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BDNF promoter I methylation correlates between post-mortem human peripheral and brain tissues

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ABSTRACT

Several psychiatric disorders have been associated with CpG methylation changes in CG rich promoters of the brain-derived neurotrophic factor (*BDNF*) mainly by extracting DNA from peripheral blood cells. Whether changes in peripheral DNA methylation can be used as a proxy for brain-specific alterations remains an open question. In this study we aimed to compare DNA methylation levels in *BDNF* promoter regions in human blood cells, muscle and brain regions using bisulfite-pyrosequencing. We found a significant correlation between the levels of *BDNF* promoter I methylation measured in quadriceps and vPFC tissues extracted from the same individuals ($n = 98$, Pearson, $r = 0.48$, $p = 4.5 \times 10^{-7}$). In the hippocampus, *BDNF* promoter I and IV methylation levels were strongly correlated (Pearson, $n = 37$, $r = 0.74$, $p = 1.4 \times 10^{-7}$). We found evidence for sex-dependent effect on *BDNF* promoter methylation levels in the various tissues and blood samples. Taken together, these data indicate a strong intra-individual correlation between peripheral and brain tissue. They also suggest that sex determines methylation patterns in *BDNF* promoter region across different types of tissue, including muscle, brain, and blood.

1. Introduction

The brain-derived neurotropic factor (BDNF) is a family member of neurotrophins, which are important regulators of neuronal development, plasticity and survival (Lu and Figurov, 1997). Alterations in BDNF related pathways are thought to be involved in various neurological and psychiatric disorders (Autry and Monteggia, 2012). For example, genetic variation in the *BDNF* gene has been associated with bipolar disorder (Neves-Pereira et al., 2002; Lohoff et al., 2005; Sears et al., 2011), Rett syndrome (Zeev et al., 2009), Parkinson disease (Chen et al., 2007), suicidal behaviours (Perroud et al., 2008; Pregelj et al., 2011) and schizophrenia (Zakharyan and Boyajyan, 2014). At an epigenetic level, environmental factors have been shown to affect the methylation status of various *BDNF* promoters among which promoter IV was the most investigated. In rodents and humans, early-life adversity has been associated with changes in the methylation status of *BDNF* (Berton et al., 2006; Ivy et al., 2008; Roth et al., 2009; Perroud et al., 2013). In rodent models of early-life stress, increased methylation of *BDNF* promoter IV was found to be associated with decreased mRNA expression of *BDNF* in the prefrontal cortex (Ivy et al., 2008; Roth et al., 2009). In addition to early-life adversity, *BDNF* methylation patterns appear to be sex-dependent (Ikegame et al., 2013). A recent study comparing *BDNF* promoter I and IV methylation levels between 100 controls and 100 schizophrenic patients reported a gender-dependant DNA methylation status in peripheral blood (Ikegame et al., 2013). A major limitation to these human studies relates to the fact that *BDNF* methylation levels are generally measured in DNA extracted from blood cells (Ikegame et al., 2013; Perroud et al., 2013). Whether methylation levels in blood cells are relevant indicators of changes occurring in the brain remains an open question. To test this hypothesis, we aimed to compare DNA methylation patterns in *BDNF* promoter regions in blood cells extracted from a cohort of living bipolar patients

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and muscle and brain regions extracted from patients who died from different causes. We also aimed at determining whether sex-differences in *BDNF* methylation status could be observed across tissues. Subjects with psychiatric disorders were selected for this study as *BDNF* has been previously shown to be dysregulated in this population affecting brain functions. Moreover, such samples are influenced by environmental factors known to contribute to epigenetic dis-regulations and psychopathology.

2. Material and methods

2.1. Subjects and clinical data

Detailed demographic information of patients used in this study is provided in supplementary material in Table 1S.

Ventral prefrontal cortex (vPFC) and quadriceps tissues of 98 individuals as well as hippocampus of another 37 subjects were collected post-mortem during medico-legal autopsy performed by forensic physicians.

Since we did not have access to the blood of deceased subjects from whom the vPFC and quadriceps tissues were collected, we used blood from one hundred and twenty-one subjects with a primary diagnosis of bipolar disorder (BD) who were recruited in our specialized mood disorder clinic. Subjects were diagnosed based on a clinical evaluation and assessed using the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) (First et al., 2012).

This study was approved by the research and ethics review board of the Department of Mental Health and Psychiatry of the University Hospitals of Geneva. Informed written consent was obtained either from patients or from their families when a patient was deceased.

2.2. Collection and dissection of post-mortem vPFC, hippocampus and muscle tissues

Human brain and muscle tissues were collected at autopsy from the Institute of Forensic Medicine, Geneva, Switzerland. The brain was removed from the cranium and was examined for neuropathological abnormalities. Dissection of the vPFC as well as the hippocampus was performed by forensic physicians and skilled technicians. Tissue blocks from the ventral prefrontal cortex (vPFC; Brodmann's area 11) were dissected in 0.5- to 1-cm coronal slices from the right hemisphere. Samples of muscle tissue were drawn from quadriceps. The samples were immediately stored at −80 °C. Brain samples were then carefully dissected on a glass surface with ice, in accordance with Brodmann's Atlas. Only grey matter was carefully isolated. Samples were then stored at −80 °C until DNA extraction.

2.3. DNA extraction and bisulfite conversion

Approximately 100 mg of vPFC, quadriceps or hippocampus tissues were homogenized using an Ultra-Turrax® T8 homogenizer (IKA, France). Genomic DNA was extracted from homogenized tissue using the Illustra Nucleon Genomic DNA Extraction kit (GE Healthcare, Dübendorf, Switzerland). The same kit was used to extract DNA from blood samples of living BD patients. Bisulfite conversion of unmethylated cytosines to uracil was performed on 2 μg of DNA using the EpiTect Bisulfite Kit (Qiagen, Germany). Bisulfite treated samples were resuspended in 20 μl EB Buffer and were stored at −20 °C.

2.4. PCR amplification and pyrosequencing

Methylation levels of 4 CpG sites were measured in *BDNF* promoter I in vPFC, quadriceps and blood samples using the pyrosequencing assay named "assay A". The original sequence is located in the coordinates chr11:27743930-27744048 on the hg19 human version on the negative strand and corresponds to: GGGCTGTTAACTCACATTTGGGAAGCCATAACCCATTAGAGCAAA[1]-(CG)CAGTCATAACTTCATTCAACTCAGC[2](CG)CT[3](CG)AGAGCT[4]-(CG)GCTTACACAGGTTCCTGT GGGCAACTAGTGGCT . Bases underlined show the binding sites of the amplification primers. The CpG sites tested are numbered and appear in parenthesis. The amplification primers to measure methylation levels in the four CpG sites within *BDNF* promoter I were designed using MethPrimer software (Li and Dahiya, 2002). Forward primer: 5′-GGG**T**TGTTAA**TTTAT**ATTTGGGAAG**T**-3′ (bold: C converted to T) and 5′-biotinylated reverse primer (5′-A**A**CCACTA**A**TT**A**CCCACA**AA**-AACC-3′ (bold: G converted to A) were used for PCR amplification of the bisulfite converted DNA leading to a 119 bp product. The product was sequenced with the forward primer. The sequence to analyze was: **T**ATAA**TTT**ATTAGAG**T**AAA[1](YG)**T**AGT-**T**ATAA**TTT**ATT**T**AA**TTT**AG**T**[2](YG)**T**T[3](YG)AGAG**TT**[4](YG)G**T**TTA**T**-A**T**AGGTT**TT**TGTGGG**T**AA**T**TAGTGG**T**T where the four "YG" (CG or TG) correspond to the four CpG sites from 1 to 4 respectively. Bold T corresponds to cytosine bisulfite-converted to thymine.

Additionally, methylation levels of 13 CpG sites in the *BDNF* promoter I, using the pyrosequencing assay named "assay B", as well as 15 CpG sites in the *BDNF* promoter IV, using the pyrosequencing assay named "assay C", were measured in hippocampal tissue. In the pyrosequencing assay named "assay B" for measurements performed on 13 CpG sites in *BDNF* promoter I, the original sequence is located in the coordinate chr11:27743698–27744046 on the hg19 human genome version and corresponds to: GGGTAAAAAA-AGGAAACTTCTTAGAAAAGTT(CG)TGCCCCTCCCCCTCCCCCATC ATGACTAAGGGTC TCCAGC[1](CG)ATGAGGT[2](CG)TGAGTGATG-ATCAAATGGGGACTGGGGGGAGGGGGG[3](CG)AGTAAGTGACTTGT-CCTTGGGAACATCTGCATG[4](CG)T[5](CG)AAG[6](CG)[7](CG)AACCAGCC-CAACAACTTTCCCTTTTCCTCTTAGGTACTGATGACTAGG[8](CG)AGAG-GCACCAAGG[9](CG)AGCCACTAGTTGCCCACAGGAACCTGTGTAAG-C[10](CG)AGCTCT[11](CG)AG[12](CG)GCTGAGTTGAATGAAGTTATGAC-TG[13](CG)TTTGCTCTAATGGGTTATGGCTTCCCAAATGTGAGTTAACA-GC. Bases underlined show the binding sites of the amplification primers for human bisulfite converted DNA and resulted in a 349 bp amplicon. The bases located in the box represent the binding sites of the sequencing primer. The CpG sites tested are numbered and appear in parenthesis. Forward (F), reverse (R) and sequencing (S) primers used to measure methylation percentages in 13 CpG sites in *BDNF* promoter I were; F: 5′-GGGTAAAAAAAGGAAA**T**TT**T**TTA-GAAAAGT-3′ (bold T corresponds to cytosine converted to thymine), R: biotin-5′-**A**CT**A**TTAACTCACATTT**AAA**AAA**A**CCATA-3′ (bold A corresponds to guanine converted to adenine), S: 5′-**TTT**-T**TTTTT**ATT**A**TGAT**T**AAGGGT**T**-3′ (bold T corresponds to cytosine converted to thymine). The sequence to analyze was T**TT**AG**T**[1](YG)-ATGAGGT[2](YG)TGAGTGATGAT**T**AAATGGGGA**T**TGGGGGGAGGGG-GG[3](YG)AGTAAGTGA**T**TTGT**TT**TTGGGAA**T**AT**T**TG**T**ATG[4](YG)T[5]-(YG)AAG[6](YG)[7](YG)AA**TT**AG**TTT**AA**T**AA**T**TTT**TTT**TTTT**TT**TTTAGG-TA**T**TGATGA**T**TAGG[8](YG)AGAGG**T**A**TT**AAGG[9](YG)AG**TT**A**T**TAGTT-G**TTT**A**T**AGGAA**TT**TGTGTAAG**T**[10](YG)AG**T**T**T**[11](YG)AG[12](YG)G**T**T-GAGTTGAATGAAGTTATGA**T**TG[13](YG)TTTG**T** where the 13 "YG" representing either CG or TG correspond to the 13 CpG sites from 1 to 13 respectively. Bold T corresponds to cytosine bisulfite-converted to thymine.

Primers used for the pyrosequencing assay named "assay C" developed to measure methylation levels in 15 CpG sites in *BDNF* promoter IV as published previously (Keller et al., 2010) were F: 5′biotine-TTTG**T**TGGGG**T**TGGAAGTGAAAA**T**-3′ (bold T correspond to cytosine converted to thymine), R: 5′-CCCATCA**A**CAA-**A**AA**A**CTCCATTT**A**ATCTC-3′ (bold A correspond to guanine converted to adenine), S: 5′-ACA**AAA**AA**A**ATTTCAT**A**CTA**A**-3′ (bold

A corresponds to guanine converted to adenine). The original sequence is located in the coordinates chr11:27723067–27723380 on the hg19 human genome version and corresponds to: CCCATCAGCGAGAAGCTCCATTTGATCT(CG)GCAGAG-GCAGGGAGATTTCATGCT AG TT[1](CG)C[2](CG)GGGGGAG[3]-(CG)GCAG[4](CG)AGAGCAGCCCTCTC[5](CG)[6](CG)GTGAATGGGAAAG-TGGGTGGGAGTCCA[7](CG)AGAGGGCTCCA[8](CG)GTGCCTTGA[9](CG)-TG[10](CG)CTGTCATATGATACCTC[11](CG)CTGCCT[12](CG)AAATAGAC-ACTCTAGTGCA[13](CG)AATTACCAGAATCAAAATTCAG[14](CG)CATTT-AAAATGATACATCTTTTATTAGAAGAGTTC[15](CG)TTCCAGGGCATT-GCATGCTTTTGCAGATGTTTTCACTTCCAGCCCCAGCAAA. Bases underlined show the binding sites of the amplification primers for bisulfite converted DNA resulting in a 315 bp product, whereas the bases located in the box represent the binding sites of the sequencing primer. CpG sites tested appeared in parenthesis and are numbered. The bisulfite modified sequence to analyze was: TT[1](CR)***Keller***=*CpG4*C[2](CR)***Keller***=*CpG3***AAAAAAA**[3](CR)***Keller***=*CpG2***A**CAA[4]-(CR)***Keller***=*CpG1*AAAACAACCCTCTC[5](CR)[6](CR)**ATA**AAT**AAA**AAA**ATA-AA**T**AAA**AATCCA[7](CR)A**AAAAA**CTCCA[8](CR)**ATA**CCTT**A**A[9](CR)T**A**[10]-(CR)CT**A**TCATAT**A**TACCTC[11](CR)CT**A**CCT[12](CR)AAATA**A**ACACTCT-A**A**T**A**CA[13](CR)AATTACCA**A**AATCAAAATTCA**A**[14](CR)CATTTAAAAT-**A**ATACATCTTTTATTA**A**AAA**A**A**A**TTC[15](CR)TTCCA**AAA**CATT**A**CAT**A**-CTTTT**A**CA**A**AT, where the 15 "CR" sites that represented either CG or CA correspond to the 15 CpG sites from 1 to 15, respectively. Bold A corresponds to guanine converted to adenine. The four CpG sites tested by Keller et al., are indicated with their respective numbering (Keller et al., 2010).

PCR reactions were performed in 25 μl final volume containing 50 ng bisulfite-treated DNA, 1× PCR buffer, 1.6 mM $MgCl_2$, 200 μM dNTP, 0.2 μM forward primer, 0.2 μM Reverse primer, 2 units of Hot Start Taq DNA polymerase (HotStart Taq DNA Polymerase Kit, Qiagen). PCR condition was as follows: 95 °C for 15 min followed by 50 cycles of 95 °C for 30 s, 58.3 °C for 30 s, 72 °C for 10 s. Optimal annealing temperature was determined experimentally by using a temperature gradient. A no-template negative control was added to each PCR plate and agarose gel electrophoresis was performed to control for nonspecific amplification. Negative controls were also pyrosequenced as well as five different dilutions (0%, 25%, 50%, 75%, 100%) from unmethylated (0%) and methylated (100%) genomic DNA standards (EpiTect PCR Control DNA, Qiagen) (Fig. 1S). PCR products were immobilized onto streptavidin-coated sepharose beads (Fisher Scientific, ref 17-5113-01) using a Vacuum preparation tool (Biotage), washed in ethanol 70%, and denatured in 0.2 M NaOH. Single stranded DNA was then washed in 10 mM Tris–acetate pH 7.6 before being dispensed into 20 mM Tris, 2 mM $MgAc_2$ containing 15 pmol of forward primer in 40 μl final volume by gently agitating the aspirator. The resulting single stranded DNA molecules were then sequenced in a PSQ 96 MA (Qiagen) using appropriate enzymes, substrates and nucleotides (PyroMark Gold Q96 SQA Reagents, Qiagen).

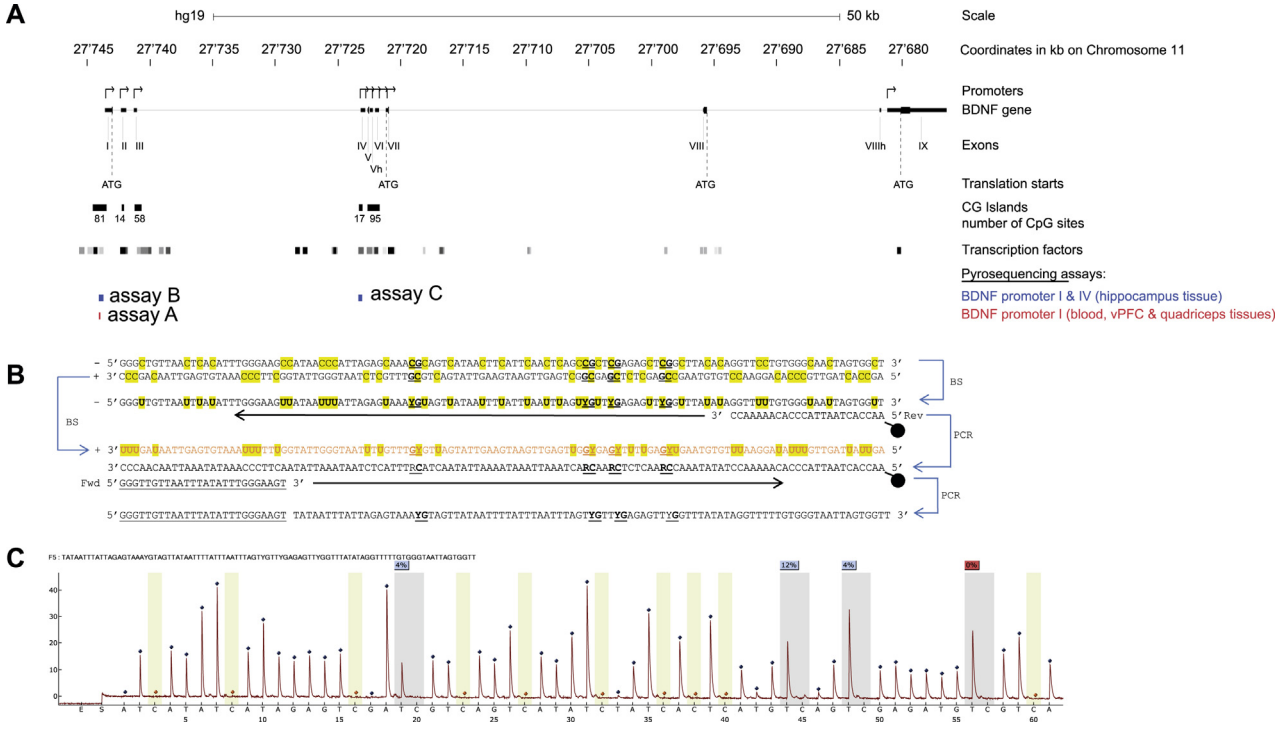

Fig. 1. Pyrosequencing analysis of DNA "methylation" in *BDNF* promoter I. (A) Structure of the human *BDNF* gene and its features relevant for this study extracted from the UCSCS website (https://genome.ucsc.edu/) with respect to their relative sizes and locations on the chromosome 11 according to the hg19 genome version. Regions coding for antisense *BDNF* are not shown. The scale bar represents 50 kb. Exons appear as black boxes numerated according to the study of Pruunsild et al. (2007). Introns appear as horizontal black lines. Arrows represent promoters. The four known AUG translation starts encoded as ATG are shown. CpG islands are shown as black boxes with the number of CpG sites they contain. The density of transcription factors presence along *BDNF* is shown with a gradient from white to black for low to high respectively according to public available ChIP-Seq experiments. Localization of the two pyrosequencing assays used for the analysis of hippocampus samples in *BDNF* promoters I and IV are shown as two blue boxes (assays B & C). Localization of the pyrosequencing assays used for the analysis of blood, vPFC and quadriceps samples in *BDNF* promoter I appeared as a red box (assay A). The boxes representing the locations of the pyrosequencing assays were generated by using the DNA BLAT alignment tool. (B) Reactions linked with the pyrosequencing assay A. CpG sites are underlined. Both negative (−) and positive (+) DNA strand are modified by bisulfite treatment (BS) leading to the substitution of unmethylated cytosine (highlighted in yellow) by uracil and to the loss of strand complementarity. PCR amplified the BS modified negative strand whereas the BS modified positive strand is lost within the PCR reaction (orange). The biotin, shown as a dark round structure, is incorporated to the newly synthetized strand by incorporation of the reverse primer (Rev). (C) The pyrogram obtained after pyrosequencing of the biotin-captured strand with the forward primer (Fwd) used as a sequencing primer for the pyrosequencing assay A. The percentages of methylation resulted to the ratio of C and T peak intensities in the grey highlighted regions. Blue percentages are of good quality and the red percentage is of bad quality. Blue diamonds indicate pics used as references. Yellow highlighted regions showed bisulfite controls added within the dispensation order.

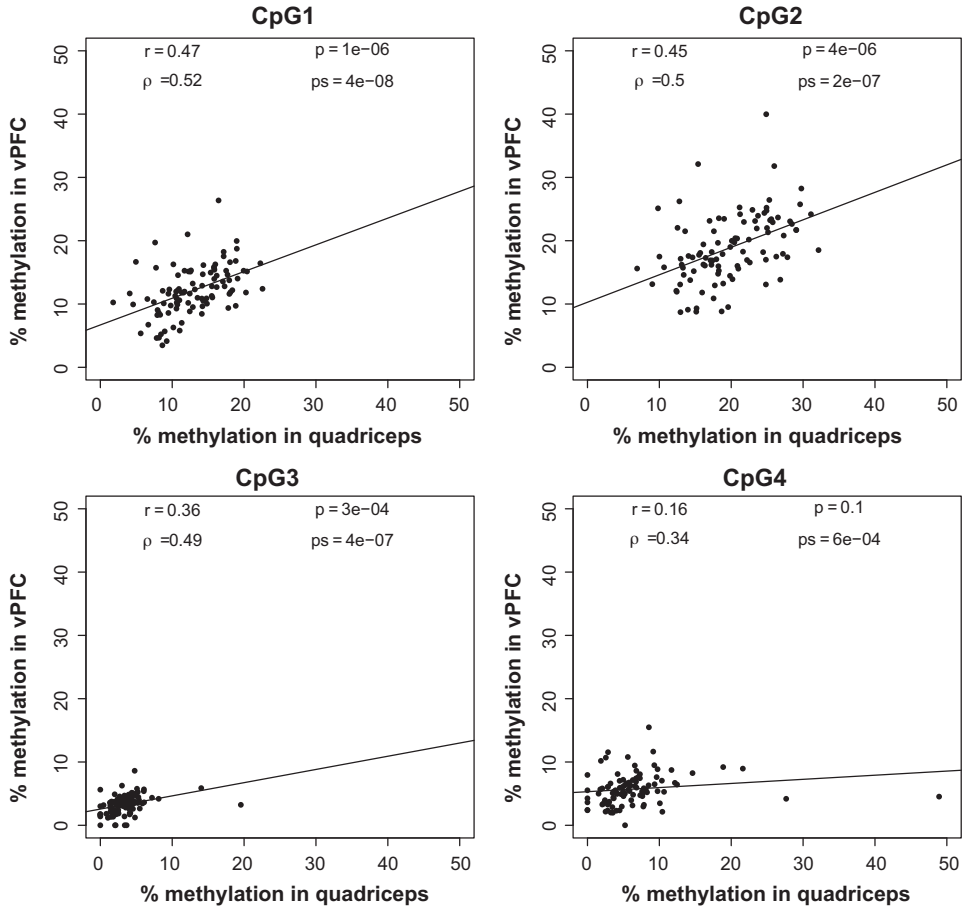

Fig. 2. Correlation of *BDNF* promoter I methylation between two tissues. Correlation analysis of methylation percentages in each of the four CpG sites tested in *BDNF* promoter I (assay A) between the vPFC tissues, shown on the *y*-axis and the quadriceps tissues, shown on the *x*-axis. Each dot represents the methylation percentages recorded in the two post-mortem tissues extracted from the same 98 individuals at the corresponding CpG site. Both Pearson correlation coefficient (r) and its associated p-value (p), as well as Spearman rho (ρ) with its corresponding p-value (p_s) are indicated on each of the four graphs.

2.5. Statistical analysis

Only the four first CpG sites were used for analysis in each of the three pyrosequencing assays (A, B and C) after validation of the recorded methylation levels using methylated standards (Supplementary material). This restriction of sites was performed in accordance with a previous work (Keller et al., 2010) and because pyrosequencing is prone to inaccuracies in the longer extension sites (Tost and Gut, 2007). Pearson's r and Spearman's rho were used to test the correlation between *BDNF* methylation levels of quadriceps and vPFC tissues. Pearson's r was used to test site-specific correlations between methylation levels of *BDNF* promoters I and IV in the hippocampus. t-Test was used to compare *BDNF* methylation levels between males and females in the different tissues. Analysis of variance (ANOVA) was used to test the effect of important demographic and clinical variables on the experimental methylation levels recorded. Missing values were not taken into account in the ANOVA.

3. Results

3.1. Intra-individual correlation in methylation of BDNF promoters

A positive correlation of *BDNF* promoter I methylation levels (assay A) was observed between two different tissues (vPFC and quadriceps) extracted from the same individuals (Pearson, $n = 98$, $r = 0.48$, $p = 4.5 \times 10^{-7}$). The correlation observed between the two tissues remains significant when considering each CpG sites individually (Fig. 2).

In addition, there was a positive correlation between the mean methylation levels of *BDNF* promoter I (assay B) and *BDNF* promoter IV (assay C) measured in the hippocampus (Pearson, $n = 37$, $r = 0.74$, $p = 1.4 \times 10^{-7}$). The correlation observed between *BDNF* promoter I and *BDNF* promoter IV remains significant when considering each CpG sites individually, except for the first CpG site in *BDNF* promoter IV that do not correlated with the 3 first CpG sites in *BDNF* promoter I (Fig. 3).

3.2. Sex-dependent differences

Methylation levels in *BDNF* promoter I (assay A) were significantly higher in males ($n = 72$) compared to females ($n = 26$) in the vPFC for CpG_2 ($t = -2.3$, df = 60.3, $p = 0.02$) and in the quadriceps for CpG_4 ($t = -2.2$, df = 95.96, $p = 0.036$). Looking at the blood of BD subjects, there was also a significant increase in *BDNF* promoter I methylation levels in males ($n = 58$) compared to females ($n = 67$) in CpG1 ($t = 2.10$, df = 107.05, p-value = 0.038) (Fig. 4). The methylation of *BDNF* promoter I and IV (assays B & C) were not significantly different between sex in hippocampus (20 males, 17 females) (data not shown).

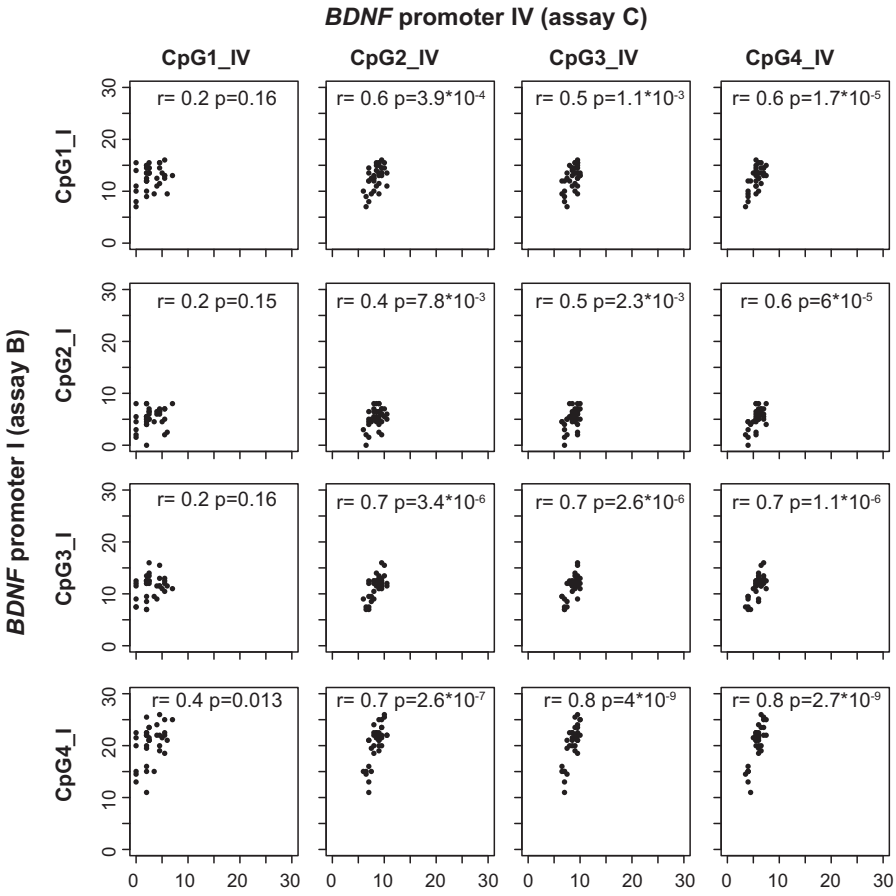

Fig. 3. Correlation of methylation between *BDNF* promoters I and IV in hippocampus. Methylation levels and correlations analysis of the four first CpG sites between both *BDNF* promoters I and IV. Promoter I was tested with assay B and promoter IV was tested with assay C. Each dot represents the methylation percentages recorded in both *BDNF* promoters (I and IV) in the hippocampus of the same 37 individuals. The methylation percentages in the four first CpG located in *BDNF* promoter I (CpG1_I to CpG4_I) are shown in the *x*-axes. The methylation percentages in the four first CpG located in *BDNF* promoter IV (CpG1_IV to CpG4_IV) are shown in the *y*-axes. Scales vary from 0% to 30% of methylation with 5% methylation increment. CpG site-specific correlation analysis is performed. Pearson correlation coefficient (*r*) and its associated *p*-value (*p*) are indicated on each of the 16 graphs.

4. Discussion

We firstly found that methylation levels measured across two tissues extracted from the same individuals were strongly correlated. Indeed, we found a strong correlation between vPFC and quadriceps for the methylation levels of *BDNF* promoter I suggesting that measures of *BDNF* promoter methylation performed in a peripheral tissue could reflect the methylation levels obtained in the brain. These results echo those found in animal studies. Blood was indeed a good proxy for brain in terms of cytosine methylation measured at the genome wide level using both methyl-binding domain enrichment and next-generation sequencing in C57BL/6 mice treated with haloperidol (Aberg et al., 2013). Using a target gene approach, methylation changes in the *Fkbp5* mouse gene were found to be correlated in brain and blood following exposition to glucocorticoids (Ewald et al., 2014).

Similar works performed in humans are rare and challenging. Nevertheless our results are also concordant with previous report identifying individual-specific methylation patterns conserved across different tissues in humans (Byun et al., 2009). Davies et al. (2012) investigating inter- and intra-individual differences of methylation across blood and multiple regions of the brains found distinct tissue-specific patterns of DNA methylation including *BDNF*. Interestingly, in this study, although between-tissue differences in DNA methylation was by far more pronounced than between-individual differences, the authors found that variation in methylation levels across brain and blood may still reflect inter-individual differences and concluded that peripheral tissues may be useful in studies of psychiatric disorders. Moreover, as highlighted by Guidotti et al. (2014) in a review on peripheral epigenetic biomarkers of schizophrenia, the fact that methylation levels of genes such as *BDNF* are altered in the same direction in both brain and blood lymphocytes strongly support the validity of using peripheral blood cells for the investigation of the impact of environmental factors on epigenetic processes.

In our study, blood samples were unfortunately not available and were kept for the purposes of autopsy. The quadriceps tissue was then chosen as the proxy to test for brain specific alteration. According to our knowledge this study consists of the largest number of human individuals tested for methylation across specific brain regions and a peripheral tissue. Furthermore, the correlation of methylation in *BDNF* promoter I represents a strong result in that field.

We secondarily found that the methylation levels of *BDNF* promoters I and IV were highly correlated in the hippocampus of a same subject. These results are compatible with previous reports testing the two *BDNF* promoter I and IV from blood samples in borderline personality disorder patients (Perroud et al., 2013) and in schizophrenia patients (Ikegame et al., 2013), suggesting that the methylation level at one promoter may predict methylation level at the other.

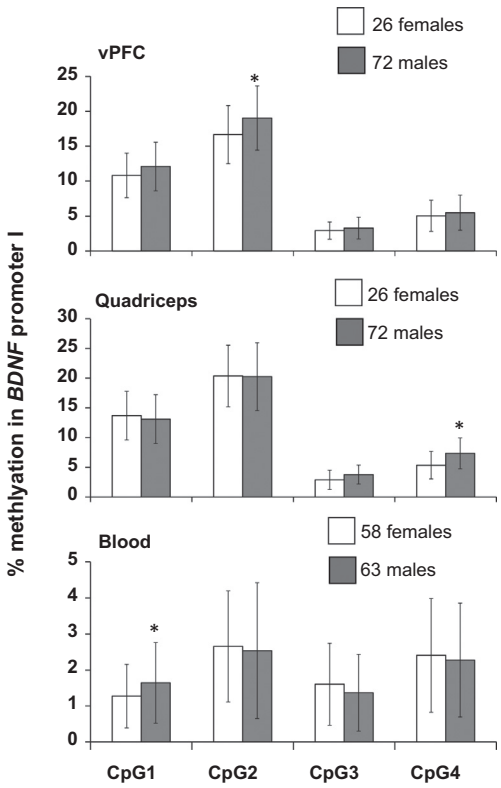

Fig. 4. *BDNF* promoter I methylation between sex and tissues. *BDNF* promoter I methylation values are shown on the *y*-axis. The corresponding four CpG sites are shown on the *x*-axis. Data generated with assay A from vPFC, quadriceps and blood are shown. * $p < 0.05$ for the difference between males and females. Error bars represent standard deviations.

The three regions analyzed in *BDNF* promoters I and IV are characterized by the presence of CpG islands (CGI), transcription factors and active state of chromatin (Fig. 1 and public available DNase-Seq data, not shown). CGIs are able to initiate transcription depending on their methylation states (Deaton and Bird, 2011), even in the absence of a TATA box (Butler and Kadonaga, 2002). The information extracted from four sites for each assay is most probably reflecting the methylation state of the whole CpG Islands. Indeed, a recent study suggests that a single CpG site could be a good proxy for the whole CpG Island to which the site belongs to in terms of methylation (Barrera and Peinado, 2012). The different promoters located at the 5′ side of the *BDNF* gene combined with different splice sites and the presence of an antisense regulatory mechanism are thought to be responsible for the production of different transcript isoforms of *BDNF* that are differentially expressed among tissues and at various development periods (Wong et al., 2009). Previous work performed in mice showed that promoter IV was active in nonneural tissues (lung, heart, and muscle), whereas promoters I, II, and III were predominantly used in the brain (Timmusk et al., 1993, 1999). Note that *BDNF* promoters were numerated differently across different studies (Pruunsild et al., 2007). Nevertheless, even if *BDNF* promoters are activated in different tissues, thus probably differentially methylated across tissues, our results still showed strong correlations of methylation in *BDNF* promoter I across two different human tissues and strong correlation between promoters I and IV when measured in the same tissue from the same individuals.

Analysis of methylation levels showed significant increased methylations in males compared to females in three different sources (blood, quadriceps and vPFC). These results are concordant with previous studies reporting a significantly higher presence of methylated cytosine in males compared to females (Fuke et al., 2004), and with a gender dependent methylation status reported in the peripheral blood when measuring *BDNF* promoter I and IV methylation levels (Ikegame et al., 2013). The sex-differences observed in blood, quadriceps and vPFC were however not observed in hippocampus. Absence of sex-specific differences in the hippocampus could be explained by the lowest quantity of subjects tested.

Interestingly the enhancer of zeste homolog 2 (EZH2) binds to the pyrosequenced region in *BDNF* promoter I. EZH2 is a transcription factor negatively regulated by androgen (Bohrer et al., 2010) and has been implicated in a complex silencing mechanism of the *BDNF* locus (Varela et al., 2012), supporting the sex-dependent epigenetic repression of *BDNF* promoter I. EZH2 binds additionally to the *SLC6A4* according to public ChIP-seq data in a region where a gender effect on cytosine methylations was reported (Philibert et al., 2008).

The main limitation in this work is that hydroxyl-methylation of cytosine is not discriminated from methylation of cytosine by the test (Huang et al., 2010). Therefore, methylation levels we detected could also be due to other kinds of DNA modifications. Another important limitation of this study is that it was not possible to perform correlation analysis between blood and brain samples derived from the same individuals.

In conclusion, using cortical and peripheral tissues from the same individuals we aimed at determining if the periphery could be used to measure epigenetic alterations occurring in the brain. Results showed that the periphery was correlated with the brain on *BDNF* promoter I methylation levels, suggesting that the periphery can reflect, albeit in a limited manner, epigenetic marks occurring in the brain. Males showed higher methylation levels compared to females in different sites and this difference was dependent on the tissue tested, supporting additional experimental evidence for a sex-dependent methylation of *BDNF* promoter I. Analysis of methylation in both *BDNF* promoters I and IV suggest that both promoters are methylated in a similar manner.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neures.2014.10.003.

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