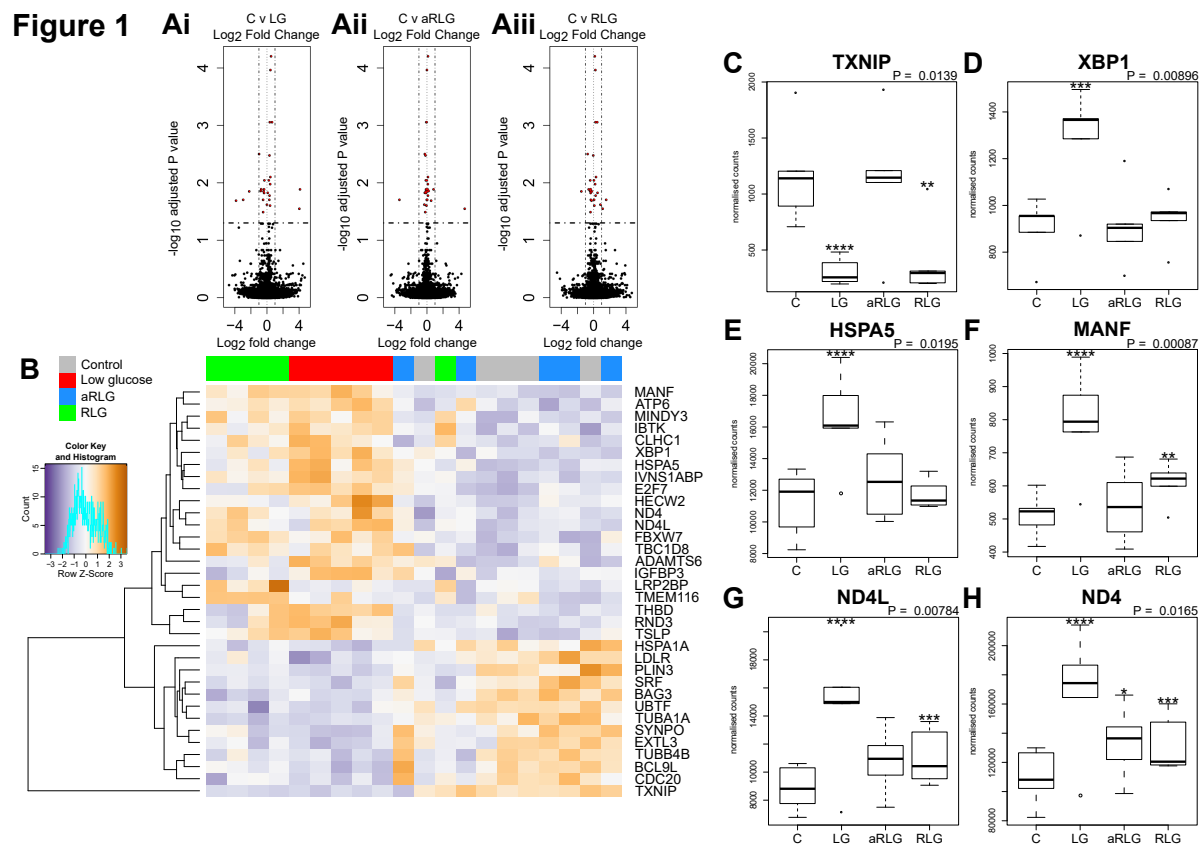
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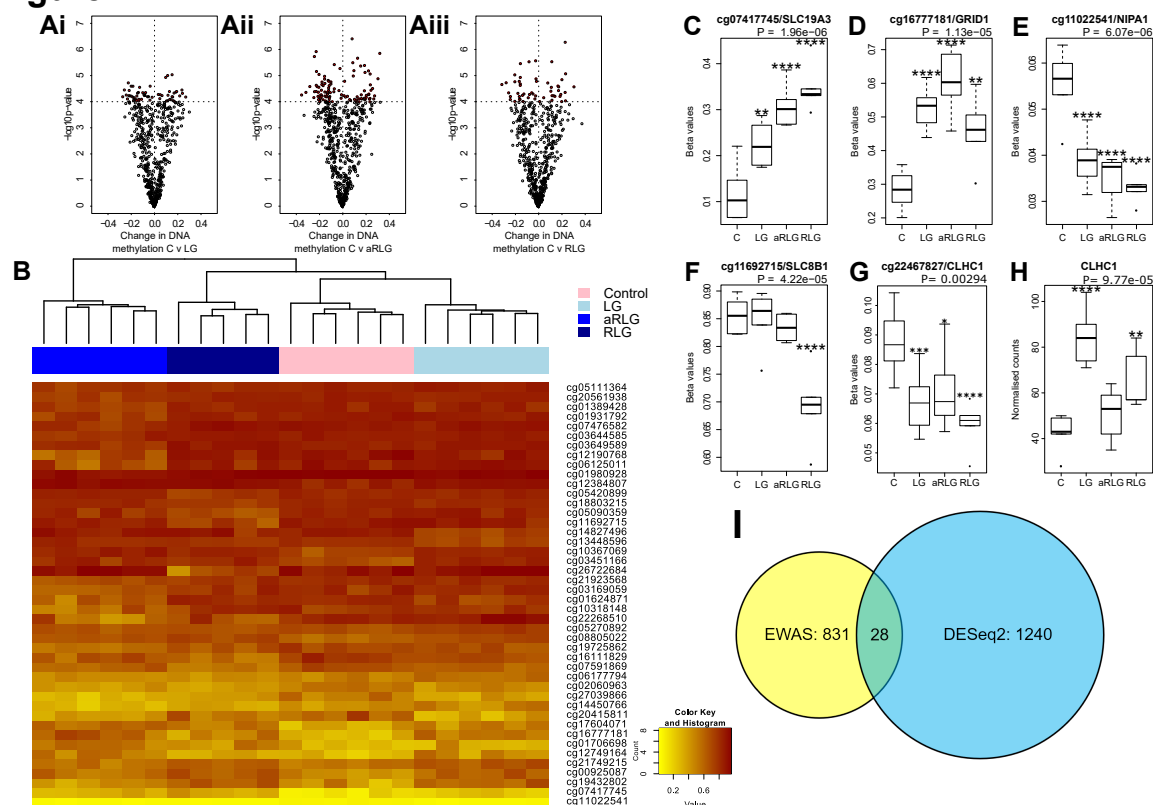
## Figure Legends

**Figure 1. Summary of the RNAseq results.** Volcano plots on the pairwise differential expression analysis between control cells (C) versus **(Ai)** low glucose (LG), **(Aii)** antecedent RLG, and **(Aiii)** recurrent low glucose (RLG), the red points on the plots represent genes  $p_{adj} < 0.05$ . **B.** Heatmap of hierarchical clustering of LRT analysis  $FDR \leq 0.1$  indicates differentially expressed genes (rows) between the four groups ( $p_{adj} < 0.1$ ). Orange indicates up-regulation and blue indicates down-regulation. The LG and RLG groups cluster together. TXNIP (**C**), XBP1 (**D**), HSPA5 (**E**), MANF (**F**), ND4L (**G**), ND4 (**H**) expression profiles, selected for their functional relevance to hypoglycaemia ( $p$ -value is the adjusted result of the likelihood ratio test).  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ . Error bars represent standard deviation.  $N=5$ .

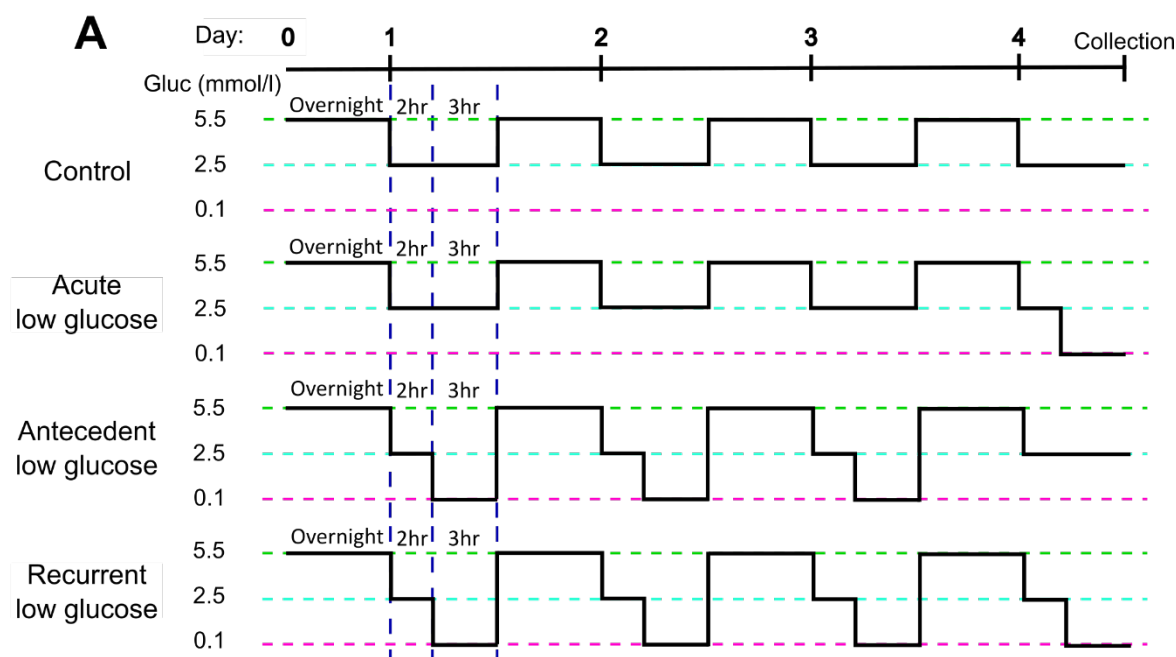
**Figure 2. Summary of DNA methylation.** **A.** the most differentially methylated genes (ANOVA  $p \leq 0.001$ ) in pairwise comparison (red points are  $p < 0.0001$ ) between control treated HPA cells (C) versus **(Ai)** low glucose (LG), **(Aii)** antecedent recurrent low glucose (aRLG), and **(Aiii)** recurrent low glucose (RLG). **B.** Heatmap of hierarchical clustering using probes ANOVA  $p < 0.001$  indicates differentially methylated cg sites (rows) between the four groups. Orange indicates hypermethylation and yellow indicates hypomethylation. Box plots of some of the most differentially methylated cg sites labelled by their associated gene, selected for functional importance **C**, cg07417745/SLC19A3, **D**, cg16777181/GRID1, **E**, cg11022541/NIPA1, **F**, cg11692715/SLC8B1 **G**, cg22467827/CLHC1 ( $p$ -value is the adjusted result of the ANOVA). **H**, CLCH1 gene expression increases. Error bars represent standard deviation **I**, Venn diagram of differentially methylated cg sites in yellow and differentially expressed genes (blue) and overlap between the two data sets, 28 genes. Error bars represent standard deviation.  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ .  $n=6$  for methylation data and  $n=5$  for gene expression changes.



**Figure 2**



# ESM Fig. 1



**ESM figure 1. Schematic of the recurrent low glucose model.** Human primary astrocytes were exposed to 0, 1, 3, or 4, three-hour long bouts of 0.1 mmol/l glucose; control (C), acute low glucose (LG), antecedent recurrent low glucose (aRLG), and recurrent low glucose (RLG) respectively. Each day cells were first incubated in 2.5 mmol/l glucose for 2 hours as a step down from overnight/stock media of 5.5 mmol/l glucose. Adapted from Weightman Potter *et al* 2019.

**ESM Table 1. Glucose variation in human primary astrocytes significantly enriched gene ontologies related to endoplasmic-reticulum stress**

GO term ID	GO term full name	Number of DEGs in the category	Total number of genes in the category	corrected <i>p</i> value
GO:0006986	response to unfolded protein	8	160	0.0159
GO:1905897	regulation of response to endoplasmic reticulum stress	6	72	0.0159
GO:0006984	ER-nucleus signaling pathway	5	45	0.0159
GO:0035966	response to topologically incorrect protein	8	179	0.0159
GO:0034620	cellular response to unfolded protein	7	125	0.0159
GO:0035967	cellular response to topologically incorrect protein	7	143	0.0329
GO:0036498	IRE1-mediated unfolded protein response	5	59	0.0440

Gene ontologies that were significantly enriched by the differentially expressed genes. All seven of the GO terms were related to endoplasmic-reticulum stress and the unfolded protein response.



**ESM Table 2. Glucose variation induces differentially expression of the following genes adjusted  $p$  value  $<0.05$  in human primary astrocytes**

Ensembl ID	Gene symbol	Count Mean	$p$ value	Adjusted $p$ value
ENSG00000138411.11	HECW2	744	3.42E-09	6.19E-05
ENSG00000116679.15	IVNS1ABP	1034	1.19E-08	0.000107
ENSG00000145777.14	TSLP	435	1.80E-07	0.00087
ENSG00000145050.15	MANF	600	1.92E-07	0.00087
ENSG00000204389.9	HSPA1A	125	8.63E-07	0.003118
ENSG00000178726.6	THBD	757	1.10E-06	0.0033
ENSG00000212907.2	ND4L	10951	3.04E-06	0.007842
ENSG00000100219.16	XBP1	983	4.43E-06	0.008959
ENSG00000151929.9	BAG3	458	4.46E-06	0.008959
ENSG00000165891.15	E2F7	1122	5.77E-06	0.010434
ENSG00000186174.12	BCL9L	190	7.79E-06	0.012794
ENSG00000108312.14	UBTF	647	8.66E-06	0.012862
ENSG00000112658.7	SRF	236	9.96E-06	0.012862
ENSG00000171992.12	SYNPO	59	1.14E-05	0.01372
ENSG00000265972.5	TXNIP	753	1.27E-05	0.013948
ENSG00000130164.13	LDLR	1075	1.31E-05	0.013948
ENSG00000198270.12	TMEM116	108	1.47E-05	0.01477
ENSG00000167552.13	TUBA1A	2944	1.56E-05	0.014874
ENSG00000198886.2	ND4	132078	1.83E-05	0.016508
ENSG00000044574.7	HSPA5	12703	2.33E-05	0.019539
ENSG00000012232.8	EXTL3	328	2.49E-05	0.019539
ENSG00000109771.15	LRP2BP	29	3.28E-05	0.023736
ENSG00000115963.13	RND3	2648	3.55E-05	0.024649
ENSG00000105355.8	PLIN3	551	4.96E-05	0.032026

Significantly differentially expressed genes from human primary astrocytes exposed to control and acute low glucose.

**ESM Table 3 Glucose variation induces differentially methylation of the following cg sites  $p$  value <0.0001 in human primary astrocytes**

Illumina probe ID	$p$ value	UCSC gene name (if known)
cg11894282	1.78E-06	
cg07417745	1.96E-06	SLC19A3
cg12190768	4.92E-06	DAPP1;DAPP1
cg10367069	6.01E-06	
cg11022541	6.07E-06	NIPA1;NIPA1
cg26217489	7.94E-06	SRI;SRI
cg22268510	8.26E-06	PRRT1
cg22147598	9.40E-06	MAF1;KIAA1875
cg08805022	1.03E-05	PVT1
cg16777181	1.13E-05	GRID1
cg12384807	1.25E-05	
cg07476582	1.29E-05	
cg27039866	1.36E-05	
cg21923568	1.86E-05	SMURF1;SMURF1;SMURF1
cg18803215	1.93E-05	
cg01624871	2.06E-05	APBB1;APBB1;APBB1;APBB1;APBB1;APBB1;APBB1;APBB1;APBB1
cg03169059	2.24E-05	HDAC4
cg00842000	2.24E-05	
cg26722684	2.44E-05	OR13A1
cg00242597	2.45E-05	RPS6
cg16991768	2.79E-05	KIAA0495
cg05090359	2.87E-05	TPO;TPO;TPO;TPO
cg11454719	3.09E-05	PAX3;PAX3;PAX3;PAX3;PAX3;PAX3
cg03649589	3.13E-05	CSGALNACT1;CSGALNACT1;CSGALNACT1
cg01980928	3.33E-05	C19orf34;CSNK1G2
cg05420899	3.34E-05	LRRFIP2;LRRFIP2;LRRFIP2;LRRFIP2
cg06177794	4.00E-05	LPL
cg02060963	4.01E-05	
cg14343062	4.21E-05	ERCC6
cg11692715	4.22E-05	SLC8B1
cg21002176	4.45E-05	
cg13448596	4.70E-05	MYOM2
cg14015441	4.77E-05	DPYS
cg07591869	4.79E-05	EREG
cg03451166	4.80E-05	GOLGA2P9
cg05270892	4.97E-05	
cg10318148	5.51E-05	CYP2W1
cg14003022	5.66E-05	
cg14827496	5.71E-05	

cg20561938	6.02E-05	RNF39;RNF39
cg14450766	6.17E-05	SLC22A2
cg07324918	6.35E-05	MGAT5B;MGAT5B;MGAT5B
cg00925087	6.53E-05	LHCGR
cg11367159	6.53E-05	
cg17604071	6.58E-05	LINC01234;LINC01234
cg06125011	6.84E-05	
cg05111364	7.38E-05	BBS1
cg20415811	7.61E-05	CCDC142;MRPL53
cg17386815	7.61E-05	
cg19432802	7.76E-05	
cg01389428	7.88E-05	
cg19725862	8.12E-05	GTF2H4
cg12749164	8.53E-05	
cg21749215	8.59E-05	UBE2F;UBE2F;UBE2F;UBE2F-SCLY;UBE2F;UBE2F;UBE2F;UBE2F;UBE2F
cg01931792	8.60E-05	MATN3
cg03249950	9.14E-05	EIF2AK1;EIF2AK1
cg02995584	9.23E-05	
cg03644585	9.26E-05	UNC84A;UNC84A
cg21321885	9.53E-05	NDE1;NDE1
cg05389803	9.87E-05	PRR3;PRR3;PRR3;GNL1;PRR3;GNL1
cg16111829	9.89E-05	LOC643339
cg01706698	9.96E-05	CETP
cg24157364	9.97E-05	
cg18945601	9.98E-05	RFX5;RFX5
cg25308231	9.99E-05	TBCEL;TBCEL

65 methylation probe sites were significantly altered ( $p < 0.0001$ ) in human primary astrocytes by control, low glucose, antecedent recurrent low glucose, or recurrent low glucose.

#### **ESM Table 4 Glucose variation induces differential methylation of 31 probe sites associated with 28 differentially expressed genes in human primary astrocytes**

Ilumina probe ID	<i>p</i> value (EWAS)	UCSC gene name	<i>p</i> value (RNAseq)
cg24453271	0.010194	ABL1	0.001024
cg13667243	0.001098	ADAM19	0.010173
cg24399080	0.008434	AP1B1	0.038462
cg08687137	0.010093	B3GAT3	0.004575
cg06146614	0.008972	CDC42EP1	0.002952
cg05471700	0.007188	CDK2	0.008905
cg23928910	0.002032	CETN3	0.012128
cg22467827	0.002938	CLHC1	9.77E-05

cg03313451	0.010718	DNAH10	0.032603
cg20130615	0.010576	DNAH10	0.032603
cg06590513	0.001668	EPHA7	0.041006
cg09121934	0.002043	ERMP1	0.002866
cg03300960	0.00795	ERMP1	0.002866
cg01155025	0.012175	EXTL3	2.49E-05
cg17446503	0.002883	GLT8D1	0.049909
cg01348055	0.009789	GRIN2A	0.037006
cg17808849	0.007487	HERPUD1	0.000658
cg01660966	0.000367	HNRNPUL2-BSCL2	0.025627
cg06320134	0.000485	KLHL21	0.036451
cg07003778	0.007396	MFSD1	0.047196
cg05712894	0.002093	PITPNC1	0.00352
cg18596621	0.013042	PITPNC1	0.00352
cg09385983	0.005945	PLXNA2	0.019505
cg13929566	0.011286	SYNE1	0.028185
cg10503992	0.00069	TRIM62	0.044432
cg04321497	0.000464	TTN	0.001563
cg13647878	0.00897	TUBA1C	0.038334
cg02334770	0.006588	UBE2J2	0.027877
cg03710176	0.006012	VPS13C	0.021884
cg01395650	0.008254	ZBTB41	0.033364
cg06681008	0.008138	ZNF26	0.020245

31 differentially methylated probe sites ( $p < 0.0001$ ) were associated with 28 differentially expressed genes ( $p < 0.05$ ) in human primary astrocytes by control, low glucose, antecedent recurrent low glucose, or recurrent low glucose.

## **Supplementary Methods**

### **Astrocyte isolation and cell culture.**

HPA cells were isolated from normal subventricular deep white matter blocks immediately post-mortem following consent from next-of-kin and with ethical approval from the North and East Devon Research Ethics Committee as previously described [5]. The recurrent low glucose (RLG) model has been previously described [6]. Each day cells were cultured in 2.5 mmol/L glucose-containing media for 2 hours before being changed for media containing 0.1 (low) or 2.5 (normal) mmol/L glucose for 3 hours. Overnight, cells were recovered in stock media containing 5.5 mmol/L glucose. This was repeated for four days. Control and low glucose (LG) treated cells had 2.5 mmol/L glucose for three days and on the fourth day the LG group received low glucose. The antecedent RLG (aRLG) and RLG groups had 0.1 mmol/L glucose for four days, except on the fourth day the aRLG groups had 2.5 mmol/L glucose. Samples were split for RNA extraction and DNA extraction, with a total of five and six replicates for RNA sequencing and DNA methylation studies, respectively. Cells were confirmed as mycoplasma free using the MycoAlert kit (Lonza, Slough, UK).

### **RNA sequencing**

Briefly, RNA was extracted using TRIzol and Direct-zol miniprep kit (Invitrogen, Carlsbad, CA, USA), according to manufacturers' instructions. cDNA libraries were generated using the TruSeq DNA HT Library Preparation Kit (Illumina Inc., San Diego, CA, USA). Sequencing reads were generated using the Illumina HiSeq 2500 and fastq sequence quality was checked using MultiQC before alignment to the human genome (Build GRCh38.p12) using STAR. Mapped reads were counted using the FeatureCounts function of the subread package. Differential gene expression was calculated using DESeq2 using the Likelihood ratio test function to analyse all groups together followed by the Wald-test for pairwise analysis. Genes with a false discovery rate (FDR)  $\leq 0.05$  were considered differentially expressed. Functional gene ontology analysis was performed using GOSep. Gene length was accounted for during GO analysis. Raw RNAseq files are available at GEOLINK.

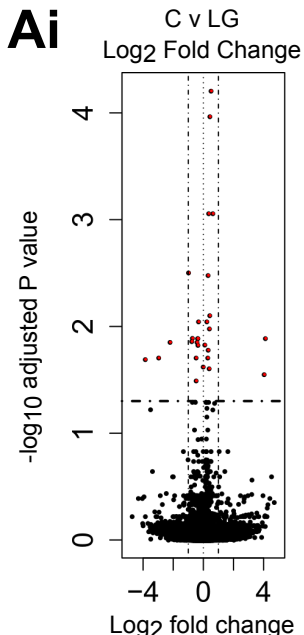
### **DNA methylation analysis**

DNA was extracted using a modified phenol:chloroform protocol. DNA methylation was measured using the Infinium MethylationEPIC BeadChip platform (Illumina Inc.)

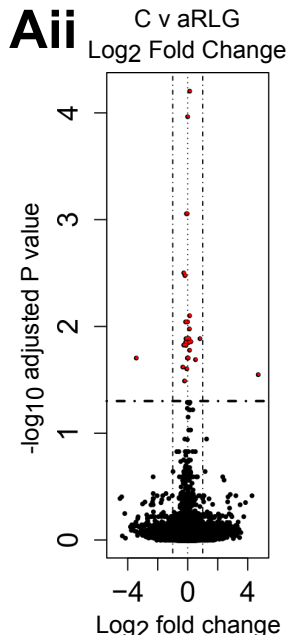
(EPIC). 729727 probes remained after QC processes. The one-way analysis of variance (ANOVA) test was used to test for differentially methylated sites associated across the three groups: LG, aRLG, RLG compared to controls. To determine which group was driving the association behind the significant ANOVA results, the *T* statistics for controls versus each of the three groups were extracted from the regression model. Power was calculated using <https://epigenetics.essex.ac.uk/shiny/EPICDNAmPowerCalcs/>.

# Figure 1

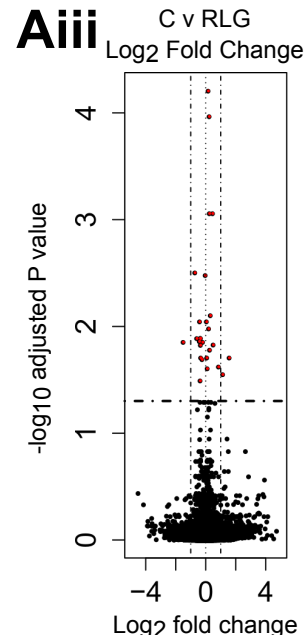
## Ai



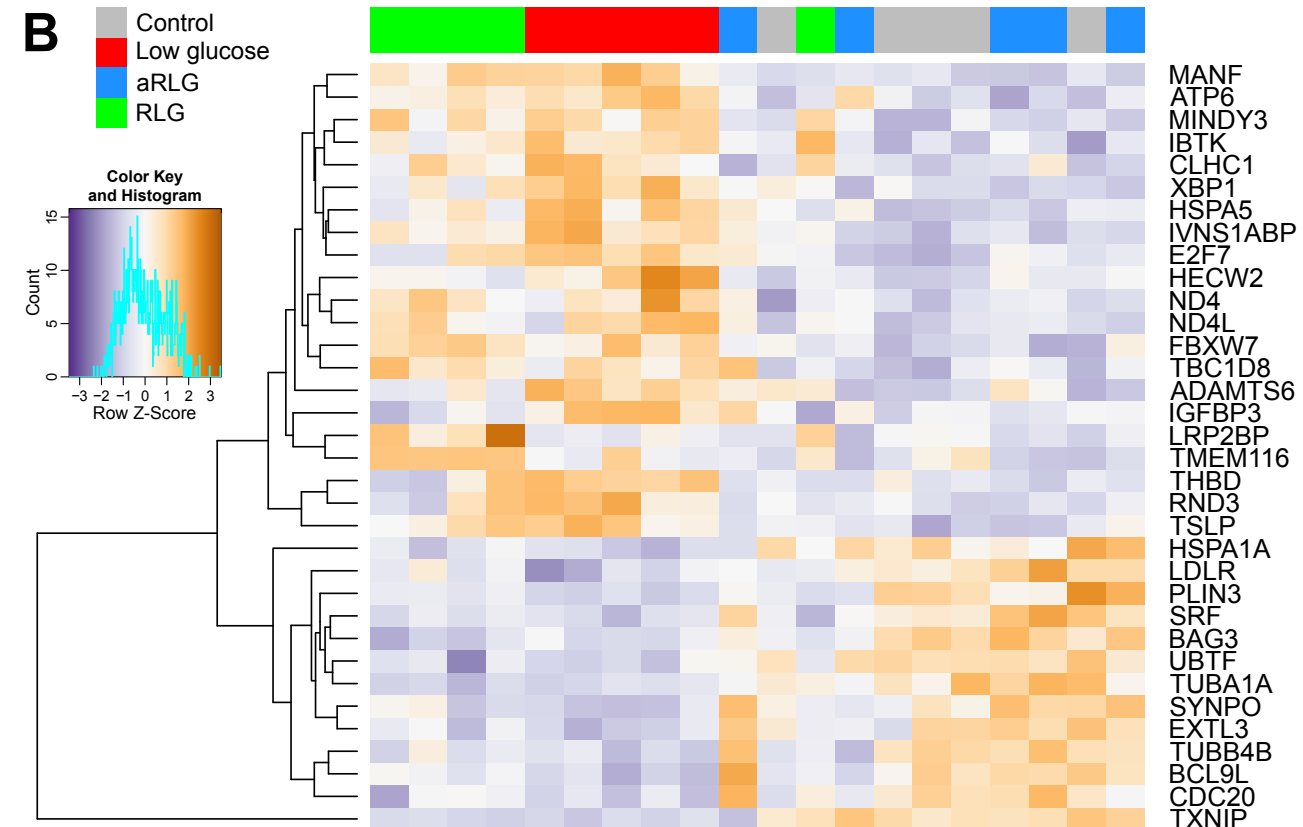
## Aii



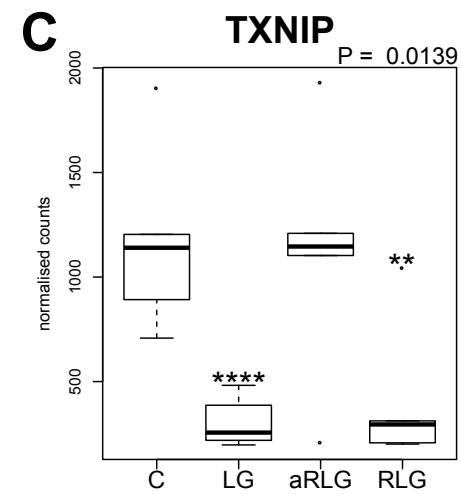
## Aiii



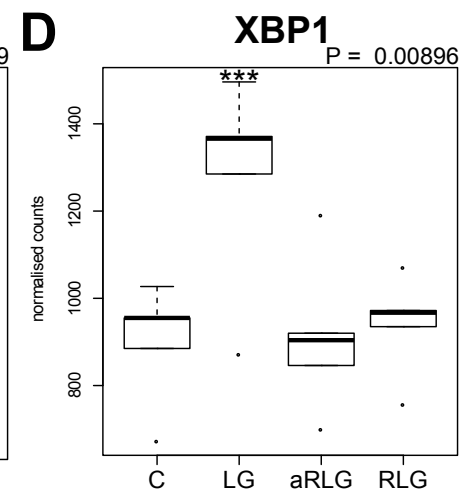
## B



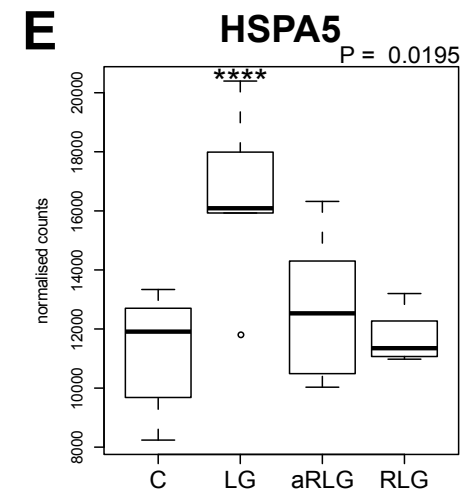
## C



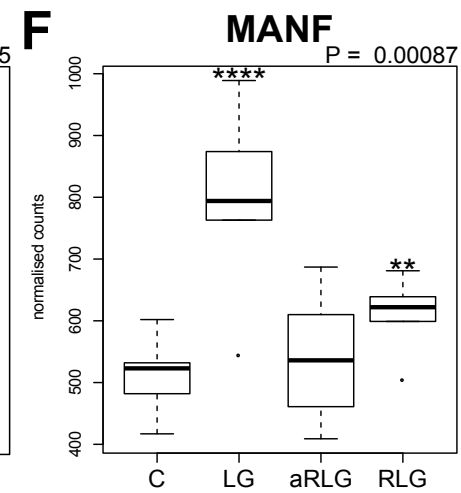
## D



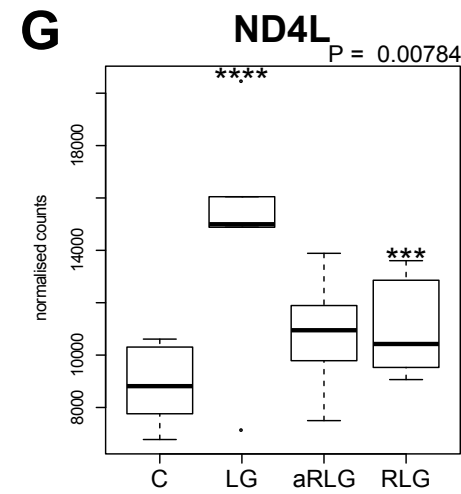
## E



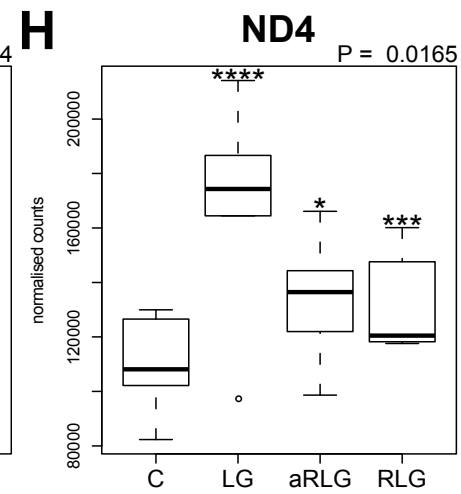
## F



## G



## H



# Figure 2

