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Alterations in glucose concentrations affect DNA methylation at *Lrg1* in an *ex vivo* rat cortical slice model of preterm brain injury.

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Abstract

Preterm birth affects 5-18% of all babies and is associated with neurodevelopmental impairment and increased neuropsychiatric disease risk. Although preterm birth associates with differential DNA methylation at neurodevelopmental genes in buccal DNA, including Leucine-rich alpha-2-glycoprotein 1 (LRG1), it is not known whether these differences also occur in the brain, or if they persist. Thus, there is a need for animal models or *in vitro* systems in which to undertake longitudinal and mechanistic studies. We used a combination of *in vivo* rat studies and *ex vivo* experiments in rat cortical slices to explore their utility in modelling the human preterm brain. We identified temporal changes in DNA methylation at LRG1 in human buccal DNA over the first year of life and found persistent differences in LRG1 methylation between preterm and term infants at 1 year. These developmental changes also occurred in rat brains *in vivo*, alongside changes in global DNA hydroxymethylation and expression of the Ten-eleven-translocation enzyme Tet1, and were reproducible in *ex vivo* rat cortical slices. Based on the observation that neonatal glucose homeostasis can modify neurodevelopmental outcome we studied whether glucose concentration affects Lrg1 methylation using cortical slices. Culture of slices in lower glucose concentration was associated with lower Lrg1 methylation, lower global 5hmC and Tet1 expression. Our results suggest that *ex vivo* organotypic cultures may be useful in the study of biological and environmental influences on the epigenome and that perturbations during early life including glucose concentration can affect methylation at specific genes implicated in neurodevelopment.

Introduction

Preterm birth affects 5-18% of all babies and is associated with alterations in the connectivity of neural systems, with long-term neurodevelopmental impairment and with an increased risk of neuropsychiatric disease (Johnson & Marlow, 2011; Blencowe *et al.*, 2013; Johnson *et al.*, 2015). Modifications of the epigenome, for example changes in DNA methylation, have been proposed as one mechanism linking the early life environment with later disease risk, including psychiatric disease (Ozanne & Constancia, 2007; Mehta *et al.*, 2013; Khulan *et al.*, 2014). Epigenetic modifications are highly dynamic during brain development and the establishment of normal DNA methylation patterns is critical for optimal neurodevelopment (Spiers *et al.*, 2015). Studies suggest that DNA methylation can be affected by environmental factors which are common following preterm birth such as stress, immune or metabolic dysregulation, and hypoxia (Volpe, 2009; Hagberg *et al.*, 2015), and we have recently shown that preterm birth is associated with differential methylation at key neurodevelopmental genes in buccal cell DNA (Sparrow *et al.*, 2016). Whether these differences are persistent, if they are present in the tissue of interest, and which potentially modifiable early life events mediate these differences are not known.

The limitations with the accessibility of tissue in human studies mean that *in vivo* animal models or *in vitro* studies may be necessary to demonstrate the relevance of changes in peripheral tissue to specific diseases and to undertake mechanistic studies. Although rodents are extremely useful for disease modelling, early delivery results in death because of lung immaturity, so that studying longitudinal neurodevelopmental effects is problematic. However, in rodents, brain development at birth resembles that of a 24-week gestation fetus, whilst at postnatal day (P)10 it is equivalent to babies at term and at P21 resembles that in the infant/young child (De Simoni *et al.*, 2003; Vannucci & Vannucci, 2005; Tucker *et al.*, 2009;

Favrais *et al.*, 2011; Semple *et al.*, 2013). Thus, the early postnatal rodent brain provides a ‘window’ in which to study factors influencing brain development in the human preterm infant.

In this study, we analysed longitudinal changes in DNA methylation in preterm infants over the first year of life. We then proceeded to use a combination of *in vivo* and *ex vivo* experiments to study the utility of *ex vivo* rat cortical brain slices to model longitudinal changes in, and the effects of a relevant insult (altered glucose concentrations) on DNA methylation. We focussed on effects at one of the genes we have previously identified as differentially methylated in preterm babies: Leucine-rich alpha-2-glycoprotein 1 (Lrg1) (Sparrow *et al.*, 2016), one of the leucine-rich repeat (LRR) family of proteins which are involved in protein-protein interactions, signal transduction, cell adhesion and which are known to be important during development.

Methods

Human studies

Samples were analysed from a cohort of infants recruited within the first week of life from the Simpson Centre for Reproductive Health, Edinburgh, UK, with informed written parental consent. Details of sample collection and the demographic details of these infants and their mothers have been reported elsewhere (Piyasena *et al.*, 2016). From an original cohort of 50 preterm (<32 weeks gestation) and 40 term infants (37-42 weeks gestation) recruited at birth and followed up for one year, buccal cell DNA was available from 32 preterm infants at term corrected age and 30 term infants at birth; and from 37 preterm infants at 1 year corrected age and 34 of the term infants at 1 year. DNA from 21 of the term babies, but none of the preterm babies, was used in an epigenome-wide association study which we have previously reported (Sparrow *et al.*, 2016). Ethical approval was obtained from the South East Scotland Research Ethics Committee (Reference 11/AL/0329 and 13/SS/0143). Infant samples were collected under the framework of the Edinburgh Reproductive Tissue BioBank (ERTBB) (West of Scotland Research Ethics Service Reference 09/S0704/3). DNA was extracted using Oragene OG-250 kits (DNAGenotek, Ottawa, Canada) and DNeasy blood and tissue kits (Qiagen, Manchester, UK) and the concentration determined using Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Paisley, UK).

Animal studies

Wistar rats (Charles-River, Tranent, UK) were maintained under conditions of controlled lighting (lights on 7:00 am to 7:00 pm) and temperature (22°C) and allowed free access to food (standard rat chow, Special Diets Services, Witham, Essex, UK) and water. All studies were conducted under licensed approval by the UK Home Office, under the Animals (Scientific Procedures) Act, 1986, and with University of Edinburgh ethical committee

approval. Animals were mated and pups killed at postnatal day (P)2 or P10 as specified by overdose of Euthatal (Merial) (250mg/kg) injected intraperitoneally. Pups were then decapitated and the brain removed for further study.

Organotypic cortical slices

For organotypic cortical slices, rat litters were killed at P2 by an overdose of anaesthetic injected intraperitoneally. The forebrain was dissected rapidly and placed in L15 media before being sliced into 300 μ M sections using a tissue chopper (McIlwain, TedPella, Redding, CA, USA) and placed into organotypic cell culture inserts (Millipore, Nottingham, UK). The day before, the inserts had been coated with poly D-lysine 4 μ g/mL (Sigma-Aldrich, Dorset, UK) in a 6 well plate for 1 hour, then washed with sterile water and coated overnight with 200 μ L of laminin from human placenta (10 μ g/mL) (Sigma-Aldrich, Dorset, UK). 2-3 slices from different animals were positioned on an insert in each well and cultured in Slice Culture Media (SCM): 25% EBSS (Thermo Fisher Scientific, Paisley, UK), 69% BME (Thermo Fisher Scientific, Paisley, UK), 5% heat inactivated horse serum (Gibco, Thermo Fisher Scientific, Paisley, UK), 1% D (+)-glucose (Sigma-Aldrich, Dorset, UK), 1% GlutaMAX-I supplement (Sigma-Aldrich, Dorset, UK), 1% penicillin/streptomycin (Sigma-Aldrich, Dorset, UK), at 37C with humidity and 5% CO₂. Slices were cultured for 10 or 21 days in 'normal' SCM (11mM glucose) or SCM with no added D(+)-Glucose (3.7mM glucose).

DNA and RNA extraction and RT-qPCR from rat cortices and cortical slices

DNA and RNA from freshly harvested rat cortices and organotypic slices were simultaneously extracted using an RNA/DNA All Prep Mini Kit (Qiagen, Manchester, UK) and quantified using the Qubit 2.0 Fluorimeter system using the DNA BR or HS Qubit kit

and RNA BR or HS Qubit kit (Thermo Fisher Scientific, Paisley, UK). RNA was DNase treated using RQ1 DNase (Promega, Southampton, UK) and reverse transcribed using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Paisley, UK). Primers for Tet3, Dnmt3a, Dnmt1 and the housekeeping gene TBP were designed using the UPL assay design system from Roche (Table 1), and Taqman assays for Tet1 (Rn01428192_m1) and Lrg1 (Rn02376998_s1) were purchased from Thermo Fisher Scientific. TBP was selected as the most stable housekeeping gene across experiments. qPCR was performed using the Roche LightCycler® 480. Data were assessed for normality using the Shapiro-Wilk test in GraphPad prism (La Jolla, CA, USA). When the data were normally distributed, unpaired Student's t-tests were performed, otherwise a Mann-Whitney U test was performed using Graphpad prism. When 2 variables (condition/treatment with time) were analysed, a 2-way ANOVA with Bonferroni post hoc test was performed using Graphpad prism.

5methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) levels

The Quest 5-hmC DNA ELISA Kit (Zymo research) and MethylFlash Methylated DNA 5mC Quantification Kit (Epigentek, Farmingdale, NY, USA) were used to measure the level of 5mC and 5hmC respectively in genomic DNA extracted from cortical slices. A total of 6 (5mC) or 8 (5hmC) slices treated independently from 3 different littermates were used for each group. 5mC and 5hmC levels from the *in vivo* study were measured by Ultra High Pressure Liquid Chromatography UPLC following the protocol: <https://assets.thermofisher.com/TFS-Assets/LSG/Application-Notes/technote-Global-And-Locus-Specific-5-Methylcytosine-Detection.pdf>.

Pyrosequencing

Pyrosequencing was used to quantify DNA methylation at individual cytosines (CpGs) in DNA from buccal swabs from babies and from DNA extracted from rat brains and cortical slices. Bisulphite conversion was performed on 500 ng of genomic DNA using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA). Converted DNA was amplified using the AmpliTaq Gold 360 Kit (Applied Biosystems, Thermo Fisher Scientific, Paisley, UK) with primers mapping to target regions containing CpGs assayed within the array from our previous study (Sparrow *et al.*, 2016). For analysis of DNA methylation in rat cortices, bisulfite converted DNA was amplified by PCR with primers mapping to the homologous promoter regions of *Lrg1* in human and rat (Table 1). PCR primers were designed using PyroMark Assay Design Software 2.0 (Qiagen, Manchester, UK) (Table 1). Pyrosequencing was performed using PyroMark Q24Gold reagents on a PyroMark Q24 Pyrosequencer (Qiagen, Manchester, UK) according to the manufacturer's instructions. Data were extracted and analysed using PyroMark Q24 1.0.10 software (Qiagen, Manchester, UK). Background non-conversion levels were ~1–3%. Shapiro-Wilk normality test and unpaired t-testing was performed using SPSS (IBM, Hampshire, UK).

Results

Differences in LRG1 DNA methylation observed in preterm babies at term age persist at 1 year.

We previously identified differences in DNA methylation at LRG1 of ~20% between term infants at birth and preterm infants at term age using the Illumina Methylation 450k beadchip array (Illumina, San Diego, CA, USA) and these differences were confirmed by Pyrosequencing (Sparrow *et al.*, 2016). Differences in DNA methylation at LRG1 were persistent at one year of age despite an overall decrease in methylation over time (Figure 1A). Persistent differences in DNA methylation at one year of age were identified at some, but not all of the genes which we validated as differentially methylated in preterm infants at term age in our previous study (Sparrow *et al.*, 2016) (Figure 1B).

Developmental changes in 5mC, 5hmC and the expression of epigenome-modifying enzymes occur in vivo in rat brain.

In rat cortices, there were no changes in global 5mC *in vivo* between days P2 and P10, however 5hmC levels increased with time (Figure 2A). The expression levels of Tet1, Tet3, Dnmt3a and Dnmt3b increased between P2 and P10 (Figure 2B). The expression of Lrg1 also increased with postnatal age *in vivo* (Figure 2C), whilst DNA methylation at Lrg1 declined between P2 and P10 (Figure 2D).

Glucose concentration affects locus-specific DNA methylation and global DNA hydroxymethylation in ex vivo rat organotypic cortical slices.

In *ex vivo* rat organotypic cortical slice cultures, exposure to lower glucose concentrations was associated with a trend towards higher global 5mC and significantly lower 5hmC (Figure 3A). Consistent with this decrease in 5hmC, the expression of Tet1 was reduced with long-

term exposure to lower glucose concentrations (Figure 3B). DNA methylation at Lrg1 in *ex vivo* cortical slices was maintained at the same levels as in the rat *in vivo*, and also resembled DNA methylation levels present at CpG2 human infants (Figure 3C). Consistent with the results in the human study and in rats *in vivo*, Lrg1 methylation decreased over time in *ex vivo* cortical slices (Figure 3C). Lower glucose concentrations were associated with lower DNA methylation at CpG2 in cortical slices at both 10 and 21 days (Figure 3C). However, Lrg1 expression in the *ex vivo* cortical slices was low and there were no changes in gene expression with exposure to lower glucose concentrations (Figure 3D).

Discussion

We had previously identified differential DNA methylation at LRG1 in buccal DNA from preterm infants at term corrected age (Sparrow *et al.*, 2016), and here we show that these differences are persistent, at least at one year of age, suggesting that, at least at some loci, DNA methylation differences are stable during early infancy. Although there were no persistent differences at a number of other sites at which we identified differential DNA methylation earlier in life (Sparrow *et al.*, 2016), these may still be relevant, for example there could be dynamic injury/repair responses in the perinatal period that are important. Longitudinal studies of DNA methylation patterns in association with developmental and health data may help establish a DNA methylation ‘signature’ that could be useful in the early diagnosis and management of preterm infants.

We chose to focus on Lrg1, which encodes a protein involved in neovascularisation and angiogenesis and is distributed throughout the brain, particularly in the deep cerebral cortex, in astrocytes and their processes associated with blood vessels (Nakajima *et al.*, 2012; Miyajima *et al.*, 2013). Recent studies have demonstrated a potential role for Lrg1 in neuronal diseases: Lrg1 expression increases with age in both humans and mice (Miyajima *et al.*, 2013), and is increased further in cerebrospinal fluid and brain tissue of patients with Alzheimer’s or Parkinson’s disease with dementia (Miyajima *et al.*, 2013). Indeed, in humans, cognitive function declines with greater cerebrospinal fluid LRG1 concentrations (Miyajima *et al.*, 2013). In mice, Lrg1 overexpression in glia and neurons results in early neuronal decline and neurodegeneration, supporting a role for Lrg1 in neuronal loss (Miyajima *et al.*, 2013). Lrg1 may regulate signalling through the TGF β pathway which is thought to be an important regulator of CNS development (Wang *et al.*, 2013). TGF β is released in response to brain injury and may play a role in post-ischemic neural stem cell

proliferation and differentiation (Sun *et al.*, 2010) and the development of post haemorrhagic hydrocephalus in preterm infants (Whitelaw, 2001).

Although there were no changes in global 5mC in the rat brain *in vivo* over time, we identified locus-specific developmental changes in DNA methylation at *Lrg1*, with a decrease over the first year of life in humans and over the equivalent course of brain development in the rat *in vivo* (21 days). The lack of change in global 5mC is perhaps not surprising since there are both increases and decreases in specific loci with early life ‘insults’ and over time (Sparrow *et al.*, 2016). The temporal changes in *Lrg1* methylation were reproduced in the *ex vivo* slices, which showed maintenance of the absolute levels of DNA methylation seen *in vivo* and a decrease over time.

Factors acting during intrauterine development which may be important in mediating ‘programming’ effects in small for gestational age (SGA) infants include undernutrition and glucocorticoid overexposure (Bayman *et al.*, 2014). In addition, preterm infants are vulnerable to these and other factors acting in early *postnatal* life (Bayman *et al.*, 2014). Importantly, *Lrg1* expression is responsive to changes in the environment *in vivo* and *in vitro*: exposure to chronic social stress increases the expression of *Lrg1* in mouse brain (Stankiewicz *et al.*, 2015) and *Lrg1* expression is affected by changes in glucose concentrations in culture, at least in the pancreas (Anderson *et al.*, 2009). Seeking to explore the potential use of *ex vivo* cortical slices to model insults which may impact on the preterm brain, we show that changes in glucose concentrations can affect site-specific DNA methylation such that lower glucose concentrations are associated with a reduction in DNA methylation at *Lrg1*. Neonatal hypoglycaemia can, if severe and prolonged, cause brain tissue injury and long term neurodevelopmental impairment (Boluyt *et al.*, 2006; Rozance & Hay,

2006; Burns *et al.*, 2008); and it accounts for a substantial proportion of costs settled by the NHS litigation authority (Hawdon *et al.*, 2017). The 3.7mM and 11mM glucose concentrations in the culture medium are higher than those normally considered hypoglycaemic or normoglycaemic for human neonates, so it is not possible to translate directly the impact of these concentrations to humans, but the data provide proof of concept that glucose homeostasis can lead to differences in DNA methylation. It is possible that the observed effect size may be greater with even lower glucose concentrations, but this is difficult to model in *ex vivo* slices, which require additional glucose to be viable since they do not obtain nutrients from the circulation.

The extent to which DNA methylation regulates the expression of Lrg1 is not known. The temporal increase in Lrg1 expression *in vivo* occurs in association with a decrease in DNA methylation, suggesting that the developmental changes in expression could be regulated by DNA methylation. However, although in the *ex vivo* slices DNA methylation levels were the same as those *in vivo*, Lrg1 expression levels were very low. Further, although exposure to low glucose concentrations was associated with a reduction in DNA methylation in *ex vivo* cortical slices, there were no effects on Lrg1 expression. Since the Lrg1 promoter is not CG rich, changes in DNA methylation may simply reflect its previous transcriptional status. It is not clear why the expression of Lrg1 in *ex vivo* slice culture is so low, however it may be that additional factors that are not present in slice culture are required for the normal expression of Lrg1. Alternative explanations might include the loss of specific cell types *in vitro* which highly express Lrg1 or which are required for the expression of Lrg1 in other cells. For example, it is known that the *in vivo* expression of some genes in astrocytes and neurons are not maintained when their normal niche is disrupted (Hasel *et al.*, 2017).

5-hydroxymethylation is a recently discovered epigenetic modification which associates with active gene transcription but which also plays a key role in the process of DNA demethylation, catalysed by the α -ketoglutarate dependent Ten-Eleven-Translocation (Tet) enzymes (Tahiliani *et al.*, 2009). Recent evidence suggests that ‘environmental’ insults can produce dynamic, reciprocal changes in 5hmC/5mC and suggest that 5hmC profiles may be a useful signature of gene transcription and a marker of cell state (Thomson *et al.*, 2012; Thomson *et al.*, 2013). Whether events experienced in early life might result in changes in 5hmC is unclear, although we have recently shown in human studies that 5hmC at imprinted loci in the placenta associates with fetal growth in term infants (Piyasena *et al.*, 2015). Here we show that there are dynamic changes in global DNA hydroxymethylation and in the expression of Tet1 *in vivo* between P2 and P10 in the rat brain. After 21 days in *ex vivo* culture, global 5hmC was decreased in low glucose conditions and consistent with this, Tet1 expression was also decreased, suggesting that glucose levels could profoundly affect 5hmC in the brain. Although we have not tested specific mechanisms, the effects of glucose availability on the abundance of key metabolic intermediates, for example in the TCA cycle, could impact on the activity of epigenetic enzymes, including the α -ketoglutarate dependent Tet enzymes. The Pyrosequencing technique we used to analyse DNA methylation uses bisulphite conversion and therefore does not distinguish between 5mC and 5hmC, so that the changes in DNA methylation we saw could reflect changes in 5mC, 5hmC or both, and further studies using more specific techniques are required to further dissect specific individual modifications.

Preterm birth is associated with a number of environmental ‘insults’ which could alter brain development, perhaps through changes in the epigenome. Our data support the hypothesis that perturbations during early life including differences in glucose homeostasis can affect

DNA methylation at specific genes implicated in neurodevelopment and that some of these changes may be persistent. Our results suggest that *ex vivo* organotypic cultures may be useful in the study of environmental influences on the brain epigenome, particularly with respect to the analysis of DNA methylation.

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Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contributions

JC conceived, designed and performed the study. CP, SAS and JPB were involved in subject recruitment, sample collection and analysis of human data. JC and AD wrote the initial manuscript draft and all authors revised it critically for intellectual content. All authors gave final approval of the version to be published.

Data accessibility

Data will be available following publication by correspondence with the corresponding author.

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Figure legends

Figure 1: (A) Pyrosequencing analysis of DNA methylation at 2 CpGs located in the promoter region of LRG1 in term babies at birth and preterm babies at term-corrected age and in term babies at 1 year of age and preterm babies at 1 year corrected age. DNA methylation at LRG1 was lower in preterm infants at term corrected age and 1 year of age and also decreased in both preterm and term infants over time. (B) DNA methylation in term babies at 1 year of age and preterm babies at 1 year corrected age at 5 genes previously reported as differentially methylated in preterm infants at term corrected age (SLC7A5, NPBWR, APOL1, QPRT and SLC1A2) (Sparrow *et al.*, 2016). Graphs are mean +/-SEM, p values from 2 way-ANOVA with Bonferroni post-hoc test (A) and unpaired Student's t-test or Mann Whitney as indicated (B).

Figure 2: (A) Percentage of 5mC and 5hmC nucleotides in cortices from P2 and P10 animals (n=8 per group) showing temporal changes in 5hmC, p values from unpaired Student's t test. (B) Temporal changes in expression of epigenome-modifying enzymes in cortices from P2 and P10 animals (n=10 per group), p values from Mann Whitney test. (C) *Lrg1* expression changes in rat cortices between P2 and P10 *in vivo* (n=10 per group), p values from Mann Whitney test. (D) DNA methylation at *Lrg1* declines between P2 and P10 *in vivo* (n=10 per group), p values from unpaired Student's t test. Graphs are mean +/-SEM

Figure 3: (A) Exposure to low glucose concentrations resulted in a non-significant increase in global 5mC and a significant decrease in 5hmC in *ex vivo* cortical slices at 21 days (n=6 (5mC) or 8 (5hmC) independent slices from 3 animals per group. (B) mRNA expression of Tet1 was decreased with exposure to low glucose in *ex vivo* cortical slices at 21 days (n=9 independent slices from 3 animals per group). (C) There was a temporal decrease in *Lrg1*

methylation in cortical slices between 10 and 21 days and lower glucose concentrations resulted in a decrease in DNA methylation at CpG2 at both 10 and 21 days (n=8 (day 10) and 9 (day 21) slices from 3 animals for all groups). (D) *Lrg1* expression in *ex vivo* cortical slices was low and was not affected by glucose concentration (n=9 slices for 11mM and 7 slices for 3.7mM from 3 animals per group). *p<0.05. Graphs are mean +/-SEM, p values from unpaired Student's t test (A) and from 2-way ANOVA with Bonferroni post-hoc test (B-C).

Table 1: Primer sequences

qPCR			
Target	Forward	Reverse	UPL probe#
TBP	caattctgggttgatcattctg	cccaccagcagttcagtagc	129
Tet3	ggaaataaatgctcgtaagga	gctgagctctgagcctgtct	81
Dnmt3a	aacggaagcgggatgagt	actgcaatcaccttgcttt	75
Dnmt1	aactcgtcttggttgagacct	gcgactgcaatacacactgaa	55
Pyrosequencing			
Target	Forward	Reverse	sequencing
rat-Lrg1	AGAGGTGGGGATTTAGTTTAGG	bio-TAACTTACTTCCTACCCACCCTTTCC	GGGGATTTAGTTTAGGAG
human LRG1 -CpG1	GTGGGGATTTTTTAGGGTTGG	bio-CTCCAAAAAACATAATAACTCTACTCTT	GTTTAGGTAGGTATAAGGTTAT
human LRG1 -CpG2	GATTTTTGGGGGTATTTAAGAG	bio-CCCTATCTCCAAAAATAATACCTTACA	ACCTTACAAACCTTAACC
human SLC1A2	GTTATTTAGTTAGAAGGTGGTGAAGAA	bio-ACCAAAAAAACCCAAATCCCAACAAA	GTGGTGAAGAATTTAAGTTT
human SLC7A5	TGTGTGTTTTTATAGGGTATGAGG	bio-AAAACACTCCTATATCCCCCTCT	TTTTTATAGGGTATGAGGT
human QPRT	TTGGGAGTTTTGGTTTTGAGT	bio-ACAAAAAAAATATCCCTTTACCTTCA	AGATAGTTGTAAGTTATTATGG
human NPBWR -CpG1	ATAGAGATAGGGGAGTTAAGATTGTTT	bio-TCTCTACTTATCCACACACTTACC	TTTTGTTAGTTTTTTTTTGTTAT
human NPBWR -CpG2-4	AGAGTTTTTTATTATTTTAGATGGAGTGAG	bio-CCCCCAAATTTAAAAAATTCCTTTC	GATGGAGTGAGGTTG
human APOL1	TTTGGTTATGAGTTGTTGGGAAGTT	bio-ACAAATCCTCCAATCCCCAAAATATAC	GGGAAGTTGTGATTTTTA

Fig 1

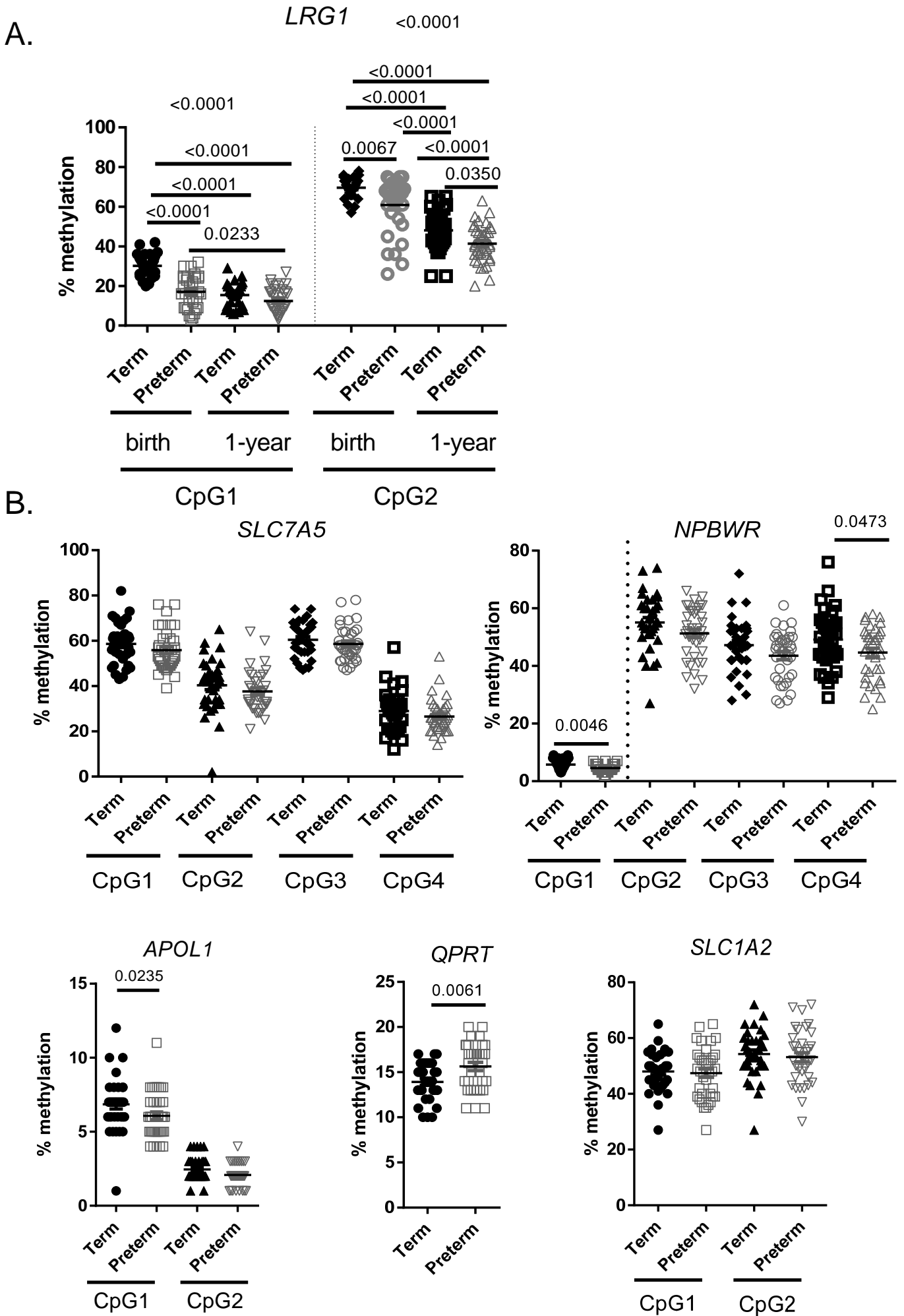
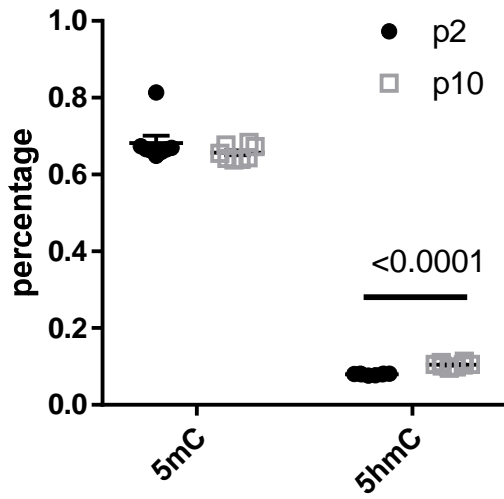
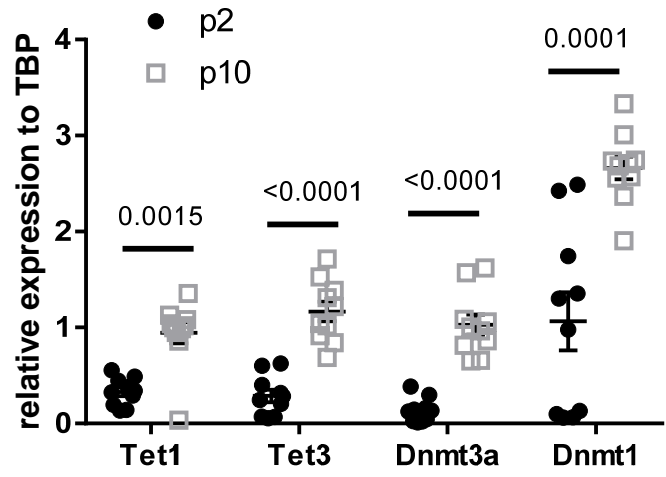


Fig 2

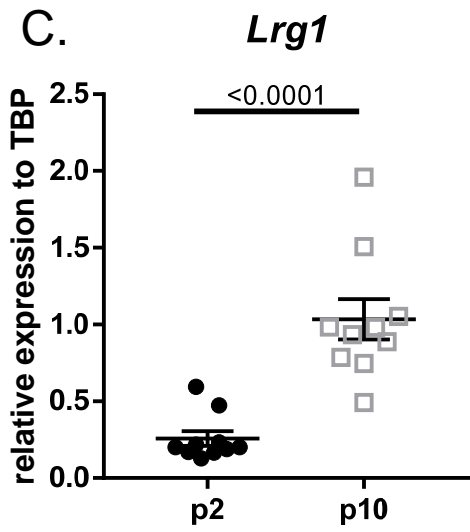
A.



B.



C.



D.

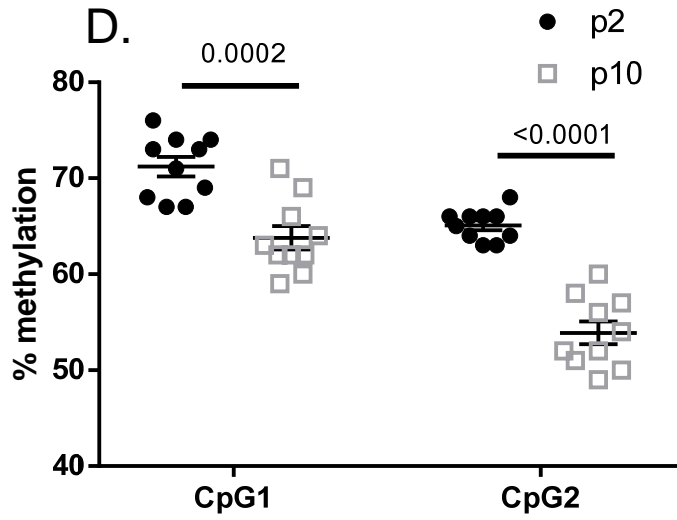


Fig 3

