Spring 2017 – Epigenetics and Systems Biology
Discussion Session (Environmental Epigenetics)
Michael K. Skinner – Biol 476/576
Week 11 (March 23)

Environmental Epigenetics

Primary Papers

1. Kaut, et al., (2017) Neurogenetics 18:7-22
2. McGowan et al., (2009) Nat Neurosci. 12(3):342-8.
3. Burdge et al., (2009) J Nutr. 139(6):1054-60.

Discussion

Student 27 – Ref #1 above

- Why are twin studies useful for epigenetic studies?
- Does the data support an environmental impact on the human epigenome and disease?
- What is the application of these epigenetic changes?

Student 28 – Ref #2 above

- What mechanism is proposed for early life effects on brain function?
- Is NGF1 the only gene effected?
- What is the impact of these epigenetic changes?

Student 29 – Ref #3 above

- How does folic acid effect epigenetics?
- Does diet effect epigenetic programming?
- What happens if you have too much folate?

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ORIGINAL ARTICLE

Epigenome-wide DNA methylation analysis in siblings and monozygotic twins discordant for sporadic Parkinson's disease revealed different epigenetic patterns in peripheral blood mononuclear cells

Oliver Kaut[1] · Ina Schmitt[1] · Jörg Tost[2] · Florence Busato[2] · Yi Liu[2] · Per Hofmann[3,4] · Stephanie H. Witt[5] · Marcella Rietschel[5] · Holger Fröhlich[6] · Ullrich Wüllner[1,7]

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Abstract Numerous studies have elucidated the genetics of Parkinson's disease; however, the aetiology of the majority of sporadic cases has not yet been resolved. We hypothesized that epigenetic variations could be associated with PD and evaluated the DNA methylation pattern in PD patients compared to brothers or twins without PD. The methylation of DNA from peripheral blood mononuclear cells of 62 discordant siblings including 24 monozygotic twins was characterized with Illumina DNA Methylation 450K bead arrays and subsequently validated in two independent cohorts: 221 PD vs. 227 healthy individuals (cohort 1) applying Illumina's VeraCode and 472 PD patients vs. 487 controls (cohort 2) using pyrosequencing. We choose a delta beta of >15 % and selected 62 differentially methylated CpGs in 51 genes from the discordant siblings. Among them, three displayed multiple CpGs per gene: microRNA 886 (*MIR886*, 10 CpGs), phosphodiesterase 4D (*PDE4D*, 2 CpGs) and tripartite motif-containing 34 (*TRIM34*, 2 CpGs). *PDE4D* was confirmed in both cohorts (p value 2.44e−05). In addition, for biomarker construction, we used the penalized logistic regression model, resulting in a signature of eight CpGs with an AUC of 0.77. Our findings suggest that a distinct level of PD susceptibility stems from individual, epigenetic modifications of specific genes. We identified a signature of CpGs in blood cells that could separate control from disease with a reasonable discriminatory power, holding promise for future epigenetically based biomarker development.

✉ Oliver Kaut
oliver.kaut@ukb.uni-bonn.de

[1] Department of Neurology, University of Bonn, 53105 Bonn, Germany

[2] Laboratory for Epigenetics and Environment (LEE), Centre National de Génotypage, CEA-Institut de Génomique, 91057 Evry Cedex, France

[3] Institute for Human Genetics, Department of Genomics, Life and Brain Center, University of Bonn, Bonn, Germany

[4] Human Genetics Research Group, Department of Biomedicine, University of Basel, Basel, Switzerland

[5] Department of Genetic Epidemiology in Psychiatry, Central Institute of Mental Health, Medical Faculty Mannheim, University of Heidelberg, 68159 Mannheim, Germany

[6] Bonn-Aachen International Center for IT (B-IT), Algorithmic Bioinformatics, University of Bonn, 53113 Bonn, Germany

[7] German Center for Neurodegenerative Diseases (DZNE), 53175 Bonn, Germany

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Abbreviations

AUC	Area under the curve
CpG	Cytosine and guanine separated by one phosphate
GWAS	Genome-wide association studies
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine
MZ	Monozygotic
PD	Parkinson's disease
PBMC	Peripheral blood mononuclear cells
ROC	Receiver-operating characteristic

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is characterized by bradykinesia, rest tremor, rigidity and postural instability. Although several recessive and dominant mutations have been identified in familial PD in distinct genes, the complex genetic contribution to sporadic PD has not been fully elucidated yet. Nevertheless, a genetic etiologic involvement is also assumed in sporadic cases. Genome-wide association studies (GWAS) have identified relatively few rather low-risk loci, and only a part of the heritable component of PD has been identified [1, 2]. Interestingly, several twin studies have pointed to a major role of non-Mendelian factors in PD, and a recent follow-up of the Swedish twin study again revealed low concordance rates for PD (13 % for monozygotic and 5 % for same-sexed dizygotic twin pairs), confirming earlier studies with similar results in the UK and the USA [3]. These low concordance rates favour non-genetic, e.g. environmental, factors.

Currently, sporadic PD is conceptualized as a consequence of various genetic variants and complex gene-environmental interactions on a background of age-related changes that constitute to individual levels of susceptibility.

Epigenetic mechanisms such as DNA methylation are considered important mediators of putative environmental factors [4] and have been implicated in ageing [5]. The DNA methylation of cytosines in CpG dinucleotides is an epigenetic mechanism for regulation of the gene expression [6].

Despite the fact that monozygotic twins are epigenetically indistinguishable in their early life, they will develop remarkable differences in their overall content and genomic distribution of 5-methylcytosine DNA over time [7]. MZ twins share nearly identical DNA with small genetic variants [8]. Thus, epigenetic changes are rather related to environmental factors that contribute to a phenotype [9]. Moreover, epigenetic variation can be induced across the lifespan in response to a broad range of environmental exposures [10, 11]. Pre- and post-natal malnutrition has an impact on the methylome of the foetus, with consequences that can persist into adulthood [12]. In addition, evidence is accumulating, stating that epigenetic marks are transmitted across generations in humans [13].

We hypothesize that early environmental influences in the perinatal period or early childhood may promote epigenetic, in particular DNA methylation changes, which later in life may predispose an individual to PD.

To elucidate the possibility that a particular epigenetic signature might distinguish PD patients from healthy controls or could point to a novel hitherto not appreciated mechanism of disease, we performed an epigenetic epigenome-wide association study (EWAS) of siblings (brothers) and monozygotic twins discordant for PD and corroborated our findings in two independent samples of sporadic PD patients.

Methods

Samples for methylation analysis using a 450K microarray (brothers)

DNA samples derived from peripheral blood mononuclear cells (PBMC) of 17 male Parkinson's disease patients vs. 21 healthy male brothers were used for a pairwise DNA methylation microarray analysis using the Illumina® 450K methylation microarray system (Table 1). In three families, two healthy brothers were paired with one PD patient.

Samples were acquired within the German network of competence for Parkinson's disease (Kompetenznetz Parkinson e.V. Bonn). The experiments were undertaken with the understanding and written consent of each subject. The study conforms to the World Medical Association Declaration of Helsinki. The Ethics

Table 1 Control case and Parkinson's disease information of study cohorts applied

	Control			PD		
	Sex: F/M, *N* (%)	Age	Number of samples	Sex: F/M, *N* (%)	Age	Number of samples
Male brothers[a]	0/23	62.2 ± 12.6	23	0/17	61.4 ± 11.4	17
MZ twins[a]	7/5	69.0 ± 9.06	12	7/5	69.0 ± 9.06	12
VeraCode cohort	90/98 (47.9/52.1)[b]	60.67 ± 8.0	227	84/137 (62/38)	65.1 ± 10.2	221
Pyrosequencing cohort	234/238	65,5 ± 7.97	472	306/181	64.24 ± 11.0	487

PD Parkinson's disease

[a] Analysed with Illumina 450K methylation array

[b] Information of age missing in 39 probes

Committee of the Medical Faculty of the University of Bonn approved this study (No. 51/00, 6 July 2000). DNA isolation and bisulphite conversion were performed as described previously [14].

Samples for methylation analysis using a 450K microarray (MZ twins)

DNA samples derived from PBMC of 12 discordant MZ twin pairs aged 69.0 ± 9.06 years were used for a pairwise DNA methylation microarray (Tables 1 and 2). MZ twin pairs were selected according to the following criteria: (i) European origin, (ii) both co-twins available for analysis and (iii) one twin of the pair diagnosed with Parkinson's disease. Nine MZ twin pairs were acquired from our outpatient clinic. Three MZ twin pairs were recruited from the UK Twins Cohort, King's College London, Great Britain. Written informed consent was obtained from all individuals prior to study entry.

Monozygosity testing

All of the twin pairs were tested for their zygosity status at the Institute of Human Genetics, University of Bonn, Germany, before proceeding with molecular typing. This analysis was performed using a PowerPlex R16 System according to the manufacturer's recommendations (Promega, Madison, WI, USA), permitting the co-amplification and three-colour detection of 16 loci.

Samples for methylation analysis using VeraCode® technology

A total of 448 DNA samples from peripheral blood mononuclear cells of 221 Parkinson's disease patients (65.1 ± 10.2 years) vs. 227 healthy individuals (60.67 ± 8.0 years) were used for a DNA methylation microarray analysis using a customized GoldenGate® Assay for methylation with the VeraCode technology to target 96 CpGs in a single reaction. For further details of the clinical phenotypes, see Tables 1 and 2. Samples were acquired within the German network of competence for Parkinson's disease (Kompetenznetz Parkinson e.V.) and from the Central Institute for Mental Health, Mannheim, Germany. To ensure assay quality, built-in controls were integrated. Multiplexed DNA methylation profiling was performed in the Central Institute of Mental Health, Mannheim, Germany, using a BeadXpress Reader.

Statistics of the 450K microarray

To analyse CpG methylation, the Illumina HumanMethylation450K BeadChip representing more than 485,000 individual CpG sites was used according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Raw signals of the 450K BeadChips were extracted using the GenomeStudio® software (v2011.1, Illumina). The DNA methylation level of each CpG was calculated as the ratio of the intensity of fluorescent signals of the methylated alleles to the sum of the methylated and unmethylated alleles (β value). All of the samples passed initial quality control and had on average

Table 2 Clinical features of Parkinson's disease study cohorts applied

		Male brothers (PD patients only)	MZ twins (PD patients only)	VeraCode cohort (PD patients only)
PD subtype[a], *N* (%)				
Hypokinetic-rigid		2	2	82 (51.2 %)
Tremor		6	0	26 (16.3 %)
Equivalent		9	6	52 (32.5 %)
H&Y	I N (%)	2	1[b]	23 (16.2 %)
	II	12	4	42 (29.6 %)
	III	1	1	32 (22.5 %)
	IV	1	0	26 (18.3 %)
	V	1	0	19 (13.4 %)
Levodopa intake	No	5	10	47 (29.4 %)
	Yes	12	2	113 (70.6 %)
Levodopa, mg/day[c]		540.90 ± 201.33	489.28 ± 182.49	424.13 ± 256.83

[a] Information of subtype missing in four patients

[b] Information of H&Y stage missing in six patients

[c] Mean dosage only of those who take levodopa

more than 98 % of valid data points (detection p value <0.01). A refined version of the subset quantile normalization (SQN) pipeline [15] including a revised annotation file [16] was used for data processing, correction and normalization. Intensity values were corrected for potential biases in fluorescent dye intensity, and the background was corrected using the *lumi* R package [17] as implemented in the SQN pipeline. The probes that were potentially influenced by SNPs underlying the entire sequence of the probe (+1 or +2 bases depending on the Infinium probe type) that were present in the EUR population of the 1000 Genome project (http://www.1000genomes.org) at a frequency of more than 5 % were removed from the analysis. Furthermore, probes that had been previously reported to map to several genomic regions were removed [18]. The SQN pipeline then used the intensity signals of high-quality (i.e. low-detection p value) Infinium I probes as "anchors" to estimate a reference distribution of quantiles for probes in a biologically similar context based on the annotation file. This reference was then used to estimate a target distribution of quantiles for InfII probes as a means to provide an accurate normalization of InfI/InfII probes and to correct for the shift. SQN was performed for each individual separately. Because a principal component analysis and hierarchical clustering did not show an overall difference in the methylation patterns between patients and controls, a quantile normalization was performed for between-sample normalization.

To compare the DNA methylation levels of CpG sites between Parkinson's disease patients and controls, CpG sites with a mean methylation difference ($\Delta\beta$) of greater than 15 % difference were considered as differentially methylated. Due to the small size of the cohorts (brothers and MZ twins) and the potential number of markers that were tested, no threshold such as p value based on a statistical test was applied. Genes were considered as differentially methylated if two CpGs fulfilled the difference of 15 %.

Analysis of leukocyte subsets using DNA methylation

To distinguish cell lineage-specific DNA methylation patterns in leukocyte subsets, we used the approach published by Houseman et al. [19].

DNA methylation analysis using VeraCode® technology

The VeraCode® chip in principle should have allowed to rapidly verify the candidate genes from the 450K analysis. Unfortunately, several CpGs were not available for technical reasons. Thus, only a subset of candidate CpGs from the 450K chips could be annotated on the VeraCode chip.

Validation data from the VeraCode platform was normalized with the methylumi Bioconductor package (http://www.bioconductor.org/packages/release/bioc/html/methylumi.html; V2.12.0); at the pre-processing level, individuals with more than 20 % of low-confidence detection p values ($p < 0.05$) were removed from the analysis (13 controls and 19 Parkinson's disease patients), and differential methylation was assessed as described for the 450K data. To compare the DNA methylation levels of CpG sites between Parkinson's disease patients and controls, we used a cut-off of $p < 0.01$ to consider CpGs as differentially methylated (Fig. 1).

Meta-analysis with the MetaPWilcoxon test

The method to calculate a meta-analysis p value from an individual p value is taken from Zaykin [20]. The weight of each individual p value is chosen to be the square root of the sample numbers in each test. Briefly, for each CpG, we have three p-values, p_1, p_2 and p_3, from the twin data set, the brother data set and the VeraCode data set, respectively. From these p values, we can construct a z-score Z_i = PHI^{−1}(1 − p_i). Then, the meta-analysis p value is calculated as p_z = 1 − PHI(\sum_i w_i Z_i / \sqrt{\sum_i w_i^2}).

Pyrosequencing

Samples for pyrosequencing (PDE4D, METRNL, NDUFA10 and MIR886)

Blood DNA was sampled from 487 PD patients (age 64.24 ± 11.07 years; 300 males, 171 females) and 472 healthy controls (age 65.5 ± 7.97 years; 234 females, 238 males) who were diagnosed and recruited in Bonn, Lübeck and Tübingen, respectively. Information about sex and age was not available for every individual.

Bisulphite treatment and pyrosequencing

Bisulphite treatment was performed using the EZ-96 DNA Methylation-Gold™ Kit (Zymo Research) following the manufacturer's instructions. For PDE4D PCR (CpG 1 and 2), 1 μl of bisulphite-treated DNA was amplified using 4 pmol of each primer (PDE4D-PF1 AGGGAGTGATTGTAAGTAGAAAA; PDE4D-PR1/Bio, CAAAACTAAAATAACCCTACCAATTCTT) and 10 μl of HotStarTaq Plus Master Mix (Qiagen) in a 20-μl reaction and the following cycling protocol: 95 °C for 5 min, 48× (95 °C for 35 s, 58 °C for 35 s, 72 °C for 35 s), and 72 °C for 5 min.

For pyrosequencing, we used 20 μl of the PCR reaction, 5 pmol of primer PDE4D-PS1 (TGTAAGTAGAAAATATTGAATTAK) and the PyroMark Gold Q24 Reagents (Qiagen) and performed sequencing on a PyroMark Q24 instrument (Qiagen) according to the manual.

For METRNL PCR (CpG 1–3), 1 μl of bisulphite-treated DNA was amplified using 4 pmol of each primer (METRNL-PF1, TTGTTGGKTGTTTTTTTTAAGTAGTTAAG; METRNL-PR1/Bio, Bio-ATTCATAATCCACATTTATA

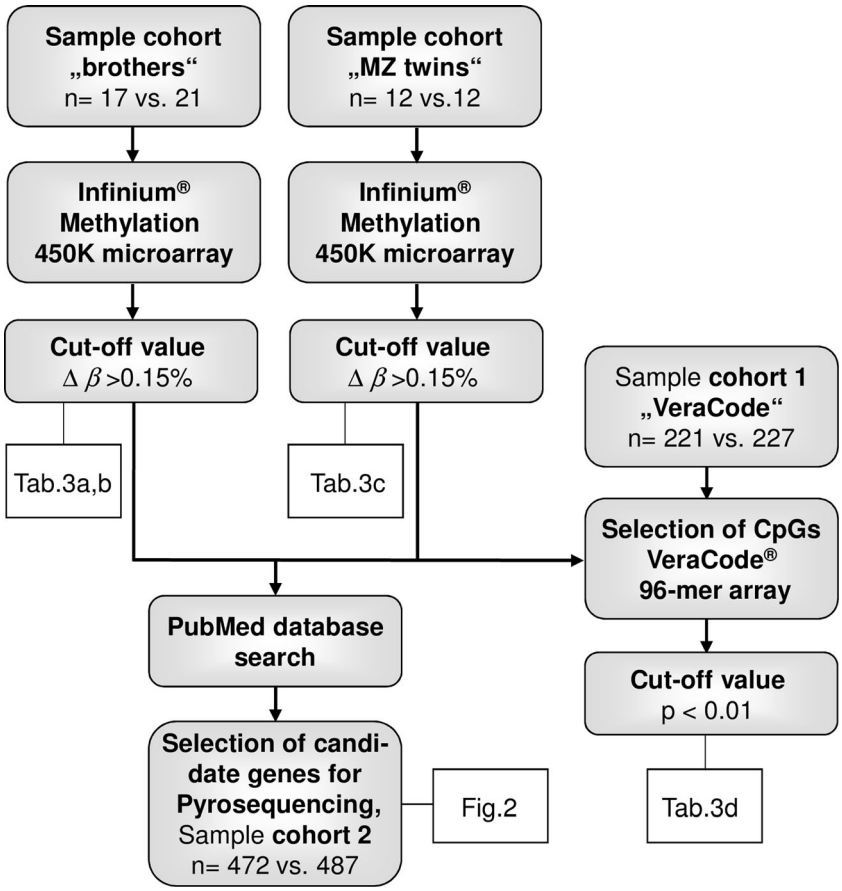


Fig. 1 Flow chart of experiments and study cohorts used. Due to the small size of the cohorts (brothers and MZ twins), no threshold such as *p* value was applied. Genes were considered as differentially methylated if two CpGs fulfilled the delta beta difference of 15 %. In a second step, candidate genes were evaluated with statistics based on *p* values in greater cohorts (cohorts 1 and 2)

AACACTAACT) and 10 μl of HotStarTaq Plus Master Mix (Qiagen) in a 20-μl reaction and the following cycling protocol: 95 °C for 5 min, 50× (95 °C for 35 s, 55 °C for 35 s, 72 °C for 35 s), and 72 °C for 5 min.

For pyrosequencing, we used 20 μl of the PCR reaction, 5 pmol of primer (METRNL-PS1, TTTYGTGGGGGGAGG) and the PyroMark Gold Q24 Reagents (Qiagen) and performed sequencing on a PyroMark Q24 instrument (Qiagen) according to the manual.

For NDUFA10 PCR (CpG 1–6), 1 μl of bisulphite-treated DNA was amplified using 4 pmol of each primer (NDUFA10-PF1, AGTAGGTTTTTAGAAAAATTTTTTAGT; NDUFA10-PR2/Bio, Bio-ATATTTCYRTAAAAATTTCCCCAT) and 10 μl of HotStarTaq Plus Master Mix (Qiagen) in a 20-μl reaction and the following cycling protocol: 95 °C for 5 min, 48× (95 °C for 35 s, 50 °C for 35 s, 72 °C for 35 s), and 72 °C for 5 min.

For pyrosequencing, we used 20 μl of the PCR reaction, 5 pmol of primer (NDUFA10-PS1, ATATTAMATAGGGTTTASAGTAG) and the PyroMark Gold Q24 Reagents (Qiagen) and performed sequencing on a PyroMark Q24 instrument (Qiagen) according to the manual.

For MIR886 PCR (CpG 1–9), 1 μl of bisulphite-treated DNA was amplified using 4 pmol of each primer (MIR886-PF1, GGAGGAATTGAGAGTTTTTTAGGA; MIR886-PR1, Bio-AAAAAAAAACRAACTACATATACTCCCC) and 10 μl of HotStarTaq Plus Master Mix (Qiagen) in a 20-μl reaction and the following cycling protocol: 95 °C for 5 min, 45× (95 °C for 35 s, 60 °C for 35 s, 72 °C for 35 s), and 72 °C for 5 min.

For pyrosequencing, we used 20 μl of the PCR reaction, 5 pmol of primer (MIR886-PS1, GGAATTGAGAGTTTTTTTAGGATA) and the PyroMark Gold Q24 Reagents (Qiagen) and performed sequencing on a PyroMark Q24 instrument (Qiagen) according to the manual.

The pyrosequencing data were evaluated using SPSS 22.0 for Windows (SPSS Inc., Chicago, IL, USA). The level of significance was set at $p < 0.05$. Descriptive results are given as the mean ± SD. To calculate the differences between control and PD, we used an ANOVA.

Results

Epigenome-wide methylation analysis of discordant brothers

The chosen conservative approach revealed 62 differentially methylated CpGs (27 CpGs hypomethylated and 35 CpGs hypermethylated in PD with a delta beta mean >0.15) in a

Table 3 *Hyper*methylated CpG sites in PD brothers and their healthy siblings

Illumina ID[a]	Gene ID[b]	Chr.[c]	Mean β value[d] Parkinson	Mean β value[d] Control	Difference[e] ($\Delta\beta$)
cg18339359	*SLC25A37*	8	78.4	56.0	+22.4
cg11784298	*HLA-DQA1*	6	73.8	53.0	+20.7
cg12307373	*DYNC2H1*	11	61.7	41.1	+20.7
cg21771569	*CDKL5*	X	64.3	44.1	+20.2
cg06270401	*DYRK4*	12	63.2	44.7	+18.5
cg21514997	*CCDC148*	2	37.1	19.5	+17.6
cg23213217	*DEGS1*	1	44.4	27.4	+17.1
cg08154963	*RAD51L3*	17	61.2	44.4	+16.9
cg13211008	*ESYT2*	7	32.3	15.5	+16.8
cg26217827	*ITGA11*	15	61.6	44.9	+16.8
cg04297507	*SGMS2*	4	60.0	43.4	+16.6
cg09786420	*ADAR*	1	74.8	58.3	+16.5
cg21685770	*OLR1*	12	61.5	45.0	+16.5
cg17442155	*PILRB*	7	58.8	42.4	+16.5
cg05492904	*CYP19A1*	15	59.3	42.9	+16.3
cg25739938	*CPSF3*	2	74.3	58.0	+16.3
cg22523050	*CREB3L2*	7	48.4	32.2	+16.2
cg09948350	*ASPRV1*	2	61.6	45.6	+16.0
cg17080697	*TRIM39*	6	72.2	56.2	+16.0
cg15410418	*PDE4D*	5	50.4	34.6	+15.7
cg00705730	*NCK2*	2	67.4	51.7	+15.7
cg25006077	*MBNL1*	3	74.2	58.5	+15.6
cg10487428	*PDE4D*	5	54.8	39.2	+15.6
cg01699630	*ARG1*	6	67.3	51.7	+15.6
cg04036920	*C11orf41*	11	73.5	57.9	+15.5
cg20817228	*LOC728855*	1	64.3	48.9	+15.5
cg16051954	*CABC1*	1	53.7	38.3	+15.4
cg11014468	*DCP1A*	3	72.6	57.3	+15.3
cg16374656	*EXT1*	8	60.1	44.9	+15.3
cg00501765	*C1orf105*	1	68.6	53.4	+15.2
cg18663897	*NDUFA10*	2	64.5	79.7	+15.1
cg06465076	*CAST*	5	64.9	49.8	+15.1
cg13640297	*WDR27*	6	71.3	56.2	+15.1
cg05171937	*STK38L*	12	59.4	44.3	+15.0
cg05501357	*HIPK3*	11	66.4	51.4	+15.0

[a] Signature of CpG dinucleotide according to Illumina 450K microarray annotation, ranked by difference of mean methylations in PD and control ($\Delta\beta$)

[b] UCSC reference gene name. Overlap of PFC and HIP candidate genes are marked grey

[c] Chromosome

[d] Methylation beta value ranging from 0.0 to 1.0 (0–100 %)

[e] Difference between mean beta value of control and PD group. Positive prefix indicates hypermethylation of Parkinson group. Negative prefix indicates hypomethylation of Parkinson group in comparison to control

genome-wide methylation analysis (Tables 3 and 4). These findings are not significant due to the fact that the small sample size does not allow statistical testing with *p* values, so it represents a first screening only to identify appropriate candidate genes that have to be evaluated by pyrosequencing.

A hierarchical clustering analysis of those 62 CpGs resulted in a rather reasonable segregation of PD vs. control, as visualized in the heat map plot (Fig. 2a).

Three genes were identified with multiple differentially methylated CpGs: phosphodiesterase 4D (*PDE4D*) with 2

Table 4 *Hypo*methylated CpG sites in PD brothers and their healthy siblings

Illumina ID[a]	Gene ID[b]	Chr.[c]	Mean β value[d] Parkinson	Mean β value[d] Control	Difference[e] ($\Delta\beta$)
cg04481923	*MIR886*	5	26.0	50.9	−24.9
cg18678645	*MIR886*	5	27.0	50.4	−23.4
cg16615357	*MIR886*	5	37.5	60.2	−22.7
cg23217386	*TRIM34*	11	45.6	67.8	−22.2
cg25340688	*MIR886*	5	29.4	51.3	−21.9
cg06536614	*MIR886*	5	29.2	50.5	−21.3
cg18391209	*CAPN8*	1	50.0	71.1	−21.2
cg18797653	*MIR886*	5	32.2	53.0	−20.8
cg00124993	*MIR886*	5	26.3	47.0	−20.7
cg26328633	*MIR886*	5	31.4	51.9	−20.5
cg08745965	*MIR886*	5	24.5	43.2	−18.8
cg22160073	*TRIM34*	11	57.1	75.8	−18.7
cg26896946	*MIR886*	5	31.6	50.1	−18.5
cg04850148	*CCL4L2*	17	45.7	64.3	−18.5
cg04850148	*CCL4L1*	17	45.7	64.3	−18.5
cg12483340	*TMEM87A*	15	60.6	42.4	−18.3
cg26296371	*FARS2*	6	55.7	74.0	−18.2
cg02902412	*ATP9B*	18	55.0	72.3	−17.3
cg25033076	*MPPE1*	18	65.9	82.6	−16.7
cg07427475	*PLEC1*	8	35.4	51.9	−16.5
cg27561099	*MAP3K13*	3	53.2	69.7	−16.5
cg07018090	*BANP*	16	56.9	73.1	−16.2
cg24136292	*INSC*	11	37.6	53.7	−16.1
cg15742700	*BLK*	8	64.2	79.8	−15.6
cg12991306	*C8orf80*	8	39.4	54.9	−15.6
cg11354682	*C19orf38*	19	48.6	63.9	−15.2
cg12856521	*DGKZ*	11	13.1	28.4	−15.2

[a] Signature of CpG dinucleotide according to Illumina 450K microarray annotation, ranked by difference of mean methylations in PD and control ($\Delta\beta$)

[b] UCSC reference gene name. Overlap of PFC and HIP candidate genes are marked grey

[c] Chromosome

[d] Methylation beta value ranging from 0.0 to 1.0 (0–100 %)

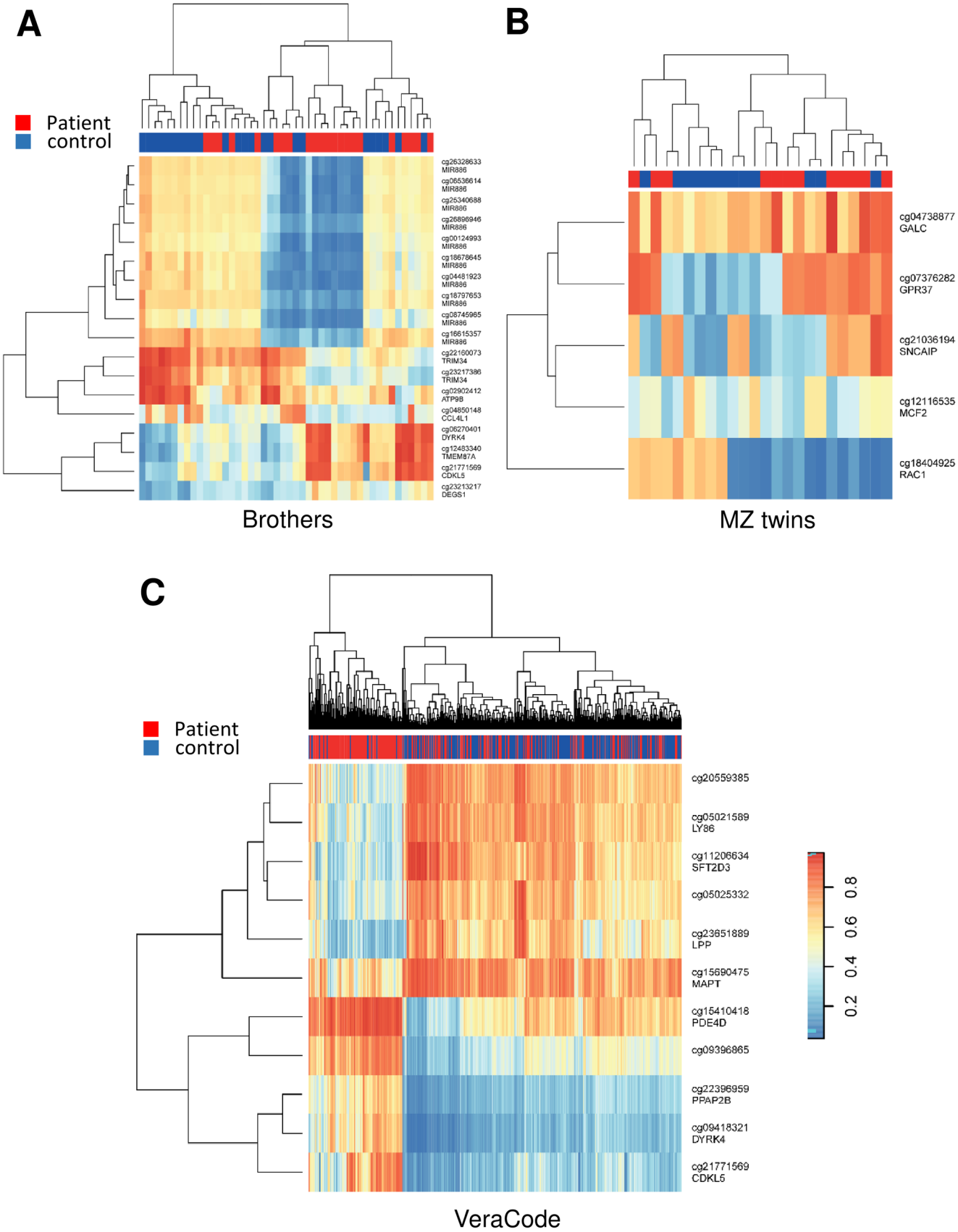
[e] Difference between mean beta value of control and PD group. Positive prefix indicates hypermethylation of Parkinson group. Negative prefix indicates hypomethylation of Parkinson group in comparison to control

CpGs hypermethylated in PD, microRNA 886 (*MIR886*) with 10 CpGs hypomethylated in PD and tripartite motif-containing 6 and tripartite motif-containing 34 (*TRIM6-TRIM34*) with 2 CpGs hypomethylated in PD together with another TRIM gene family member, *TRIM39*, which is hypermethylated in PD.

Among the genes with the largest difference (delta beta 22.4 %) in the degree of methylation (Table 3), we found the solute carrier family 25 (mitochondrial iron transporter), member 37 (*SLC25A37*) encoding mitoferrin-1, a major iron transporter that imports ferrous iron from the intermembrane space of the mitochondria for the synthesis of mitochondrial heme [21]. The MHC class II *HLA-DQA1* gene which encodes an essential cell surface receptor to form the DQ heterodimer was found hypermethylated in PD and cyclin-dependent kinase-like 5 (*CDKL5*) encoding serine/threonine kinase 9 (*STK9*) which is highly expressed in the brain, mainly in neurons [22].

The differential methylation of *MIR886* was previously identified in PD (and in Friedreich's ataxia), which prompted us to investigate this putative candidate gene in greater detail).

Fig. 2 Heat maps with hierarchical clustering of differential DNA methylation in PD vs. control. **a** Display of top CpGs in the comparison of male PD patients with their healthy brothers. **b** Display of top CpGs in the comparison of siblings of monozygotic twin pairs discordant for PD. **c** VeraCode cohort comparing PD and control CpGs with adjusted *p* value <0.01. DNA methylation (0–100 %) is visualized using a colour spectrum (*blue*, low methylation; *red*, high methylation). (Colour figure online) ▶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Patient
control
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cg18797653 MIR886
cg06745965 MIR886
cg16615357 MIR886
cg22160073 TRIM34
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Brothers
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cg04738877 GALC
cg07376282 GPR37
cg21036194 SNCAIP
cg12116535 MCF2
cg18404925 RAC1
MZ twins
C
Patient
control
cg20559385
cg05021589 LY86
cg11206634 SFT2D3
cg05025332
cg23651889 LPP
cg15690475 MAPT
cg15410418 PDE4D
cg09396865
cg22396959 PPAP2B
cg09418321 DYRK4
cg21771569 CDKL5
0.2
0.4
0.6
0.8
VeraCode

Displaying the individual methylation levels of *MIR886* of each proband, we observed a very homogeneous methylation state of all of the CpGs in a given individual, which was either low (i.e. 10 % methylation) or medium-high (i.e. 60 % methylation). Interestingly, the methylation of *MIR886* was either low or medium-high. The skewed distribution within our small screening cohort using the 450K array suggested a difference of PD vs. control (Fig. 3a). But when we investigated *MIR886* using pyrosequencing at the single-base level in a greater cohort, this was not confirmed, and the apparent difference between PD and control turned out to be random (see below and Fig. 3b).

Regression analysis

We investigated whether age, sex, disease state (Hoehn and Yahr stage) or PD subtype and other clinical items, such as L-DOPA intake and dosage, correlated with the observed differential methylation in the 64 candidate genes. Only in the PD group was DNA methylation in the gene body of *TRIM6_TRIM34* (cg 23217386) inversely correlated with age (R^2 0.22; p value of regression 0.42).

Epigenome-wide methylation analysis of MZ twins

The same conservative approach was used to compare discordant twins, resulting in a single hypermethylated gene, *GPR37* (NM_005302), encoding for an orphan G protein-coupled receptor for prosaposin, which is predominantly expressed in the nervous system and interacts with parkin [23]. Using a less stringent approach, we also found SNCA-interacting protein (*SNCAIP*) hypermethylated in PD and *PDE4D* hypomethylated in PD (Tables 5; Fig. 3c).

Regression analysis

Performing the same set of multivariate regression analyses, we found no correlation with age, sex, disease state (Hoehn and Yahr stage) or PD subtype or L-DOPA intake or dosage.

Analysis of leukocyte subsets in brothers and MZ twins

No significant difference concerning the leukocyte subsets was found comparing control and PD cases. The mean proportion of NK cells was higher in the control group without reaching the level of significance (Fig. 4).

Candidate gene methylation analysis with the Illumina *VeraCode* array

Candidates from the array data and additional candidate genes that had been identified previously in GWAS [2] were investigated using the *VeraCode* in cohort 1 (227 healthy individuals vs. 221 PD patients).

Fig. 3 Detailed evaluation of methylation patterns. **a** DNA methylation of *MIR886* analysed using the 450K methylation chip in PD brothers and their healthy siblings (n = 17 vs. 21). The 10 CpGs annotated on the chip are displayed for each individual. **b** DNA methylation of *MIR886* analysed using pyrosequencing in an independent cohort of individuals with PD compared with healthy individuals (n = 47 vs. 47). Methylation values show either low or medium-high values, independently from disease state. **c** The methylation of *PDE4D* is analysed using three different methods (VeraCode, 450K methylation chip, pyrosequencing) in different study cohorts. Interestingly, all methods demonstrated consistent hypermethylation of *PDED4* in Parkinson's disease. **d** Predicting Parkinson's disease via CpG signature. Evaluations of model predictions were made on the basis of the area under the ROC curve and AUC, indicating a predictive performance clearly above random chance level. The L1-penalized logistic regression model resulted in a signature of eight CpGs (*IRF8*, *DHX37*, *GPX1*, *PRF1*, *MAPT*, *PIP5K1A*) and CpGs not associated with a known protein such as cg05025332 and cg01747796. The weighted composition of these CpGs showed that cg09100014 (IRF8) had the greatest impact

PDE4D hypermethylation in PD was corroborated (corrected p value 1.47e−10) (Fig. 3c). In addition, several genes such as microtubule-associated protein tau (*MAPT*), *GPX4* and *GPX1*, which had been shown to be risk loci for PD in GWAS were detected significantly differentially methylated (2 CpGs of *MAPT*, with corrected p values of 2.8e−10 and 1.4e−06), *GPX4* (corrected p value 0.0001) and *GPX1* (2 CpGs, with corrected p values of 0.0003 and 0.0006) (Table 6).

Regression analysis

A multivariate regression analysis of age, sex, L-DOPA intake, PD subtype and Hoehn and Yahr stage revealed that the hypomethylation of GPX1 (cg02758552), which protects the haemoglobin in erythrocytes from oxidative breakdown, was correlated with control and PD females (control group: p value 0.0001, Pearson 0.48; PD group: p value 0.0001, Pearson 0.34). PD-related phenotypes such as PD subtype or Hoehn and Yahr stage were not relevant for the DNA methylation of any of the 96 CpG sites annotated on the VeraCode chip. The methylation of four of eight CpGs of *SNCA* annotated on the VeraCode array suggested a correlation with L-DOPA dosage in the PD group. Higher L-DOPA intake was associated with lower SNCA methylation (R^2 0.042–0.056).

MetaPWilcoxon test

Using all three cohorts for meta-analysis, we confirmed selected candidate genes like SFT2D3 (cg 11206634, p value 3.10e−15; cg08726863 p value 1.32e−14), TNFRSF17 (cg02368508, p value 1.36e−15), PDE4D (cg15410418, p value 4.49e−13), MAPT (cg15690475, p value 8.88e−15; cg20840174, p value 2.0e−06), DYRK4 (cg09418321, p value 3.11e−11), LY86 (cg10129493, p value 1.36e−10) and CD33 (cg10129493, p value 1.45e−05).

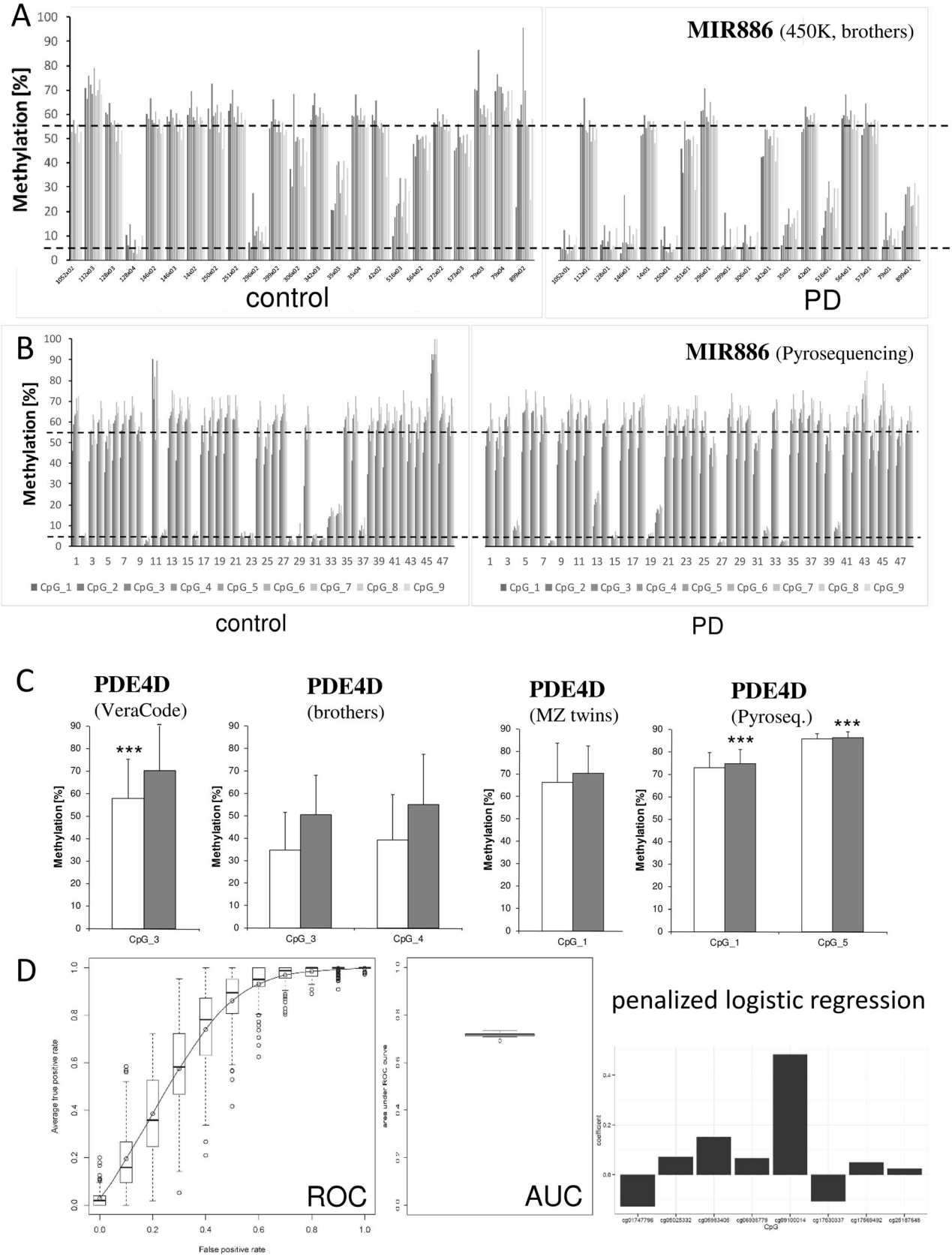

A
MIR886 (450K, brothers)
Methylation [%]
control
PD
B
MIR886 (Pyrosequencing)
Methylation [%]
CpG_1 CpG_2 CpG_3 CpG_4 CpG_5 CpG_6 CpG_7 CpG_8 CpG_9
control
PD
C
PDE4D (VeraCode)
PDE4D (brothers)
PDE4D (MZ twins)
PDE4D (Pyroseq.)
Methylation [%]
***
CpG_3
CpG_4
CpG_1
CpG_5
D
ROC
AUC
Average true positive rate
False positive rate
area under ROC curve
penalized logistic regression
coefficient
CpG

Table 5 Differentially methylated CpG sites in PD MZ twins and healthy siblings

Illumina ID[a]	Gene ID[b]	Chr.[c]	Mean β value[d] Parkinson	Mean β value[d] control	Difference[e] ($\Delta\beta$)
cg07376282	*GPR37*	7	69.80	44.21	+25.60
cg21036194	*SNCAIP*[f]	5	53.74	41.63	+12.11
cg04738877	*GALC*[f]	14	76.31	64.68	+11.63
cg18404925	*RAC1*[f]	7	27.19	38.67	−11.49
cg12116535	*MCF2*[f]	X	38.84	49.71	−10.88
cg18704047	*PDE4D*[f]	5	70.23	66.28	+3.95[f]

[a] Signature of CpG dinucleotide according to Illumina 450K microarray annotation, ranked by difference of mean methylations in PD and control ($\Delta\beta$)

[b] UCSC reference gene name. Overlap of PFC and HIP candidate genes are marked grey

[c] Chromosome

[d] Methylation beta value ranging from 0.0 to 1.0 (0–100 %)

[e] Difference between mean beta value of control and PD group. Positive prefix indicates hypermethylation of Parkinson group. Negative prefix indicates hypomethylation of Parkinson group in comparison to control

[f] Failed to fulfil the cut-off value of methylation difference >15 %

Identification of Parkinson's disease via CpG signature

We asked whether a subset of the 96 measured CpGs would not only discriminate but might also predict PD, i.e. could serve as biomarker signatures. For this purpose, we first transformed the beta values measured on the VeraCode array into M values, as previously suggested [24]. Based on these transformed data, we trained an L1-penalized logistic regression model [25] within a 10-times-repeated 10-fold cross-validation procedure. Briefly, we randomly split the whole dataset into 10 subsets (folds) and sequentially trained the penalized regression model on 9 of these subsets, leaving out one for testing, i.e. comparing predicted against known disease. Importantly, the regularization hyperparameter for the classification model was tuned in an inner 10-fold cross-validation procedure, resulting in a nested cross-validation. Evaluations of model predictions were based on the area under the ROC curve (AUC). The cross-validated AUC is defined as the AUC averaged over the 10 folds and ranges from 0 to 1, where 0.5 indicates chance level. The whole nested, 10-fold cross-validation procedure was repeated 10 times, and the distribution of the cross-validated AUC was depicted as a boxplot, indicating a prediction performance clearly above chance level (Fig. 3d). Furthermore, we plotted the full ROC curve of the classifier based on the predictions that were made on all 100 test sets that were considered during the repeated cross-validation procedure (Fig. 3d).

In the last step, we trained and optimized the L1-penalized logistic regression model on the whole dataset, resulting in a signature of eight CpGs (Fig. 3d). Those eight CpGs were *IRF8*, *DHX37*, *GPX1*, *PRF1*, *MAPT*, *PIP5K1A* and CpGs that were not associated with a known protein such as cg05025332 and cg01747796 according to Illumina's annotation on the array. Notably, not all of these CpGs showed a significant difference between PD and healthy controls; only the weighted composition of these CpGs (weights given by model coefficients are shown in Fig. 3d) resulted in a discriminative model.

Methylation analysis of *PDE4D*, *METRNL*, *NDUFA10* and *MIR886* with pyrosequencing

We found highly significant, but rather minor *PDE4D* hypermethylation of the two CpGs in PD (control 72.7 ± 6.9; PD 74.5 ± 6.5, *p* value 2.44e−05, ANOVA; and control 85.6 ± 2.4; PD 86.3 ± 2.4; *p* value 4.02e−06) (Fig. 3c).

Moreover, we replicated several other candidate genes identified in the 450K arrays with pyrosequencing. All three of the CpGs of *METRNL* that were analysed showed significant hypomethylation (*p* value <0.0001) in PD compared to the control (CpG 1 corresponding to cg21088259 of Illumina's 450K methylation chip: mean control 30.43 ± 10.40; mean PD 26.46 ± 9.58. CpG 2; not annotated on 450K chip: mean control 29.33 ± 9.72; mean PD 25.56 ± 9.31; CpG 3 not annotated on 450K chip: mean control 22.40 ± 8.07; mean PD 19.41 ± 7.81).

A multivariant regression analysis revealed no correlation between sex, age or L-DOPA dosage and *PDE4D* hypermethylation or *METRNL* hypomethylation (data not shown).

Several other candidate genes that were identified in the array experiments were not confirmed using pyrosequencing (*RAC1*, *DYRK4*) or, like *NDUFA10*, reached significance only at single CpGs (control 78.85 ± 3.71; PD 75.58 ± 3.39, *p* value 0.025, ANOVA).

Table 6 Differentially methylated CpG sites in PD and control (VeraCode)

Illumina ID[a]	Gene ID[b]	Chr.[c]	Mean β value[d] Parkinson	Mean β value[d] control	Difference[e] ($\Delta\beta$)	p value[g]
cg05025332	–	2	53.90	66.58	−11.31	5.74e−12
cg11206634	*SFT2D3*	2	54.91	70.33	−14.44	6.06e−12
cg02368508	*TNFRSF17*	16	87.79	92.74	−2.84	9.63e−11
cg15410418	*PDE4D*	5	70.12	57.81	+11.90	1.47e−10
cg15742700	*BLK*	8	81.98	89.48	−3.42	1.47e−10
cg09100014	*IRF8*	16	58.91	68.63	−6.75	1.47e−10
cg01747796	–	1	92.64	88.43	+3.67	2.19e−10
cg21331947	*CPNE5*	6	86.45	92.92	−3.61	2.60e−10
cg15690475	*MAPT*	17	67.46	77.35	−8.46	2.84e−10
cg05983405	*PRF1*	10	86.58	91.19	−3.46	4.30e−10
cg09396865	–	2	55.40	41.29	+10.94	4.46e−10
cg09418321	*DYRK4*	12	36.75	20.38	+9.52	1.80e−09
cg23651889	*LPP*	3	47.25	60.29	−12.20	2.77e−09
cg22396959	*PPAP2B*	1	38.16	24.77	+7.84	3.81e−09
cg21771569	*CDKL5*	X	41.75	26.96	+9.20	1.22e−08
cg05021589	*LY86*	6	58.00	70.68	−8.05	1.22e−08
cg12547959	*TRIO*	5	78.59	71.21	+7.72	1.22e−08
cg08726863	*SFT2D3*	2	82.22	88.11	−4.25	1.45e−08
cg20518096	*AKR1D1*	7	39.15	47.48	+6.68	3.58e−08
cg03722295	–	19	28.76	37.63	−10.16	5.06e−08
cg20559385	–	6	59.33	70.93	−6.68	1.02e−06
cg20840174	*MAPT*	17	74.90	68.77	+9.48	1.43e−06
cg00125455	*PCIF1*	20	34.54	39.35	−4.86	6.60e−06
cg10129493	*CD33*	19	14.57	11.53	+1.52	4.58e−05
cg01613691	*GPX4*	19	85.53	82.57	+3.97	0.0001
cg17530337	*DHX37*	12	75.07	66.20	+7.24	0.0002
cg05055782	*GPX1*	3	7.90	6.98	+0.49	0.0003
cg14341177	*BICD2*	9	92.84	91.17	+1.61	0.0003
cg25187648	*GPX1*	3	48.52	55.87	−3.79	0.0006
cg08767460	*SNCA*	4	20.28	17.54	+2.11	0.002
cg12015991	*HLA-DRB5*	6	77.12	69.28	+11.25	0.002
cg08218101	*BST1*	4	10.07	8.64	+1.13	0.002
cg10208370	*SNCA*	4	17.56	17.30	+1.87	0.01

[a] Signature of CpG dinucleotide according to Illumina 450K microarray annotation, ranked by difference of mean methylations in PD and control ($\Delta\beta$)

[b] UCSC reference gene name. Overlap of PFC and HIP candidate genes are marked grey

[c] Chromosome

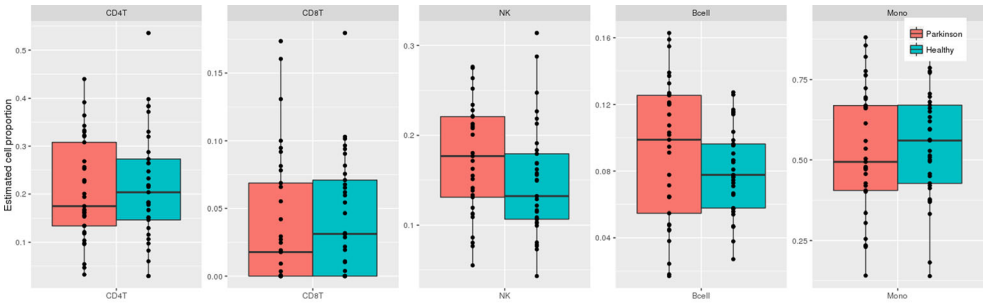
[d] Methylation beta value ranging from 0.0 to 1.0 (0–100 %)

[e] Difference between mean beta value of control and PD group. Positive prefix indicates hypermethylation of Parkinson group. Negative prefix indicates hypomethylation of Parkinson group in comparison to control

[f] Failed to fulfil the cut-off value of methylation difference >15 %

[g] Adjusted p value

We also analysed nine CpGs of *MIR886* using pyrosequencing, five of which were annotated on the 450K array. Interestingly, the DNA methylation of *MIR886* appeared only in two distinct, almost binary states of either low or medium-high levels in a given individual (Fig. 3b). This unique dichotomy was present in similar frequencies both in healthy individuals and in PD patients: the percentage of low vs. high *MIR886*-methylated individuals in the control group was 27 vs. 73 %, while that in the PD group was 21 vs. 79 %.



	CD4T	CD8T	NK	Bcell	Mono
Wilcoxon	0.685	0.574	0.07079	0.296	0.7376
t-test	0.7614	0.7047	0.0664	0.2189	0.5685

Fig. 4 Quantitative reconstruction of leukocyte subsets of brothers and MZ twins using DNA methylation. The estimated proportion of leukocyte subsets was unchanged comparing healthy individuals with Parkinson's disease patients. NK cells were higher in controls than in PD cases without reaching the level of significance

Discussion

We present here a comprehensive analysis of the methylation pattern in PD peripheral blood mononuclear cells comparing initially discordant sibling pairs (brothers) with MZ twin pairs and exploring the candidate genes in two larger independent cohorts. We found *PDE4D* significantly hypermethylated in PD in all cohorts, while the DNA methylation of *MIR886* was randomly distributed over groups. Furthermore, we found several known risk loci for PD such as *MAPT*, *GPX4* and *GPX1* identified in GWAS significantly differentially methylated.

Hypermethylation of PDE4D

We identified differentially methylated CpGs of several genes. Among these CpGs, the dysmethylation of various CpGs located in the 5′-UTR and intron 3 of the *PDE4D* gene was the main finding as it was consistently hypermethylated in all sample cohorts.

PDE4D is a member of the intracellular phosphodiesterase family (*PDE*s) and acts as an essential contributor to intracellular signalling [26]. *PDE4D* is inhibited by rolipram, a compound that attenuates 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP) neurotoxicity and dopaminergic cell death [27]. *PDE4* inhibition protects neurons against the α-synuclein-induced loss of synaptophysin and Aβ-induced synapse damage [28]. Caffeine is a broad-spectrum *PDE* inhibitor, and epidemiological data consistently showed that caffeine consumption reduces the risk of PD in Asian, US and European populations [29]. Possibly, coffee consumers modulate their PD risk not only by the action of caffeine on adenosine receptors but also by *PDE* inhibition.

Hypomethylation of *MAPT*

In our study, *MAPT* was significantly hypomethylated in PD at one CpG and hypermethylated in another CpG. The MAPT locus is a susceptibility gene for idiopathic PD in Caucasian studies [30]. The methylation of *MAPT* was not correlated with sex, age, Hoehn and Yahr stage or L-DOPA dosage in the PD group. In the control group, females had a slightly higher methylation (R^2 = 0.029, p value =0.032; multiple linear regression). This pattern has been observed by others at different CpG sites of *MAPT*, also [31] confirming our results.

Several genes are dysmethylated in PD

CDKL5 was hypermethylated in PD. *CDKL5* is highly expressed in the brain, mainly in neurons [32, 33]. The substrates of this kinase that have been identified so far are DNA methyltransferase 1 (*DNMT1*) and *MeCP2* [32, 34], indicating that *CDKL5* may epigenetically regulate targets.

In addition, several known risk loci for PD such as *MAPT*, *GPX4* and *GPX1* identified in GWAS studies were detected in our array as significantly differentially methylated, too. These findings are in line with a recent large-scale meta-analysis [2].

Hypermethylation of *GPR37*

GPR37 was hypermethylated in MZ twins only. This might suggest an acquired alteration. *GPR37*, also known as parkin-associated endothelin-like receptor (Pael-R) [35], is an orphan G protein-coupled receptor that is predominantly expressed in the nervous system, is found in Lewy bodies and accumulates in the brains of PD patients [36]. The proteins prosaptide and prosaposin bind to the *GPR37* receptor and protect primary

astrocytes against oxidative stress [37]. The observed hypermethylation of *GPR37* might weaken a neuroprotective component in PD patients.

MZ twins share nearly identical genetics, shared the same interuterine environment and shared early childhood period, but are exposed to different influences later on [38]. Epigenetic differences in MZ twins might thus reflect mainly late-life influences, whereas dizygotic brothers differ both in genetics *and* late-life influences, suggesting that the hypermethylation of *GPR37* represents environmentally induced changes that in turn are associated with PD.

Immune dysfunction

Neuroinflammation and immune dysfunction is a putative pathway that is involved in PD pathogenesis [39–41]. Interestingly, several candidate CpGs in our study were associated with immune functions, such as the MHC class II *HLA-DQA1*. On the other hand, *TRIM* proteins have been implicated in antiviral response and *TRIM34* has also been found to be up-regulated by interferons [42], while *TRIM6* mediates antiviral response [43] suggesting that PD patients display a decreased antiviral response capacity with ageing. *TRIM6-TRIM34* and *LY86*, when deficient, lead to increased susceptibility to viral infection [44]. *LY86* was found hypomethylated in PD in the gene body probably resulting in the down-regulation of the protein. *HLA-DQB1* (rs9275326) has been listed on the top risk loci for PD in GWAS [2], and here we observed the dysmethylation of a related HLA-gene, i.e. *HLA-DQA1*.

The observed dysmethylation of those immune-associated genes is expected to result in a reduced ability of antiviral defence in PD patients. One might speculate about the impact of viral infection and inflammation to induce PD, corresponding to the epidemiological data that the risk of PD is decreased in individuals who take non-steroidal anti-inflammatory drugs [45].

Dysmethylation and PD

Surprisingly, many of the identified differentially methylated genes played no known role in peripheral blood cells, but rather were expressed predominantly in the nervous system. Most strikingly, we found in twins' genes encoding proteins which interact with synuclein (*SNCA interacting protein*; *SNCAIP*) and parkin (*GPR37*) respectively, which are associated with familial Parkinson's disease. *SNCAIP* transcripts have been detected in many human tissues, particularly enriched in brain, including substantia nigra. Its protein, synphilin-1, contains several protein-protein interaction domains and has been reported to interact with alpha-synuclein in vivo [46].

The DNA methylation pattern may reflect a complex phenotype susceptibility. This susceptibility seems to be independent of a particular methylation level threshold, because there is no clear-cut methylation level that is associated with the disease in a single individual, and only mean values of the group are valid as separators. This extensive heterogeneity in each patient may lead to the conclusion that sporadic PD patients are unique in the causation of PD (if the dysmethylation contributes to the disease at all) or that a *combination* of several dysmethylated genes represents the risk factor.

The different methylation patterns in PD and controls are not caused by differences of leukocyte subsets, because CD4+ T, CD8+T, NK cells, B cells and monocytes were distributed equally in both groups. Only the proportion of NK cells was higher in controls than PD cases without significance.

Our study design cannot enlighten whether the identified methylation changes identified are causal or secondary to the disease. Methylation changes are not applicable as markers for the disease progression, because disappointingly none of the multivariate regressions showed a clear correlation between DNA methylation and the Hoehn and Yahr disease state.

In general, differences in DNA methylation show greater discordance in dizygotic twins than in monozygotic twins [47]. This may explain why we found many more differentially methylated CpGs in brothers than in our MZ twin cohort.

MIR886 is unchanged in PD

We also initially identified a microRNA (*MIR886* also termed *VTNRA2*), previously shown to be elevated in Friedreich's ataxia and PD [48, 49] as differentially methylated gene, when either the Illumina-based analyses were used or the *mean* methylation values were compared. In the brothers' cohort, 10 CpGs of *MIR886* were found hypomethylated in PD compared to the control, but in an independent and larger cohort, this result was not confirmed using pyrosequencing. Detailed analysis of the methylation levels revealed a clear-cut dichotomy in both brothers and MZ twins who displayed either low or medium-high methylation of all *MIR886* CpGs. The mean values of PD vs. control masked this noticeable distribution pattern. Therefore, the supposed hypomethylation or hypermethylation of *MIR886* in the blood of PD patients was probably based on the random recruitment of low- or high-methylated individuals, but is not related to the disease state, in contrast to the findings of Masliah [48].

Biomarker development

Finally, we asked whether differential methylation patterns in blood monocytes could be used for discrimination of cases and control. Using the penalized logistic regression model, we identified eight CpGs as classifiers with an AUC value

of 0.77, which performed better than an SNP-based ROC of a recent GWAS meta-analysis [2], but is not yet appropriate for clinical use. Both signatures combined may allow an even better discrimination of PD vs. healthy individuals.

Mashlia and Desplats also performed a comparative study of DNA methylation profiles of brain and blood in PD patients and healthy controls [49]. They observed changes in a number of genes which were associated to PD in the NextBio Disease Atlas and suggested the possibility that common risk factors for PD and age-associated changes may mediate their effects by methylation of specific genes. Although this study investigated only five PD patients with different pharmacological treatments, the data are important in two ways: the majority of CpGs were hypomethylated (at least 88 % of the analysed genes) and the overall methylation patterns of blood- and brain-derived DNA were similar, suggesting that blood holds the promise as a surrogate for brain tissue in the search for a biomarker of PD. Although absolute levels of DNA methylation at specific loci differed between brain and blood, Davies et al. [50] in a functional annotation of the brain methylome observed a correlation between blood and brain, too.

Conclusions

Our findings suggest that a distinct level of PD susceptibility stems from individual, epigenetic modifications of specific genes. Some of our top-ranked candidate genes (CpGs resp.) such as *PDE4D*, *MAPT* and *GPR37* have apparent biological relevance and/or are in line with previous findings of huge GWAS. This existing overlap with GWAS implies that the observed dysmethylation pattern can be partially linked to the aetiology of the disease.

In addition, although the individual effects of the methylation variants are rather small, we identified a signature of CpGs in blood cells to separate control from disease with a reasonable discriminatory power, holding promise for future epigenetically based biomarker development.

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Compliance with ethical standards The experiments were undertaken with the understanding and written consent of each subject. The study conforms to the World Medical Association Declaration of Helsinki. The Ethics Committee of the Medical Faculty of the University of Bonn approved this study (No. 51/00, 6 July 2000).

Conflict of interest The authors declare that they have no conflict of interest.

Authors' contributions OK conceived the study, collected MZ twin probes, participated in pyrosequencing, performed statistical analysis and interpretation of data and wrote the manuscript.

IS participated in the sequence alignment, designed primers and performed pyrosequencing.

JT performed statistical analysis and provided figures.

FB performed statistical analysis.

YL performed statistical analysis.

PH participated in the sequence alignment and helped to draft the manuscript.

SW collected the data and probes and performed the VeraCode array.

MR participated in the study design and helped to draft the manuscript.

VV collected MZ twin probes, contributed to data interpretation and helped to draft the manuscript.

HF performed statistical analysis and provided figures.

UW conceived the study, contributed to data interpretation and wrote the manuscript.

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Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse

Patrick O McGowan[1,2], Aya Sasaki[1,2], Ana C D'Alessio[3], Sergiy Dymov[3], Benoit Labonté[1,4], Moshe Szyf[2,3], Gustavo Turecki[1,4] & Michael J Meaney[1,2,5]

Maternal care influences hypothalamic-pituitary-adrenal (HPA) function in the rat through epigenetic programming of glucocorticoid receptor expression. In humans, childhood abuse alters HPA stress responses and increases the risk of suicide. We examined epigenetic differences in a neuron-specific glucocorticoid receptor (*NR3C1*) promoter between postmortem hippocampus obtained from suicide victims with a history of childhood abuse and those from either suicide victims with no childhood abuse or controls. We found decreased levels of glucocorticoid receptor mRNA, as well as mRNA transcripts bearing the glucocorticoid receptor 1_F splice variant and increased cytosine methylation of an *NR3C1* promoter. Patch-methylated *NR3C1* promoter constructs that mimicked the methylation state in samples from abused suicide victims showed decreased NGFI-A transcription factor binding and NGFI-A–inducible gene transcription. These findings translate previous results from rat to humans and suggest a common effect of parental care on the epigenetic regulation of hippocampal glucocorticoid receptor expression.

There are maternal effects on the development of individual differences in behavioral and HPA stress responses in rodents and nonhuman primates[1,2]. Maternal behavior alters the development of HPA responses to stress in the rat through tissue-specific effects on gene transcription[3,4], including forebrain glucocorticoid receptor expression, the activation of which inhibits HPA activity through negative-feedback inhibition[5]. Thus, selective knockdown of glucocorticoid receptor expression in the corticolimbic system in rodents is associated with increased HPA activity under both basal and stressful conditions[6,7]. Conversely, glucocorticoid receptor overexpression is associated with a dampened HPA stress response[8].

Familial function and childhood adversity are linked to altered HPA stress responses in humans, which are associated with an increased risk for multiple forms of psychopathology[9–11]. There is evidence for decreased hippocampal glucocorticoid receptor expression in several psychopathological conditions associated with suicide, including schizophrenia and mood disorders[12–14]. Suicide is also strongly associated with a history of childhood abuse and neglect, and this effect is independent of that associated with psychopathology[15,16]. Thus, environmental events that associate with decreased hippocampal glucocorticoid receptor expression and increased HPA activity enhance the risk of suicide.

The effects of maternal care on hippocampal glucocorticoid receptor expression, and therefore HPA responses to stress, in the adult rodent are associated with an epigenetic modification of a neuron-specific exon 1_7 glucocorticoid receptor (*Nr3c1*) promoter[4,17]. We attempted to translate these findings to humans. We examined glucocorticoid receptor expression and *NR3C1* promoter methylation in hippocampal samples obtained from suicide victims and control subjects who died suddenly of unrelated causes. The focus of our examination was the *NR3C1* (also known as $GR1_F$) promoter, the homolog of the exon 1_7 region in the rat[18], which is highly expressed in human hippocampus[19]. Suicide victims were either positive or negative for history of childhood abuse (sexual contact, severe physical abuse and/or severe neglect), allowing for the separation of the effects associated with childhood abuse from those associated with suicide *per se*. Our controls were all negative for a history of childhood abuse.

RESULTS

Hippocampal glucocorticoid receptor expression

The human glucocorticoid receptor gene *NR3C1* covers a region of more than 80 kb in chromosome 5, containing eight coding exons (exons 2–9) and alternative 5′ noncoding exon 1s[19–21]. The 5′ untranslated region (UTR) of exon 1 of the *NR3C1* gene determines the tissue-specific expression. The 5′ UTR of *NR3C1* contains 11 exon 1 splice variants, all of which bear unique splice donor sites and share a common exon 2 splice acceptor site[19]. Exon 1_F of *NR3C1* is similar to the rat exon 1_7, which reveals a maternal effect on cytosine methylation and expression[8,18,22]. Because individuals with severe forms of major depression show decreased glucocorticoid receptor

[1]Douglas Mental Health University Institute, 6875 LaSalle Boulevard, Montreal, Quebec, H4H 1R3, Canada. [2]Sackler Program for Epigenetics & Developmental Psychobiology, McGill University, 3655 Promenade Sir William Osler, Montreal, Quebec, H3G 1Y6, Canada. [3]Department of Pharmacology and Therapeutics, McGill University, 3655 Promenade Sir William Osler, Montreal, Quebec, H3G 1Y6, Canada. [4]McGill Group for Suicide Studies, 6875 LaSalle Boulevard, Douglas Mental Health University Institute, Montreal, Quebec, H4H 1R3, Canada. [5]Singapore Institute for Clinical Sciences, Brenner Centre for Molecular Medicine, 30 Medical Drive, Singapore 117609. Correspondence should be addressed to M.J.M. (michael.meaney@mcgill.ca), G.T. (gustavo.turecki@mcgill.ca) or M.S. (moshe.szyf@mcgill.ca).

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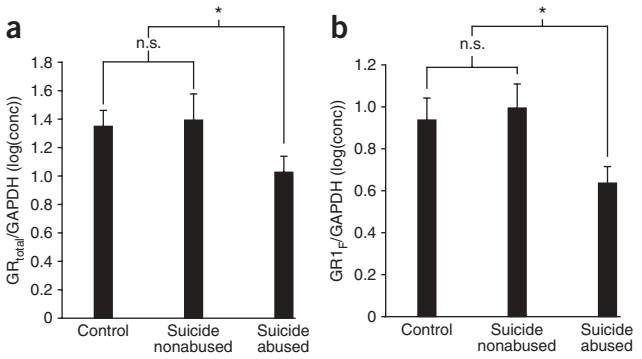

Figure 1 Hippocampal glucocorticoid receptor expression. (**a**,**b**) Mean ± s.e.m. expression levels of total glucocorticoid receptor (GR) mRNA (**a**) and glucocorticoid receptor 1_F (GR1_F) in 12 suicide victims with a history of childhood abuse, 12 nonabused suicide victims and 12 control subjects (**b**). Outliers excluded from analysis included $n = 2$ control subjects, $n = 1$ suicide victims with a history of childhood abuse for glucocorticoid receptor 1_F and an additional $n = 1$ suicide victim with a history of childhood abuse, and $n = 3$ nonabused suicide victims for overall levels of glucocorticoid receptor. * indicates $P < 0.05$; n.s. indicates not statistically significant.

expression and increased HPA activity, we hypothesized that suicide victims would show decreased expression both of glucocorticoid receptor and glucocorticoid receptor 1_F compared with control subjects.

We examined the expression of total glucocorticoid receptor and glucocorticoid receptor 1_F using quantitative reverse transcription PCR (qRT-PCR) with RNA extracted from hippocampal tissue of suicide completers with ($n = 12$) and without ($n = 12$) a history of childhood abuse and from controls ($n = 12$). There was a significant effect on glucocorticoid receptor expression ($F = 3.17$, $P = 0.05$). *Post hoc* tests showed that expression of total glucocorticoid receptor mRNA was significantly reduced in suicide victims with a history of childhood abuse relative to nonabused suicide victims or controls ($P < 0.05$); there was no difference between nonabused suicide victims and controls ($P > 0.05$; **Fig. 1a**). There was also a significant effect on the expression of transcripts containing the exon 1_F *NR3C1* promoter ($F = 3.58$, $P < 0.05$). *Post hoc* tests revealed that glucocorticoid receptor 1_F expression was significantly lower in samples from suicide victims with a history of childhood abuse compared with suicide victims without childhood abuse or controls ($P < 0.05$). Similar to the findings with total glucocorticoid receptor mRNA expression, there was no difference between nonabused suicide victims and controls ($P > 0.05$; **Fig. 1b**).

We examined the relationship between glucocorticoid receptor expression and psychiatric diagnoses (**Table 1**). Mood disorders and substance abuse disorders are risk factors for suicide and have been linked to alterations of glucocorticoid receptor expression[12]. There were no significant effects of psychopathology, even after controlling for childhood abuse status, on overall glucocorticoid receptor or glucocorticoid receptor 1_F expression ($P > 0.05$).

Genotyping and methylation analysis

Because alterations in glucocorticoid receptor 1_F activity could be derived from nucleotide sequence variation and/or epigenetic modifications, we sequenced the *NR3C1* promoter region from each subject. No sequence variation was seen among subjects and all of the sequences were identical to those published previously[19]. Moreover, for each subject, the genomic sequences targeted for binding by the primers used for bisulfite mapping were identical to the published sequence[19], thus eliminating potential primer bias between subjects in sodium bisulfite mapping.

The rat homolog of the exon 1_F *NR3C1* promoter, the exon 1_7 region, is differentially methylated as a function of variations in maternal care[4,17,22]. Cytosine methylation is a highly stable epigenetic mark that regulates gene expression via its effects on transcription factor binding[23,24]. We used sodium bisulfite mapping[25] to examine the methylation status of individual CpG dinucleotides in the *NR3C1* promoter sequence extracted from the hippocampal tissue of the same subjects used for glucocorticoid receptor expression analysis. Sodium bisulfite mapping revealed a significant effect on the percentage of methylated clones (that is, the number of clones with at least one methylated CpG site divided by the total number of clones) between groups ($F = 3.47$, $P < 0.05$). *Post hoc* tests revealed a significant difference between suicide victims with a history of childhood abuse compared with nonabused suicide victims ($P = 0.05$) or controls ($P < 0.05$). There was no difference in the percentage of methylated clones between suicide victims without childhood abuse and controls ($P > 0.05$; **Fig. 2a**). Methylation was limited to specific sites, with no clone showing global methylation (**Fig. 2b**). There were no significant correlations between levels of exon 1_F methylation and age at death ($r = 0.15$, $P > 0.05$), brain pH ($r = 0.08$, $P > 0.05$) or postmortem interval (PMI, $r = 0.24$, $P > 0.05$; **Table 1**).

Patch methylation of the *NR3C1* promoter

DNA methylation of a limited number of sites in the exon 1_F *NR3C1* promoter was associated with decreased expression of the glucocorticoid receptor 1_F variant and of total glucocorticoid receptor mRNA in suicide victims with a history of childhood abuse. Defining a causal relation between the methylation status and transcriptional efficiency of the *NR3C1* promoter is therefore of great importance. We hypothesized that DNA methylation regulates the expression of the *NR3C1* promoter through alterations in transcription factor binding. The transcription factor NGFI-A regulates the expression of *Nr3c1* promoter in the rat, an effect that is inhibited by DNA methylation[17]. To our knowledge, the regulation of NGFI-A (also known as Zif268, EGR1, Krox-24 and ZENK) has not been studied in the human hippocampus, although there is evidence that its expression is downregulated in the prefrontal cortex in schizophrenia[26]. The *NR3C1* promoter contains a number of canonical and noncanonical NGFI-A recognition elements (**Fig. 3a**). We wondered whether, as in the rat[17], NGFI-A could regulate gene transcription through the *NR3C1* promoter and whether this effect might be influenced by the methylation status of the promoter. We used a transient transfection assay in human HEK293 cells to examine transcriptional activity of a *NR3C1* promoter ligated to a promoter-less firefly luciferase expression vector (pGEM-LUC, Promega; **Fig. 3a**) in the presence or absence of ectopic NGFI-A

Table 1 Demographic characteristics and psychiatric diagnoses

	Abused suicide	Nonabused suicide	Control
Male/female	12/0	12/0	12/0
Age (years)	34.2 ± 10	33.8 ± 11	35.8 ± 12
PMI (h)	24.6 ± 5.8	39.0 ± 25.7	23.5 ± 6.0
pH	6.3 ± 0.24	6.5 ± 0.29	6.5 ± 0.22
Childhood abuse/neglect	12/0 (100%)	0/12 (0%)	0/12 (0%)
Mood disorder	8/12 (67%)	8/12 (67%)	0/12 (0%)
Alcohol/drug abuse disorder	9/12 (75%)	6/12 (50%)	5/12 (42%)

Data are presented as mean ± s.d.

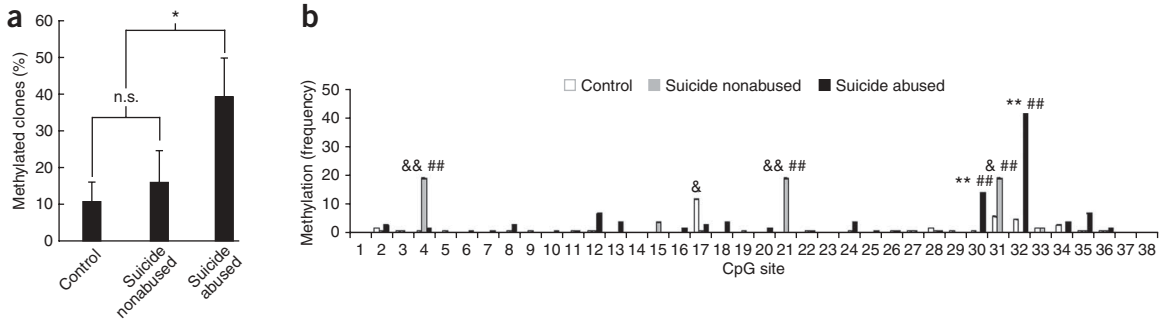

Figure 2 Methylation of the *NR3C1* promoter in the hippocampus. Twenty clones were sequenced for each subject for methylation mapping. (**a**) Mean ± s.e.m. percentage of methylated clones for suicide victims with a history of childhood abuse ($n = 12$), suicide victims without a history of childhood abuse ($n = 12$) and controls ($n = 12$). The methylation percentage was calculated as the number of clones with at least one methylated CpG site divided by the total number of clones (* indicates $P \leq 0.05$; n.s. indicates not statistically significant). (**b**) Methylation of the *NR3C1* promoter region, showing the frequency of methylation observed at each CpG site for suicide victims with a history of childhood abuse, suicide victims with no history of childhood abuse and control subjects (*$P < 0.05$, **$P < 0.001$, abused suicides versus controls; $^{\&}P < 0.05$, $^{\&\&}P < 0.001$, non-abused suicides versus controls; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$, abused suicides versus non-abused suicides; Bonferroni *post hoc* comparisons).

expression. The use of HEK293 cells allowed us to concurrently transfect a number of expression vectors with high efficiency. The absence of plasmid replication during the transient transfection assay precludes the loss of methylation via passive demethylation[27].

Luciferase expression was measured in the presence or absence of NGFI-A from the unmethylated *NR3C1* promoter plasmid compared with a methylated version. There was a significant effect of testing condition on the transcriptional activity of the exon 1_F *NR3C1* promoter ($F = 110.6$, $P < 0.0001$; **Fig. 3b**). *Post hoc* analysis revealed that the transcriptional activity of the unmethylated *NR3C1* promoter was significantly increased in the presence of the NGFI-A expression vector (*NR3C1*versus *NR3C1* + *EGR1*; $P < 0.0001$). Furthermore, methylation of the *NR3C1* promoter (the entire *NR3C1* construct was methylated *in vitro* and ligated to an unmethylated vector before transfection, *NR3C1-M*) reduced basal transcriptional activity of the *NR3C1* construct (*NR3C1* versus *NR3C1-M*, $P < 0.05$). Methylation of the *NR3C1* construct also blunted NGFI-A induction of transcription (*NR3C1* + *EGR1* versus *NR3C1-M* + *EGR1*, $P < 0.0001$).

These results indicate that methylation attenuates NGFI-A induction of gene expression through the *NR3C1* promoter. However, the decreased glucocorticoid receptor transcription observed in suicide victims with a history of childhood abuse was associated with differences in methylation levels occurring only at specific sites in the exon 1_F *NR3C1* promoter (**Fig. 2b**). An ANOVA examining the methylation of CpG dinucleotides across the exon 1_F *NR3C1* promoter revealed a significant effect of CpG site ($F = 13.86$, $P < 0.0001$), a significant effect of group ($F = 17.12$, $P < 0.0001$) and a significant interaction between CpG site and group ($F = 13.44$, $P < 0.0001$). In NGFI-A recognition elements, methylation was observed at CpG sites 12, 13, 30, 31 and 32 (**Fig. 2b**). We therefore examined whether such selective, site-specific differences in methylation could alter transcriptional activation through the *NR3C1* promoter. Two deletion constructs of the *NR3C1* promoter were generated in which selected CpG dinucleotides were patch-methylated (**Fig. 3a**). CpG sites 12 and 13 were methylated in the 255-bp construct, whereas the 125-bp promoter construct was methylated at CpG sites 30, 31 and 32. Thus, each deletion construct included at least one known or putative NGFI-A binding site[28].

We used patch methylation to examine whether selective methylation at specific sites reduces NGFI-A binding to and transactivation through the exon 1_F *NR3C1* promoter. We found an effect of methylation status on transcription factor–induced gene expression from the *NR3C1* promoter (**Fig. 3c**). For the 125-bp construct, there was a significant effect of methylation status ($F = 57.6$, $P < 0.0001$) and

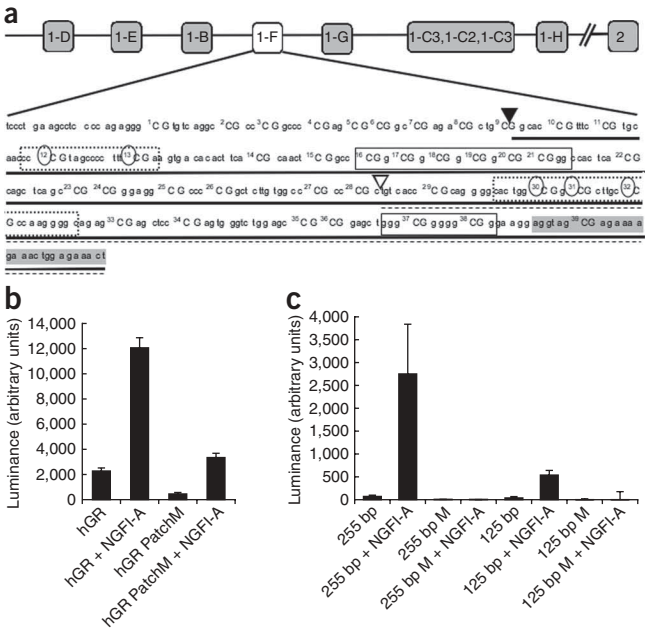

Figure 3 *In vitro* analysis of *NR3C1* promoter methylation. (**a**) The relative position of the *NR3C1* variant and the promoter sequence, showing the location of the CpG dinucleotides. The 255-bp (▼, solid underline) and 125-bp (▽, broken underline) deletion constructs are shown, along with specific CpG dinucleotides that were methylated in each deletion construct, as indicated by circles. Boxes represent known or putative canonical (solid-lined box) and noncanonical (broken-lined box) NGFI-A–binding sites, with the shaded area indicating the beginning of the exon. (**b**,**c**) Mean ± s.e.m. levels of luciferase expression in HEK293 cells. Results are shown after the subtraction of expression of the promoter in the antisense orientation. (**b**) The full *NR3C1* promoter was either unmethylated (hGR) or completely patch methylated (hGR PatchM) and transfected in the presence or absence of NGFI-A. (**c**) The 255-bp and 125-bp *NR3C1* deletion construct were either unmethylated (255 bp or 125 bp) or methylated (255 bp M or 125 bp M), as shown in **a**, and transfected either in the presence or absence of NGFI-A.

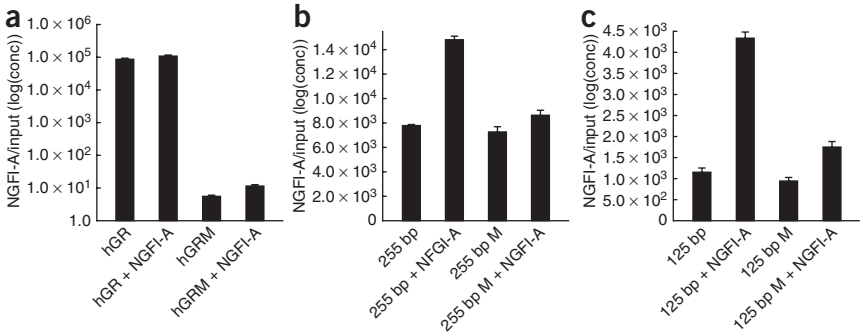

Figure 4 NGFI-A association with exon 1_F *NR3C1* promoter constructs. (**a**–**c**) Quantification of *NR3C1* promoter immunoprecipitated with NGFI-A antibody and normalized to input DNA for the full human *NR3C1* promoter (**a**), the 255-bp deletion construct (**b**) and the 125-bp deletion construct (**c**), each of which was either unmethylated or methylated and transfected in the presence or absence of NGFI-A. Data are presented as means ± s.e.m.

NGFI-A treatment ($F = 6.3$, $P < 0.05$). As predicted, there was also a significant interaction between methylation status and the NGFI-A expression ($F = 48.7$, $P < 0.0001$). *Post hoc* analysis of the 125-bp *NR3C1* promoter construct revealed that the effect of NGFI-A on gene transcription was significantly ($P < 0.001$) greater in the presence of the unmethylated rather than the patch-methylated *NR3C1* promoter construct. The same pattern of results was observed for the 255-bp *NR3C1* promoter construct. Thus, for the 255-bp *NR3C1* promoter construct, there was a significant effect on transcriptional activity of methylation status ($F = 555.4$, $P < 0.0001$) and NGFI-A expression ($F = 387.3$, $P < 0.0001$). There was also a significant interaction between methylation status and the presence of NGFI-A ($F = 489.1$, $P < 0.0001$). *Post hoc* analysis revealed a significantly ($P < 0.001$) greater effect of NGFI-A on gene transcription in the presence of the unmethylated compared with the patch-methylated 255-bp *NR3C1* promoter construct.

NGFI-A association with the *NR3C1* promoter

Site-selective differences in methylation of CpG regions in promoters can alter gene transcription by affecting transcription factor binding[24]. We carried out chromatin immunoprecipitation assays on samples that were obtained using the transfection model described above to examine the association of NGFI-A with the methylated and the nonmethylated *NR3C1* promoter constructs. The results revealed a significant effect of methylation status on NGFI-A association with the exon 1_F *NR3C1* promoter constructs transfected with *EGR1* ($F = 242.92$, $P < 0.0001$; **Fig. 4a**). *Post hoc* tests showed that, in comparison with the nonmethylated construct, patch-methylation inhibited NGFI-A binding to the exon 1_F *NR3C1* promoter ($P < 0.005$; **Fig. 4a**). For the 125-bp promoter construct, there was a significant effect of methylation status ($F = 102.28$, $P < 0.001$) and NGFI-A treatment ($F = 209.99$, $P < 0.0005$) and a significant interaction between methylation status and the presence of NGFI-A ($F = 72.71$, $P < 0.005$). Similarly, for the 255-bp promoter construct, there was a significant effect of methylation status ($F = 95.18$, $P < 0.001$) and NGFI-A treatment ($F = 152.13$, $P < 0.0005$) and a significant interaction between methylation status and the presence of NGFI-A ($F = 67.75$, $P < 0.005$). *Post hoc* testing revealed that, in comparison with the nonmethylated version, patch-methylation inhibited NGFI-A binding to either the 255-bp or 125-bp promoter constructs ($P < 0.01$; **Fig. 4b**,**c**). These findings suggest that the site-selective methylation of the exon 1_F *NR3C1* promoter construct, mimicking the differences observed between samples obtained from suicide victims with a history of childhood abuse, reduces NGFI-A binding and transcriptional activation through the exon 1_F *NR3C1* promoter.

DISCUSSION

Our findings indicate that hippocampal *NR3C1* gene expression is decreased in samples from suicide victims with a history of childhood abuse compared with controls (victims of sudden, accidental death with no history of abuse). In contrast, we found no differences in glucocorticoid receptor expression between suicide victims without a history of childhood abuse and controls. The pattern of results for hippocampal expression of the glucocorticoid receptor 1_F variant was identical to that of total glucocorticoid receptor expression. Our findings suggest that changes in glucocorticoid receptor expression are closely associated with a developmental history of familial adversity, in this case a history of childhood abuse, than with suicide completion. These results are also similar to those of earlier reports in which childhood abuse was associated with an increase in pituitary adrenocorticotropic hormone (ACTH) responses to stress among individuals with or without concurrent major depression[11]. These findings are particularly relevant, as pituitary ACTH directly reflects central activation of the HPA stress response and hippocampal glucocorticoid receptor activation dampens HPA activity.

Our findings are also consistent with those from studies with rodent and nonhuman primates showing that persistent disruptions of mother-infant interactions are associated with increased hypothalamic corticotrophin-releasing hormone expression and increased HPA responses to stress[1,2,29]. Variations in maternal care in the rat influence hippocampal glucocorticoid receptor expression, as well as methylation of the rat fetal calf serum *Nr3c1* promoter, the homolog of the human exon 1_F *NR3C1* promoter[3,4,17,22]. Hippocampal samples from suicide victims showed increased methylation of the exon 1_F *NR3C1* promoter in comparison with samples from controls, but only in cases with a history of childhood abuse. Neither hippocampal glucocorticoid receptor expression nor the methylation status of the exon 1_F *NR3C1* promoter was altered in suicide victims with no history of abuse. These findings suggest that variation in the methylation status of the exon 1_F *NR3C1* promoter, similar to that for glucocorticoid receptor 1_F and total glucocorticoid receptor mRNA expression, associates with childhood adversity and not with suicide *per se*.

A recent study of human cord blood found a correlation between maternal mood and neonatal methylation status of glucocorticoid receptor 1_F[30]. This study reported that increased site-specific methylation of an NGFI-A response element of glucocorticoid receptor 1_F is linked to an enhanced cortisol stress response in infants. Maternal mood disorders are associated with decreased maternal sensitivity and impaired mother-infant interactions[31], as well as with an increased risk for depression in the offspring[32]. Decreased hippocampal glucocorticoid receptor expression associates with depression[12], and psychotic and severe forms of depression are commonly associated with increased HPA activity[13,33]. Thus, our findings suggest that the transmission of vulnerability for depression from parent to offspring could occur, in part, through the epigenetic modification of genomic regions that are implicated in the regulation of stress responses.

One limitation of our design is the absence of samples from control subjects with a history of child abuse. Notably, child abuse predicts ACTH responses to stress[11]. However, the best predictor of HPA

responses to stress is the interaction between a developmental history of child abuse and stress in adulthood. One interpretation for such findings is that childhood adversity might alter the development of systems that serve to regulate stress responses, such as hippocampal glucocorticoid receptor expression, and thus enhance the effect of stress in adulthood and vulnerability for mood disorders[11]. Rodent models provide evidence for a direct effect of variations in forebrain glucocorticoid receptor expression and the behavioral characteristics of depression. Mice bearing a brain-specific glucocorticoid receptor knockdown show behavioral alterations that mimic some of the features of depression[6,7,34].

The data reported here are consistent with previous reports of alterations in cytosine methylation associated with psychopathology[35–37]. We found increased site-specific methylation of the exon 1_F *NR3C1* promoter in suicide victims with a history of childhood abuse (**Fig. 2**). Our transfection studies with constructs that replicated the *in vivo* methylation profiles indicated that there was a relationship between cytosine methylation, transcription factor binding and gene expression. Variations in maternal care in the rat produce differential methylation of the exon 1_7 *Nr3c1* promoter, the rat homolog of the exon 1_F *NR3C1* promoter, which regulates hippocampal glucocorticoid receptor expression[4,17,22] and HPA responses to stress[3,4]. Increased CpG methylation of the *Nr3c1* promoter decreased NGFI-A binding and reduced hippocampal glucocorticoid receptor expression. Manipulations that reversed the differences in exon 1_7 methylation eliminated the maternal effect on NGFI-A binding, glucocorticoid receptor expression and HPA activity[4,22]. Likewise, our *in vitro* data reveal that differential methylation of the human *NR3C1* promoter altered NGFI-A binding and NGFI-A–induced gene transcription. These findings suggest that selective differences in methylation status at certain sites affect transcription factor binding and gene expression.

Because cytosine methylation is a highly stable, the differences in CpG methylation are unlikely to be a consequence of events immediately preceding death or during the postmortem period. Therefore, changes in brain pH do not affect DNA methylation[38]. The PMI did not differ between the groups and was uncorrelated with the methylation of the *NR3C1* promoter. The intermittent pattern of methylation and the fact that only a portion of the neuronal population was methylated in each subject is consistent with a model in which alterations in methylation occur at later stages in development, after the completion of embryogenesis and neuronal differentiation. Indeed, the maternal effect on the methylation status of the exon 1_7 *Nr3c1* promoter in the rat occurs during early postnatal life[4].

There are precedents for the apparent developmental origins for the observed differences in DNA methylation and glucocorticoid receptor expression. Childhood abuse in humans is associated with altered hippocampal development[39], enhanced HPA activity[9,11] and an increased risk for psychopathology[15,16]. Similarly, children exposed to childhood adversity are more likely to engage in suicidal behavior[40,41]. Variations in the parental care of children are linked with individual differences in HPA and sympathetic and central catecholamine responses to stress[1,11,42,43]. Interventions that target parental care of high-risk children alter HPA activity[44]. Thus, it is tempting to speculate that epigenetic processes might mediate the effects of the social environment during childhood on hippocampal gene expression and that stable epigenetic marks such as DNA methylation might then persist into adulthood and influence the vulnerability for psychopathology through effects on intermediate levels of function, such as HPA activity.

In summary, our data reveal increased site-specific methylation in the exon 1_F *NR3C1* promoter in suicide victims with a history of childhood abuse and suggest that there is a relationship between cytosine methylation, transcription factor binding and gene expression. Our results are consistent with evidence from studies using psychological autopsy methods[40] and epidemiological longitudinal designs[45], which suggest that suicide has a developmental origin. We acknowledge that such conclusions are based on samples that differ along a wide range of experiential and potentially genetic dimensions. Our data certainly do not exclude alternative mechanisms of vulnerability. Indeed, the challenge for the future is to understand how epigenetic variation overlaying that occurring in nucleotide sequence might explain the developmental origins of vulnerability for chronic illness. Our data are merely consistent with observations from animal studies investigating epigenetic regulation of the *Nr3c1* gene and with the hypothesis that early life events can alter the epigenetic state of relevant genomic regions, the expression of which may contribute to individual differences in the risk for psychopathology.

METHODS

Postmortem sample preparation and subject characteristics. We used hippocampal samples from the Quebec Suicide Brain Bank (http://www.douglasrecherche.qc.ca/suicide), which included 12 suicide victims with a history of childhood abuse, 12 suicide victims with a negative history of childhood abuse (matched for psychiatric diagnoses) and 12 controls. All subjects were matched for PMI, gender and age (**Table 1**). Samples were processed as described previously[46] and consisted of hippocampus tissue from male suicides and control subjects of French-Canadian origin, dissected at 4 °C and stored at –80 °C. All subjects died suddenly with no medical or paramedical intervention. Suicides were determined by the Quebec Coroner's Office and the control subjects were individuals who had died suddenly from causes other than suicide. Homogenates of tissue samples were used for genomic DNA (DNeasy, Qiagen) and RNA (Trizol, Invitrogen) extraction. Possible confounds that were examined included PMI, brain pH and the age of the donor at death. Samples were processed and analyzed blindly with respect to demographic and diagnostic variables. Signed informed consent was obtained from next of kin.

Psychological autopsies. Psychiatric diagnoses were obtained using Structured Clinical Interviews for DSM-III-R I (ref. 47) adapted for psychological autopsies, which is a validated method for reconstructing psychiatric history by means of extensive proxy-based interviews, as described elsewhere[48]. History of severe childhood sexual and/or physical abuse or severe neglect was determined by means of structured interviews using the Childhood Experience of Care and Abuse[49] questionnaire adapted for psychological autopsies[45].

Genotyping. Genomic DNA was extracted (DNeasy, Qiagen) according to the manufacturer's protocol. For PCR, we used a 5′-GGG TTC TGC TTT GCA ACT TC-3′ sense primer and a 5′-CCT TTT TCC TGG GGA GTT G-3′ antisense primer that were directed to the *NR3C1* promoter (Genebank accession number AY436590). Primers were selected that covered a 536-bp region that included the region for sodium bisulfite analyses. The resulting PCR products for each subject were sequenced bidirectionally using the forward and the reverse primer on an ABI 3100 genetic analyzer (Applied Biosystems) following the manufacturer's instructions. Genetic variation was assessed throughout the *NR3C1* promoter region used for bisulfite analysis by alignment of genomic DNA with the previously published *NR3C1* promoter sequence[19] using freely available software (CLC Workbench, CLC bio).

Methylation mapping and expression analyses. Sodium bisulfite mapping was performed as previously described[25,50] for 12 suicide victims with a history of childhood abuse, 12 suicide victims with a negative history of childhood abuse and 12 controls. Individual clones were extracted and sequenced (Cequation (8800), Beckman-Coulter). We obtained 20 clones per subject for sequencing from 2–3 independent PCR reactions.

RNA extraction was performed using Trizol (Invitrogen) and was followed by Dnase I treatment, and cDNA conversion was performed using oligo(dT) primers (Invitrogen) according to manufacturers instructions (Roche Molecular Biochemicals). The same subjects used for methylation analysis were studied

for expression analyses using quantitative RT-PCR. Outliers with expression values that differed more than 1.5 s.d. from the mean were excluded from analysis ($n = 2$ control subjects, $n = 1$ suicide victims with a history of childhood abuse for glucocorticoid receptor 1_F and $n = 2$ control subjects, $n = 2$ suicide victims with a history of childhood abuse and $n = 3$ nonabused suicide victims for overall levels of glucocorticoid receptor; see **Supplementary Methods** online).

HEK293 cell cultures and transient transfection assays. To prepare and transfect (and see ref. 17) the unmethylated and methylated plasmids, we subjected the exon 1_F *NR3C1* promoter to PCR amplification and cloned the resulting PCR product into a PCR2.1 plasmid (Original TA cloning kit, Invitrogen). The *NR3C1* promoter was then ligated into the PCR2.1 plasmid. For patch methylation, the glucocorticoid receptor 1_F plasmid was incubated (2 h, 37 °C) with SssI DNA methyltransferase (20 U, New England Biolabs) in a buffer containing S-adenosylmethionine, and this procedure was repeated until full protection from *Hpa*II digestion was observed. Following digestion with *Hin*dIII and EcoRV restriction enzymes, each fragment was then ligated into a pGEM-luc vector (Promega) at the *Hin*dIII and *Bam*HI or XbaI and *Bam*HI sites in the 5′ to 3′ (sense) or 3′ to 5′ (antisense) orientation, respectively. The concentration of each ligation product was determined by fractionation on a 1.5% agarose gel, by comparing bands of the expected ligation product size against a standard curve of known DNA concentration (ten fivefold serial dilutions of 2 $\mu g/\mu l^{-1}$ micrococal nuclease DNA) to control for possible unequal efficiency of ligation and to ensure that equal amounts of correctly ligated plasmids were used in the transfections. The ligated plasmid was directly transfected into HEK293 cells and was not subcloned to avoid loss of methyl groups from CG dinucleotides during growth in *E. coli*, which do not express CG DNA methyltransferases. For deletion constructs of the exon 1_F *NR3C1* promoter plasmids were prepared and ligations verified in the same manner as described above, except that oligonucleotides for *NR3C1* promoter amplification were designed that incorporated *Hin*dIII and EcoRV restriction sites, obviating the need for ligation into PCR2.1 vector before ligation into the pGEM-luc vector (also see **Supplementary Methods**).

For the NGFI-A plasmid, we subcloned the *EGR1* coding sequence into a TOPO–His expression vector (pEF6/V5-His TOPO TA Expression kit, Invitrogen)[4]. In co-transfection studies, human embryonic kidney HEK293 cells were plated at a density of 6×10^4 in six-well dishes and transiently co-transfected with a total amount of 1.5 μg of plasmid DNA (1.0 μg of *NR3C1* promoter ligated into the pGEM-luc plasmid and 0.5 μg of NGFI-A expression plasmid or 0.5 μg of control pEF6/V5 plasmid) using the calcium phosphate method. HEK293 cells were maintained as a monolayer in DMEM (Invitrogen) containing 10% fetal calf serum (Colorado Serum Company). The cells were harvested 72 h after transfection and lysed, and luciferase activity was assayed using the Luciferase Assay System (Promega) according to the manufacturer's protocol.

Chromatin immunoprecipitation assay for NGFI-A. We carried out chromatin immunoprecipitation assays[30] by postfixing cells in 37% formaldehyde and then pelleted them before lysis and sonication. We reserved one tenth of the sample as 'input' to quantify the amount of DNA before immunoprecipitation. For the remainder of each sample, extracted chomatin was immunoprecipitated using rabbit polyclonal antibody to NGFI-A (antibody) or normal rabbit IgG (nonspecific; both from Santa Cruz Biotechnology). All of the DNA was then uncrosslinked and subjected to qRT-PCR, using primers directed at the luciferase gene immediately downstream of the transfected *NR3C1* promoter (sense, 5′-AGA GAT ACG CCC TGG TTC C-3′; antisense, 5′-CCA ACA CCG GCA TAA AGA A-3′; $T_m = 54$ °C). The signal for each sample was calculated by dividing the value of the antibody by the input. Each resulting value was multiplied by a constant (1×10^6) to plot the values obtained from the experiments on logarithmic axes.

Statistical analyses. Statistical analyses were conducted using Statview or JMP 7 (SAS Institute). Data are presented as mean ± s.e.m. For DNA methylation analysis, the percentage of methylated clones for each subject was tabulated by dividing the number of clones with at least one methylated CpG site by the total number of clones. A factorial ANOVA was used to compare the percentage of methylated clones for each subject as the dependent variable and group (suicide abused, suicide nonabused or control) as the between groups factor. To examine differential methylation across CpG sites methylated *in vitro*, we compared the total number of methylated CpG sites across the *NR3C1* promoter ($n = 33$) for all clones per group (that is, 12 subjects × 20 clones = 240 clones per group) using ANOVA followed by Bonferroni corrected *post hoc* comparisons. For RNA expression analysis, ANOVA followed by PLSD *post hoc* tests were used to examine differences between the suicide victim and control group. Unpaired *t* tests were used to compare groups of subjects with different clinical diagnoses (for example, subjects with mood disorders versus subjects without mood disorders). The relationships between DNA methylation, expression, age at death, PMI and brain pH were analyzed using linear regression analysis. Factorial ANOVA were used for *in vitro* analyses of *NR3C1* promoter, followed by *post hoc* analyses. Statistical significance was determined at $P \leq 0.05$ and analyses included type 1 error correction for multiple comparisons where applicable.

Note: Supplementary information is available on the Nature Neuroscience website.

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Folic Acid Supplementation during the Juvenile-Pubertal Period in Rats Modifies the Phenotype and Epigenotype Induced by Prenatal Nutrition[1–3]

Graham C. Burdge,[4]* Karen A. Lillycrop,[5] Emma S. Phillips,[6] Joanne L. Slater-Jefferies,[6] Alan A. Jackson,[4] and Mark A. Hanson[6]

[4]Institute of Human Nutrition, [5]Development and Cell Biology, and [6]Institute of Developmental Sciences, University of Southampton, Southampton SO16 6YD, UK

Abstract

Prenatal nutritional constraint is associated with increased risk of metabolic dysregulation in adulthood contingent on adult diet. In rats, folic acid supplementation of a protein-restricted (PR) diet during pregnancy prevents altered phenotype and epigenotype in the offspring induced by the PR diet. We hypothesized that increasing folic acid intake during the juvenile-pubertal (JP) period would reverse the effects of a maternal PR diet on the offspring. Rats were fed a control (C) or PR diet during pregnancy and AIN93G during lactation. Offspring were weaned on d 28 onto diets containing 1 mg [adequate folate (AF)] or 5 mg [folic acid-supplemented (FS)] folic acid/kg feed. After 28 d, all offspring were fed a high-fat (18% wt:wt) diet and killed on d 84. As expected, offspring of PR dams fed the AF diet had increased fasting plasma triglyceride (TAG) and β-hydroxybutyrate (βHB) concentrations. The FS diet induced increased weight gain, a lower plasma βHB concentration, and increased hepatic and plasma TAG concentration compared with AF offspring irrespective of maternal diet. PPARα and glucocorticoid receptor promoter methylation increased in liver and insulin receptor promoter methylation decreased in liver and adipose tissue in FS compared with AF offspring, with reciprocal changes in mRNA expression irrespective of maternal diet. These findings show that increased folic acid intake during the JP period did not simply reverse the phenotype induced by the maternal diet. This may represent a period of plasticity when specific nutrient intakes may alter the phenotype of the offspring through epigenetic changes in specific genes. J. Nutr. 139: 1054–1060, 2009.

Introduction

Environmental constraint during development, including undernutrition, is causally associated with increased risk of metabolic disease (1). It has been suggested that the developing fetus responds to environmental cues via developmental plasticity in a manner that sets the phenotype in prediction of the future environment, conferring a Darwinian fitness advantage (2). In humans, mismatch between the predicted and future environments has been suggested to underlie the early life origins of metabolic disease (2). Several animal models show that suboptimal nutrition during critical periods in early development induces metabolic dysregulation that resembles human cardiometabolic disease, especially if the offspring are fed a high-fat diet (3,4). In humans and in animal models, the nature of the phenotype induced by maternal dietary constraint differs according the developmental stage of the fetus at the time of exposure (5,6). Thus, the response of individual tissues to nutrient constraint is dependent upon the stage of maturation at the time of exposure.

Moderate maternal dietary protein restriction in rats is a well-established model of induction of an altered phenotype in the offspring (7). The offspring are characterized by persistent hypertension (7,8), dyslipidemia, and impaired glucose metabolism (4). Supplementation of the maternal protein-restricted (PR)[7] diet with folic acid or glycine prevents induction of

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[3] Supplemental Tables 1 and 2, and Supplemental Figure 1 are available with the online posting of this paper at jn.nutrition.org.
* To whom correspondence should be addressed. E-mail: g.c.burdge@southampton.ac.uk

[7] Abbreviations used: AF, folic acid-adequate; AOX, acyl-CoA carboxylase; βHB, β-hydroxybutyrate; C, control group; CPT-1, carnitine palmitoyl transferase-1; C/AF, control/adequate folate; C/FS, control/folate supplemented; Dnmt, DNA methyltransferase; FS, folic acid-supplemented; GR, glucocorticoid receptor; HSL, hormone-sensitive lipase; IR, insulin receptor; JP, juvenile-pubertal; LPL, lipoprotein lipase; NEFA, nonesterified fatty acid; PEPCK, phosphoenolpyruvate carboxykinase; pn, postnatal; PR, protein-restricted, PR/AF, protein-restricted/adequate folate; PR/FS, protein-restricted/folate supplemented; TAG, triglyceride.

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hypertension and endothelial dysfunction in the offspring (9,10), indicating that 1-carbon metabolism is central to the mechanism underlying induction of an altered phenotype. However, increasing the folic acid content of a protein-sufficient maternal diet also induced dyslipidemia and hyperglycemia in the offspring (11). This suggests that the nature of the induced phenotype is contingent upon the nutrient balance of the maternal diet.

Persistent changes in metabolism imply stable alterations to gene transcription. Epigenetic regulation of individual genes during specific periods in the developmental program, primarily by modification of the DNA methylation status of specific cytosines in CpG dinucleotides within their 5′-regulatory regions confers such stable changes in the level of transcription (12). However, stability of the epigenome is reduced during specific periods during the life course that are associated with more intensive changes in tissue function, namely prenatal and neonatal development, puberty, and aging (13). In rodents, manipulation of maternal dietary intakes of folic acid and methyl donors (14), differences in maternal nursing behavior (15), and constricted uterine blood flow (16) induce an altered phenotype in the offspring. We have shown that induction of an altered phenotype by a maternal PR diet during pregnancy involves changes in the epigenetic regulation by DNA methylation (17–20) and by covalent modifications of histones of specific genes, including the glucocorticoid receptor (GR) and PPARα in the liver of juvenile (17) and adult offspring (18). Such epigenetic changes are associated with altered mRNA expression of these genes and of their target genes. Induction of hypomethylation of GR involves downregulation of DNA methyltransferase (Dnmt)-1 mRNA expression and reduced binding to the $GR1_{10}$ promoter (20). This suggests that hypomethylation of the PPARα and GR promoters may involve progressive loss of methyl groups from CpG dinucleotides following mitosis rather than active demethylation (18). These epigenetic changes and altered Dnmt-1 expression were prevented by increasing the folic acid content of the maternal PR diet (17–19).

There is some information that changes in phenotype induced by nutritional constraint during early life can be reversed by subsequent interventions. Vickers et al. (21) have shown that leptin administration to neonatal rats reversed metabolic dysregulation induced by global maternal undernutrition during pregnancy. It is not known whether phenotypes induced by nutritional constraint during pregnancy can be reversed by dietary manipulation after the neonatal period. Folic acid supplementation in pubertal (22) and aging (23) rats altered hepatic whole-genome DNA methylation, although the effect on the epigenetic regulation of individual genes was not reported. Because the stability of the epigenome is decreased during the pubertal period (13), interventions during this time may have the potential to modify phenotypes induced in early life and so change life-long risk of disease. We therefore, tested in rats the hypothesis that increasing folic acid intake of the offspring during their juvenile-pubertal (JP) period could reverse the phenotype and epigenotype induced by the maternal PR diet. Puberty occurs at about postnatal (pn) d 40 in male rats (24) and d 35 in female rats (25). Offspring of control (C) or PR dams were fed a folic acid-adequate (AF) or -supplemented (FS) diet from weaning on d 28 until d 56. Because increased fat intake after weaning exasperates the effect of the maternal PR diet on fat and glucose metabolism (11), offspring were then challenged with a high-fat diet for a further 28 d. We measured the effect of the maternal and JP diets on growth and on markers of lipid and glucose metabolism and on the epigenetic regulation of candidate genes in liver, skeletal muscle, and adipose tissue.

Materials and Methods

Rats and diets. The study was carried out in accordance with the Home Office Animals (Scientific Procedures) Act (1986) and approved by internal ethical review. Virgin female Wistar rats (body weight 200–250 g) were mated and fed either C or PR isocaloric diets (**Supplemental Table 1**) (n = 8 per dietary group) from conception to spontaneous delivery on approximately d 21. Dams were fed the AIN93G semipurified diet (Supplemental Table 1) from delivery until the offspring were weaned on pn d 28. Litters were reduced to 8 (approximately equal males and females) within 24 h of delivery. At weaning, offspring were randomly assigned to an AF (1 mg/kg feed) or FS (5 mg/kg feed) diet (Supplemental Table 1) and were maintained on these diets until pn d 56. This produced 4 dietary groups (maternal diet/JP diet); C/AF, PR/AF, C/FS, and PR/FS. The increment in folate intake was comparable to that currently recommended for women in the UK before pregnancy and during the first trimester (26). Offspring were then fed a high-fat diet (Supplemental Table 1) for a further 28 d and were killed on pn d 84. Body weights were recorded at 7-d intervals throughout the postweaning period. Offspring were food deprived for 6 h before they were killed by CO_2 asphyxiation at ~1400 h. Liver, mesenteric adipose tissue, and biceps femoris muscle from the posterior right leg were collected and frozen immediately in liquid nitrogen and stored at −80°C. Blood was collected by cardiac puncture into tubes containing lithium heparin, and plasma was separated from cells by centrifugation and stored at −20°C. Samples from 10 male or female rats in each dietary group were selected for measurements of metabolite concentrations in plasma and for molecular biology analysis.

Measurement of metabolites in plasma. Plasma triglyceride (TAG), nonesterified fatty acid (NEFA), β-hydroxybutyrate (βHB), cholesterol, and glucose concentrations were measured using a Konelab 20 autoanalyzer (27).

Measurement of TAG concentration in liver. Liver TAG concentration was measured by GC (28,29) using triheptadecanoin as internal standard on a 6890 gas chromatograph (Agilent) equipped with a 30-m × 0.25-μm × 0.25-mm BPX-70 fused silica capillary column and flame ionization detection.

Measurement of mRNA expression by real-time RT-PCR. Measurement of the levels of specific mRNA transcripts was carried out using the primers listed (**Supplemental Table 2**). Total RNA was isolated from liver, adipose tissue, and skeletal muscle using Tri Reagent (Sigma) according to the manufacturer's instructions. cDNA was prepared and amplified using real-time RT-PCR (18,30). Samples were analyzed in duplicate and the expression of the individual transcripts were normalized to tissue-specific housekeeping genes, which did not differ in transcript level between groups of offspring (Supplemental Table 2) (18,31).

Measurement of DNA methylation of specific genes by real-time PCR. DNA methylation was conducted using the PCR primers listed (Supplemental Table 2). Genomic DNA was isolated from liver and muscle as described (17) and from adipose tissue using the Wizard SV Genomic DNA Purification system (Promega). Purified DNA was incubated with the methylation-sensitive restriction endonucleases *Aci*I and *Hpa*II according to the manufacturer's instructions (New England Biolabs) (17,18). The resulting DNA was amplified in duplicate using real-time PCR. A region of the PPARγ2 promoter that does not contain *Aci*I or *Hpa*II cleavage sites was used as an internal control (17,18).

Statistical analysis. Data are presented as mean ± 1 SD. Because the PCR measurements of samples from male and female offspring could not be compared directly, all other outcome variables were analyzed separately for males and females. The effect of the different dietary

regimens on change in body weight over time was assessed using a general linear model with time as a repeated measure and maternal diet and JP diet as fixed factors with weight at weaning as a covariate. Post hoc comparisons were by Bonferroni's method. Comparisons between groups of the effect of different dietary regimens were by a general linear model with maternal diet and JP diet as fixed factors and Bonferroni's post hoc correction for multiple comparisons.

Results

Growth after weaning. Groups of offspring did not differ in body weight at weaning (data not shown). There was an interaction between time and JP diet in male and female offspring (both $P < 0.0001$) but no interaction between time and maternal diet or interaction between time, maternal diet, and JP diet. Weight gain did not differ between C/AF and PR/AF offspring or between C/FS and PR/FS male or female offspring. In males, weight gain was greater in C/FS offspring from pn d 63 and PR/FS offspring from pn d 70 than in C/AF offspring, although body weight tended to be higher ($P < 0.1$) in both C/FS and PR/FS offspring than in C/AF or PR/AF offspring from pn d 49 (**Supplemental Fig. 1**). The mean weight gain at pn d 84 of the C/FS group was 54 g higher than the C/AF group and the PR/FS group was 31 g more than the PR/AF group (Supplemental Fig. 1). In females, weight gain was significantly greater in C/FS than in C/AF offspring from pn d 35 and in PR/FS than in PR/AF offspring from pn d 42 (Supplemental Fig. 1). The mean weight gain at pn d 84 of the C/FS group was 35 g higher than the C/AF group and the PR/FS group was 37 g more than the PR/AF group (Supplemental Fig. 1).

Liver weight and hepatic TAG concentration. The JP diet affected liver weight and liver weight as a proportion of body weight in male offspring but not in females (**Table 1**). There was no effect of maternal diet or interaction between maternal and JP diet in either males or females. The livers of male C/FS and PR/FS offspring were significantly heavier (~25%) and weighed more as a proportion of body weight (~20%) than in C/AF and PR/AF offspring. The JP diet affected liver TAG concentration in males and females, but there was no effect of maternal diet or interaction between maternal diet and JP diet (Table 1). In males and females, liver TAG concentrations were greater in C/FS than C/AF offspring and in the PR/FS than PR/AF offspring.

Concentrations of specific metabolites in plasma. Maternal diet and JP diet induced selective changes in the concentrations of metabolites in plasma. In males, the maternal PR/AF diet increased plasma TAG and βHB concentrations compared with C/AF offspring (Table 1). The NEFA concentration tended to be higher ($P < 0.1$) in PR/AF offspring than in C/AF offspring. The plasma TAG concentration was higher in C/FS and PR/FS offspring than in C/AF offspring (Table 1). In contrast, the βHB concentration was significantly lower in C/FS and PR/FS offspring than in C/AF offspring (Table 1). Maternal diet and JP diet did not affect plasma cholesterol or glucose concentrations.

In females, maternal diet affected plasma TAG and NEFA concentrations such that these metabolites were significantly higher in PR/AF than in C/AF offspring (Table 1). The plasma βHB concentration was significantly higher in PR/AF than in C/AF offspring. Plasma TAG and NEFA concentrations were significantly higher in C/FS and PR/FS offspring than in C/AF and PR/AF offspring, whereas the βHB concentration was lower than in C/AF and PR/AF offspring (Table 1). Maternal diet or JP diet did not affect plasma cholesterol or glucose concentrations.

mRNA expression of specific genes in liver. It was not possible to compare the level of expression or DNA methylation between males and females in the same PCR and so results are presented relative to the C/AF group for each sex. The effects of maternal and JP diet on mRNA expression are summarized (**Tables 2** and **3**). Hepatic PPARα, acyl-CoA oxidase (AOX), carnitine-palmitoyl transferase (CPT-1), GR and phosphoenol-

TABLE 1 Liver weight and TAG and plasma metabolites in rats exposed to diets differing in protein before birth and folic acid in their JP period[1]

	Dietary group				ANOVA		
	C/AF	PR/AF	C/FS	PR/FS	JP diet (*P*)	Maternal diet (*P*)	JP diet × maternal diet (*P*)
Males							
Liver weight							
g	9.4 ± 2.0[a]	9.5 ± 1.6[a]	12.1 ± 1.6[b]	11.9 ± 2.1[b]	<0.0001	—	—
g/100 g body weight	2.5 ± 0.5[a]	2.5 ± 0.6[a]	3.0 ± 0.3[b]	3.0 ± 0.6[b]	<0.0001	—	—
Liver TAG, *μmol/g liver*	12 ± 4[a]	14 ± 3[a]	26 ± 9[b]	21 ± 7[b]	<0.0001	—	—
Plasma TAG, *mmol/L*	1.5 ± 0.7[a]	2.1 ± 0.5[b]	2.7 ± 0.9[b]	2.6 ± 0.8[b]	<0.0001	0.038	—
Plasma βHB, *μmol/L*	193 ± 43[a]	284 ± 79[b]	81 ± 45[c]	58 ± 33[c]	<0.0001	0.003	0.002
Plasma glucose, *mmol/L*	10.7 ± 3.0	9.0 ± 1.6	12.7 ± 4.0	8.8 ± 1.8	—	—	—
Plasma NEFA, *μmol/L*	346 ± 122	606 ± 438	683 ± 587	584 ± 413	—	—	—
Plasma cholesterol, *mmol/L*	2.1 ± 0.3	2.3 ± 0.3	2.8 ± 0.6	2.2 ± 0.3	—	—	—
Females							
Liver weight							
g	7.7 ± 1.3	7.3 ± 1.0	8.3 ± 1.0	8.7 ± 1.2	—	—	—
g/100 g body weight	3.1 ± 0.6	3.0 ± 0.6	3.2 ± 0.3	3.2 ± 0.4	—	—	—
Liver TAG, *μmol/g liver*	14 ± 3[a]	11 ± 6[a]	17 ± 6[b]	17 ± 2[b]	<0.001	—	—
Plasma TAG, *mmol/L*	1.0 ± 0.2[a]	1.4 ± 0.4[b]	1.8 ± 0.7[c]	1.9 ± 0.4[c]	0.001	0.022	—
Plasma βHB, *μmol/L*	229 ± 53[a]	291 ± 64[b]	35 ± 24[c]	49 ± 42[c]	<0.0001	0.019	—
Plasma glucose, *mmol/L*	9.6 ± 2.2	9.3 ± 1.7	7.9 ± 2.6	8.4 ± 2.5	—	—	—
Plasma NEFA, *μmol/L*	362 ± 84[a]	441 ± 115[b]	425 ± 96[b]	484 ± 212[b]	0.011	0.017	—
Plasma cholesterol, *mmol/L*	1.7 ± 0.2	1.6 ± 0.2	2.0 ± 0.3	1.7 ± 0.31	—	—	—

[1] Values are means ± SD, $n = 10$. Means in a row with superscripts without a common letter differ, $P < 0.05$.

TABLE 2 mRNA expression of genes in liver, adipose tissue, and skeletal muscle in male offspring exposed to diets differing in protein before birth and folic acid in their JP period[1]

	Dietary group				ANOVA		
	C/AF	PR/AF	C/FS	PR/FS	JP diet (*P*)	Maternal diet (*P*)	JP diet × maternal diet (*P*)
Liver			*% of C/AF*				
PPARα	100.0 ± 43.7[a]	176.0 ± 44.3[b]	32.3 ± 14.0[c]	24.4 ± 3.1[c]	<0.0001	0.002	<0.0001
AOX	100.0 ± 16.6[a]	136.0 ± 36.7[b]	63.7 ± 14.0[c]	73.6 ± 13.4[c]	<0.0001	0.03	—
CPT-1	100.0 ± 33.3[a]	139.0 ± 39.8[b]	71.8 ± 24.1[a]	77.3 ± 23.9[a]	<0.0001	0.029	—
GR	100.0 ± 45.6[a]	174.1 ± 43.3[b]	318.5 ± 81.3[c]	297.3 ± 86.2[c]	<0.0001	0.041	—
PEPCK	100.0 ± 27.3[a]	203.6 ± 41.9[b]	256.3 ± 65.5[b]	185.4 ± 45.8[b]	<0.0001	—	—
IR	100.0 ± 23.3[a]	72.8 ± 18.9[a]	229.4 ± 43.2[b]	245.0 ± 58.4[b]	<0.0001	—	—
Adipose tissue							
PPARγ2	100.0 ± 21.0[a]	101.7 ± 37.9[a]	240.5 ± 34.6[b]	218.7 ± 48.3[b]	<0.001	—	—
IR	100.0 ± 38.7[a]	84.3 ± 18.8[a]	281.7 ± 77.9[b]	278.6 ± 82.9[b]			
HSL	100.0 ± 28.8[a]	147.0 ± 44.8[a]	35.0 ± 19.4[b]	41.1 ± 20.4[b]			
LPL	100.0 ± 34.6[a]	107.6 ± 59.6[a]	144.8 ± 56.6[b]	149.5 ± 69.7[b]	0.047	—	—
Skeletal muscle							
PPARα	100.0 ± 34.2[a]	119.7 ± 33.4[a]	224.4 ± 69.4[b]	184.8 ± 46.8[b]	<0.0001	—	—
AOX	100.0 ± 28.9[a]	117.1 ± 29.1[a]	318.7 ± 23.1[b]	317.2 ± 29.0[b]	<0.0001	—	—
CPT-1	100.0 ± 38.2	98.4 ± 29.1	88.2 ± 36.8	87.8 ± 30.2	—	—	—
LPL	100.0 ± 35.6	99.5 ± 66.6	73.4 ± 19.5	76.1 ± 10.1	—	—	—

[1] Values are means ± SD, $n = 10$. Means in a row with superscripts without a common letter differ, $P < 0.05$.

pyruvate carboxykinase (PEPCK) expression were higher in male PR/AF offspring than in C/AF offspring (Table 2). The expression of these genes did not differ between the C/FS and PR/FS offspring. However, PPARα, AOX, and CPT-1 mRNA levels were lower in male C/FS and PR/FS than in C/AF and PR/AF offspring. In contrast, GR and PEPCK expression was higher in C/FS and PR/FS offspring than in C/AF offspring but were expressed at a similar level to PR/AF offspring (Table 2). Insulin receptor (IR) expression was higher in FS offspring than in AF offspring but did not differ between maternal dietary groups (Table 2).

PPARα, AOX, CPT-1, GR, and PEPCK expression was significantly higher in livers of female PR/AF offspring than in C/AF offspring (Table 3). The expression of these genes did not differ between C/FS and PR/FS offspring. PPARα, AOX, and CPT-1 mRNA levels were lower in female C/FS and PR/FS offspring than in C/AF and PR/AF offspring. GR expression was higher in C/FS and PR/FS than in C/AF offspring but at a similar level to PR/AF offspring (Table 3). PEPCK mRNA levels were higher in C/FS and PR/FS offspring than C/AF offspring but were lower than in PR/AF offspring. IR mRNA expression did not differ significantly between PR and C offspring. However, the level of IR mRNA was significantly higher in FS offspring than in C/AF offspring (Table 3).

mRNA expression of specific genes in adipose tissue. Maternal diet did not affect PPARγ2, lipoprotein lipase (LPL), or IR expression in adipose tissue in male or female offspring, although hormone-sensitive lipase (HSL) expression in PR/AF

TABLE 3 mRNA expression of genes in liver, adipose tissue, and skeletal muscle in female offspring exposed to diets differing in protein before birth and folic acid in their JP period[1]

	Dietary group				ANOVA		
	C/AF	PR/AF	C/FS	PR/FS	JP diet (*P*)	Maternal diet (*P*)	JP diet × maternal diet (*P*)
Liver			*% of C/AF*				
PPARα	100.0 ± 45.0[a]	185.6 ± 45.5[b]	29.3 ± 12.7[c]	23.2 ± 8.5[c]	<0.0001	0.001	<0.0001
AOX	100.0 ± 16.7[a]	160.4 ± 45.1[b]	71.1 ± 16.5[a]	75.8 ± 24.3[a]	<0.0001	0.01	0.004
CPT-1	100.0 ± 36.4[a]	168.4 ± 55.3[b]	48.4 ± 26.8[c]	48.6 ± 28.2[c]	<0.0001	0.008	0.008
GR	100.0 ± 39.3[a]	396.4 ± 189.0[b]	394.8 ± 127.5[b]	254.5 ± 83.4[b]	<0.0001	0.032	—
PEPCK	100.0 ± 28.4[a]	305.3 ± 77.6[b]	121.6 ± 56.8[c]	150.1 ± 34.3[c]	<0.0001	<0.0001	<0.001
IR	100.0 ± 26.1[a]	81.4 ± 5.5[a]	169.4 ± 44.6[b]	166.6 ± 46.6[b]	<0.0001	—	—
Adipose tissue							
PPARγ2	100.0 ± 26.5[a]	65.7 ± 18.9[a]	275.4 ± 35.4[b]	373.1 ± 88.1[c]	<0.0001	—	—
IR	100.0 ± 62.5[a]	62.3 ± 52.9[a]	290.1 ± 98.7[b]	202.6 ± 131.4[b]	0.048	<0.0001	—
HSL	100.0 ± 47.2[a]	121.6 ± 41.4[a]	27.8 ± 22.0[b]	13.0 ± 8.4[b]	<0.0001	—	—
LPL	100.0 ± 31.9	91.8 ± 51.1	98.1 ± 45.2	87.0 ± 34.5	—	—	—
Skeletal muscle							
PPARα	100.0 ± 56.8[a]	181.4 ± 72.9[b]	194.8 ± 57.9[b]	233.8 ± 69.4[b]	0.001	0.006	—
AOX	100.0 ± 42.8[a]	123.4 ± 45.4[a]	363.9 ± 49.2[b]	277.6 ± 52.0[b]	<0.0001	—	—
CPT-1	100.0 ± 24.9[a]	86.2 ± 28.7[a]	56.7 ± 13.3[b]	62.0 ± 25.3[b]	<0.0001	—	—
LPL	100.0 ± 46.6	117.4 ± 49.9	78.0 ± 25.7	65.2 ± 25.7	—	—	—

[1] Values are means ± SD, $n = 10$. Means in a row with superscripts without a common letter differ, $P < 0.05$.

offspring tended be higher ($P < 0.1$) than in C/AF offspring (Tables 2 and 3). PPARγ2 and IR mRNA levels were higher in C/FS and PR/FS male and female offspring than in C/AF and PR/AF offspring (Tables 2 and 3), but HSL expression was lower in C/FS and PR/FS offspring than in C/AF male and female offspring (Tables 2 and 3). LPL mRNA expression was higher in C/FS and PR/FS male offspring than in C/AF and PR/AF offspring but did not differ between C/AF female offspring and FS offspring (Tables 2 and 3).

mRNA expression of specific genes in skeletal muscle. In skeletal muscle, PPARα, AOX, and CPT-1 expression did not differ significantly between male and female PR/AF offspring or between C/FS and PR/FS offspring (Tables 2 and 3). The mRNA levels of PPARα and AOX were significantly higher in FS offspring than in AF offspring, but CPT-1 expression did not differ between these groups (Tables 2 and 3). LPL mRNA expression did not differ between maternal dietary or JP dietary groups in male offspring but was significantly lower in female PR/FS offspring than in C/AF offspring (Tables 2 and 3).

DNA methylation status of specific genes in liver and adipose tissue. In males and females, the maternal diet affected hepatic PPARα and GR, but not IR or methylation and the JP diet affected PPARα, IR, and GR methylation (**Table 4**). In male and female liver, PPARα methylation was significantly lower in PR/AF than C/AF offspring but was greater in FS than C/AF offspring (Table 4). GR promoter methylation was significantly lower in PR/AF and FS offspring than in C/AF offspring (Table 4). IR methylation was lower in FS male and female offspring than in C/AF offspring (Table 4).

Maternal diet did not affect adipose tissue IR or HSL methylation in male or female offspring. JP diet decreased IR methylation in male and female offspring (Table 4) but did not alter HSL methylation (Table 4).

Discussion

Our findings show that in contrast to folic acid supplementation of the maternal PR diet (11,17), supplementation during the JP period did not normalize changes in metabolism and epigenotype induced by the PR diet. Rather, JP folic acid supplementation induced distinct changes in the phenotype and epigenotype of the offspring. Together with our previous observations (11,17), these findings suggest that the effect of increased folic acid intake on phenotype is contingent on the timing of folic acid supplementation relative to the developmental stage of the organism and the nutrient pattern within the diet. One possible mechanism for the difference between the effects of providing the same amount of folic acid to pregnant dams and to JP offspring is that the supply of folic acid to the offspring in utero would have been buffered by maternal metabolism, whereas the JP offspring would have been exposed directly to amount of folic acid in the diet.

Although the maternal dietary groups did not differ in offspring weight gain after weaning, folic acid supplementation increased weight gain in both male and female offspring. Because increased growth started during the pubertal period, this suggests that the JP period is one of plasticity in the regulation of weight gain, which responds positively to increased folic acid intake. Puberty in rats and humans is preceded by a proliferation of preadipocytes (32,33). One possible mechanism by which folic acid supplementation may increase weight gain is by increasing the formation of adipocytes. This is consistent with upregulation in the FS offspring of PPARγ2 expression, which induces adipogenesis (34).

The coordinated activities of metabolic pathways in liver, skeletal muscle, and adipose tissue are critical for maintaining lipid and glucose homeostasis. We therefore measured the epigenetic regulation and mRNA expression of candidate genes in each of these tissues. We did not attempt to provide a comprehensive assessment of the changes in metabolic pathways that underlie the induced phenotypes. The changes in mRNA expression and DNA methylation of hepatic PPARα and GR and in the mRNA levels of their respective targets AOX and CPT-1, and PEPCK in the PR/AF offspring are consistent with our previous findings (20,17,19,18) and, together with higher plasma βHB concentrations, suggests increased capacity for fatty acid β-oxidation. The absence of an effect of maternal diet on glucose concentration suggests that the increased GR and PEPCK expression was a

TABLE 4 Promoter methylation of genes in liver and adipose tissue in the offspring exposed to diets differing in protein before birth and folic acid in their JP period[1]

	Dietary group				ANOVA		
	C/AF	PR/AF	C/FS	PR/FS	JP diet (*P*)	Maternal diet (*P*)	JP diet × maternal diet (*P*)
Male offspring	*% of C/AF*						
Liver							
PPARα	100.0 ± 34.2[a]	47.6 ± 32.1[b]	172.2 ± 43.3[c]	158.7 ± 33.8[c]	<0.0001	0.042	—
GR	100.0 ± 44.1[a]	45.7 ± 24.0[b]	27.7 ± 15.5[b]	30.1 ± 29.2[b]	<0.0001	0.01	<0.0001
IR	100.0 ± 47.0[a]	108.8 ± 24.8[a]	55.9 ± 21.9[b]	62.3 ± 32.6[b]	<0.0001	—	—
Adipose tissue							
IR	100.0 ± 7.9[a]	98.7 ± 6.5[a]	75.2 ± 8.0[b]	73.7 ± 7.1[b]	—	<0.0001	—
HSL	100.0 ± 25.8	104.1 ± 32.0	93.4 ± 21.4	91.8 ± 23.9	—	—	—
Female offspring							
Liver							
PPARα	100.0 ± 44.4[a]	55.1 ± 45.9[b]	141.5 ± 35.7[c]	157.5 ± 61.4[c]	<0.0001	0.027	0.049
GR	100.0 ± 41.4[a]	33.9 ± 20.0[b]	11.4 ± 8.5[b]	15.2 ± 10.8[b]	<0.0001	0.01	<0.0001
IR	100.0 ± 33.5[a]	62.8 ± 14.8[a]	35.9 ± 20.1[b]	38.8 ± 35.3[b]	<0.0001	—	—
Adipose tissue							
IR	100.0 ± 11.3[a]	99.6 ± 6.8[a]	84.8 ± 9.2[b]	84.8 ± 14.1[b]	—	0.003	—
HSL	100.0 ± 17.1	102.2 ± 29.2	92.0 ± 46.1	93.0 ± 24.6	—	—	—

[1] Values are means ± SD, $n = 10$. Means in a row with superscripts without a common letter differ, $P < 0.05$.

relatively minor determinant of plasma glucose. PPARγ2 and LPL mRNA expression in adipose tissue and PPARα, CPT-1, and AOX expression in skeletal muscle were not altered in the PR/AF offspring, indicating differential sensitivity of liver, adipose tissue, and skeletal muscle to induction of altered transcription by maternal protein restriction.

JP folic acid supplementation induced differential changes in the methylation status and/or mRNA expression of individual genes that were associated with a shift in lipid metabolism in the food-deprived state. These are summarized in the model presented in **Figure 1**. In adipose tissue, the changes in PPARγ2, IR, LPL, and HSL mRNA expression suggest that JP folic acid supplementation increased capacity for deposition of fatty acids in adipocytes, which is consistent with greater weight gain. Downregulation of HSL and increased LPL expression may reflect increased IR mRNA levels leading to upregulation of the insulin signaling pathway. Although HSL expression was reduced, the plasma NEFA concentration tended to be higher in FS offspring. One possible explanation is that greater fat mass and inefficient entrapment of fatty acids from TAG-rich lipoproteins (35) would tend to increase NEFA flux to the liver.

Lower plasma βHB concentration in FS offspring is consistent with lower expression of PPARα and of the target genes AOX and CPT-1 and suggests constraint in hepatic fatty acid β-oxidation (Fig. 1). Deposition of TAG in liver and increased plasma TAG concentration is consistent with constraint in fatty acid β-oxidation and a shift in fatty acid metabolism toward TAG synthesis, which may be exacerbated by increased NEFA flux. Reduced intakes of folic acid or 1-carbon donors are associated with fatty liver due to reduced phosphatidylcholine synthesis, which is required for VLDL secretion (36,37). Our results suggest that increased folic acid intake after weaning can also induce fatty liver by altering the metabolic partitioning of fatty acids from β-oxidation toward TAG synthesis. Thus, FS supplementation appears to induce opposite changes in equivalent metabolic pathways in different tissues.

PPARα expression is directionally dependent upon the methylation status of its promoter (17,38). Thus, hypermethylation of the PPARα promoter is consistent with its lower expression. In contrast, the methylation status and expression of GR and mRNA expression of PEPCK in FS offspring was similar to PR/AF. Thus, within a single tissue, increased JP folic acid intake induces gene-specific changes in promoter methylation and expression, although the mechanism for such targeting is not known. Furthermore, in skeletal muscle from FS offspring, increased PPARα and AOX expression suggests upregulation of peroxisomal β-oxidation, which suggests the in JP animals, the effects of folic acid supplementation differ between tissues.

Overall, these findings suggest that increased folic acid intake in the JP period induced a change in the partitioning of fatty acids between different metabolic fates. It remains to be determined whether changes in metabolism induced by altered nutrition during the JP period persist throughout the life course and if these observations are influenced by the background diet. Although the effects of increased folic acid intake operate through alterations in the epigenome, the mechanism by which such changes occur cannot be deduced from the present findings. One implication of our findings is that folic acid supplementation as a strategy to reverse the adverse effects of poor nutrition during early life on future health in humans may need to be undertaken with caution, particularly with respect to the timing of the period of supplementation and the composition of the background diet. However, demonstration of plasticity during the JP period supports the possibility of resetting an inappropriate phenotype induced in early life to one better adapted to meet the challenge of the prevailing environment.

Puberty has been identified as a period of relative instability in the epigenome associated with altered epigenetic regulation of genes associated with sexual maturation (13) and variation in nutrition in the prepubertal period in grandparents has been associated with patterns of mortality in the grandchildren (39). Furthermore, exposure during this period to hormones, including estrogens, induces changes in the epigenome in a manner that affects subsequent disease risk, which differs from the effects of hormonal exposure during the neonatal period (40). This may involve changing the DNA demethylation and remethylation cycles that occur in nondividing cells (41), e.g. by altering the balance of Dnmt-1 and demethylase activities (18,41). The JP period is also a time of increased growth and cell proliferation in specific tissues (32,33) that may facilitate epigenetic changes during mitotic cycles (12). Overall, our data support the view that, during the life course, specific periods of plasticity are associated with reduced stability of the epigenome that allow the phenotype of an organism to adapt to environmental cues, such as nutrition. It has been suggested that the phenotype induced in the fetus by environmental cues can promote later reproductive

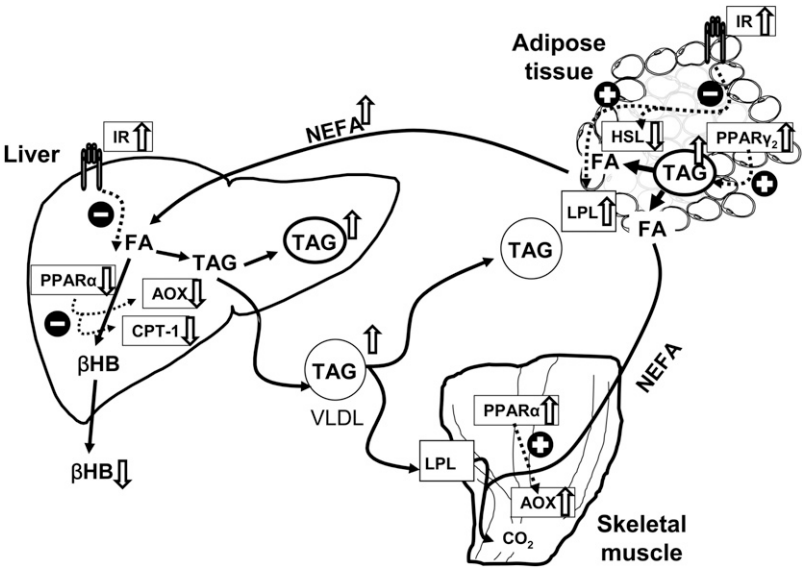

FIGURE 1 A model for the effect of altered gene expression induced in liver, adipose tissue, and skeletal muscle by increased PP folic acid intake. A detailed explanation is provided in the text. Black arrows indicate flux of metabolites. Broken arrows indicate effects of various genes on other genes or on metabolic processes. White arrows indicate the direction of change relative to C/AF offspring. Circle with horizontal bar indicates negative effect on transcription. Circle with cross indicates positive effect on transcription.

fitness by predicting the future environment and that an incorrect prediction is associated with increased risk of later chronic disease (2). Our findings suggest this hypothesis could be extended to include the phenotypic changes induced during the subsequent period of plasticity in puberty, perhaps enhancing the organism's life course strategy.

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