



## Original Article

# Epigenetic dysregulation of genes related to synaptic long-term depression among adolescents with depressive disorder and sleep symptoms

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## ARTICLE INFO

## Article history:

Received 10 August 2018

Received in revised form

18 January 2019

Accepted 23 January 2019

Available online xxx

## Keywords:

Adolescence

Depression

Epigenetics

Mood

Polysomnography

Sleepiness

## ABSTRACT

**Objectives:** This study aimed to test the hypothesis that sleep and depression have independent effects on brain development and plasticity in adolescents, and that these changes are reflected in changes in the epigenome.

**Methods:** Participants were 17 medication-free adolescent boys (age  $16.05 \pm 0.80$  years, mean  $\pm$  standard deviation (SD); eight cases with depression and sleep symptoms, nine healthy controls). Sleep was assessed by polysomnography recordings and the Pediatric Daytime Sleepiness Scale (PDSS) and Athens Insomnia Scale (AIS). Participants underwent a clinical evaluation. DNA methylation of blood leukocytes was measured by Illumina 450K array, and Ingenuity Pathway analysis was applied to identify the most significant pathways with differentially methylated positions (DMPs). Secondary analysis of the identified loci included linear correlations between methylation and the subjectively rated scales of sleep, depression and sleep microarchitecture.

**Results:** Due to small sample size, we found no genome-wide significant differences in methylation between cases and controls. However, pathway analysis identified the synaptic long-term depression (LTD) canonical pathway ( $p = 0.00045$ ) when the best 500 DMPs from the original case–control design were included. A flattened dissipation of slow wave sleep, tiredness and depression severity values correlated with five of 10 sites from the LTD pathway (IGF1R, PLAG16, PLA2R1, PPP2C5 and ERK12) in the secondary analysis when the case–control status was controlled for.

**Conclusion:** Among adolescents, depressive disorder with sleep symptoms is associated with a distinctive epigenetic pattern of DNA methylation in blood leukocytes. The enrichment of DMPs on genes related to synaptic LTD emphasizes the role of sleep in synaptic plasticity and the widespread physiological consequences of disturbed sleep.

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## 1. Introduction

Adolescence is characterized by major changes in sleep patterns. Despite being a period of rapid increases in physical and mental capabilities, it is paradoxically also a period of augmented vulnerability to psychiatric and sleep disorders, and the incidence of both insomnia and major depression increases simultaneously during adolescence [1].

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Symptomatology in insomnia and depression are tightly linked and share many common features as well as common mechanisms [2,3]. Disordered sleep also increases the risk of depression [4,5]. The majority of patients with depressive disorder have symptoms of disturbed sleep (insomnia or hypersomnia), which also belong to the diagnostic criteria of the disorder [6]. Further, disturbances in cognitive functions are common to both conditions. A diminished ability to think or concentrate are hallmarks of clinical depression [6], while sleep deprivation and chronic sleep disturbance lead to reduced attention and vigilance, as well as impairments in memory and learning. Information acquisition takes place during waking, most effectively during high vigilance, while sleep promotes memory consolidation. The cellular mechanisms for memory formation comprise the activity-dependent plasticity processes of long-term potentiation (LTP) and long-term depression (LTD). They involve a number of biological processes ranging from changes in gene expression to synthesis of new proteins and comprising hundreds of molecules and molecular pathways [7]. Sleep deprivation, on the other hand, may impact structural plasticity via synaptic downscaling or by reducing spine density and attenuating synaptic efficacy in the hippocampus [8], which might be one of the cellular mechanisms linking depression, disturbed sleep and cognitive functions [9,10].

Similarities between depression and disturbed sleep extend beyond signs and symptoms from the brain. Both conditions co-occur with low-level systemic inflammatory reactions and corresponding changes in gene expression in cells of the immune system, including blood leukocytes [11,12]. They are also frequently comorbid with other medical conditions associated with systemic inflammation, such as obesity or metabolic syndrome [13,14]. Thus, despite a neural origin, depression and insufficient sleep can be considered systemic disorders affecting functions of the whole body.

Insomnia and depression are both frequently triggered by environmental stressors, such as psychological stress or trauma [15–17]. DNA methylation is an epigenetic mechanism that allows interaction between gene expression and the environment. The role of the epigenetic regulation of sleep and emotions is emerging, and changes in DNA methylation have been linked to different forms of depression as well as to antidepressant response [18]. Furthermore, altered expression of histone deacetylases, major methylating enzymes, was found in depressive states, but not after remission, suggesting that methylation-driven changes in gene expression can play a role in the pathophysiology of depression [19]. In mice, sleep deprivation causes marked

alterations in DNA methylation in the cortex, especially in the genes involved in synaptic plasticity, and concordant RNA expression changes [20]. These methylation changes are possibly driven by LTP, a major component in synaptic plasticity [21]. Additionally, in young healthy men, total sleep deprivation caused marked changes in the methylation of white blood cells and RNA expression [20]. In a large cohort of older women, insomnia symptoms, but not shortened sleep, were associated with altered methylation of peripheral blood leukocytes [22].

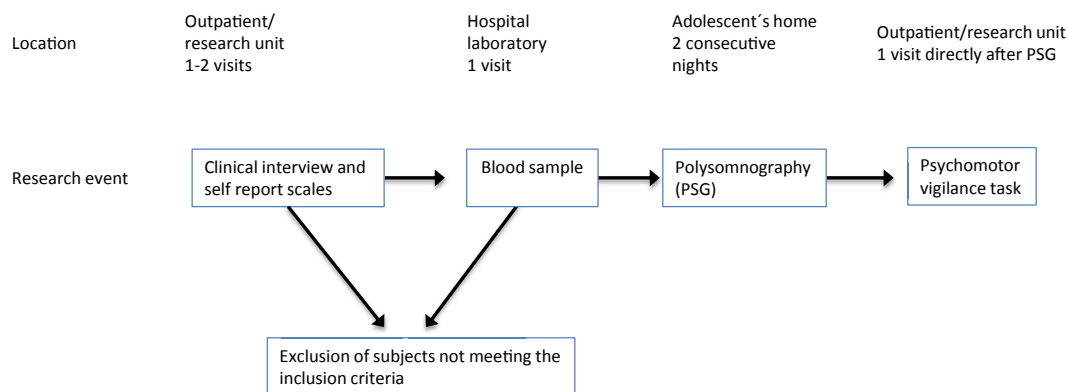
Given the systemic physiological effects of sleep insufficiency and depression, we hypothesized that sleep difficulties and depression have an effect on DNA methylation in peripheral blood leukocytes. To our knowledge, no studies in human clinical samples exist concerning the effect of sleep disturbances and depression on DNA methylation. We studied this in a sample of depressed adolescents with sleep difficulties compared to age- and gender-matched controls. We were particularly interested in identifying deviating patterns of DNA methylation in genes from pathways related to disturbed sleep. Finally, given the impact of sleep difficulties on depression in the short and long term, we hypothesized that sleep difficulties could show an effect on DNA methylation that is not entirely explained by depression severity.

## 2. Methods

### 2.1. Subjects

A total of 20 non-medicated adolescent boys aged 14.70–17.40 years (mean 16.05 years) participated in this study. Ten were cases all suffering from depressive and sleep symptoms recruited from the Helsinki University Central Hospital Department of Adolescent Psychiatry outpatient units, while 10 were healthy controls recruited via advertisements in a newspaper for the hospital staff. Due to dropout, genetic blood samples were not available for two subjects in the case group and one subject in the control group, leaving a total of 17 subjects in the analyses presented in this paper. The adolescents took part in a larger research project focusing on adolescent depression, sleep and brain maturation (the ADSLEEP project) [23–25]. Both the subjects and their parents or legal guardians gave written informed consent for participating in the study, and the study protocol (Fig. 1) was approved by the ethics committee of the Helsinki University Central Hospital.

Exclusion criteria for all subjects included mental retardation, chronic somatic illness, current use of medication, substance abuse/dependence, age over 17.5 or under 14.5 years, insufficient



**Fig. 1.** The study protocol. Exclusion criteria were: mental retardation, chronic somatic illness, current use of medication, substance abuse/dependence, age over 17.5 or under 14.5 years, insufficient knowledge of Finnish language, principal Diagnostic and Statistical Manual of Mental Disorders, fourth Edition (DSM-IV) diagnosis other than depressive disorder, and any contraindications for brain Magnetic Resonance Imaging (MRI). Timeline between different study events varied from case to case, except for Psychomotor vigilance task, which was done directly after PSG.

knowledge of Finnish language, principal Diagnostic and Statistical Manual of Mental Disorders, fourth Edition (DSM-IV) diagnosis other than depressive disorder, and any contraindications for brain magnetic resonance imaging (MRI). All subjects remained free of psychotropic and other medications during the entire study period. The presence of somatic conditions was ruled out based on blood samples, which included measurements of blood cell count, serum testosterone, serum luteinizing hormone, C-reactive protein, fasted blood sugar levels, and liver, kidney and thyroid function measurements. No structural pathologies were found in their brain anatomy according to brain MRI evaluated by a neuroradiologist. The subgroups of cases and controls did not differ in terms of their age, body mass index (BMI), or serum testosterone levels (independent samples *t*-tests  $p > 0.05$ ; Table 1). All subjects consumed less than three cups of coffee daily (or the equivalent amount of other caffeinated products). Subject characteristics are described in detail in Supplementary Table S1.

## 2.2. Psychiatric evaluation

The present and lifetime episodes of DSM-IV axis I disorders were assessed with a semi-structured diagnostic interview, the Schedule for Affective Disorders and Schizophrenia for School-Age Children: Present and Lifetime version (K-SADS-PL) [26]. The same clinician (A.S.U.) performed all of the interviews, and the diagnoses were confirmed in a diagnostic meeting with a senior clinician (M.M.). No axis I diagnoses were found among the controls. The cases were confirmed to suffer from depressive disorder (lifetime first depressive episode for six, second episode for one of the cases; mean length of the current depressive episode at the time of interview,  $58 \pm 53$  weeks), except for one case who only suffered from a circadian rhythm sleep disorder with minor mood symptoms (pessimistic views of the future, feelings of failure, disappointment in oneself, self-criticism, fatigue). None of the cases manifested psychotic features or suffered from bipolar disorder.

The majority of cases (6/8) did not have comorbid axis I disorders. Comorbid anxiety disorder was present in one case and comorbid disruptive behaviour disorder in another.

As part of the DSM-IV axial diagnostic procedure, the global assessment of functioning scale (GAF; numeric range of 0–100) was used according to DSM-IV guidelines to assess overall psychosocial functioning [6]. GAF has previously been used in studies among adolescents [27,28].

Depression symptom severity was assessed with the 21-item Beck Depression Inventory (BDI-21), a standardized 21-item self-rating questionnaire [29] which has been well studied in adolescents [30,31]. In the BDI-21, subjects are asked to rate each of the symptoms on a four-point scale (range 0–3) according to the severity of the symptom (sum score range 0–63). In correlation analyses with differentially methylated positions (DMPs), a shorter version of the BDI, excluding the sleep and tiredness items, annotated here as BDI-19 (sum score range 0–57), was applied.

## 2.3. Psychomotor vigilance task

The psychomotor vigilance task (PVT) is a widely used neuro-behavioural test to assess sustained attention [32]. As part of a larger neuropsychological test battery, a 5-min version of the PVT task was performed with a PVT-192 unit (Ambulatory Monitoring, Inc., Ardsley, NY, USA). During the task, a digital millisecond counter starts to scroll on a small computer screen at random intervals of 2–10 s, and the subjects have to respond by pressing a button as quickly as possible to stop the counter. After each press, immediate feedback on performance is displayed on the screen.

The tests that differed between cases and controls ( $p < 0.05$ , one-way analysis of variance (ANOVA)) were median reaction time (Median RT) ( $p = 0.022$ ), minimum reaction time (MIN RT) ( $p = 0.007$ ) and fastest 10% mean reaction time (FRT) ( $p = 0.010$ ). We chose Median RT to represent vigilance in the secondary analysis.

**Table 1**

Sample characteristics presented for the total sample ( $N = 17$ ), and separately for the cases ( $N = 8$ ) and controls ( $N = 9$ ).

Variable	Mean	SD	Range
Age ( $N = 17$ )	<b>16.05</b>	<b>0.80</b>	<b>14.70–17.40</b>
	Cases ( $N = 8$ )	15.93	14.70–17.30
	Controls ( $N = 9$ )	16.16	14.80–17.40
BMI ( $N = 17$ )	<b>21.22</b>	<b>2.77</b>	<b>16.78–27.55</b>
	Cases ( $N = 8$ )	21.89	16.78–27.55
	Controls ( $N = 9$ )	20.62	17.82–23.80
S-Testo ( $N = 17$ )	<b>19.79</b>	<b>3.77</b>	<b>15.00–27.60</b>
	Cases ( $N = 8$ )	20.28	15.00–26.30
	Controls ( $N = 9$ )	19.36	15.30–27.60
BDI-21 ( $N = 17$ )	<b>9.65</b>	<b>11.05</b>	<b>0–33</b>
	Cases ( $N = 8$ )	17.63 <sup>a</sup>	6–33
	Controls ( $N = 9$ )	2.56 <sup>a</sup>	0–12
GAF ( $N = 17$ )	<b>66.76</b>	<b>16.57</b>	<b>43–90</b>
	Cases ( $N = 8$ )	50.62 <sup>b</sup>	43–59
	Controls ( $N = 9$ )	81.11 <sup>b</sup>	75–90
AIS ( $N = 17$ )	<b>6.12</b>	<b>6.11</b>	<b>0–18</b>
	Cases ( $N = 8$ )	10.75 <sup>b</sup>	1–18
	Controls ( $N = 9$ )	2.00 <sup>b</sup>	0–7
PDSS ( $N = 16$ )	<b>15.94</b>	<b>6.07</b>	<b>6–26</b>
	Cases ( $N = 7$ )	19.71 <sup>a</sup>	12–26
	Controls ( $N = 9$ )	13.00 <sup>a</sup>	6–21
TST ( $N = 17$ )	<b>454.35</b>	<b>69.10</b>	<b>312.5–529.5</b>
	Cases ( $N = 8$ )	412.31 <sup>a</sup>	312.5–517.0
	Controls ( $N = 9$ )	491.72 <sup>a</sup>	469.0–529.5

AIS, Athens Insomnia Scale; BDI-21, 21-item Beck Depression Inventory; BMI, body mass index; GAF, Global Assessment of Functioning Scale; PDSS, Pediatric Daytime Sleepiness Scale; S-Testo, serum testosterone level (nmol/L); SD, standard deviation; TST, total sleep time (min). In bold both groups combined.

<sup>a</sup> Statistically significant differences between the cases and controls at a statistical threshold of  $p < 0.05$ .

<sup>b</sup> Statistically significant differences between the cases and controls at a threshold of  $p < 0.001$  (independent samples *t*-tests).

## 2.4. Assessment of sleep

The sleep symptoms were assessed as part of the K-SADS-PL interview attachment for affective disorders, which contains six items on sleep (initial insomnia, middle insomnia, terminal insomnia, sleep–wake rhythm disturbance, non-restorative sleep, and hypersomnia). The interviewer rates each symptom as non-existent, sub-threshold, or clinically significant according to standard criteria, explained in detail previously [33]. The patients' clinically significant sleep symptoms are listed in [Supplementary Table S1](#). No clinically significant sleep symptoms were found among the controls.

The Athens Insomnia Scale (AIS) was used to assess insomnia symptoms during the past month based on International Classification of Diseases 10th edition (ICD-10) criteria [34]. The AIS is a validated self-report questionnaire consisting of eight items rated on a scale of 0–3 (total sum score range 0–24) on sleep induction, awakenings during the night, final awakening, total sleep duration, sleep quality, daytime well-being, daytime functioning capacity and sleepiness during the day.

The Pediatric Daytime Sleepiness Scale (PDSS) was used to measure daytime sleepiness [35]. The PDSS is a self-rated sleepiness scale specially designed for use among school-aged adolescents. It consists of eight items rated on a scale of 0–4 (total sum score range 0–32) related to daytime alertness, daytime tiredness/grumpiness, trouble waking up, frequency of falling asleep/drowsiness in class and while doing homework, falling back to sleep after being awakened, need for outside help to wake up and thoughts of needing more sleep.

Sleep architecture was measured with polysomnography including electroencephalogram (EEG), electrooculogram (EOG) and chin electromyogram (EMG) recordings. Polysomnography was performed in the adolescents' home environment using portable recording devices (Embla, Flaga Hf. Medical devices, Reykjavik, Iceland; EEG derivations F4-M1, C4-M1, O2-M1 and backup derivations F3-M2, C3-M2, and O1-M2; sampling rate 200 Hz). The electrode positions were determined according to the International 10–20 system. Polysomnography was performed for two consecutive nights and, for the purposes of this study, the first night (adaptation night) was discarded and the second night of polysomnography was used in the analyses. One of the most important functional parameters of the sleeping EEG, slow wave sleep (SWA), was calculated using a power spectral analysis according to a previously described procedure [23]. Because in previous work with the same sample of adolescents we detected a flattening of SWA dissipation associated with depression especially in the frontal electrodes [23], we also wanted to explore its effect on methylation in this study.

## 2.5. Epigenetic analyses

Blood samples were obtained from peripheral venous sampling and DNA was extracted according to standard laboratory methods. The blood samples were taken at varying intervals from the clinical measurements (average time between clinical interview and blood sampling was 25 days  $\pm$  51; range 3–218 days), but since the same investigator also conducted the post-sampling procedures (A.S.U.) and there were no apparent changes in the clinical status of the cases and controls, we can assume that they were symptomatic or asymptomatic, respectively. For depressed cases, the average time between the beginning of depression episode and blood sampling was 415 days (range 78–1078 days; standard deviation (SD)  $\pm$  377 days). We therefore explored the association between time spent depressed and level of methylation for those loci of special interest.

## 2.6. Illumina 450K workflow

DNA was bisulfite converted with the EZ-methylation kit (ZYMO research, Irvine, CA, USA). Bisulfite-converted DNA was then analysed with the Illumina Infinium HumanMethylation450 BeadChip Kit (Illumina Inc., San Diego, CA, USA) at the Estonian Genome Center. Quality control (QC) was performed using R software ( $R \geq 3.0.1$ ) with specific algorithms implemented in R packages 'minfi', 'IlluminaHumanMethylation450kmanifest' and 'limma' [36]. At this stage, probes with a detection of  $p > 0.01$  for any DNA sample or those missing measurements, known as cross-reactive probes, probes on X and Y chromosomes, and probes that contained either a single nucleotide polymorphism (SNP) at the CpG interrogation site or at the single nucleotide extension were excluded from subsequent analysis. Altogether, 46,384 probes were excluded, leaving 439,128 probes for subsequent analysis. Normalization of the 450K data was performed using the subset-quantile within-array normalization (SWAN) function in R-package 'minfi', which takes into account the fact that the Illumina 450k chip uses two types of assays [37,38] with two different types of chemistry, making different probes comparable to each other. We calculated estimates for white blood cell populations of each sample with 'limma' and compared estimated populations for cases and controls with  $t$ -tests ([Supplementary Table S2](#)) [39,40]. No significant differences ( $p < 0.05$ ) were found in cell population distributions between cases and controls. All raw values in 450K were then converted to M-values in order to take into account the non-linear Bernoulli-type distribution of the raw values in the array, which creates biased methylation values, especially at highly methylated probes [41]. The M-value is calculated as the  $\log_2$  ratio of the intensities of a methylated probe versus unmethylated probe, and describes a measurement of how much more a probe is methylated than unmethylated. A positive M-value, hence, means that more than half of a probe is methylated. M-values were then used in data analysis.

## 3. Statistical analyses

### 3.1. Genome-wide analysis of DNA methylation in cases compared to controls

In genome-wide analysis of the data from the Illumina 450K chip, we used an empirical Bayes moderated  $t$ -test in probe-wise comparisons to compare the methylation values between cases and controls and to thereby identify DMPs. This approach was used because it is especially well-suited for experiments with a small number of arrays and for both one- and two-channel microarray experiments, such as the Illumina 450K [42]. To correct for multiple testing, we used the Benjamini–Hochberg procedure (BH) in R-package 'limma' [43], which is based on false discovery rate (FDR) calculations using a  $p < 0.05$  cut-off. We set genome-wide significance to  $5.0 \times 10^{-8}$  [36].

### 3.2. Pathway analysis

The 500 sites that differed the most between cases and controls according to the genome-wide analysis were entered into Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA, USA) to assess the biological relevance of dysregulated DMPs in cases compared to controls. This threshold was chosen to yield a manageable number of sites for pathway analysis. After removing sites that did not pass the QC procedure described above, there were 486 sites remaining. Of those sites, IPA could annotate 332 to a known gene in its pathway repository. A list of those genes is shown in [Supplementary Table S3](#). IPA compares a given set of genes to



previously known biological pathways and uses a right-tailed Fisher's exact test to evaluate whether this gene set is enriched to some pathway more than would be expected by chance. It does not correct for multiple testing, but correction for multiple testing would only affect the magnitude of a detected  $p$ -value, not which pathways arise from the analysis or the order of pathways.

Our top canonical pathway was the synaptic LTD pathway ( $p = 0.00045$ ) followed by the Nitric Oxide Signaling pathway (NOS) signalling in skeletal muscle cells pathway and netrin signalling pathway both with nearly 10-fold higher  $p$ -value ( $p = 0.0042$ ). All  $p$ -values are nominal.

A canonical pathway refers to a molecular pathway that is an idealized or generalized pathway representing common properties of a module or pathway [44]. We chose to continue with canonical pathways as they are the most generalizable across species and tissues and are considered to be the most common pathways of a module or pathway.

### 3.3. Correlation between methylation and depression, indices of sleep and neuropsychological performance

We used linear regression between methylation and mood, tiredness and sleep, and neuropsychological measurements. Since original case–control status was expected to have an effect on results in these secondary analyses, we denoted whether the association was also significant after controlling for the case–control status by including the status as a covariate in the analysis. In addition, we denoted whether the association survived Bonferroni correction for multiple testing. All analyses were performed using SPSS v.24 (IBM, Armonk, NY, USA).

## 4. Results

### 4.1. Epigenome-wide analysis between cases and controls

We first compared the genome-wide DNA methylation levels of depressed adolescents with healthy controls using the Illumina 450K array. There were no genome-wide significant differences in methylation levels between cases and controls (all  $p > 5 \times 10^{-8}$ ).

Next, we proceeded to pathway analysis by including 500 sites that differed most in cases vs controls upon initial analysis, comprising 332 sites that annotated to a known gene. Our top canonical pathway was the synaptic LTD pathway (10/165 sites) ( $p = 0.00045$ ), followed by the NOS signalling in skeletal muscle cells pathway and Netrin signalling pathway, both with a nearly 10-fold higher  $p$ -value ( $p = 0.0042$ ).

Sites in the LTD pathway that were present in the top 500 DMPs were cg16841761 at Calcium Voltage-Gated Channel Auxiliary

Subunit Gamma 1 (*CACNG1*), cg22025854 at Calcium Voltage-Gated Channel Auxiliary Subunit Gamma 6 (*CACNG6*), cg08364956 at a heterochromatic area of Glutamate Receptor, Metabotropic 6 (*GRM6*), cg05110803 at a weak enhancer region of the Insulin Like Growth Factor 1 (*IGF1R*), cg19161850 at a promoter region of the gene encoding for Extracellular Signal-Regulated Kinase 1 and 2 (*ERK12*) (also known as Mitogen-Activated Protein Kinase 1, *MAPK1*), cg12066398 at a promoter region of Phospholipase A2 Group XVI (*PLA2G16*), cg04367351 at Phospholipase A2 Receptor 1 (*PLA2R1*), cg02263165 at a promoter region and downstream transcription start site of Protein Phosphatase 2 Regulatory Subunit 'B' gamma' (*PPP2R5C*), cg18823846 at Protein Kinase, CGMP-Dependent, Type I (*PRKG1*) and cg25405123 at a bivalent promoter region of Ryanodine Receptor 3 (*RYR3*).

Among these sites, DMPs at *GRM6*, *CACNG1* and *IGF1R* showed lower, and all other sites higher, levels of methylation in cases compared to controls (relative hypo- and hypermethylation, respectively) (Table 2).

### 4.2. Secondary analysis with three domains and methylation of sites related to the LTD pathway

Given overall statistical evidence from the pathway analysis and previous evidence on the intimate connection between sleep, mood and cognition [45,46], we focused the secondary analysis on the DMPs identified from the LTD pathway.

Secondary analyses utilized linear regression to model associations between the 10 methylation loci for the LTD pathway and the subjective and objective measures for depressed mood (BDI-19), tiredness (PDSS) and vigilance (median RT), and sleep (AIS, SWA dissipation) in the whole sample. Since only one site, cg12066398 (*PLA2G16*), showed correlation between methylation and depression length ( $\beta = 0.860$ ,  $p = 0.013$  uncorrected for multiple testing), we decided not to adjust analyses by depression length. Adjusting would have abolished controls from the analysis, leaving the sample size very low (Table 3).

In linear regression models with no covariates, we found associations between mood (BDI-19) and *PLA2G16* ( $\beta = 0.77$ ,  $p = 0.00031$ ) and with tiredness (PDSS) and *IGF1R* ( $\beta = -0.79$ ,  $p = 0.00029$ ), *ERK 12* ( $\beta = 0.72$ ,  $p = 0.002$ ) and *PLA2R1* ( $\beta = 0.77$ ,  $p = 0.00047$ ). We also found an association with sleep (AIS) and *RYR3* ( $\beta = 0.76$ ,  $p = 0.00041$ ). Finally, we found an association between *PPP2R5C* and SWA dissipation ( $\beta = -0.67$ ,  $p = 0.004$ ). Of these, results from *PLA2G16*, *IGF1R*, *PLA2R1* and *RYR3* survived Bonferroni correction (Table 3).

In linear regression models where original case–control status was taken into account as a covariate, we found several nominal associations between certain sites and parameters. Nominal

**Table 2**

Relative methylation status of long-term depression pathway methylation sites that appeared in the 500 sites that differed the most between cases and controls according to the genome-wide analysis their respective genes, and values in cases and controls.

Locus	Gene	$p$ -value in 450K array	Methylation level in cases vs controls <sup>a</sup>	Average M-value <sup>b</sup> for cases/controls
cg1684176	<i>CACNG1</i>	0.000381	Lower	3.18/3.49
cg22025854	<i>CACNG6</i>	0.000527	Higher	-1.17/-1.57
cg08364956	<i>GRM6</i>	0.000838	Lower	3.29/3.56
cg05110803	<i>IGF1R</i>	0.000306	Lower	1.47/1.95
cg19161850	<i>ERK12</i>	0.000663	Higher	-3.88/-4.13
cg12066398	<i>PLA2G16</i>	0.000540	Higher	-3.70/-4.07
cg04367351	<i>PLA2R1</i>	0.000659	Higher	-4.14/-4.46
cg02263165	<i>PPP2R5C</i>	0.000207	Higher	-3.14/-3.35
cg18823846	<i>PRKG1</i>	0.000903	Higher	-3.95/-4.20
cg25405123	<i>RYR3</i>	0.000427	Higher	-3.61/-3.89

<sup>a</sup> 'Lower' indicates that methylation at the CpG site is lower in cases vs controls and 'higher' means higher methylation in cases vs controls.

<sup>b</sup> M-values indicate normalized methylation values.

**Table 3**  
Linear regression results between long-term depression pathway methylation sites and mood, vigilance and tiredness, and sleep.

Locus (gene)	Mood		Vigilance and tiredness		Sleep					
	BDI-19		Median RT		PDSS		AIS		SWAdiss	
	B	p	B	p	B	p	B	p	B	p
cg1684176 ( <i>CACNG1</i> )	-0.69	0.002	-0.44	0.08	-0.58	0.02	-0.68	0.003	0.38	0.14
cg22025854 ( <i>CACNG6</i> )	0.44	0.08	0.40	0.11	0.19	0.49	0.55	0.02	-0.25	0.36
cg08364956 ( <i>GRM6</i> )	-0.47	0.06	-0.26	0.31	-0.60	0.01	-0.75	0.001	0.38	0.15
cg05110803 ( <i>IGF1R</i> )	-0.50	0.04	-0.28	0.29	-0.79	0.00029 <sup>a,b</sup>	-0.68	0.003	0.24	0.38
cg19161850 ( <i>ERK 12</i> )	0.55	0.023	0.21	0.43	0.72	0.002 <sup>a</sup>	0.65	0.005	-0.47	0.67
cg12066398 ( <i>PLA2G16</i> )	0.77	0.00031 <sup>b,a</sup>	0.66	0.004	0.33	0.21	0.70	0.002	-0.32	0.23
cg04367351 ( <i>PLA2R1</i> )	0.48	0.053	0.17	0.51	0.77	0.00047 <sup>a,b</sup>	0.68	0.003	-0.30	0.26
cg02263165 ( <i>PPP2R5C</i> )	0.64	0.006	0.51	0.035	0.32	0.23	0.65	0.005	-0.67	0.004 <sup>a</sup>
cg18823846 ( <i>PRKG1</i> )	0.57	0.018	0.49	0.045	0.50	0.051	0.60	0.010	-0.16	0.56
cg25405123 ( <i>RYR</i> )	0.64	0.006	0.42	0.095	0.44	0.091	0.76	0.00041 <sup>b</sup>	-0.54	0.031

AIS, Athens Insomnia Scale; BDI-19, Beck Depression Inventory with questions concerning sleep and tiredness removed; Median RT, median reaction time in psychomotor vigilance task (PVT); PDSS, Pediatric Sleepiness Scale; SWAdiss, slow wave dissipation during first episode of the night measured from frontal electrodes.

<sup>a</sup> Association is significant at <0.05 level when case-control status is controlled for (nominal).

<sup>b</sup> Association is significant after Bonferroni-correction.

correlations of  $p < 0.05$  (not corrected for multiple testing) were observed for cg05110803 (from *IGF1R*), cg19161850 (*ERK 12*) and cg04367351 (*PLA2R1*) to subjective tiredness ( $p = 0.007, 0.041$  and  $0.011$ , respectively), cg12066398 (*PLA2G16*) to depressed mood ( $p = 0.031$ ) and cg02263165 (*PPP2R5C*) to slower SWA dissipation ( $p = 0.02$ ) (Table 3, Fig. 2(a)–(e)).

Although the statistical significance was limited due to small sample size, these findings make biologic sense, with similar trends observed for both cases and controls. For example, lower methylation levels at cg05110803 (*IGF1R*), which showed relative hypomethylation in cases compared to controls in the initial case-control analysis, also associated with more pronounced tiredness in both cases and controls (Fig. 2(a)). Similarly, higher methylation levels at cg02263165 (*PPP2R5C*), which showed relative hypermethylation in cases in the initial analysis, associated with slower SWA dissipation among both cases and controls (Fig. 2(c)).

## 5. Discussion

In this study we searched for deviating methylation patterns in adolescents with depressive disorder and sleep difficulties, as compared to age- and gender-matched controls. Due to small sample size, this was aimed as a hypothesis-generating rather than hypothesis-confirming study. However, we found enrichment of DMPs at genes from a canonical pathway essential for learning and memory consolidation, the synaptic LTD pathway. This finding adds to the systematic evidence of the connection between depressive disorder, sleep and cognition, and supports earlier results concerning the role of sleep in synaptic plasticity, especially during brain development [46].

LTD acts in memory consolidation and synaptic plasticity together with long-term potentiation. LTD can deactivate memories that are activated by LTP and, thus, learning can be seen as a result of a balance between these two processes [47]. LTD also plays a crucial role in the formation of new memories [48]. Sleep deprivation can significantly increase LTD in rat hippocampus [49], and the possible mechanism could be related to an altered expression of  $\gamma$ -aminobutyric acid (GABA) B receptors [50].

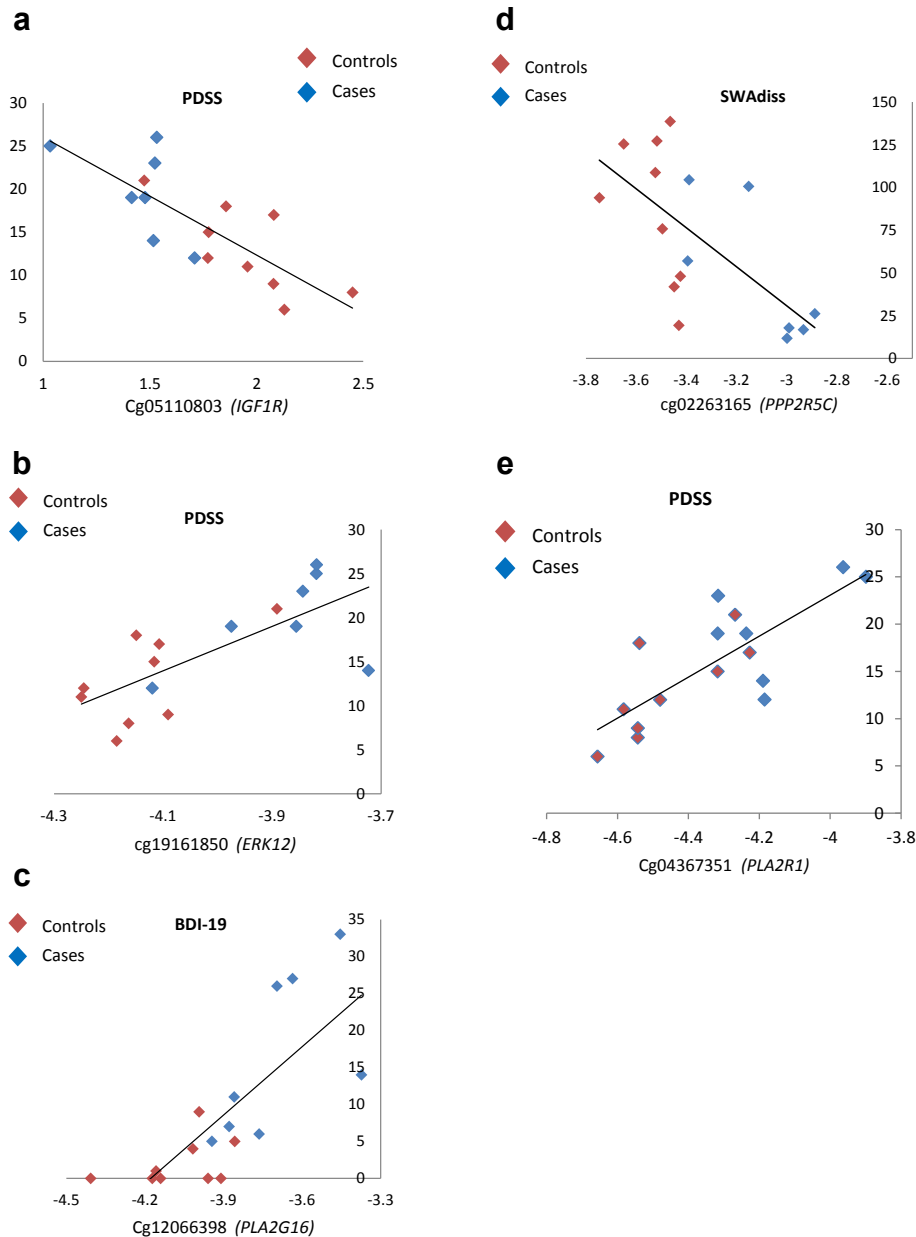
It is postulated that the decrease in SWA during the course of sleep is associated with reduced synaptic strength and is thus related to memory consolidation [51]. Our previous results on the same sample of adolescents indicated that this SWA dissipation that occurs normally during healthy sleep is affected by depression

and related sleep disturbances [23]. The results of the current study further propose a link between SWA dissipation and changes in methylation of the Protein Phosphatase 2 regulatory subunit B'gamma (*PPP2R5C*).

According to an epigenome consortium [52], certain tissues have common methylation patterns across each other, while others do not. With the Epigenome browser (<http://epigenomegateway.wustl.edu/browser/>) (WashU), we explored methylation values and expression levels of *PPP2R5C*'s cg12066398, located at chromosome 14; 102,276,300. It appeared that methylation levels of different histone methylation arrays were comparable between peripheral blood leukocytes and brain hippocampal cells, but no data concerning CpG methylation was available from brain tissue and peripheral blood leukocytes for comparison.

*PPP2R5C* belongs to the phosphatase 2A regulatory subunit B family. This phosphatizing enzyme is implicated in the negative control of cell growth and division (RefSeq, Jul 2008). Mutations in this enzyme family are related to human overgrowth syndromes accompanied by intellectual disabilities [53]. This family of enzymes is highly and widely expressed in the human brain [54]. Protein phosphatases are responsible for the induction of LTD and initiating the cascade with a slow  $\text{Ca}^{+}$  ion flow into neurons [55,56]. We found that those patients with the highest levels of methylation in the promoter region of *PPP2R5C*, suggesting slower LTD induction, also had the flattest SWA dissipation pattern throughout the night. The association between methylation and gene expression can either be active, where methylation directly affects gene expression, or passive, meaning that methylation is just a by-product of expression regulation. Either way, it can be regarded as an indicator of gene expression regulation [57,58]. It has also been shown that even small changes in DNA methylation may result in marked differences in phenotype [59].

The other genes of the LTD pathway found to correlate with sleep and depression in the current study were *ERK12*, *PLA2G16*, *IGF1R* and *PLA2R1*. Extracellular signal-regulated kinase (ERK) is a group of protein kinases that belong to a larger family of mitogen-activated protein kinases (MAPK). *ERK12* is related to the formation of LTP and is partially regulated by phosphatases, such as *PPP2R5C* [60,61]. Notably, we found correlation between daytime sleepiness and *ERK12*, which is in line with findings that this enzyme is involved in the daytime enhancement of LTP [62]. There is evidence that for synaptic plasticity, LTP is favoured during the daytime and contributes to the strengthening of synapses, whereas LTD is induced by slow wave sleep (SWS) [62]. This creates a balance



**Fig. 2.** a. Correlation between methylation of cg05110803 (*IGF1R*) and tiredness (Pediatric Daytime Sleepiness Scale, PDSS) ( $\beta = -0.79$ ,  $p = 0.00029$ ). b. Correlation between methylation of cg19161850 (*ERK12*) and tiredness (Pediatric Daytime Sleepiness, PDSS) ( $\beta = 0.72$ ,  $p = 0.002$ ). c. Correlation between cg12066398 (*PLA2G16*) methylation and depression (Beck Depression inventory with questions concerning sleep and tiredness removed, BDI-19) ( $\beta = 0.77$ ,  $p = 0.00031$ ). d. Correlation between cg02263165 (*PPP2R5C*) methylation and slow wave dissipation (SWAdiss) during first episode of the night measured from frontal electrodes ( $\beta = 0.67$ ,  $p = 0.004$ ). e. Correlation between methylation of cg04367351 (*PLA2R1*) and tiredness (Pediatric Daytime Sleepiness, PDSS) ( $\beta = 0.77$ ,  $p = 0.00047$ ).

called synaptic homeostasis between synaptic strengthening and weakening, allowing new information to be consolidated while preserving previous information. *PLA2G16* (Phospholipase A2 Group XVI) mediates the *ERK12* pathway [63]. *IGF1R* (Insulin-like Growth Factor 1 Receptor) is a protein coding gene that plays a crucial role in central nervous system (CNS) development, maturation and neuronal plasticity [64]. Blocking *IGF1R* in mouse Purkinje cells leads to cessation of LTD [65]. *PLA2R1* (Phospholipase A2 Receptor 1) is a protein-coding gene that has a variety of roles depending on tissue. Its role in LTD is somewhat unclear, but it is identified as a part of both pre- and postsynaptic cascades related to regulation of LTD and is activated by *ERK 12*. Neuronal activity during SWS has been shown to promote LTD in neurons [47,51], and

the slow oscillation that happens in neurons during SWS favours LTD over LTP [48], suggesting that the SWS stage is strongly related to LTD. In contrast, other sleep stages, especially REM sleep, have a stronger effect on LTP [56]. Different sleep disturbances can therefore affect LTP and LTD differently [56,66]. Since in our sample there was no marked difference in REM sleep between cases and controls [23], it is plausible that there were no differences in LTP genetic pathways either.

We did not find marked differences in cognitive performance between cases and controls (unpublished data), except for a longer mean reaction time, minimum reaction time, and fastest 10% reaction time in cases vs controls, implying lower vigilance among cases. This is in line with previous studies concerning the effect of

depression on adolescent cognitive performance [65] and the effect of sleep difficulties on cognition [67]. This is somewhat in contrast to findings in adults, however, putatively reflecting the specific developmental stage involving marked changes in the reorganizing of neural networks (synaptic pruning) and consolidation of cortical neural layers [68,69]. Given the variable but, on average, quite long duration of depressive disorder (mean length from beginning of episode to sampling:  $415 \pm 377$  weeks), the cases in our sample may have been able to compensate for the effect of depression and sleep disturbance on cognition. Although the changes in methylation of the LTD pathway would suggest compromised synaptic plasticity, it is thus possible that altered epigenetic regulation of the LTD pathway is a compensatory mechanism for changes in SWA dissipation, acting to protect individuals from memory and other cognitive impairments during adolescence.

The strengths of the study include the carefully selected and homogeneous sample: all participants were boys, they were all non-medicated, the age range of the sample was narrow, and the participants underwent careful clinical assessment. Further, the use of polysomnography to objectively assess sleep is also one of the major strengths of the study. An evident shortcoming in our study includes the small sample size. Because of this, we were unable to conduct reliable analyses for possible methylation quantitative loci (mQTLs). mQTLs are SNPs that can have an effect on the methylation of one or several distant CpG sites. Thus, the results need to be duplicated and extended in larger samples before more extensive interpretation.

We did not adjust analyses for depression length but, based on our analyses, that would lead to exploration of cases only and thus an even more limited sample size. All participants remained free from psychotropic and other medication during the study period. The patients received, however, "treatment as usual" at the Helsinki University Hospital adolescent psychiatric outpatient unit, which usually consists of individual (frequency typically once a week) and family appointments (frequency typically once a month) and thus we cannot rule out the effect of psychosocial treatment on the findings from the secondary analysis. We analysed epigenetic alterations from peripheral blood leukocytes, and it is known that many epigenetic alterations are tissue specific [52]. It is not, however, an easy task to obtain living brain tissue for epigenetic studies. Peripheral blood leukocytes serve as a good option to study sleep-related changes in the CNS since the brain's lymphatic drainage system connects the immune system, CNS and sleep tightly together [70]. Therefore, it is exciting that we found an association between sleep and methylation of the LTD pathway. Some databases exist where the methylation level of peripheral blood leukocytes and brain tissues can be found (eg, Epigenome browser), but they do not cover all sites or all methylation arrays. Moreover, comparable samples from the same individual, drawn at the same time point, do not exist, making comparison even more uncertain [71]. Further, only boys were included in our sample, limiting the generalizability of the findings to girls.

Our Pathway analysis did not correct for multiple testing, but correction would not change which pathways arise as most likely to associate with a set of genes derived from DMPs. This approach allows the generation of a new hypothesis concerning the interplay between sleep, depression and cognition.

In conclusion, our work presents new insights into the systematic effects of disordered sleep and depression, resulting in a deviating pattern of DNA methylation in blood leukocytes and providing evidence of compromised synaptic plasticity among a group of adolescents with depressive disorder and disturbed sleep. However, further studies involving more detailed neurobiological analysis in both experimental and naturalistic settings should be conducted in order to further explore the link between systematic

and brain-specific epigenetic modifications of LTD pathway genes and their relationships with depression and disordered sleep.

## Acknowledgements

Financial support for the study includes a special federal grant (TYH 2013342) to T.P. and funding from the Academy of Finland (grant number 276612 to A.S.U. and number 290039 to T.P.), Emil Aaltonen Foundation, Finnish Medical Foundation, Finnish Brain Foundation, Orion-Farmos Research Foundation, Päivikki and Sakari Sohlberg Foundation, and the Foundation for Psychocultural Research. We also wish to thank Auli Toivola for her valuable contribution regarding laboratory work.

## Conflict of interest

None of the authors reported conflict of interest.

The ICMJE Uniform Disclosure Form for Potential Conflicts of Interest associated with this article can be viewed by clicking on the following link: <https://doi.org/10.1016/j.sleep.2019.01.050>.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.sleep.2019.01.050>.

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