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The effect of season of birth on brain epigenome-wide DNA methylation of older adults

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Original Article

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Abstract

Perinatal light exposure predisposes towards health and behaviour in adulthood. Season of birth is associated with psychiatric, allergic, cardiovascular and metabolic problems. It has been proposed that early-life environmental light disrupts the development of biological rhythms which, in turn, influence later-life health. However, the mechanisms linking perinatal seasonal light to later-life biological rhythm and health in humans are unknown. In this study, we investigated the association between season of birth and epigenome-wide DNA methylation of two post-mortem human brain regions (16 hypothalamus, 14 temporal cortex). We did not find statistically significant differences at the whole epigenome level, either because we lacked statistical power or that no association exists. However, when we examined 24 CpG sites that had the highest significance or differential methylation, we identified regions which may be associated with circadian rhythm entrainment, cholinergic neurotransmission and neural development. Amongst methylation of the core clock genes, we identified that hypothalamus *Neuronal PAS Domain Protein 2* (*NPAS2*) gene has hypermethylated regions in long photoperiod-born individuals. In addition, we found nominal associations between season of birth and genes linked to chronotype and narcolepsy. Season of birth-related brain DNA methylation profile was different than a previously reported blood methylation profile, suggesting a tissue-specific mechanism of perinatal light programming. Overall, we are the first to analyse the relationship between season of birth and human brain DNA methylation. Further studies with larger sample sizes are required to confirm an imprinting effect of perinatal light on the circadian clock.

Introduction

The concept of The Developmental Origins of Health and Disease states that early-life environmental exposures may influence an individual's behaviours and health in adulthood.[1] For example, maternal care and malnutrition are well-established as being associated with later-life mental health and metabolism.[2,3] Early-life light exposure has also been suggested to predispose towards health issues in adulthood. Epidemiological studies have found that season of birth is associated with psychiatric conditions,[4] allergies,[5] cardiovascular diseases,[6] cancer,[7] metabolic problems[8] and mortality.[9] The Perinatal Light Imprinting of Circadian Clocks and Systems (PLICCS) hypothesis suggests that environmental light exposure may disrupt the development of biological rhythms which in turn influence later-life health.[10] In line with this, day length at birth was associated with chronotype, which is the timing preferences of daily behaviours and which represents the biological rhythm of an individual.[11,12] Moreover, animal models have shown that seasonal daylight duration can modify developing circadian clocks.[13] If mice are reared under a summer solstice-like (16 h light:8 h dark) photoperiod environment, the period of their circadian rhythm of gene expression, the period of their free-running activity rhythm in constant darkness and the period of individual neurons of their principal pacemaker, the suprachiasmatic nucleus (SCN) in the brain, all become shorter in adulthood compared to animals reared under a winter solstice-like (8 h light:16 h dark) environment.[13]

Epigenetic regulations are highly dynamic during embryonic and postnatal development, and it has been hypothesised that early-life programming is due to lasting epigenetic remodelling during development.[14] Season of birth effects may also include early-life epigenetic

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programming. In the hippocampus and olfactory bulb of mice, long day length was associated with lower concentrations of total 5-Methylcytosine and *DNA methyltransferase* (*DNMT*) transcription, and higher DNA hydroxymethylation and *Ten-eleven translocation* (*TET2*) transcription, which is an indicator of DNA demethylation.[15] SCN genome-wide DNA methylation and transcription were compared between 22- and 26-hour day length entrained mice.[16] Differently methylated DNA regions were enriched in pathways of synapse formation, neuroendocrine s ystem, axon pathfinding and neurophysiology.[16] A methyltransferase inhibitor drug prevented adaptation to day length cycle.[16] This study is important as it provides evidence supporting circadian epigenetic programming in the mammalian SCN during entrainment to new period length. However, the effect of seasonal day length on epigenetics of the SCN is still largely unknown. In the only human study, blood samples from 367 individuals (age 18) were analysed for global methylation variations in relation to their season of birth.[17] Allergic diseases were found associated with autumn-born participants in this cohort, and 92 seasonal differentially methylated CpG sites were identified, two of which were causally associated with allergy.[17] Involvement of seasonal epigenetic programming in PLICCS has not been characterised in the human hypothalamus.

In this study, we investigate epigenome-wide DNA methylation differences associated with seasonal photoperiod at birth in the human hypothalamus, which includes biological rhythm and sleep centres, and the inferior temporal gyrus (ITG), which has previously been reported as a candidate brain region for season of birth effect in a brain imaging study.[18]

Methods

Subjects and sample preparation

Subjects in this study were participants of The University of Manchester Longitudinal Study of Cognition in Normal Healthy Old Age (UMLCHA) cohort.[19] Starting in 1983, this study recruited 6375 healthy individuals without dementia aged between 40 and 90 years. The participants were followed for up to 35 years for changes in self-reported health, cognition, demographics and lifestyle data including diurnal sleeping habits[11] under the approval of The University of Manchester research ethics committee with written consent from all participants. Participants were approached in 2003 for consent to brain donation and 312 individuals agreed to donate their brain after death.[20] One hemisphere of the postmortem brains was fresh frozen at −80°C and is stored by The Manchester Brain Bank (https://www.bmh.manchester.ac.uk/research/domains/neuroscience-mental-health/manchester-brain-bank), which has ethical approval to distribute tissue and data to researchers. The present study was approved by the Manchester Brain Bank Management Committee (REC reference 19/NE/0242; application number TR83).

Amongst the donated brains, only 16 samples had intact hypothalamus and ITG regions because of tissue sectioning and storing procedures, and usage of the tissues in other studies. These 16 individuals were unrelated with 10 being female. The demographic and brain pathology data of subjects are summarised in Table S1. Hypothalamic tissue blocks were cut into sections with 300 µm thickness using a cryostat (Leica CM 1950). ITG tissue blocks and hypothalamic sections were stored in a −80°C freezer until use. The hypothalamus region was identified by means of a human brain atlas,[21] and 10 punches (1 mm diameter) per participant were obtained. From the ITG tissue blocks, 30–50 mg of tissue was cut. Genomic DNA was isolated with a PureLink Genomic DNA mini kit (Invitrogen, USA). Agarose gel electrophoresis was performed to confirm that DNA was not degraded. For all participants, quantity of extracted DNA was verified to be >500 ng. Bisulphite conversion of DNA was performed using the EZ DNA Methylation™ Kit, following the manufacturer's protocol (Zymo Research, USA). Epigenome-wide methylation analysis was conducted using the Infinium MethylationEPIC BeadChip using the protocol provided by the manufacturer (Illumina Inc., USA). The arrays were scanned using the iScan System, and signal intensities were recorded as IDAT files by GenomeStudio (Illumina Inc., USA).

Data filtering

Quality control and analyses were carried out in R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). IDAT files were imported and preprocessed using the R package *minfi* (version 1.34.0).[22] Two ITG samples were excluded because their mean detection *P*-values were above 0.01. Sex chromosomes were removed from the analysis. Probes that had a mean detection *P*-value above 0.01 were also excluded. Probes which were cross reactive, or which contained a single nucleotide polymorphism (SNP) on the CpG of interest, were removed.[23] Genomic locations were reported in Genome Reference Consortium Human Build 37 (GRCh37). After quality control, 771,382 CpG probes in 30 samples remained. β-values, the ratio of methylated intensity to total intensity, and M-values, the logit transformed β, were calculated and normalised using SWAN.[24] Density plots of β-values were controlled for bimodal distributions (Fig. S1). Principal component analysis was performed using the R package *pcaMethods* (version 1.80.0) to identify potential confounders such as batch effect and tissue type.[25] Cell type heterogeneity was inspected using the R packages *RefFreeEWAS* (version 2.2.0)[26] and *FlowSorted.DLPFC.450k* (version 1.24.0).[27]

Epigenome-wide association analysis

Linear models were calculated to examine differentially methylated probes (DMPs) between hypothalamus and ITG using the R package *limma* (version 3.44.3).[28] Since different tissue samples were taken from the same individual, a paired analysis was performed to adjust within-individual differences. *P*-values were adjusted for multiple testing using the Benjamini and Hochberg method to control the false discovery rate (FDR). Significant hypothalamus-specific methylation profiles (FDR < 0.05) were compared with human lateral hypothalamus DNA methylation,[29] human hypothalamus gene expression in The Genotype-Tissue Expression (GTEx) database,[30] and mice suprachiasmatic nucleus gene expression[31] using Spearman's correlation (Stata Statistical Software: Release 14. College Station, TX: StataCorp LP.).

Season of birth was categorised as long photoperiod (LP), March 21–September 22, and short photoperiod (SP), September 23–March 20. Postmortem brains from 8 SP-born and 8 LP-born subjects were used in this study. Linear models were fitted to find DMPs in season of birth groups. Two separate models were fitted for hypothalamus and ITG. Sex and age at death were included as covariates in the models. DMPs with adjusted *P*-value < 10^{-5} were accepted as a suggestive association. Power calculations for DNA methylation studies using the Illumina EPIC microarray were performed using the University of Essex EPIC Array Power Calculations.[32] In addition, probes that had a

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log_2 fold change (logFC) >3 or <−3 were further investigated. Differentially methylated regions (DMR), where neighbouring CpGs are differentially methylated, were calculated using the R package *DMRcate* (version 2.2.2).[33]

Season of birth-associated CpGs and chronotype

With season of birth being correlated with later-life midsleep time,[11] we investigated whether nominally CpGs associated with season of birth predict later-life sleep time. Self-reported sleep timing data were available for 15 subjects. Midsleep time, a measure of chronotype, was used as an outcome for the linear regression model. Candidate CpGs ($P < 10^{-5}$ or $P < 0.05$, logFC < −3, logFC > 3) were included in the regression models as the main predictor, adjusted for age and sex. *P*-value below 0.0017 was accepted as significant with Bonferroni correction of multiple testing.

Pathway analysis and correlations with previous findings

The top 1000 CpGs in terms of logFC from the season of birth epigenome-wide results were tested for potential enrichment in biological pathways, molecular function or cellular components using the Gene Ontology database.[34] The pathway analysis was performed using the R package *missMethyl* (version 1.22.0) with a *P*-value threshold $<10^{-5}$ used to determine statistical significance.[35]

Hypothalamus and ITG methylation profiles that were related to season of birth in this study were compared with previously reported season of birth DNA methylation in blood[17] using Spearman's correlation (Stata Statistical Software: Release 14. College Station, TX: StataCorp LP.). In addition, overlapping methylated CpGs between our season of birth methylation ($P < 0.05$) and season of birth-associated disorders (Frontal cortex DNA methylation in schizophrenia[36] and Lateral Hypothalamus DNA methylation in narcolepsy[29]) were investigated using a chi-squared test.

Candidate probes were further investigated for their association with other phenotypes using the EWAS Atlas.[37] Our candidate probes were also compared with primate brain diurnal or seasonal gene expression and DNA methylation,[38,39] fetal and early-life DNA methylation in blood and brain,[36,40,41] and season of birth-associated placenta gene expression.[42]

MeQTL analysis

Blood samples of the UMLCHA cohort were previously genotyped and imputed to The Haplotype Reference Consortium.[43] A total of 15 subjects had both DNA methylation and genotype data. Using PLINK v2, SNPs located within 1 million bases around the CpGs that had *P*-value $<10^{-5}$ were extracted. Cis-acting methylation quantitative trait loci (meQTL) were investigated using the R package *MatrixEQTL* (version 2.3.0) with a *P*-value threshold $<10^{-5}$ to determine statistical significance.[44] In addition, previously reported meQTLs in the developing human brain were investigated for our candidate loci.[45]

Technical replication using pyrosequencing

Amongst the strongest associated CpG regions, four hypothalamus probes (cg12323699, cg18445760, cg12833267 and cg25628433) and four ITG probes (cg01839373, cg03370588, cg16402757 and cg14859874) were selected as target regions for technical replication using pyrosequencing. Predesigned PyroMark CpG Assays for validating results from Infinium MethylationEPIC BeadChip arrays were used (Qiagen, Germany). Before pyrosequencing, PCR amplification was performed using 10 ng of bisulphite-converted DNA and the PCR product was cleaned using Streptavidin Sepharose High Performance Beads (GE Healthcare, UK). Pyrosequencing was performed using PyroMark Q24 (Qiagen, Germany). β-values from epigenome-wide arrays were compared with the methylation percentage from pyrosequencing using Spearman's correlation.

Results

Tissue validation

Average age at death was 88.56 (SD = 5.49) years, and average postmortem delay of brain removal was 92.78 (SD = 45.62) hours. We performed principal component analysis using all 30 samples, and the first three principal components were used to separate tissue types (Fig. S2). We identified 37,402 hypermethylated and 81,832 hypomethylated probes that were significantly (adjusted $P < 0.05$) different between the hypothalamus and ITG (Table S2: https://doi.org/10.48420/14679996). Tissue-specific DMPs in our results positively correlated (Spearman's Rho = 0.64; $P < 0.0001$; $N = 115{,}124$) with DMPs specific to the lateral hypothalamus compared to the temporal cortex[29] (Fig. S2). Furthermore, tissue-specific DMPs located upstream of genes (5'UTR, TSS200, TSS1500), which may include promoter regions, were negatively correlated (Spearman's Rho = −0.31; $P < 0.0001$; $N = 7078$) with gene expression differences between human hypothalamus and cortex[30] (Fig. S2). Similarly, these DMPs estimated downregulated gene expression (Spearman's Rho = −0.22; $P < 0.0001$; $N = 2355$) in mice hypothalamic SCN compared to whole brain[31] (Fig. S2).

Cellular heterogeneity

Since brain samples include different cell types, association analysis may be confounded. Therefore, we used a reference-free deconvolution method to predict cell type proportions using methylation profiles. The estimation revealed that there were two main cell types in the tissue. A proportion of neural cells was subsequently estimated using publicly available data, *FlowSorted.DLPFC.450k* (version 1.24.0).[27] One of the two estimated cell types clustered with neural cells, while the other type clustered with non-neural cells. We found the estimated proportion of neurons to be higher in ITG (26%) compared to hypothalamus (18%) ($P = 5.4 \times 10^{-3}$). The higher glia:neuron ratio of the hypothalamus compared to cortex has been previously shown in primates.[46] The proportion of neurons did not significantly differ between SP and LP season of birth groups (Fig. 1) in the hypothalamus ($P = 0.33$) and ITG ($P = 0.78$).

Season of birth and DNA methylation

There was no evidence of epigenome-wide significant (FDR < 0.05) DMP for any tissue (Table S3: https://doi.org/10.48420/14680170, Table S4: https://doi.org/10.48420/14680281, Fig. S3). We observed that two CpGs in the ITG had suggestive significance (Table 1). Probes cg01839373 ($P = 1.7 \times 10^{-6}$) and cg16791508 ($P = 7.8 \times 10^{-6}$) were hypomethylated in SP-born individuals (Fig. 2). Amongst nine suggestively significant CpGs in the hypothalamus, the most significant association was with an open sea probe cg12323699 ($P = 8.1 \times 10^{-7}$) and the highest methylation change was at cg00773142 (13% less methylation in

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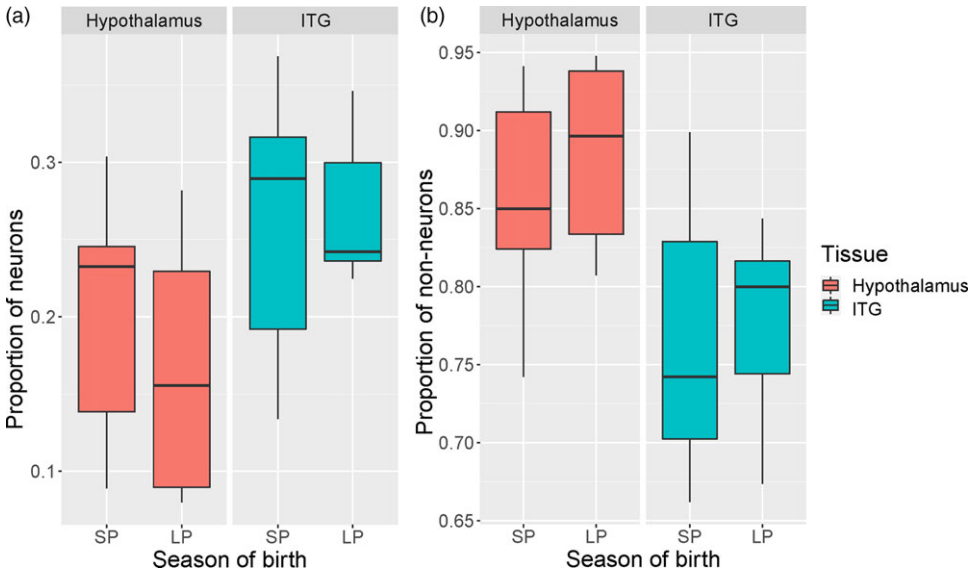

Fig. 1. Comparisons of the estimated proportions of (a) neurons and (b) non-neurons between season of birth groups in the hypothalamus and inferior temporal gyrus (ITG). SP, Short photoperiod season of birth; LP, Long photoperiod season of birth.

SP-born individuals; $P = 3.0 \times 10^{-6}$), which is at the intron of *Phospholipase C Gamma 2* (*PLCG2*) (Table 1). These nine CpGs were located within either intergenic or intronic regions, but cg12833267 ($P = 5.6 \times 10^{-6}$) is located within the 5'UTR of *cAMP-Dependent Protein Kinase Inhibitor Beta* (*PKIB*) (Fig. 2), and cg17733353 ($P = 6.0 \times 10^{-6}$) is in a CpG island of the first exon on *Vav Guanine Nucleotide Exchange Factor 2* (*VAV2*) (Fig. 2). Ten CpGs had a high fold change in the ITG and eight in the hypothalamus ($P < 0.05$, logFC < -3, logFC > 3). Five of these DMPs were common in both tissues: cg03370588 in a CpG island at the transcription start site of *Myosin VA* (*MYO5A*), cg16402757 at the intron of *Cullin 2* (*CUL2*), cg14859874 at the intron of *Ubiquitin Associated Protein 2 Like* (*UBAP2L*), and cg06405219 and cg10993517, which are both located at intergenic regions (Table 1).

Since we found no epigenome-wide significant DMP after correcting for multiple testing, we searched for DMRs using our arbitrary threshold ($P < 5 \times 10^{-8}$) for smoothed estimates (Table 2). There were 4 DMRs in the ITG and 16 DMRs in the hypothalamus showing weak associations. Amongst these DMRs, chr20:57425979–57426931 had the highest number of CpGs ($N = 29$; $P = 1.7 \times 10^{-9}$) that were annotated to the gene *Guanine Nucleotide-Binding Protein G(S) Subunit Alpha Isoforms* (*GNAS*) (Fig. S4). The DMP cg18445760 has also showed regional differential methylation at chr6:31837439–31837445, which is located within the *Solute Carrier Family 44 Member 4* (*SLC44A4*) gene ($N = 2$; $P = 2.6 \times 10^{-13}$) (Fig. S4).

A total of 75 CpGs in our analysis have been previously reported as season of birth-related blood DNA methylation markers.[17] Neither the hypothalamus (Spearman's Rho = −0.12; $P = 0.30$) nor the ITG (Spearman's Rho = 0.15; $P = 0.19$) results were correlated with blood (Fig. S5). In addition, schizophrenia frontal cortex DMPs[36] were not enriched in our hypothalamus and ITG results. Narcolepsy lateral hypothalamus DMPs[29] were not enriched in our ITG results, but they were associated with our hypothalamus season of birth DMPs (OR = 1.53; chi2(1) = 102.66; $P = 4.0 \times 10^{-24}$). 6458 DMPs ($P < 0.05$ and $|\Delta\beta| > 0.05$) have been previously reported. Amongst these, 6143 CpGs were available in our data set, in which 630 probes had *P*-value lower than 0.05 in season of birth analysis. In addition, *GNAS* is one of the top ten DMRs for narcolepsy (Table S5).

Season of birth and clock gene methylation

A total of 699 CpGs in our analysis were annotated to the core clock genes (*CLOCK*, *ARNTL*, *NPAS2*, *PER1-3*, *CRY1-2*, *RORA*, *NR1D1*, *CSNK1D* and *CSNK1E*).[47] After Bonferroni correction for multiple testing ($0.05/699 = 7.15 \times 10^{-5}$), *P*-values did not provide enough evidence of association. Amongst these, 48 CpGs in the hypothalamus and 24 CpGs in the ITG had a *P*-value <0.05 (Table S6). The strongest effect size in the hypothalamus was hypomethylation of cg25628433 in SP-born individuals, which is at the intron of the *Neuronal PAS Domain Protein 2* (*NPAS2*) gene (logFC = −0.65; $P = 2.4 \times 10^{-3}$). Nine of the 10 CpGs within the *NPAS2* were also hypomethylated ($P < 0.05$) (Fig. S6). The strongest effect size in the ITG was hypermethylation of cg09161455 in SP-born individuals, which is located within a CpG island at the intron of the *Nuclear Receptor Subfamily 1 Group D Member 1* (*NR1D1*) gene (logFC = 0.57; $P = 8.0 \times 10^{-4}$) (Fig. S6). The second strongest CpG of ITG was cg22387253, which is at the intron of the *RAR Related Orphan Receptor A* (*RORA*) gene (logFC = −0.49; $P = 1.1 \times 10^{-3}$). Ten of the 12 CpGs within *RORA* were also hypomethylated in SP-born individuals ($P < 0.05$) (Fig. S6).

Season of birth-associated CpGs and later-life chronotype

The UMLCHA cohort has data available for sleep timing (mean age 58.76 (SD = 4.14) years). The average midsleep time was 3:30 am (SD = 26 min). After Bonferroni correction for multiple testing ($0.05/29 = 0.0017$), *P*-values did not provide enough evidence of association. Methylation of cg14859874 within the *UBAP2L* gene in both tissues showed nominal significance to estimate later-life chronotype (Fig. 3). Increased DNA methylation in the hypothalamus was associated with a 30 min earlier chronotype (B = −0.09; SE = 0.04; p = 0.04) and in the ITG, it was associated with a 45 min earlier chronotype (B = −0.13; SE = 0.05; $P = 0.02$).

Pathway analysis results

We found no significant enrichment for any biological pathways. The most significant *P*-value for hypothalamus season of birth-related genes belonged to the biological function of "Specification of animal organ identity" (GO:0010092, $P = 4.6 \times 10^{-4}$). The top

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Table 1. Differentially methylated CpG probes (DMP) associated with season of birth

CpG site	Chr	Pos	Tissue	Log FC	Δmethyl % (SP-LP)	***P*-value**	CpG Island	Relation to nearest gene	Nearest gene
cg12323699	5	40521514	Hypo.	−1.02	−12.01	8.13E-07	OpenSea		
cg00773142	16	81898406	Hypo.	−0.92	−13.82	2.96E−06	OpenSea	Body	PLCG2
cg19640581	5	501343	Hypo.	−0.71	−4.99	3.98E-06	*N*-Shore	Body	SLC9A3
cg18445760	6	31837445	Hypo.	−0.68	−6.25	4.28E-06	OpenSea	Body	SLC44A4
cg02519718	8	11251277	Hypo.	−0.69	−4.31	4.71E-06	OpenSea	Body	C8orf12
cg12833267	6	122907893	Hypo.	−0.68	−6.48	5.57E-06	OpenSea	5'UTR	PKIB
cg17733353	9	136857387	Hypo.	0.87	1.72	6.04E-06	Island	1stExon	VAV2
cg25760005	18	61100522	Hypo.	1.08	4.44	7.63E-06	OpenSea		
cg01589327	7	4603669	Hypo.	−0.63	−6.70	9.98E-06	OpenSea		
cg06405219	7	30219135	Hypo.	3.80	43.43	7.85E-04	OpenSea		
cg27586797	5	13664584	Hypo.	3.94	37.47	1.41E-03	OpenSea		
cg03689146	7	52341810	Hypo.	−3.44	−19.63	1.86E-03	Island		
cg03370588	15	52821290	Hypo.	3.58	16.30	9.38E-03	Island	TSS200	MYO5A
cg16402757	10	35311004	Hypo.	−3.46	−38.64	1.22E-02	OpenSea	Body	CUL2
cg17641943	1	11919010	Hypo.	4.33	19.55	2.43E-02	*N*-Shore	TSS200	NPPB
cg14859874	1	154238265	Hypo.	−3.07	−25.88	2.48E-02	OpenSea	Body	UBAP2L
cg10993517	12	4810234	Hypo.	−3.80	−26.30	2.75E-02	OpenSea		
cg01839373	11	82343201	ITG	−0.93	−8.11	1.66E-06	OpenSea		
cg16791508	12	52715691	ITG	−0.65	−4.97	7.82E-06	S-Shore	TSS1500	KRT83
cg23327483	22	24110105	ITG	3.14	3.15	1.12E-03	Island	5'UTR; 1stExon	CHCHD10; CHCHD10
cg06405219	7	30219135	ITG	3.85	43.45	1.27E-03	OpenSea		
cg19618634	17	69085963	ITG	−3.60	−35.63	4.60E-03	OpenSea		
cg16402757	10	35311004	ITG	−4.20	−43.95	4.84E-03	OpenSea	Body	CUL2
cg03370588	15	52821290	ITG	4.10	18.13	5.49E-03	Island	TSS200	MYO5A
cg06638795	2	42719933	ITG	−4.25	−19.26	6.10E-03	Island	Body	KCNG3
cg18710053	5	126409061	ITG	4.35	17.30	1.48E-02	Island	5'UTR	FLJ44606
cg10993517	12	4810234	ITG	−4.34	−31.36	1.85E-02	OpenSea		
cg14859874	1	154238265	ITG	−3.34	−31.17	2.19E-02	OpenSea	Body	UBAP2L
cg21963178	10	75571738	ITG	3.34	15.11	4.81E-02	Island	TSS200	NDST2

Chr, chromosome; DMP, differentially methylated probe; Hypo, hypothalamus; ITG, inferior temporal gyrus; LP, long photoperiod season of birth; Methyl, methylation; N/S-Shore, the 2kb sequences upstream or downstream of CpG islands, respectively; Pos, position; SP, short photoperiod season of birth; TSS1500/200, 1500/200 base pairs upstream of the transcription start site; 5'UTR, 5'-untranslated region.
Comparison of DNA methylation between participants born in short photoperiod and long photoperiod season of birth ($P < 1E\text{-}5$ or $P < 0.05$, logFC < -3, logFC > 3).

results for ITG season of birth-related genes were the biological function of "Proteasome assembly" (GO:0043248, $P = 5.1 \times 10^{-5}$), biological function of "Retina development in camera-type eye" (GO:0060041, $P = 1.8 \times 10^{-4}$), molecular function of "Phosphatidylinositol-3,5-bisphosphate binding" (GO:0080025, $P = 6.2 \times 10^{-4}$) and cellular component of "Proteasome regulatory particle, base subcomplex" (GO:0008540, $P = 7.8 \times 10^{-4}$).

MeQTL analysis results

Methylation β-values of the DMPs that had a *P*-value lower then 10^{-5} were interrogated for cis-meQTL separately for each tissue. No SNPs significantly estimated DNA methylation in our study. We searched our target CpGs ($P < 10^{-5}$ or $|\Delta\beta| > 3$) in imputed Bonferroni significant meQTLs in fetal brain. We found that methylation of cg18445760, which is located within the *SLC44A4* gene, was associated with 39 linked SNPs. The strongest associated variant rs660550 was found to be associated with ITG methylation in our data after adjusting for age and sex ($B = -0.018$; $P = 6.0 \times 10^{-5}$).

Replication using pyrosequencing

Amongst the remaining bisulphite-converted DNA samples, 24 samples (9 Hypothalamus, 15 ITG) had enough DNA and successfully sequenced using pyrosequencing (Table S7). Methylation

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Table 2. Differentially methylated regions (DMR) associated with season of birth

Chr	Start pos	End pos	Tissue	Number of CpGs in the DMR	Minimum FDR of the smoothed estimate	Maximum differential within the DMR	Mean differential across the DMR	Nearest genes
chr6	28583655	28584464	Hypo.	14	6.58E-16	−0.11	−0.06	SCAND3
chr12	47219626	47220197	Hypo.	13	4.37E-14	−0.07	−0.04	SLC38A4
chr6	31837439	31837445	Hypo.	2	2.56E-13	−0.11	−0.09	SLC44A4
chr10	16561202	16561605	Hypo.	6	9.56E-10	−0.13	−0.09	C1QL3
chr2	30669385	30669863	Hypo.	5	1.36E-09	−0.23	−0.16	
chr10	118892423	118893528	Hypo.	9	1.36E-09	−0.15	−0.09	VAX1
chr2	239139911	239140369	Hypo.	8	1.49E-09	0.18	0.13	AC096574.4, AC016757.3
chr16	88717134	88717850	Hypo.	14	1.72E-09	0.12	0.06	CYBA
chr20	57425979	57426931	Hypo.	29	1.72E-09	−0.09	−0.03	GNAS
chr4	1808376	1808646	Hypo.	3	8.24E-09	−0.06	−0.05	FGFR3
chr10	123781212	123781476	Hypo.	4	8.29E-09	−0.14	−0.11	TACC2
chr1	19600471	19601069	Hypo.	7	9.42E-09	−0.10	−0.06	AKR7L
chr1	78511235	78511713	Hypo.	9	1.08E-08	−0.09	−0.05	GIPC2
chr10	29811530	29811669	Hypo.	5	1.83E-08	−0.11	−0.09	SVIL
chr7	27170313	27170554	Hypo.	6	2.89E-08	−0.07	−0.04	HOXA-AS2, HOXA-AS3, HOXA3, HOXA4
chr1	196866322	196866477	Hypo.	2	3.53E-08	−0.09	−0.09	CFHR2, CFHR4
chr6	30094960	30095802	ITG	25	9.34E-15	−0.09	−0.04	
chr1	67600172	67601159	ITG	11	1.12E-14	−0.13	−0.08	C1orf141
chr2	30669385	30670170	ITG	11	1.86E-14	−0.29	−0.09	LCLAT1
chr7	117854280	117854634	ITG	5	2.98E-08	−0.10	−0.06	

Chr, chromosome; DMR, differentially methylated region; FDR, false discovery rate; Hypo, hypothalamus; ITG, inferior temporal gyrus; LP, long photoperiod season of birth; Pos, position; SP, short photoperiod season of birth.
Comparison of DNA methylation between participants born in short photoperiod and long photoperiod season of birth ($P < 5E\text{-}8$).

array β-values were significantly correlated with pyrosequencing methylation % at the eight target regions (Spearman's Rho = 0.83; $P < 0.0001$; $N = 24$).

Discussion

We investigated associations between photoperiod at birth and human hypothalamus and ITG epigenome-wide DNA methylation patterns. This study is the first of its kind that presents prospective results in the field of early-life seasonal programming via human brain epigenetic modifications. Small sample size may have limited our analyses and results. Nevertheless, suggestive weak associations in this study insinuated that season of birth may generate lasting epigenetic changes, with distinct mechanisms, in different tissues. These results are also in line with the PLICCS hypothesis, since season of birth was weakly associated with hypothalamus DNA methylation, which includes the principal biological clock, and the suggestive significant genes we found may be involved in circadian rhythm pathways. Moreover, reported candidate CpG regions were also enriched in narcolepsy-related DNA methylation, which suggests a relationship between season of birth and narcolepsy in adulthood. Despite limitations, this study is the first to examine the imprinting effects of perinatal light on the human brain and presents unique data that can be used in future meta-analyses and an in-depth evaluation studies of early-life seasonal programming.

Season of birth is associated with traits and behaviours such as height, pubertal timing, educational attainment, biological rhythms and lifetime disease risk.[5,9,12,48] Day length sunlight exposure is the main seasonal cue, but there are multiple seasonal factors that may be involved in season of birth effect such as temperature, exposure to infectious agents and nutrition.[49–52] Seasonal factors may convey their effects via lasting epigenetic modifications, but the exact mechanism of how seasonal cues regulate specific targets is unknown. The strongest candidates are melatonin, thyroid hormone and vitamin-D pathways, all of which are seasonal and have been shown to regulate epigenetic modifier enzymes.[53–56] Depressive behaviour in mice is associated with postnatal short day length, but when *Melatonin receptor 1* was silenced, the mutant animals did not show any postnatal programming in their adulthood depressive behaviour, or midbrain concentration of serotonin and norepinephrine.[57] In humans, blood epigenome-wide DNA methylation was shown to be postnatally modified by season of birth.[17] Specifically, season of birth-associated differentially methylated CpG sites were enriched in development, the cell cycle and apoptosis pathways and were causally related to allergic diseases.[17] Neither hypothalamus nor ITG DNA methylation in our study was correlated with season of birth blood DNA methylation patterns. Taken together, these results indicate that early-life

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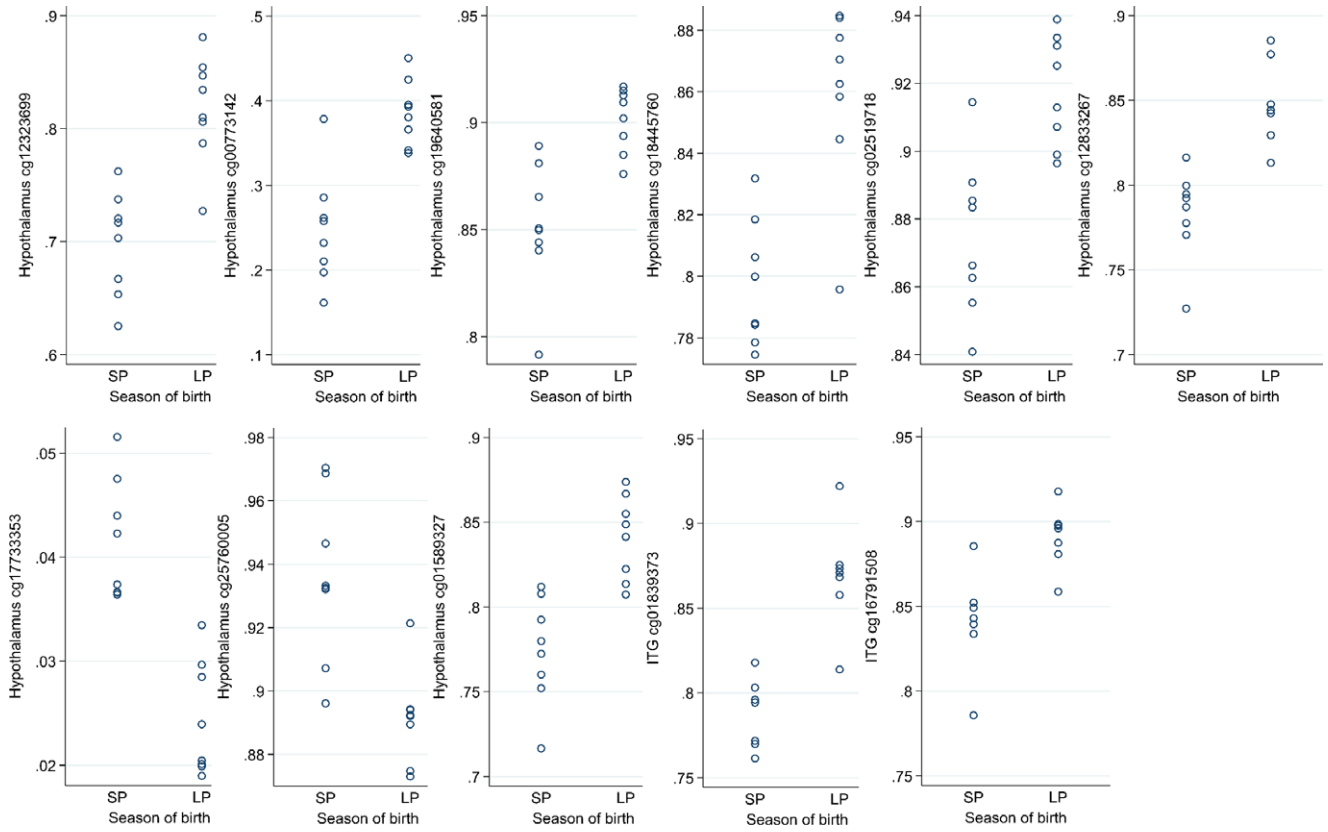

Fig. 2. Plot of season of birth-associated CpG methylation beta values for the top 9 differentially methylated positions in hypothalamus and the top 2 differentially methylated positions in inferior temporal gyrus (ITG). Scatter plot shows DNA methylation beta values. SP, Short photoperiod season of birth; LP, Long photoperiod season of birth.

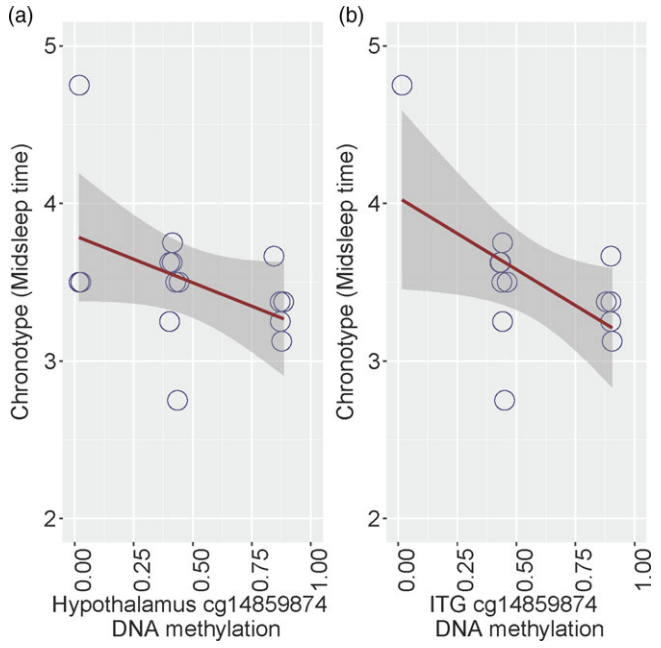

Fig. 3. Association between midsleep time (chronotype) with DNA methylation of cg14859874 in (a) hypothalamus and (b) inferior temporal gyrus (ITG). The fit line shows the predicted sleep midpoint (am) in the linear regression after adjusting for age and sex. Scatter plot shows all available sleep midpoint data. The grey area shows 95% CI.

seasonal factors can influence epigenetic regulation but there is still little evidence about the exact mechanisms. Furthermore, perinatal programming mechanisms may vary in different tissues.

The circadian biological clock is an endogenous pacemaker that regulates 24-hour rhythms in physiology and behaviour and has the ability to synchronise to the outer rhythmic cues such as light.[58] A negative feedback loop of the molecular clock, located within the neurons of the hypothalamic suprachiasmatic nucleus in mammals, maintains the self-oscillation.[58] Photic input from the eyes entrains the circadian pacemaker and sets the phase of the oscillation.[58] The circadian system is not completely mature at birth; therefore, environmental exposures may programme the later-life function of the biological clock.[59] The PLICCS hypothesis suggests that the predisposition of the biological rhythm to be programmed by perinatal factors may contribute towards the association between season of birth and later-life health problems.[10] In line with this, epidemiological studies have shown associations between season of birth and later-life circadian rhythms, and circadian rhythms and health. Narcolepsy is associated with spring season of birth,[60] insomnia with spring and summer season of birth,[18] and evening chronotype with summer season of birth.[12] Furthermore, evening-oriented chronotype is associated with psychiatric and metabolic diseases.[11] Similarly, disruption of biological rhythms and sleep is associated with negative health outcomes and mortality.[61,62] Therefore, to examine the perinatal seasonal programming of biological rhythms, we investigated the human hypothalamus.

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We found that the *NPAS2* gene was the clock gene in the hypothalamus to be most affected by season of birth, with nine CpGs being hypermethylated in LP-born individuals, which points towards a downregulated gene expression in individual born in the summer/spring. The NPAS2 protein is a paralogue of CLOCK, and after dimerisation with BMAL1, it triggers transcription of the *PER* and *CRY* clock genes in brain.[63] Knock-out of *NPAS2* in mice results in a shortened circadian period, altered response to light and sleep disturbances.[63] In line with this, LP-reared mice show shortened periods of behavioural and SCN *PER1* gene expression rhythms compared to SP-reared animals.[13] Another factor that influences circadian rhythm period is stability of clock proteins due to degradation.[64] A suggestive significant gene in our analysis was *CUL2*, which targets proteins for degradation by ubiquitination.[65] Cullins are scaffold proteins in the ubiquitin ligases complex and have been shown to work in circadian regulation.[64,66] These findings support the idea that the early-life photoperiod may influence the period of the biological rhythms in adulthood via epigenetic regulations.

Another characteristic of the circadian clock is its ability to entrain to environmental light cycles. However, responses of individuals to the same environmental input vary. Interindividual variations in sensitivity and response to environmental light have been described in humans, where evening light exposure suppressed melatonin expression differently between individuals.[67] Furthermore, phase differences of circadian genes significantly predicted chronotype.[68] In addition, LP-reared mice had invariant rhythm across different seasons compared to SP-reared animals.[13] Therefore, if early-life light experiences imprint future sensitivity of synchronisation with natural cues, this may explain the association between season of birth and later-life circadian rhythms. Photic entrainment of the circadian clock starts with the retinohypothalamic track conveying light input from the eyes to the SCN through glutamate and PACAP neurotransmission.[69] Conveyed light input activates Ca^{2+} influx and cAMP signalling cascades, in turn light-induced response genes, carrying cAMP response elements (CRE) in their promoters, undergo expression and provide the phase shift.[69] One of the differentially methylated regions in our analysis was the *GNAS* gene, which was generally hypermethylated in LP-born individuals and is known for its high expression in seasonality-related tissues such as thyroid, pituitary and hypothalamus. GNAS is one of the major regulators of adenylyl cyclases that produce cAMP.[70] Through Ca^{2+}/cAMP-dependent circadian entrainment pathways, several protein kinases are involved and another nominal significant gene in our analysis is *PKIB*, which is a peptide that can inhibits cAMP-dependent protein kinase function.[69,71] Taken together, lasting epigenetic modifications during postnatal period may alter GNAS and PKIB expression in adulthood and may influence later-life sensitivity to light.

Narcolepsy is a sleep disorder which is characterised by a loss of orexin neurons, likely due to an autoimmune process.[29,60] Genome-wide associations support the involvement of the immune system, and epigenetic analyses indicate differential methylation in myelin production.[29] Narcolepsy is a season of birth-associated disease.[60] Season of birth is associated with blood DNA methylation, immune responses and allergies[17]; therefore, it is possible that narcolepsy may also be imprinted via seasonal epigenetic programming. We found that previously described narcolepsy-associated DNA methylation in the lateral hypothalamus[29] has a significant overlap with our season of birth methylation analysis in the hypothalamus. These findings point towards a causative role of season of birth methylation in narcolepsy. In addition, one of the candidate CpGs in our analyses, cg14859874 within the *UBAP2L* gene, was associated with both season of birth and later-life chronotype. This may indicate a role of *UBAP2L* gene methylation by season of birth on later-life circadian behaviour. UBAP2L protein is responsible for the formation of stress granules, which are cytoplasmic dense membraneless structures of mRNA and proteins formed after translation inhibition due to a stress exposure.[72] Stress granules are associated with Alzheimer's disease pathology and show circadian rhythmic characteristics.[73] This may also be a link between season of birth and neurodegenerative disorders. Taken together, these findings suggest that early-life seasonal epigenetic programming may have a role in mechanisms linking season of birth and disease.

Early-life light exposure is associated with neurotransmitter levels in adulthood, such as serotonin, norepinephrine and dopamine.[57,74,75] This differential neurotransmitter expression may be due to epigenetic imprinting and may alter health and behaviours in adulthood. In mice, different day length exposure during development resulted in hyper or hypomethylation of genes enriched in synapse formation, neuroendocrine system, axon pathfinding and neurophysiology in the SCN.[16] In line with this, a candidate differentially methylated gene in our analyses was *SLC44A4*, which expresses a choline transporter protein, and was hypermethylated in LP-born individuals. The cholinergic neurotransmission is involved in modulation of wakefulness and REM sleep.[76] Even if *SLC44A4* has low transcription in brain tissue, it may have a role in brain development and neural tissue.[77,78] Season of birth-associated CpG at the *SLC44A4* gene was shown to be significantly more methylated in postnatal human brain compared to fetal.[36] The *SLC44A4* is also differentially expressed in the placenta in different seasons of birth.[42] Taken together, season of birth may programme neurotransmitter levels in adulthood.

Some of the candidate genes in the hypothalamus have been previously reported to have roles in brain development. MYO5A is a motor protein that is abundant in neurons and carries synaptic neurotransmitter vesicles, and deficiency of it is associated with developmental brain abnormalities.[79,80] The *Ventral Anterior Homeobox 1* gene is responsible for the development of the anterior ventral forebrain and visual system.[81,82] The *Homeobox A3* gene has a role in the development of the thyroid gland.[83] These studies suggest a role of season of birth epigenetic modulation in brain development.

Season of birth has previously been associated with grey matter volume of the left superior temporal gyrus in adult human males.[84] The temporal fusiform cortex and inferior temporal gyrus were also shown as suggestively significant with season of birth, with a summer peak.[18] A reduction in the grey matter volume of the temporal gyri has been linked to schizophrenia and narcolepsy,[85,86] both of which are associated with season of birth.[4,60] Therefore, it is possible that season of birth modifies the brain structure in these regions and in turn influences later-life health. We hypothesised that the epigenome of the ITG may be influenced by photoperiodic history and associated with later-life health. We found that neither schizophrenia nor narcolepsy-associated DNA methylation was correlated with ITG season of birth methylation levels.

One of the nominal significances of biological pathways in the ITG was 'Retina development in camera-type eye'. The ITG has a role in visual processing of faces, colours and shape.[87] This may indicate that season of birth epigenetic programming may influence later-life role of the ITG. In addition, when we examined the probes that had the highest fold change, we observed an overlap between ITG and hypothalamus. We found that the CpGs at *CUL2*,

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MYO5A and *UBAP2L* genes had the highest methylation differences in both ITG and hypothalamus, which indicates a possible common mechanism for these regions.

There are some limitations in our study. First, the small sample size limited the statistical power. This study has over 80% power to detect a mean difference of 10% in only 33% of tested sites using a 10^{-5} *P*-value threshold.[32] Season of birth effect has a small effect size which may make it difficult to detect in a small sample.[49,88] Even if we adjusted our analyses for age and sex, other sociocultural, genetic, environmental and latitudinal factors may confound the analyses.[7,43,49,88] We categorised the photoperiod of birth as LP and SP, but using real seasonal day length would provide better insight to postnatal light exposure. Our hypothalamus DNA methylation is correlated with hypothalamus gene expression and DNA methylation in the literature, which support the validity of our tissue blocks. However, the human brain tissue used in the study was slow-frozen, which made it difficult to identify hypothalamus subregions. The hypothalamus comprises many nuclei with distinct functions, cellular and molecular compositions. Therefore, to understand the real association between circadian rhythms and DNA methylation, SCN-specific methylation should be investigated. Furthermore, cellular heterogeneity is one of the major limitations when analysing whole tissue. Nevertheless, we show that neuron and non-neuron proportions were similar in season of birth groups. Finally, DNA methylation may be daily or seasonally rhythmic.[39] However, none of the reported suggestive significant genes or CpG probe showed circadian or seasonal characteristics in the diurnal transcriptome atlas of baboon SCN[38] and human prefrontal cortex.[39]

In summary, perinatal light exposure may predispose physical and mental health in adulthood. Despite the limitations of the present study, our results suggest that season of birth may programme later-life health and behaviour via brain DNA methylation. In addition, different mechanisms appear to underlie season of birth effects in different tissues and health disorders. Importantly, the photoperiod around birth may influence the development of the circadian rhythm system, which is essential for health. Further studies are required to fully understand the mechanisms behind early-life programming, as a key first step to preventing, diagnosing and treating future health problems in predisposed individuals.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S2040174421000453

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Conflicts of interest. The authors have no conflict of interest.

Author contributions. AD, MMC and NN designed the study. AP and NP collected behavioural and genotype data of the cohort. ACR and FR collected pathological data and dissected the required tissue blocks. NN and AD scanned the methylation arrays and performed the statistical analysis. AD wrote the manuscript. NN, AP, MMC and NP supervised the project. All authors contributed to the writing and have approved the final manuscript.

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