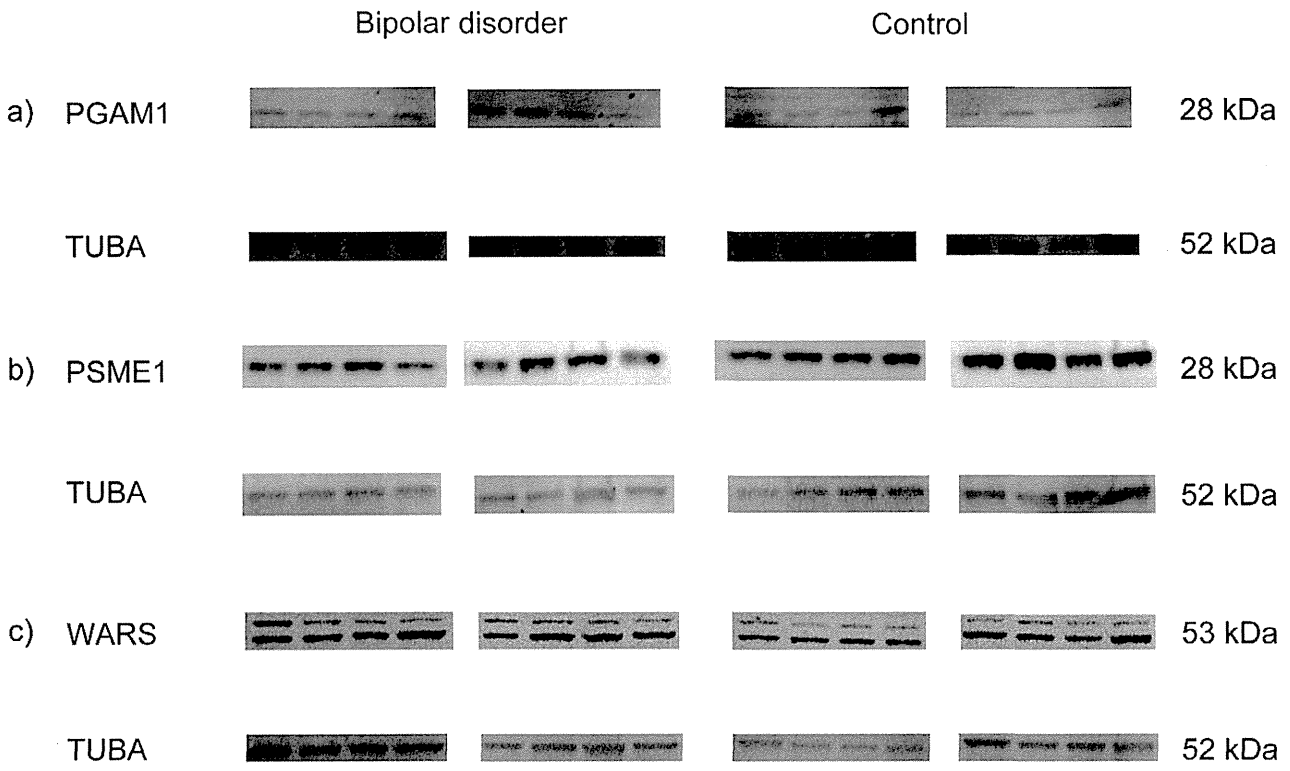

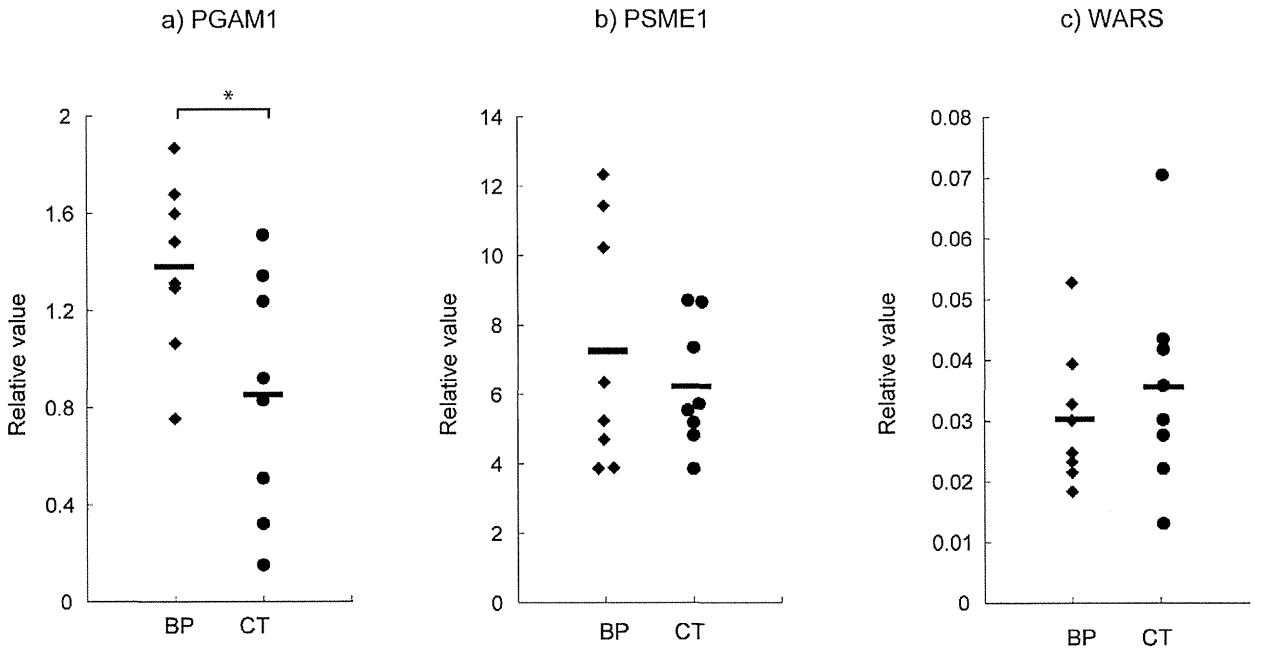

Figure 3. Verification of the alternation of PGAM1 using the other internal protein, tubulin alpha (TUBA). The methods are similar to the Figure 2, except that tubulin alpha (TUBA) was used for the internal standard. a) PGAM1, b) PSME1, c) WARS. Scatter plots show the ratio of each protein to an internal standard protein, TUBA, measured by densitometric scanning of the band intensities. The PGAM1/TUBA ratio was significantly

higher in patients with bipolar disorder compared with controls ($p<0.05$). Number of the subjects is 8 for bipolar disorder and 8 for controls, respectively.
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pathway. The categories pertained to carbohydrate metabolism (8.38E-10–3.27E-02, 14 molecules) and cell death (2.04E-05–4.72E-02, 20 molecules) (Fig. 1B). Pathway analysis and gene ontology classification using PANTHER and DAVID were conducted on the same protein IDs. These analyses also showed pathways and categories associated with glycolysis and anti-apoptosis. Taken together, most proteins identified in the present study were related to glycolysis and neurological diseases.

Case–control study by Western blot analysis

We postulated that the differentially expressed proteins might be candidate biomarkers for bipolar disorder. To validate the findings from the proteomic profiling study and to examine the possibility of biomarkers for bipolar disorder, Western blot analyses were performed using a case–control sample set consisting of eight subjects with bipolar disorder and eight healthy control subjects. To compare the protein levels across individuals, protein concentration was measured by the Bradford method, and equal amounts of proteins were loaded onto the gels. Commercially available antibodies for the candidate 7 proteins (PSME1, RPLP0, TPI1, ALDOC, ANXA4, PGAM1, and WARS) were searched for, and among available antibodies, those against PSME1, WARS, and PGAM1 showed good performance, and thus they were chosen for quantification by Western blot analysis. The levels of PGAM1, PSME1, and WARS were quantitatively investigated by Western blot analysis using NM23A as a standard (Fig. 2). Expression of PGAM1 was recognized by the presence of a single band at around 28 kDa and its protein expression was increased by 197% in bipolar disorder compared with controls ($p<0.05$). However, the levels of the other proteins were similar between bipolar disorder and controls in this case–control sample set (Fig. 2).

The absolute band intensity for the PGAM1 was also significantly higher in patients with bipolar disorder (0.93 ± 0.23 [mean $\pm$ standard deviation] [arbitrary unit]) than control subjects (0.39 ± 0.18, $p<0.0005$). In addition, we also performed an independent experiment using the other, more popular housekeeping protein, tubulin alpha (TUBA), as an internal standard. This analysis also showed higher PGAM1 levels in patients with bipolar disorder than controls ($p<0.05$) (Figure 3).

Discussion

In this study, we identified 53 proteins that were differentially expressed between a pair of monozygotic twins discordant for bipolar disorder; 34 were up-regulated and 19 were down-regulated. The differentially expressed proteins included those previously implicated in psychiatric disorders, such as ALDOC, ENO1, and PRDX2 [10,33]. Differences for ALDOC, ANXA4, PGAM1, PSME1, RPLP0, TPI1, and WARS between twins were regarded as robust because they were identified in three of four experiments with high scores.

To evaluate whether identified proteins might be biomarkers for bipolar disorder, we performed a case–control study for several proteins by Western blot analysis using available antibodies. An increased level of PGAM1 was observed in samples from patients with bipolar disorder. PGAM1 is an enzyme of the glycolytic pathway that catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate [34]. This enzyme also promotes glycolysis and ATP production via the TCA cycle and the electron transport system. Although previous studies using postmortem brains of patients with bipolar disorder and schizophrenia suggested altered protein expression of glycolysis enzymes, including PGAM1 [10,35], the results were controversial. The differentially expressed proteins between bipolar disorder and healthy control including PGAM1, might be a clue to understand the biological basis of bipolar disorder.

To examine whether the 53 identified proteins were related to each other and constituted a global molecular network, pathway, or category, we applied IPA to our data. The results showed that the networks having a high score belonged to cell death, energy production, and glucose metabolism categories. The cell death category included the following proteins: NPM1, P4HB, LGALS3, CASP3, PDIA3, ATP5A1, GAPDH, ANXA4, HSPA5, RPLP0, UCHL1, STMN1, ENO1, ANXA5, MZB1, PSMB1, ALDOA, VDAC1, LDHA, HSPB1, and PRDX2 (Fig. 1). These results are consistent with previous studies. Benes *et al.* [36] showed increased expression of pro-apoptotic gene transcripts in postmortem brains of bipolar disorder patients. Furthermore, Herbeth *et al.* [21] indicated altered cell death and inflammation-related proteins in peripheral blood mononuclear cells and serum from patients with euthymic bipolar disorder. Brain imaging studies demonstrated reductions in the mean gray matter volume of brains from patients with bipolar disorder [37]. Previous studies reported a decreased density of nonpyramidal neurons in layer II of the anterior cingulate and a lower number of glial cells in layer III with bipolar disorder [38]. Meta-analyses of volumetric magnetic resonance imaging studies showed reduced volume of gray matter in the anterior cingulate and bilateral insula [39,40]. Neuropathological studies of bipolar disorder showed decreases of each brain field and neuronal cells. Because mood stabilizers and antidepressants, which are used for treatment of bipolar disorder, have neuroprotective actions [5,41,42], it has been suggested that cells derived from patients with bipolar disorder are more vulnerable to factors related to cell death than those from controls. Patients with unipolar or bipolar depression exhibit decreased brain-derived neurotrophic factor levels [43]. Moreover, mood stabilizers have neuroprotective effects by increasing bcl-2 levels [42,44,45]. These findings suggest cellular vulnerability has a role in the pathology of bipolar disorder. Dysregulation of the apoptotic process found in the monozygotic twins discordant for bipolar disorder might be relevant to this hypothesis.

We examined the relationship of the identified proteins with canonical pathways and found that the proteins were related to the glycolysis pathway. The proteins included PKM2, ALDH2, ENO1, PGAM1, GAPDH, ALDOA, LDHA, and ALDOC. Glycolysis, or anaerobic respiration, is a fundamental metabolic process that produces energy for all cells. In order to maintain its functions, the brain needs an enormous amount of energy compared with other tissues. ALDOC is a brain-specific glycolysis enzyme that catalyzes the reversible aldol cleavage of fructose-1,6-biphosphate and fructose-1-phosphate to dihydroxyacetone phosphate and either glyceraldehyde-3-phosphate or glyceraldehyde [46]. In the present study, we found a decrease of the ALDOC protein level in the affected twin. However, previous reports showed that protein expression level of ALDOC was increased in the frontal cortex of patients, including those with mood disorder [35,47]. This discrepancy might reflect differences between tissues. Moreover, we found differential expression of many essential enzymes of glycolysis such as TPI1, ALDOA, and PGAM1. A

previous report using positron emission tomography showed that familial bipolar depressive patients had decreased blood flow in the cerebrum and a decreased rate of glucose metabolism in the ventral anterior cingulate cortex [37]. As indicated by an alteration in energy metabolism, compromised metabolic function has been reported in bipolar disorder [48,49]. In these studies, alteration of mitochondrial proteins was reported. Mitochondria are involved in processes including the TCA cycle, glycolysis and gluconeogenesis, lipogenesis, and malate-asparate shuttle [50]. Thus, changes in these proteins may lead to major alterations in the energy pathways, thus affecting ATP production. Recently, many reports have suggested that mitochondrial dysfunction is involved in bipolar disorder and other psychiatric disorders [51,52,53]. Mitochondria are also involved in other essential processes such as apoptosis, oxidative stress, and calcium regulation [50]. Thus, a decrease in energy production due to mitochondrial dysfunction in the brains of patients with bipolar disorder may be compensated for by an increase in energy production by glycolysis. It is possible that mitochondrial dysfunction affects neuronal cell death. Further study is needed to know whether these alterations in glycolysis-related proteins are a cause or consequence of the disease process.

This is the first study to our knowledge to apply proteomics for the analysis of monozygotic twins discordant for bipolar disorder, and it has major limitations. First of all, we analyzed only a single pair of monozygotic twins. Thus, results cannot be applied to bipolar disorder in general. Another limitation is the tissue examined; that is, lymphoblastoid cells. Although brain samples may be optimal to identify molecules directly related to bipolar disorder, brain samples of twins are difficult to access. In addition, accessible tissues such as body fluid and peripheral cells such as serum, plasma, cerebrospinal fluids, saliva, urine, and peripheral blood cells should be used for biomarkers. In this study, we used lymphoblastoid cells and avoided a possible effect of medication by culturing the cells in drug-free media. However, a possibility that the effect of medication at the collection of blood last even after culturing the cells in drug-free media for a month cannot be totally ruled out. The other major limitation is the small number of case–control samples.

In summary, we performed a proteomic analysis of lymphoblastoid cells in a pair of monozygotic twins discordant for bipolar disorder. The identified proteins were mainly categorized as those involved in cell death and glycolysis. In a case–control study, protein expression of PGAM1, which is related to glycolysis, was significantly higher in patients than in healthy controls. The present findings suggest future new targets that may be relevant to the pathology of bipolar disorder. The present results need to be tested in a larger, independent sample set to reach a valid conclusion.

Author Contributions

Conceived and designed the experiments: AK TK. Performed the experiments: AK K. Ohtawa K. Otsuki MU. Analyzed the data: AK TK. Contributed reagents/materials/analysis tools: HS YO. Wrote the paper: AK TK.

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Invited review

Comprehensive DNA methylation and hydroxymethylation analysis in the human brain and its implication in mental disorders

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ABSTRACT

Covalent modifications of nucleotides, such as methylation or hydroxymethylation of cytosine, regulate gene expression. Early environmental risk factors play a role in mental disorders in adulthood. This may be in part mediated by epigenetic DNA modifications. Methods for comprehensive analysis of DNA methylation and hydroxymethylation include DNA modification methods such as bisulfite sequencing, or collection of methylated, hydroxymethylated, or unmethylated DNA by specific binding proteins, antibodies, or restriction enzymes, followed by sequencing or microarray analysis. Results from these experiments should be interpreted with caution because each method gives different result. Cytosine hydroxymethylation has different effects on gene expression than cytosine methylation; methylation of CpG islands is associated with lower gene expression, whereas hydroxymethylation in intragenic regions is associated with higher gene expression. The role of hydroxymethylcytosine is of particular interest in mental disorders because the modification is enriched in the brain and synapse related genes, and it exhibits dynamic regulation during development. Many DNA methylation patterns are conserved across species, but there are also human specific signatures. Comprehensive analysis of DNA methylation shows characteristic changes associated with tissues, brain regions, cell types, and developmental states. Thus, differences in DNA methylation status between tissues, brain regions, cell types, and developmental stages should be considered when the role of DNA methylation in mental disorders is studied. Several disease-associated changes in methylation have been reported: hypermethylation of *SOX10* in schizophrenia, hypomethylation of *HCG9* (HLA complex group 9) in bipolar disorder, hypermethylation of *PRIMA1*, hypermethylation of *SLC6A4* (serotonin transporter) in bipolar disorder, and hypomethylation of *ST6GALNAC1* in bipolar disorder. These findings need to be replicated in different patient populations to be generalized. Further studies including animal experiments are necessary to understand the roles of DNA methylation in mental disorders.

This article is part of a Special Issue entitled 'Neuroepigenetic disorders'.

1. Introduction

It is well known that DNA contains the code for the amino acid sequence of proteins. In addition, the DNA molecule has information on the regulation of gene expression, which is mediated by DNA-protein or DNA–RNA interactions. The amino acid sequence is determined by the sequence of four nucleotides. Similarly, DNA-protein interactions that mediate gene expression regulation are regulated by the covalent modifications of nucleotides. The most studied covalent modification of nucleotides in mammals is methylation of the cytosine residue (Suzuki and Bird, 2008). The process of DNA methylation has been well studied; however, mechanisms of DNA demethylation are still not completely understood (Franchini et al., 2012).

DNA damage produces several types of oxidative DNA adducts including 8-oxoguanine and 8-hydroxyguanine (Cadet et al., 2003), and 5-hydroxymethylcytosine (5hmC) had also been regarded as one of such DNA adducts. Identification of ten-eleven translocation (TET) proteins as the enzymes that catalyze hydroxymethylation (Ito et al., 2010; Tahiliani et al., 2009) and enrichment of 5hmC in brain cells (Kriaucionis and Heintz, 2009) suggested its role in brain function and neuropsychiatric diseases. 5hmC is involved in the demethylation of cytosine (Cortellino et al., 2011; Guo et al., 2011; Hackett et al., 2013; He et al., 2011; Ito et al., 2011; Shen et al., 2013), it also plays a functional role by binding to methyl CpG biding protein 3 (Mbd3) (Yildirim et al., 2011), methyl CpG biding protein 3 (MeCP2) (Mellen et al., 2012), and Uhrf2 (Spruijt et al., 2013). TET proteins further oxidize 5-hmC into 5-formylcytosine (5fC) and 5-carboxylcytosine

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(5caC) (Ito et al., 2011). Recently, 5fC was found to be enriched at poised enhancers in mouse embryonic stem (ES) cells, suggesting its functional significance in gene regulation (Song et al., 2013).

The role of DNA methylation in mental disorders has long been suggested (Petronis, 2010). Epidemiological studies show a role for both genetic and environmental factors in mental disorders. Among environmental factors, early adversities such as childhood abuse or maltreatment are suggested in depression or post-traumatic stress disorder, whereas perinatal problems such as virus infection, malnutrition, and perinatal complication, are suggested in psychoses. However, it is unknown how these early environmental factors affect behavioral phenotypes in adulthood. DNA methylation can be affected by environmental factors, and methylation remains relatively stable over time. Thus, the role of DNA methylation as a mechanism of the effect of early environmental factors on adult mental disorders has drawn attention.

However, there have been no well-replicated findings of altered DNA methylation of candidate genes in mental disorders. It is suggested that genetic association studies of candidate genes frequently encounter false positive findings (Hirschhorn et al., 2002). Recently, there has been a greater focus on genome-wide association analysis rather than candidate gene approaches. The genome-wide approach can also be applied to the study of DNA methylation and hydroxymethylation.

In this review, recent studies on the comprehensive analysis of DNA methylation and hydroxymethylation in the human brain are summarized. A particular focus on the roles for methylation in mental disorders is given. Therefore, animal experiments and studies regarding brain tumors are not discussed here.

2. Methods for the comprehensive analysis of DNA methylation and hydroxymethylation

There are several approaches for genome-wide analysis of DNA methylation or hydroxymethylation. A common method utilizes the modification of cytosine to uracil by sodium bisulfite (Hayatsu et al., 1970). Methylcytosine (mC) is not converted into uracil by sodium bisulfite, allowing the identification of methylated cytosine. Bisulfite sequencing has been widely used for DNA methylation analysis since its discovery in 1970. However, this method cannot discriminate 5hmC from mC. A modified method, however, enables this discrimination (Fig. 1). For mC-specific analysis, called oxidative bisulfite sequencing (oxBS-Seq), 5hmC is selectively oxidized to 5fC, which is then converted to uracil after bisulfite treatment (Booth et al., 2012). Tet-assisted bisulfite sequencing (TAB-Seq) specifically analyzes 5hmC (Yu et al., 2012). In this method, all cytosine modifications except for glucose-protected 5hmC are converted to uracil by first treating with the Tet enzyme followed by bisulfite modification. In addition, specific analysis of 5caC has also been developed by protecting 5caC with 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride before bisulfite modification (chemical modification assisted bisulfite sequencing; CAB-seq) (Lu et al., 2013). Similarly, a method for base-pair level analysis of 5fC has also been reported wherein 5fC is protected with O-ethylhydroxylamine before modification with bisulfite (Song et al., 2013). Bisulfite-modified DNA is subject to analysis by next-generation sequencing or bead arrays (Bibikova et al., 2006). Bead arrays can determine predefined representative CpG sites for each gene. Reduced representation bisulfite sequencing (RRBS), which can selectively analyze CpG-rich regions, is also often used. RRBS is popular because the cost of whole genome bisulfite sequencing analysis is still high (Meissner et al., 2005). For the analysis of specific CpG sites, other methods such as Sanger sequencing, pyrosequencing, or mass-spectrometry are used.

In the other type of comprehensive analysis method, methylated, hydroxymethylated, or unmethylated DNA is collected using specific binding proteins or antibodies. For example, MBD2b conjugated beads are used to collect methylated DNA. Similarly, DNA containing 5hmC can be collected using streptavidin magnetic beads after glucosylation of 5hmC and subsequent biotinylation. To collect unmodified DNA, unmethylated DNA-specific binding

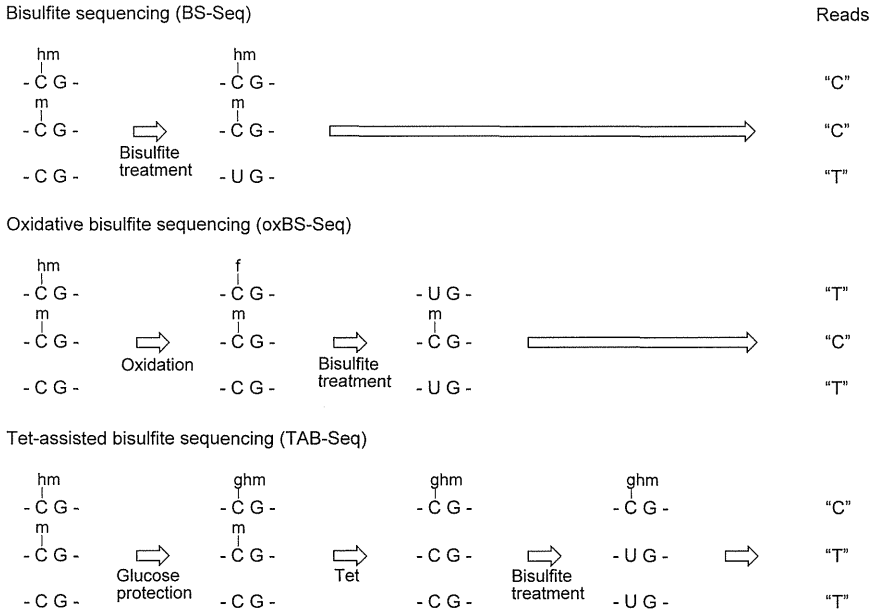

Fig. 1. Specific analysis of methylcytosine and hydroxymethylcytosine. Recently developed modified versions of bisulfite (BS) sequencing enable mC or 5hmC-specific analysis at the base pair resolution. In oxidative BS sequencing (oxBS-Seq) (Booth et al., 2012), 5hmC is selectively oxidized to 5fC. 5fC is converted to uracil after BS treatment. 5hmC reads as T instead of C when sequenced. In TAB-Seq (Yu et al., 2012), genomic DNA is pretreated with glucosyltransferase to modify 5hmC with glucose, which is resistant to BS treatment. Genomic DNA is then treated with the Tet enzyme. Sequence reads of all cytosine modifications except 5hmC would be T, and 5hmC would be C. Note that after BS treatment two other cytosine modifications, 5-carboxylmethycytosine and 5-formylcytosine, would be read as T. mC, methylcytosine; 5hmC, 5-hydroxymethycytosine; C, cytosine; T, thymine; BS, bisulfite; oxBS-Seq, oxidative bisulfite sequencing; TAB-Seq, Tet-assisted bisulfite sequencing.

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proteins can be used. Similarly, anti-mC antibody or anti-5hmC antibody can be used to collect methylated or hydroxymethylated DNA, respectively. Selective digestion of non-methylated DNA by methylation-sensitive restriction enzymes can also be used to enrich methylated DNA. On the other hand, McrBC, a restriction enzyme that can selectively digest methylated DNA, is used for the enrichment of unmethylated DNA. Glucosylation of 5hmC inhibits the activity of methylation sensitive restriction enzymes, and thus this can also be used to discriminate 5hmC and mC. After the collection of DNA, the samples are subjected to tiling arrays or deep sequencing analysis. The resolution of these methods is not at the base pair level; therefore, tiling arrays and deep sequencing would be equally useful for this analysis.

Both bisulfite sequencing and collection of methylated DNA have strengths and weakness. Bisulfite sequencing can reveal DNA modifications (methylation or hydroxymethylation) at the base pair level, but cannot discriminate between the types of modifications through a standard method. Collection of methylated DNA followed by sequencing can analyze methylation and hydroxymethylation separately, but not at the base pair level. Thus, both methods can be combined to obtain a genome-wide picture of DNA methylation and hydroxymethylation. As discussed above, sodium bisulfite sequencing cannot discriminate DNA methylation and hydroxymethylation, and, therefore, it would be appropriate to refer to these results as "DNA modification" or "DNA (hydroxy)methylation." In the following sections, the results of this method are written as "DNA methylation" for simplicity.

In summary, a number of different experimental techniques are available to analyze DNA methylation and hydroxymethylation status. The results of these analyses are dependent on the methodologies used. Methodological differences should be carefully considered when results are interpreted and compared across different studies.

3. DNA methylation

3.1. Functional significance

DNA methylation has important roles in the regulation of gene expression, imprinting, and X-chromosome inactivation (Bird, 1980). Cytosine methylation predominantly occurs at, but is not restricted to, the CpG site in genomic DNA in mammals. CpG site is less frequent than simple mathematical probability predicts because methylated cytosine can be mutated to thymine during evolution (Bird, 1980). CpG-rich genomic regions, called CpG islands, are frequently found around the transcription start sites. CpG islands of house-keeping genes are generally unmethylated; lower DNA methylation at CpG islands on the promoter is usually associated with higher gene expression (Suzuki and Bird, 2008). Methylation at the region surrounding a CpG island, called the CpG island shore, is involved in tissue differentiation (Doi et al., 2009). DNA methylation of CpG islands in intragenic or intergenic regions is associated with alternative promoter usage (Maunakea et al., 2010). DNA methylation in gene body is related to enhanced transcription (Ball et al., 2009).

In conclusion, DNA methylation can affect a wide-range of cellular functions, and it is hypothesized to play a role in diseases.

3.2. DNA methylation signature of tissues, brain regions, and cell types

An early study using a BAC (bacterial artificial chromosome) microarray identified tissue-specific DNA methylation of *SHANK3*. *SHANK3* was found to be unmethylated and highly expressed in the human brain but not in peripheral blood lymphocyte (Ching et al., 2005).

Using Restriction Landmark Genomic Scanning (RLGS), Ghosh and colleagues searched for brain-specific DNA methylation differences and identified loci showing differential methylation in the human brain. RLGS is a traditional method of comprehensive DNA methylation analysis involving digestion using methylation-sensitive restriction enzymes, followed by two-dimensional electrophoresis. This study demonstrated that *LHX2* is methylated and *CNPY1* is hypomethylated in cerebellum (Ghosh et al., 2010). This study also found clear differential methylation of several loci between gray matter and white matter. The authors suggest that this might be mediated by differential methylation between neurons and glial cells (Ghosh et al., 2010). However, the loci showing differential methylation between gray and white matter were not identified.

Using bead arrays that can examine 1505 CpG sites from 807 genes, Ladd-Acosta and colleagues studied the DNA methylation status of 76 human brain samples including patients with autism and bipolar disorder (Ladd-Acosta et al., 2007). By hierarchical clustering analysis, they clearly showed that DNA methylation status is different between brain regions including cerebral cortex, cerebellum, and pons. DNA methylation differences of five genes, *RASSF1*, *HDAC7A*, *GABRB3*, *EN2*, and *HTR2A* between the cerebral cortex and cerebellum were confirmed in an independent cohort.

To identify the differences in methylation signature between neurons and non-neuronal cells such as glial cells, we separated neurons and non-neurons from human postmortem brains and performed comprehensive DNA methylation analyses using bead arrays of bisulfite modified DNA and tiling array analysis of DNA collected by MBD-conjugated beads (Iwamoto et al., 2011). We found that neurons are hypomethylated, and the DNA methylation status of bulk cortex mostly reflects non-neurons. Genes expressed in astrocytes were methylated in neurons, and genes related to neuronal function were methylated in non-neurons. Interestingly, inter-individual difference of DNA methylation is larger in neurons than in non-neurons. This difference might reflect environment-dependent changes of DNA methylation in neurons.

Considering the differences in DNA methylation status between tissues, brain regions, and cell types, the tissues and cell types to be analyzed are crucial when the role of DNA methylation in mental disorders is studied.

3.3. Developmental aspects

DNA methyltransferases (DNMTs) and MBDs play an important role for de novo and maintenance DNA methylation as well as the recruitment of proteins involved in transcriptional regulation. Expression of these genes undergo complex regulation from early neuronal development to the adult brain, establishing a developmental and cell-type-specific DNA methylation signature in the brain (Yao and Jin, 2013). Importantly, mutations within DNMTs or MBDs are known to cause neurological disorders. For example, mutations in *MECP2* causes Rett syndrome (Chahrour and Zoghbi, 2007) and those in *DNMT3B* lead to immunodeficiency-centromeric instability-facial anomalies syndrome, which is characterized by mental retardation (Hansen et al., 1999). DNA methylation profiles in brain are drastically altered throughout development. Siegmund and colleagues performed a real-time PCR-based quantitative methylation assay of 50 genes in 125 postmortem brains. They identified four typical patterns of changes during development: age-dependent linear increase, biphasic distribution, stochastic accumulation, and a decrease in DNA methylation (Siegmund et al., 2007). This study also found higher DNA methylation of *PAX8* in patients with schizophrenia than in

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controls (Siegmund et al., 2007). Numata and colleagues examined about 27,000 CpG sites from 14,500 genes using bead arrays in the prefrontal cortex of 108 human subjects of various ages from fetal to elderly. DNA methylation showed drastic changes during the prenatal period, but showed continuous changes during aging. Typical alterations were characterized by prenatal demethylation and increase of methylation with aging (Numata et al., 2012). It was suggested that sex differences in methylation observed in this study are attributable to cross reactions to the sex chromosomes (Chen et al., 2012). Recently, Lister and colleagues performed genome-wide bisulfite sequencing analysis of the mouse and human brain (Lister et al., 2013). This study identified developmentally regulated DNA methylation changes, and found that genome-wide reconfiguration of the DNA methylation pattern occurs during the fetal to young adult stage. They also identified age-dependent accumulations of non-CpG methylation in neurons, but not in non-neurons. Although presence of non-CpG methylation in brain has been previously suggested (Xie et al., 2012; Varley et al., 2013), finding a specific accumulation in neuronal cells implies a unique epigenetic regulation in the brain. This underscores the importance for the consideration of the complexity of brain cell-types in future studies.

These studies show that data should be interpreted in the context of developmental- and aging-associated changes when we study DNA methylation in mental disorders.

3.4. Evolutionary aspects

Xin and colleagues performed comprehensive DNA methylation analysis in human and mouse brains by digestion using methylation-sensitive restriction enzymes, followed by deep sequencing. They identified that DNA methylation is evolutionally conserved in CpG dense regions, regardless of sequence conservation across species (Xin et al., 2011). DNA methylation patterns on the CpG island shore of promoters were different between the prefrontal cortex and auditory cortex. The authors of this study built a database named "MethylomeDB" with their data of DNA methylation in human and mouse brains (Xin et al., 2012). Wang and colleagues identified 150 differentially methylated regions (DMRs) between human and rhesus macaque using the Chip-Seq approach (Wang et al., 2012a). Through extensive validation experiments, they identified four DMRs (*K6IRS2*, *ProSAPiP1*, *ICAM1*, and *RNF32*). Among them, *ICAM1* and *ProSAPiP1* encode neuronal function-related proteins. Another study compared whole-genome bisulfite sequencing data of the prefrontal cortex between humans and chimpanzees (Zeng et al., 2012). They revealed extensive differences in the DNA methylation profile. These changes mostly consisted of hypomethylated genes in the human brain. Importantly, they found enrichment of DMRs in genes related to neurological and psychological disorders.

These studies show partial conservation of DNA methylation patterns across species; however, there are also human specific signatures. Comparative evolution studies of DNA methylation profiles will not only provide insight into the evolution of human-specific traits, but also important candidate genes for neuropsychiatric disorders.

3.5. Disease-associated changes

DNA methylation analysis of the candidate genes have been widely performed using postmortem brains of patients with mental disorders. These included genes coding for BDNF, COMT, serotonin receptors, glutamate receptors, dopamine transporters, and serotonin transporters. Results from these studies have been reviewed elsewhere (Dempster et al., 2013; Nishioka et al., 2012).

Comprehensive gene expression analyses in patients with schizophrenia consistently identified downregulation of oligodendrocyte-related genes. Thus we searched for DNA methylation changes of transcription factors that can explain the global downregulation of oligodendrocyte genes. We found that higher DNA methylation of *SOX10* is related to lower gene expression of many oligodendrocyte-related genes. DNA methylation of *SOX10* was higher in the gray matter than in the white matter (Iwamoto et al., 2005). Consistent with the initial findings, subsequent analysis showed a marked difference in DNA methylation status of *SOX10* between neuronal and non-neuronal cells (Iwamoto et al., 2011).

Mill and colleagues performed comprehensive DNA methylation analysis using DNA microarrays to study human postmortem brains obtained from patients with schizophrenia and bipolar disorder as well as control subjects (Mill et al., 2008). CpG island microarray analysis of DNA after restriction enzyme-based enrichment revealed disease-specific methylation differences in numerous loci, including genes involved in glutamatergic and GABAergic neurotransmission and brain development. Genes involved in mitochondrial function, brain development, and stress response were differentially methylated between groups. The strongest candidate gene obtained from this comprehensive analysis was HLA complex group 9 (*HCG9*). The authors confirmed lower DNA methylation of *HCG9* in bipolar disorder (Kaminsky et al., 2012). Sabunciyan and colleagues performed a comprehensive analysis of DNA methylation in the frontal cortex of patients with major depression ($N = 39$) and controls ($N = 26$) using Comprehensive High-throughput Arrays for Relative Methylation (CHARM), a methylation-sensitive restriction enzyme-based method (Sabunciyan et al., 2012). Among the 224 genes showing robust differential methylation, genes related to neuronal growth and development were enriched. Among the 10 genes that were experimentally validated by pyrosequencing, hypermethylation of *PRIMA1* in patients under depression was most robust. *PRIMA1* encodes a protein that anchors acetylcholinesterase in the neuronal membrane, and thus its decrease might cause enhanced cholinergic neurotransmission. The authors confirmed that acetylcholinesterase-like immunoreactivity was decreased in postmortem brains of patients with major depression. These findings are compatible with the cholinergic hypothesis of depression (Sabunciyan et al., 2012).

Several candidate genes in the brain have been identified from the comprehensive analysis of DNA methylation differences between monozygotic twins discordant for mental disorders. We enriched methylated DNA using MBD-conjugated beads and searched for DNA methylation differences between monozygotic twins discordant for bipolar disorder using tiling arrays (Sugawara et al., 2011). We found that the CpG island shore of *SLC6A4*, which encodes a serotonin transporter, was differentially methylated between twins. Hypomethylation of *SLC6A4* was verified in lymphoblastoid cells and postmortem brain samples of patients with bipolar disorder. Dempster and colleagues found altered DNA methylation of *ST6GALNAC1*, which encodes an enzyme that transfers sialic acid to O-linked N-acetylgalactosamine residues, in monozygotic twins discordant for bipolar disorder or schizophrenia (Dempster et al., 2011). Hypomethylation of this gene was also found in postmortem brains of patients.

As discussed above, comprehensive DNA methylation studies found interesting candidate genes. However, these studies are only a start point to identify the pathophysiological significance of these methylation changes. These findings need to be replicated in different patient populations to be generalized. Further studies

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including animal experiments should be performed to understand the roles of DNA methylation in mental disorder.

4. Hydroxymethylation

4.1. Changes during development

In contrast to DNA methylation studies, the functional and pathophysiological roles of hydroxymethylation have only recently been proposed. Therefore, there are few studies in human brain on hydroxymethylation. The majority of studies on 5hmC focus on ES cells (Ficz et al., 2011; Pastor et al., 2011; Stroud et al., 2011; Wu et al., 2011; Yu et al., 2012). ES cells were found to contain high levels of 5hmC that decreases after differentiation (Kinney et al., 2011; Szwagierczak et al., 2010). 5hmC increases with age in neuronal cells (Szulwach et al., 2011b).

4.2. Location of 5hmC in genome

Glucosylation-mediated enrichment of hydroxymethylated DNA and subsequent deep sequencing has been used to examine DNA derived from mouse cerebellum. The authors found that 5hmC is enriched in gene bodies and proximal upstream and downstream regions relative to transcription start sites, transcription termination sites, and distal regions (Song et al., 2011). Higher hydroxymethylation in intragenic and proximal regions is associated with higher gene expression. Hydroxymethylation in these genomic regions was higher in the cerebellum of adults compared to postnatal day 7 mice. Increases in hydroxymethylation during aging were enriched in genes related to neurodegenerative disorders, angiogenesis, and hypoxia response. These findings suggest that hydroxymethylation might play a role in age-related neurodegeneration.

Jin and colleagues mapped 5hmC in the frontal lobe by immunoprecipitation with an anti-5hmC antibody (Jin et al., 2011). In human brains, 5hmC was enriched at promoters and gene bodies but absent in non-genic regions. Enrichment of 5hmC in gene bodies was correlated with higher gene expression. This correlation was more prominent than that between mC and gene expression.

4.3. 5hmC in the brain

Consistent with the initial report that 5hmC is enriched in brain cells (Kriaucionis and Heintz, 2009), 5hmC was most abundant in the brain than in other human tissues (Li and Liu, 2011).

Szulwach and colleagues mapped 5hmC using a glucosylation-based enrichment method (Szulwach et al., 2011a) in the human and mouse cerebellum. The level of 5hmC was increased with development, from around 1% (postnatal day 7) to 2.5–5% (one year) in adult mice. 5hmC was enriched in the 5′-UTR (untranslated region) and exons but was depleted in introns. 5hmC was affected by the gene dosage of *MeCP2*. These studies were confirmed and extended in the developing human cerebellum (Wang et al., 2012b). This study found that 5hmC is enriched in exons and 5′-UTRs but depleted in introns. Fetus-specific or adult-specific differentially hydroxymethylated regions overlapped with genes that are enriched with the target sequence of FMRP (fragile X mental retardation protein) and CpG island shores.

Immunohistochemistry analysis detected 5hmC in various cell types in the brain (Orr et al., 2012). However, while 5hmC is robustly detected in neuronal nuclei, some oligodendrocyte nuclei lack in 5hmC immunoreactivity.

Khare and colleagues examined the genomic distribution of 5hmC using enzyme digestion of glucosylated DNA followed by microarray analysis. They found that 5hmC is enriched in genes with synapse related functions in the human and mouse brain. They also found tissue-specific differential distribution of 5hmC at the exon–intron boundary. Constitutive exons contained higher levels of 5hmC than alternatively spliced exons (Khare et al., 2012).

In summary, the role of 5hmC is of particular interest in mental disorders because it is enriched in the brain and in synapse-related genes. Hydroxymethylation of cytosine can occur in positions of the genome different from methylation, and it can regulate gene expression in several ways. 5hmC modifications are also regulated by development and aging. The possible role of 5hmC in mental disorders is a contemporary area of research.

5. Future directions

The role of DNA methylation in neuropsychiatric disorders is currently an active area of investigation. However, the role of hydroxymethylation and other cytosine modifications in neuropsychiatric disorders should be studied as well. As discussed above, patterns and regulation of cytosine modifications in brain cells are more complex than previously expected. Comprehensive studies of cytosine modifications in the human brain have recently begun. The role of epigenetic regulation in the physiology and pathology of the brain should be further studied in detail in the coming decade.

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HIGHLIGHT

Epigenetic Regulation of Serotonin Transporter in Psychiatric Disorders

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SLC6A4 (solute carrier family 6, member 4) gene encodes a serotonin transporter (5-hydroxytryptamine transporter, HTT), which transports synaptic serotonin into presynaptic terminal. *SLC6A4* is known to be the target of antidepressants such as selective serotonin reuptake inhibitors (SSRIs). Inhibition of HTT increases synaptic serotonin concentration and thereby exerts antidepressant efficacy. A large number of genetic studies suggest the contribution of genetic variations of *SLC6A4* to various psychiatric disorders. The most studied genetic variation, HTT-linked polymorphic region (HTTLPR), is located at the promoter region of *SLC6A4*. It consists of two major alleles: short (S) and long (L). Each allele contains further variations (Nakamura et al., 2000). The HTTLPR has been reported to affect the gene expression level of *SLC6A4* (Heils et al., 1996; Lesch et al., 1996; Bradley et al., 2005), and individuals carrying the low-expressing S allele of HTTLPR revealed anxiety-related personality trait (Lesch et al., 1996). Furthermore, it was reported that the HTTLPR moderates the influence of stressful life event on depression (Caspi et al., 2003; Kendler et al., 2005). These results suggest the contribution of gene–environment (G × E) interaction involving *SLC6A4* to psychiatric disorders.

Epigenetic factors also contribute to the mechanism of G × E interaction. DNA methylation is affected by environmental factors (Feinberg, 2007; Petronis, 2010). Epigenetic gene regulation by DNA methylation contributes to long-lasting gene expression changes (Bird, 2002). Here, we searched for recent articles relevant to DNA methylation of *SLC6A4* (Table 1), and focused on recent progress in the research on the roles of epigenetic regulation of *SLC6A4* by DNA methylation of *SLC6A4* in psychiatric disorders such as mood and anxiety disorders.

THE INTERACTION OF DNA METHYLATION AND GENOTYPE ON GENE EXPRESSION LEVEL OF *SLC6A4*

The majority of DNA methylation occurs at the fifth position of cytosine residue in the dinucleotides CpG sequences in mammals. While cytosine residues in the dinucleotides are generally methylated, CpG-rich regions, which are called "CpG island" and located within and around the regulatory promoter regions, are less methylated. Usually, the extent of methylation at the promoter region CpG island inversely correlates with the extent of gene expression.

The S allele of HTTLPR has been shown to have the lower promoter activity compared with L allele, which is associated with decreased mRNA expression (Heils et al., 1996; Lesch et al., 1996; Bradley et al., 2005). Philibert et al. (2007) examined the relationship between DNA methylation at the promoter region CpG island and gene expression level of *SLC6A4* using lymphoblastoid cell lines (LCLs). There was no significant association between total DNA methylation and mRNA levels. However, DNA methylation was associated with decreased mRNA levels under the control of HTTLPR genotype (Philibert et al., 2007). They could not replicate this finding in the second study (Philibert et al., 2008). On the other hand, in infant rhesus macaques, carriers of the S allele exhibited higher methylation of CpG island, and this was associated with lower gene expression of *SLC6A4* in peripheral blood mononuclear cells (PBMCs) (Kinnally et al., 2010).

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Table 1
Articles relevant to *SLC6A4* methylation

Reference	Subject	*N*	Source	Examination		
				Environmental factors	Biological factors	Methods
Wang et al., 2012	Healthy adult male	25	T cells and monocytes	Childhood-limited aggression	Methylation	Pyrosequencing
Vijayendran et al., 2012	Female adoptee	158	LCLs	Child abuse	Methylation	Beadchip
					HTTLPR	Real-time PCR
					Expression	RT-PCR
Sugawara et al., 2011a	MZ twins discordant for BD	2 pairs	LCLs	–	Methylation	Tiling array, bisulfite sequencing, pyrosequencing
	BD and C	BD = 20, C = 20	LCLs	–	Methylation	Pyrosequencing
					HTTLPR	PCR
					Expression	RT-PCR
	BD and C	BD = 35, C = 35	Brains	–	Methylation	Pyrosequencing
Park et al., 2011	AD and C	AD = 27, C = 15	PBMCs	–	Methylation	Pyrosequencing
Koenen et al., 2011	PTSD	100	PBMCs	Traumatic events	Methylation	Beadchip, pyrosequencing
					HTTLPR	RFLP
Kinnally et al., 2011	Female bonnet macaques	20	PBMCs	Early life stress	Methylation	Pyrosequencing
Beach et al., 2011	Adoptee	192	LCLs	Child sex abuse	Methylation	Mass spectroscopy
Devlin et al., 2010	Pregnant woman and infant	82	Maternal peripheral leukocytes, umbilical cord leukocytes	–	Methylation	Pyrosequencing
van Ijzendoorn et al., 2010	Adoptee	143	LCLs	Unresolved loss or trauma	Methylation	Mass spectroscopy
					HTTLPR	PCR
Wong et al., 2010	MZ and DZ twins	MZ twin-pairs = 46, DZ twin pairs = 45	Buccal cells	–	Methylation	Mass spectroscopy
Kinnally et al., 2010	Infant rhesus macaques	87	PBMCs	Early life stress	Methylation	Pyrosequencing
					HTTLPR	RFLP
					Expression	RT-PCR
Olsson et al., 2010	MD and C	MD = 25, C = 125	Buccal cells	–	Methylation	Mass spectroscopy
					HTTLPR	PCR
Beach et al., 2010	Adoptee	155	LCLs	Child abuse	Methylation	Mass spectroscopy
					HTTLPR	PCR
Philibert et al., 2008	Adoptee	192	LCLs	–	Methylation	Mass spectroscopy
					HTTLPR	PCR
					Expression	RT-PCR

(*continued on next page*)

Table 1 (*continued*)

Reference	Subject	*N*	Source	Examination		
				Environmental factors	Biological factors	Methods
Philibert et al., 2007	Human	49	LCLs	–	Methylation	Mass spectroscopy
					HTTLPR	PCR
					Expression	RT-PCR

MZ: monozygotic; BD: bipolar disorder; C: control; AD: alcohol dependence; PTSD: posttraumatic stress disorder; DZ: dizygotic; MD: major depression; ASPD: antisocial personality disorder; PBMCs: peripheral blood mononuclear cells; LCLs: lymphoblastoid cell lines; HTTLPR: serotonin transporter-linked promoter region; PCR: polymerase chain reaction; RT-PCR: reverse transcribed-PCR; RFLP: restriction fragment length polymorphism.

Moreover, we found that DNA methylation of the CpG island shore of *SLC6A4* was significantly correlated with mRNA level in individuals with the S/S genotype (Sugawara et al., 2011a). The other group also reported that DNA methylation of the CpG island of *SLC6A4* was associated with total gene expression, whereas that in the CpG island shore was associated with gene expression of a specific splice variant (Vijayendran et al., 2012). In an *in vitro* study, the HTTLPR genotype was found to affect the transcription factor binding and chromatin modifications of *SLC6A4* in response to cocaine in JAr cells (Vasiliou et al., 2012).

These findings collectively suggest that the DNA methylation level of the CpG island and/or CpG island shore of *SLC6A4* controls its mRNA expression by interacting with HTTLPR. Further studies are needed to elucidate the molecular mechanism underlying this interaction.

EFFECTS OF ENVIRONMENTAL FACTORS ON DNA METHYLATION OF *SLC6A4*

Weaver et al. (2004) reported that hippocampal hypermethylation of the glucocorticoid receptor gene induced by low maternal care may play a role in stress vulnerability in rats. This suggested that DNA methylation might play a role as an epigenetic mark of G × E interaction. In rhesus macaques, higher methylation of *SLC6A4* was associated with higher reactivity in adults that experienced early life stress as infants (Kinnally et al., 2011), whereas DNA methylation level was not associated with rearing condition (Kinnally et al., 2010). In humans, maternal depressed mood was associated with decreased DNA methylation of promoter region of *SLC6A4* in leukocytes of both maternal peripheral blood and neonatal cord blood (Devlin et al., 2010). Furthermore, Beach et al. (2010) reported that DNA methylation level of the CpG island was increased in the subjects who had a history of childhood physical abuse. This result was replicated in an independent study in women (Beach et al., 2011). The other group reported that higher levels of *SLC6A4* promoter methylation were observed in both T cells and monocytes in the adult males with high childhood-limited aggression, who had lower *in vivo* serotonin synthesis in the orbitofrontal cortex detected by positron emission tomography (Wang et al., 2012). Therefore, environmental factors might affect the methylation status of *SLC6A4*, though the direction of the alteration is not consistent.

The number of traumatic events is reportedly associated with posttraumatic stress disorder (PTSD), and this association was suggested to be modified by the methylation level of *SLC6A4*. Subjects with more traumatic events were at increased risk for PTSD if they showed the lower methylation level of *SLC6A4*. On the other hand, those who showed the higher methylation level were considered to be protected from PTSD (Koenen et al., 2011). The other report showed that DNA methylation of *SLC6A4* promoter affected the impact of HTTLPR genotype on psychological sequelae. Higher levels of methylation predicted more unresolved loss or trauma in the subjects with L/L allele, whereas they were associated with

less traumatic experience in the subjects with S/S allele (van Ijzendoorn et al., 2010). Vijayendran et al. (2012) reported that sexual abuse influenced the methylation of *SLC6A4* by interacting with HTTLPR.

In summary, there is a complex interaction between DNA methylation status of *SLC6A4*, HTTLPR, and environmental factors, with regard to psychiatric disorders.

DNA METHYLATION OF *SLC6A4* IN PSYCHIATRIC DISORDERS

A vast amount of genetic studies of *SLC6A4* have been reported in various psychiatric disorders. A meta-analysis showed a significant association of HTTLPR and alcohol dependence (Feinn et al., 2005). On the other hand, there was no difference in the methylation status of *SLC6A4* promoter region of PBMCs between patients with alcohol dependence and control subjects (Park et al., 2011). Using LCLs, Philibert et al. (2008) reported that the DNA methylation level of *SLC6A4* promoter tended to be higher in subjects with a lifetime history of major depression than those without a history of major depression. History of alcohol dependence did not affect the DNA methylation status of *SLC6A4*.

A role of G × E interaction between *SLC6A4* and stress has been reported in depression (Caspi et al., 2003; Kendler et al., 2005). Olsson et al. (2010) examined the DNA methylation status of *SLC6A4* promoter in buccal cells. DNA methylation status of the buccal cells, which are derived from ectoderm, might be more similar to that of neuronal cells compared with peripheral blood leucocytes (PBLs), which are derived from mesoderm (Olsson et al., 2010). Whereas there was no association between depressive symptoms and methylation level or HTTLPR genotype, depressive symptoms were more common among those with elevated methylation levels in S allele carriers (Olsson et al., 2010). This result implicated that an interaction of epigenetic and genetic factors involving *SLC6A4* is related to depressive symptoms.

Because DNA methylation differences between monozygotic (MZ) twins discordant for a disease might be relevant to the discordant phenotype, we performed a comprehensive analysis of DNA methylation profiles of promoters in LCLs of MZ twins discordant for bipolar disorder (BD) (Sugawara et al., 2011a). After careful filtering, the only robust DNA methylation difference between twins was the hypermethylation at the CpG island shore of *SLC6A4* in a bipolar twin. Causal relationship between such DNA methylation difference and discordant phenotype is unknown, because the differences of methylation patterns between MZ twins reportedly increase with age (Fraga et al., 2005). However, hypermethylation of *SLC6A4* in BD was confirmed in a case-control study. DNA methylation level of *SLC6A4* was negatively correlated with gene expression in an HTTLPR genotype-specific manner. Importantly, hypermethylation of *SLC6A4* at the same CpG sites was also found in the postmortem prefrontal cortices of patients with BD. In a study of MZ twins, Wong and colleagues showed that the variation of DNA methylation in *SLC6A4* was attributable to unique environmental factors rather than heritable factors (Wong et al., 2010). Taken together, these results suggest that epigenetic modification of *SLC6A4* might be implicated in the G × E interaction involved in the pathophysiology of BD.

PERSPECTIVE

In some studies, methylation level of *SLC6A4* is reportedly higher in females than in males (Philibert et al., 2008; Beach et al., 2010; Koenen et al., 2011). The molecular basis and consequence of the gender difference remain unclear. Further studies are needed to elucidate the gender difference of *SLC6A4* methylation. Meanwhile, we should pay a careful attention to the gender in designing the case-control association study.

Many of the studies presented here have focused on methylation at the CpG island of *SLC6A4* promoter region, where DNA methylation levels are relatively low. In contrast, the region identified in our study was located about 300 bp downstream of the CpG island. Such a region, known as a CpG island shore, plays an important role in tissue specific regulation of gene expression (Irizarry et al., 2009). Therefore, DNA methylation status of the CpG island shore of *SLC6A4* might serve as a more sensitive marker for G × E interaction.

Most of the studies have examined *SLC6A4* methylation using the peripheral tissues, and only one study examined in postmortem brains (Sugawara et al., 2011a). Among the peripheral tissues, LCLs are often used for the epigenetic studies, which were established through transformation of B lymphocyte by Epstein–Bar (EB) virus. This process can alter the epigenetic status of B lymphocyte (Antequera et al., 1990). In our study, we comprehensively analyzed the genomic regions whose methylation status was affected by the transformation (Sugawara et al., 2011b). In order to avoid the artifacts caused by the EB virus transformation, these regions were excluded from the analysis.

In addition, most of patients with psychiatric disorders are taking medication, which can affect the DNA methylation status. Indeed, cocaine reportedly alters chromatin modifications and DNA-binding activity of selected transcription factors depending on the HTTLPR genotype *in vitro* in the JAr cell lines (Vasiliou et al., 2012). In the case of patients treated with drugs, culturing the LCLs in drug-free medium for several weeks after blood sampling might eliminate the effect of medication. However, it is not known to what extent preexisting drug-induced epigenetic changes are reversed by culturing in drug-free medium. The possible effect of medication, including antidepressants, mood stabilizers and antipsychotics, on the DNA methylation pattern of *SLC6A4* should be assessed in the future studies.

In conclusion, recent studies suggested the important role of DNA methylation of *SLC6A4* in G × E interaction leading to psychiatric disorders. Further studies are needed to understand the molecular basis of G × E interaction and to develop a biological marker of psychiatric disorders.

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LETTER

Tipepidine in adolescent patients with depression: a 4 week, open-label, preliminary study

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Dear editor

Depression in children and adolescents is a common, recurrent, and debilitating condition associated with increased psychosocial, and medical morbidity and mortality.[1]

The global prevalence of depression in children and adolescents is 1%–2% and 3%–8%, respectively.[2] Depressive symptoms are also associated with significant functional impairment in school and the work place (often requiring legal interventions),[1–5] and an increased risk for substance abuse and suicide.[6–9] Clinical guidelines suggest the use of two selective serotonin reuptake inhibitors (SSRI), namely fluoxetine and escitalopram, both of which are effective with generally acceptable safety profiles in the treatment of adolescent depression.[10] Additionally, combination treatment with an SSRI and psychotherapy, typically cognitive behavioral therapy (CBT), has shown benefit in this cohort.[10] However, caution is warranted since antidepressants therapy in children and adolescents is associated with increased rates of suicidal ideation[11–13] and adverse effects, characterized by excessive emotional arousal or behavioral activation.[14] These results highlight the need for new therapies in adolescent patients with depression, particularly therapies with fewer side effects.

Tipepidine (3-[di-2-thienylmethylene]-1-methylpiperidine) has been used as a non-narcotic antitussive in Japan since 1959. The safety of short-term tipepidine use in children and adults has already been established. Furthermore, no suicide related side effects have been documented for tipepidine. It appears to act by inhibiting G-protein-coupled inwardly rectifying potassium (GIRK) channel currents.[15] The activation of the GIRK channels causes membrane hyperpolarization through potassium efflux. This inhibition is thought to modulate monoamine levels in the brain, since GIRK channels are coupled with G-protein-coupled receptors, such as 5-hydroxytryptamine $(5\text{-HT})_{1A}$, adrenaline α_2 and dopamine D_2 receptors.[15] Using in vivo microdialysis, Kawaura et al demonstrated that tipepidine increases levels of 5-HT and catecholamines, including dopamine, in the prefrontal cortex of rats.[16] Furthermore, Kawaura et al[17] showed that tipepidine produces antidepressant-like effects in rats subjected to the forced swimming test (a model of depression), by modulating these monoamine systems. Furthermore, our recent preliminary study suggests that tipepidine therapy may prove to be an effective alternative treatment for pediatric patients with ADHD.[18] Considering these results, we hypothesize that tipepidine can improve adolescent depressive symptoms by modulating monoaminergic neurotransmission, through the inhibition of GIRK channel coupling to monoamine receptors in the brain.

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We report six cases where tipepidine treatment (30 mg/day) proved effective in treating the symptoms of adolescent depression. The ethics committee of Chiba University Graduate School of Medicine approved the study protocol (G24062), which was performed in accordance with the Declaration of Helsinki II. All subjects and their parents provided written informed consent for study participation, after receiving a full explanation of the study, as well as any potential risks and benefits. This trial was registered on the official database of clinical research (Clinical Trials.gov), on April 17, 2013.[19] Statistical analyses were performed using the software package SPSS Version 21.0, for Macintosh (SPSS Statistics Desktop; IBM Corporation, Armonk, NY, US).

We recruited a total of ten outpatients from Chiba University Hospital, who were diagnosed according to the ICD-10 criteria for depressive episodes.[20] However, four subjects dropped out of the trial, because of feelings of mild irritation (n=2) and mild skin eruptions (n=2) less than 2 weeks into the study. These symptoms disappeared several days after the discontinuation of tipepidine. Overall, six subjects received tipepidine hibenzate tablets (Asverin; Mitsubishi Tanabe Pharma Corporation, Osaka, Japan), taken orally at 30 mg/day (10 mg after breakfast, 10 mg after lunch, and 10 mg after supper), for 4 weeks. Six adolescent subjects with depression (66% female, mean age 15.7 years, standard deviation (SD) ±2.2 years; mild depressive episode subtype, n=1; moderate depressive episode subtype, n=1; severe depressive episode subtype, n=4) were studied. The six subjects were Japanese adolescents. The mean height (cm), weight (kg), and tipepidine hibenzate dosage (mg/kg/day) of the six subjects were 158.2 cm±9.3; 57.3 kg±4.9; and 0.527 mg/kg/day ±0.044 mg, respectively. Four subjects were receiving drug treatment before entry into this trial, namely, quetiapine (25 mg/day, 500 mg/day, n=2), milnacipran (100 mg/day, n=1), and a combination of lamotrigine and blonanserin (400 mg/day and 4 mg/day, respectively, n=1), while two subjects were drug-naïve. These treatment regimes were stable for at least 4 weeks prior to enrollment and remained stable through the duration of the trial.

The Mini International Neuropsychiatric Interview for Children and Adolescents (MINI-KID)[21] was conducted to document any current or past, personal or familial history of mental illness. One subject had a family history of depression in their mother, one subject had a family history of bipolar disorder in their mother, while four subjects had no family history of psychiatric disease. The six subjects completed the Children's Depression Rating Scale-Revised (CDRS-R).[22] As a result, no significant changes were revealed in general state, weight, height, blood pressure, or heart rate, during the 4 week follow-up period in the six subjects who completed the trial. In the six subjects who completed the trial, a comparison of baseline and the 4 week endpoint showed that CDRS-R total scores (baseline score, 58.83±10.83; 4 week endpoint score, 38.87±3.33; P=0.003, df=5, t=5.384) and subscores for Difficulty Having Fun (baseline score; 4.33±0.82, 4 week endpoint score; 2.67±0.52; P=0.011, df=5, t=3.953), Social Withdrawal (baseline score; 4.00±0.89, 4 week endpoint score; 2.83±0.75; P=0.013, df=5, t=3.796), Appetite Disturbance (baseline score; 3.00±0.63, 4 week endpoint score; 2.00±0.89; P=0.012, df=5, t=3.873), Physical Complaints (baseline score; 3.67±0.82, 4 week endpoint score; 1.50±0.84; P=0.006, df=5, t=4.540), Excessive Guilt (baseline score; 3.17±0.75, 4 week endpoint score; 1.83±0.75; P=0.010, df=5, t=4.000), Low Self-Esteem (baseline score; 4.00±0.89, 4 week endpoint score; 2.33±1.03; P=0.011, df=5, t=3.953), Depressed Feelings (baseline score; 4.17±1.47, 4 week endpoint score; 2.33±1.21; P=0.038, df=5, t=2.803), Excessive Weeping (baseline score; 3.83±2.40, 4 week endpoint score; 1.67±0.82; P=0.027, df=5, t=3.081); and Depressed Facial Affect (baseline score; 3.83±1.17, 4 week endpoint score; 2.17±0.75; P=0.004, df=5, t=2.524), improved significantly using paired t-test. Wilcoxon signed rank test also detected statistical significance in the CDRS-R total score (P=0.027), as well as subscores for Difficulty Having Fun (P=0.039), Social Withdrawal (P=0.038), Appetite Disturbance (P=0.034), Physical Complaints (P=0.038), Excessive Guilt (P=0.020), Low Self-Esteem (P=0.039), and Depressed Facial Affect (P=0.026). However, a comparison between baseline and the 4 week end-point found subscores for Impaired Schoolwork, Sleep Disturbance, Excessive Fatigue, Irritability, Morbid Ideation, Suicidal Ideation, Listless Speech, and Hypoactivity showed no significant changes. The Wilcoxon signed rank test also failed to detect any statistical significance in subscore changes for Impaired Schoolwork, Sleep Disturbance, Excessive Fatigue, Irritability, Depressed Feelings, Morbid Ideation, Suicidal Ideation, Excessive Weeping, Listless Speech, or Hypoactivity. Tipepidine was well tolerated in the six subjects who completed the trial, with no further dropouts due to side effects. Furthermore, three patients with adolescent depression have been continuing the oral use of tipepidine (30 mg/day) for its efficacy against depressive symptoms for 3 months or more after this trial.

Tipepidine improved symptoms of adolescent depression in the six subjects who completed the trial, as shown by CDRS-R scores. To our knowledge, this is the first report

demonstrating a beneficial effect for tipepidine in adolescent depression. Tipepidine inhibits GIRK channels and is predicted to modulate brain monoamine levels, in a similar manner to SSRIs, serotonin and norepinephrine reuptake inhibitors (SNRIs) and other antidepressants. Four subjects with depression dropped out during the trial due to feelings of mild irritation and mild skin eruptions. One possible reason for having dropout patients is diagnostic error. There is a chance that some of these children with depression may have been suffering from the onset of bipolar disorder or schizophrenia. Therefore, a follow-up check may be required for the dropout patients to examine their progression.

Recent mapping the of c-Fos-like immunoreactivity (FLI) induction in rat brains identified FLI-positive neurons in several brain areas after acute dosing with different classes of antidepressants.[23] Very recently Kawahara et al[24] showed that a single injection of tipepidine (20 mg/kg or 40 mg/kg) in rats, increased FLI-positive neurons in the central nucleus of the amygdala (CeA) in a manner similar to the tested antidepressants, as well as inducing the characteristic increase in FLI-positive neurons in six other brain regions, including the nucleus accumbens (NAcc). This latter effect was not observed with other antidepressants. Therefore, further detailed studies investigating tipepidine induced dopamine activation in the CeA, NAc, and its neural pathways are warranted.

The main limitation of our study is its small sample size (n=6 evaluable subjects). Another is the low proportion of drug naïve subjects. Additional trials are needed to evaluate the efficacy and safety for tipepidine use in adolescent depression. Although tipepidine is widely used, however, there are reports[25–27] indicating a possible toxic effect like agitation, fixed drug eruption and toxic epidermal necrolysis also in pediatric populations. So we must pay attention to the mood symptoms, especially irritability and cutis symptoms, because there were patients for whom a feeling of irritation and a cutis symptom appeared in this tipepidine study. Also, future studies with greater analytical power, using larger sample sizes and more drug naïve subjects will be necessary to determine tipepidines efficacy and safety.

In conclusion, our pilot study suggests that tipepidine therapy may prove to be an effective alternative treatment for adolescent patients with depression. However, the long-term safety of tipepidine still needs to be assessed, as a cough suppressant therapy is usually completed within 1 week. In addition, the side effects detected here need careful evaluation, as part of more detailed randomized, double-blind studies into this encouraging finding for tipepidine in adolescent depression.

Disclosure

The authors report no conflicts of interest in this work.

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