(SAM) by DNA methyltransferase (DNMT) enzymes. Methylation of the promoter region of genes is generally associated with the inhibition of transcription factor binding to *cis*-acting regulatory sequences and the recruitment of repressor complexes, including methyl CpG binding proteins (MBDs), resulting in transcriptional repression [9, 10]. Histone modifications confer what has been called a 'histone code' on the genome, defining parts of the genome that are accessible to transcription in a given tissue type at a given time [11]. For example, acetylation of the ninth lysine residue on histone 3 (H3–K9) is classically associated with active transcription and open chromatin [12]. The role of histone methylation is less clear, as it can either enhance or repress transcription depending on the histone modified [8]. Because enzymes that deacetylate histones (HDACs) are known to recruit transcriptional repressors, such as MBDs, patterns of methylation and acetylation are intimately linked. Pharmacological manipulations, including drugs of abuse, such as cocaine and alcohol, as well as a mood stabilizer, such as valproate, have been shown to modulate chromatin function by influencing the activity of these enzymes. Recent evidence also suggests that chromatin remodeling as a result of environmental perturbations plays a role postnatally in processes that affect behavior.

In this review, evidence suggesting that epigenetic factors might influence the development of mood disorders, such as depression and bipolar disorder, are introduced.

Epigenetic studies relevant to depression

Clinical evidence suggesting the role of epigenetics in depression

Depression is not only one of the most prevalent causes of mental suffering [13, 14] but also places an enormous economic burden on society [15]. The accumulated evidence from epidemiological studies suggests that genetic predispositions interact with the environment in potentiating depression [16, 17]. Aversive life events potentiate depression in some individuals and resiliency in others. Such interindividual variation may be mediated by variations of neurotrophic and neurotransmitter systems. A polymorphism in the brain-derived neurotrophic factor (BDNF) coding region, producing pro-BDNF with either a methionine or a valine in position 66, has been associated with depression in several populations [18, 19]. With respect to neurotransmitter systems, particular emphasis has been placed on serotonin dysfunction in depression. For example, individuals carrying a common short variant of a repetitive sequence in the serotonin transporter gene in a region controlling transcription (5-HTTLPR, the serotonin transporter gene-linked polymorphic region) show increased neuroticism or harm avoidance relative to individuals homozygous for the long variant of the 5-HTTLPR. There is some controversy as to whether or not the polymorphic region confers greater risk of depression per se. A longitudinal study found that individuals carrying the short 5-HTTLPR variant showed more depressive symptoms as a function of stressful life events, while the carriers of the long 5-HTTLPR variant did not show increase of depression associated with stress. This result suggested a possible gene environmental interaction [20], although this is still controversial [21]. In the brain, these changes might be reflected in differential functional connectivity between areas, including the hippocampus, as a function of life stress [22].

Finally, there is evidence in a variety of species, including humans, nonhuman primates, and rodents, that early postnatal care (or early life stress) influences the risk for depression in adulthood. In humans, there is considerable evidence that early childhood abuse or neglect increases the risk of depression as well as other psychopathologies [23–25]. Patterns of abuse and neglect may be transmitted intergenerationally from mother to daughter in both humans and in non-human primates [26]. In rhesus macaques, infants cross-fostered from non-abusive mothers to abusive mothers, and infants cross-fostered from abusive mothers to non-abusive mothers showed levels of abuse in adulthood similar to that of their adoptive mothers, suggesting transmission via an epigenetic mechanism that remains to be defined [27]. Other studies have shown that early childhood adversity in humans [17] and non-human primates [28] enhances stress reactivity in adulthood. These data imply that resiliency to stress has a protective effect against depression. Similar studies in rodents have recently shed light on possible molecular mechanisms of these effects.

Epigenetic regulation of stress vulnerability in rodents

The laboratories of Michael Meaney and Moshe Szyf, working with a rodent model of maternal care, were the first to demonstrate a mechanism of epigenetic regulation of stress [12]. In rats, naturally occurring variations in maternal care have been shown to regulate the expression of the glucocorticoid receptor (GR) in the hippocampus of offspring. This effect is stable into adulthood, and recent evidence suggests that it is epigenetically regulated [6]. Rat mothers show large individual differences in licking and grooming (LG) of pups during the first week of life. Pups reared by 'high' LG mothers (at least 1 SD above the mean) show less anxiety-like behavior and a more rapid recovery from stress than do pups reared by 'low' LG (at

least 1 SD below the mean) mothers [29]. Interestingly, these differences appear to be transmitted non-genomically, as female pups exhibit maternal behavior characteristic of their foster mother [30]. In addition, pups from high LG litters cross-fostered to low LG litters, and vice versa, exhibit behavioral and physiological responses to stress in adulthood that are characteristic of their foster environment [30]. These effects are mediated, at least in part, by alterations in the hypothalamic–pituitary–adrenal axis function, including enhanced glucocorticoid negative feedback sensitivity due to an increase in GR in the offspring of high LG mothers. Glucocorticoid receptor expression is regulated at the level of RNA, through splice variation in the 5′ untranslated region (UTR) of exon 1 [31].

Alterations in maternal behavior are associated with alterations in the expression of GR, including differences in the expression of the exon 1_7 transcript [31, 32]. DNA methylation of the $GR1_7$ promoter has recently been shown to be greater in the offspring of low LG mothers than in those of high LG mothers [6]. These differences in DNA methylation emerge during the first week of life in parallel with differences in maternal behavior, and they are remarkably stable into adulthood. Nevertheless, this epigenetic programming is reversible by pharmacological manipulations later in life. Specifically, infusion of Trichostatin A (TSA), an HDAC inhibitor, eliminated the hypermethylation of $GR1_7$ in rats from low LG litters [6], whereas central infusion of the methyl donor L-methionine enhanced the methylation of $GR1_7$ in rats from high LG litters [33]. These data provide a first example of epigenetic programming by the social environment and suggest that DNA methylation may be malleable in postmitotic neurons.

Role of histone modification in antidepressive treatment action

A complete understanding of the mechanisms of action of common physiological interventions used to treat depression, including monoamine oxidase (MAO) inhibitors, tricyclic antidepressants, such as imipramine, and electroconvulsive shock (ECS) therapy has remained elusive, in part because of the relative stability of symptoms and the delayed behavioral response to treatment with these methods [8]. All antidepressants are known to increase levels of monoamine neurotransmitters at the synapse [34]. In addition, several treatments have recently been implicated in chromatin remodeling. For example, the histone demethylase BHC110/LSD1, which bears a strong sequence homology with MAO, was shown to target dimethyl Histone 3 at lysine 4 (H3K9) for demethylation in vitro [35]. Furthermore, transcriptional activity at genes targeted by BHC110 was enhanced by increases in dimethyl H3K9. These results implicate epigenetic mechanisms in the activity of MAO inhibitors.

Eric Nestler and colleagues have experimental documentation of the associations between histone modifications and changes in behavioral function in response to antidepressant treatment and ECS in the hippocampus of rodents, a brain region implicated in depression [8, 36, 37]. In mice subjected to chronic social defeat stress, chronically administered imipramine produced a selective hyperacetylation of histone H3 at the BDNF III and BDFN IV promoters as well as increased H3K4 dimethylation at the BDNF III promoter [37]. These changes were concomitant with an enhancement of BDNF transcription in these mice. In contrast, such hyperacetylation was not observed in nondefeated control mice. In addition, levels of the histone deacetylase HDAC5 decreased and HDAC9 increased with social defeat stress in imipramine-treated mice relative to controls, whereas drug treatment or stress alone had no effect. Finally, the overexpression of HDAC5 blocked the effect of imipramine in the social defeat paradigm. Thus, although BDNF expression increased in both the control and the socially defeated mice, the observed histone and HDAC modifications with imipramine treatment occurred only in the context of social defeat stress. The data provide a new explanation for the delayed onset of action of antidepressants in the treatment of depression. It should be noted that an observed increase in dimethylation of H3K27 as a result of social defeat stress was not reversed by imipramine.

In another study, Tsankova et al. [36] examined histone modification 30 min, 2 h and 24 h after acute or repeated ECS in rats. These researchers observed significant differences in the levels of H4 acetylation in both the c-Fos and cAMP regulatory element binding protein (CREB) promoter regions, and a significant decrease in acetylated H4 after 24 h in the chronic ECS only, together with decreases in expression. Electroconvulsive shock leads to H3 phosphoacetylation in the c-Fos promoter in both the acute and chronic conditions, but only in the chronic condition for the BDNF. The regulation of BDNF also differed between chronic and acute ECS conditions. For the BDNF II promoter, acetylated H4 decreased significantly 24 h after chronic ECS treatment, whereas during the same time interval there was an increase in acetylated H4 in the acute condition. However, there was no change in the levels of acetylated H4 in the BDNF II promoter. In addition, in the chronic condition only, phosphoacetylated H3 increased in the BDNF II promoter but decreased in the BDNF III promoter. These changes accompanied increased transient levels of mRNA in the acute condition and 24 h after the chronic condition. Interestingly, acetylated H3 was observed only in the chronic ECS condition for both the BDNF II and BDNF III promoters. These differences in

histone modifications accompanying acute versus chronic ECS may be instructive because the stimulus was identical and differed only in the frequency of its application. Much as is the case of antidepressant medication, a significant response to ECS treatment in depression depends upon chronic administration in patients. The findings demonstrate that several long-lasting histone modifications occur in response to chronic ECS conditions, leading to long-term effects on gene regulation. In addition, the histone modifications that accompanied ECS were idiosyncratic – not only to the type of ECS treatment but also dependent on the gene promoter analyzed. These results provide further credence to the aforementioned notion that a histone code of specific modifications determines the regulation of a specific gene (and of specific promoters within a given gene [11]. The characterization of this histone code for other genes affected by depression should lead to further advances in treatment.

Epigenetics in bipolar disorder

Genomic imprinting

In the genomic imprinting phenomenon, a maternally or paternally transmitted allele is inactivated by DNA methylation and hemiallelic expression is observed. Many imprinted genes are related to development. Transmission patterns of genetic diseases caused by the mutation of imprinted genes are complex, because their influence on offspring depends on the gender of the parent who transmitted the mutated allele. Such gender differences of transmission observed in the transmission of diseases caused by mutations of imprinted genes are referred to as a parent-of-origin effect (POE) [38–40].

Bipolar disorder may be transmitted from a mother more often than from a father [40]. When bipolar disorder is transmitted from the father, the offspring tends to have a more severe form of the illness [41] and an earlier age of onset [42] than when it is transmitted from the mother; in addition, there is a higher prevalence with the former [43]. Based on evidence suggesting the role of genomic imprinting in bipolar disorder, Gershon et al. [44] performed a linkage analysis in which they assessed the gender of the transmitting parent. These researchers found a linkage with chromosome 18 only in paternal transmission. Nothen et al. [45] confirmed that the linkage of bipolar disorder with 18p11.2 could only be seen in paternally inherited pedigrees, and McInnis et al. [46] reported that they observed linkage with 18q22 only in paternally transmitted pedigrees.

These pieces of evidence prompted the search for imprinted genes on chromosome 18. Corradi et al. [47] found that GNAL at 18p11.2, which encodes a G protein alpha subunit, may be a candidate of an imprinted gene. Although they showed that the promoter region of this gene is methylated, no evidence of allele specific methylation was shown.

Recent linkage analyses have used software to calculate the linkage based on the assumption of paternal or maternal imprinting. Such studies have detected several additional linkage loci suggestive of imprinting: 13q12, 1q41 [46], 2p24-21, 2q31-q32, 14q32, and 16q21-q23 [48]. Transmission disequilibrium tests in trio samples also revealed the association of several genes when the gender of the transmitting parent was taken into consideration [49, 50]. None of these linkages or findings in association studies suggestive of imprinting have as yet been validated by molecular biological experiments to show allele-specific expression or methylation. Apparent POE does not always represent genomic imprinting but can be caused by several mechanisms [40]. Indeed, although we found the association of polymorphisms of HSPA5 at 9q33-34.1 with bipolar disorder only in paternal transmission, this gene showed biallelic expression in the brain, which ruled out the possibility that HSPA5 is imprinted in the brain, at least in the prefrontal cortex [51].

Using a machine learning approach, Luedi et al. searched for imprinted genes in the mouse genome and reported that several genes on the candidate loci of bipolar disorder (13q13 and 18q22) might be imprinted [52]. However, this prediction also awaits experimental validation. In addition, DNA methylation status may differ between mice and humans.

In summary, genomic imprinting in bipolar disorder has only been suggested by statistical genetics and, to date, there has been no molecular biological evidence to support this possibility.

None of the findings in linkage analysis and genetic association studies in bipolar disorder have been consistently replicated. One possible explanation for the lack of consistency is false positive findings due to multiple statistical testings. It should be cautioned that an analysis considering the possibility of genomic imprinting not only provides a clue to understanding the pathophysiology of the illness but also increases the probability of false positive results, if the results are not adequately validated by experiments.

Pharmacology

Lithium is the best established mood stabilizer, having antimanic, antidepressive, and prophylactic effects on bipolar disorder. Valproate is the next most widely used mood stabilizer, having robust antimanic effects, putative prophylactic effects, but no antidepressive effects.

Although the mechanism of action of valproate on bipolar disorder remains controversial, HDAC inhibition is proposed as one of mechanisms [53]. Neuroprotective effects are common to these two major mood stabilizers, lithium and valproate [54], and the neuroprotective effect of valproate may be mediated by HDAC inhibition [55, 56]. Valproate also enhances neuronal differentiation in neural progenitor cells by HDAC inhibition [57]. These findings suggest a possible role of histone acetylation, which is coupled with DNA methylation, in the pathophysiology of bipolar disorder. However, the role of other mechanisms, for example inositol depletion and the increase of bcl-2 on the mitochondrial membrane, have also been suggested, and it is still not known whether HDAC inhibition is crucial for the effect of valproate on bipolar disorder.

S-adenosyl methionine supplies a methyl residue in a DNA methylation reaction. Many studies have found SAM to have antidepressive effects [58]. Interestingly, Carney et al. [59] reported that nine of 11 patients with bipolar depression treated with SAM switched to mania, suggesting a specific effect of SAM on bipolar depression. As mentioned above, central infusion of L-methionine, a precursor of SAM, increased DNA methylation of the promoter of the GR gene. Methionine treatment was found to abolish the effect of a high LG mother on the offspring, as shown by the decreased DNA methylation status of GR and the inhibition of behavioral despair [33]. The fact that SAM, which similarly enhances DNA methylation, is effective in the treatment of depression is apparently contradictory to the effect of methionine. *S*-adenosyl methionine is a methyl-residue donor not only for the DNA methylation reaction but also for other enzymatic reactions. For example, creatine is produced from SAM and guanidinoacetate, and SAM treatment increases the phosphocreatine level in the brain [60]. This effect may also contribute to the antidepressive effect of SAM because decreased phosphocreatine levels have been reported in bipolar depression [61].

The antimanic effect of valproate, which inhibits HDAC and decreases DNA methylation, and the antidepressant effect of SAM, which increases DNA methylation, together indirectly suggest a role for DNA methylation in the symptoms of mania and depression in bipolar disorder. However, this is still a preliminary hypothetical mechanism, and to date there has been no study focusing on the role of DNA methylation in mania and depression.

DNA methylation analysis in postmortem brains

Abdolmaleky et al. recently examined the DNA methylation status of the promoter of membrane-bound catechol-*O*-methyltransferase (COMT) [62], an enzyme which regulates the level of dopamine and which is regarded as a candidate gene in bipolar disorder. A methionine to valine substitution at site 158 (Val158Met), which alters enzyme activity, was reported to be associated with bipolar disorder [63, 64], although this result is still controversial [65]. The COMT gene has two promoters, each generating its own mRNA isoform: the membrane-bound isoform (MB-COMT) and the soluble isoform (S-COMT), respectively. Abdolmaleky et al. [62] examined the methylation status of the MB-COMT promoter in the prefrontal cortex (Brodmann's area 46) by means of a methylation-specific PCR analysis. Although this region of the genome was predominantly unmethylated, a weak methylation signal could be detected. While 60% of 35 controls in the Stanley Microarray Collection samples showed some PCR product obtained from the methylated allele, only 29% of 35 patients with bipolar disorder and 26% of 35 patients with schizophrenia showed a methylation signal. This difference was statistically significant. Subjects with a methylation signal showed significantly lower expression levels of MB-COMT than those not showing a methylation signal in postmortem brain samples obtained from the Harvard Brain Tissue Resource Center [62]. This study suggested the possible role of hypomethylation of the promoter of MB-COMT in bipolar disorder and schizophrenia.

In contrast, Dempster et al. [66] analyzed the DNA methylation status of the promoter of S-COMT using Pyrosequencing in 60 postmortem brain samples obtained from the Stanley Neuropathology Consortium. These researchers analyzed two CpG sites, site 1 and site 2, corresponding to cytosine 27 and 23, respectively, of an earlier study [67] because these two sites, among the six sites studied, were found be partially methylated in many brain regions. Site 1 showed 45.4% methylation, while site 2 showed 34.5% methylation. Dempster et al. [66] found that although the methylation status of the two CpG sites analyzed showed a high correlation ($r = 0.8$, $P < 0.001$), there was no difference in DNA methylation status between diagnoses, and DNA methylation status was not correlated with the mRNA level of COMT.

DNA methylation differences between discordant twins

In spite of extensive linkage and association studies of bipolar disorder during the past two decades, the results are still not conclusive. No causative gene nor genetic risk factor has been established for bipolar disorder. In an attempt to identify the molecular pathogenesis of bipolar disorder, we have been focusing on monozygotic twins discordant for bipolar disorder.

Within this framework, we performed gene expression analysis of lymphoblastoid cell lines obtained from two

pairs of monozygotic twins discordant for bipolar disorder and found that 17 genes, including XBP1, HSPA5, ECGF1, and ATF5, were commonly down-regulated in both of the twins. Because XBP1 is a endoplasmic reticulum (ER) stress response-related transcription factor which regulates HSPA5, we focused on XBP1 [68]. Although we initially reported that a functional polymorphism of XBP1 was associated with bipolar disorder, this association was not replicated in subsequent studies [69, 70]. We also reported a weak but significant association of bipolar disorder with HSPA5 [51]. The induction of XBP1 upon ER stress was diminished in lymphoblastoid cells derived from patients with bipolar disorder [68]; this result was recently replicated in larger number of samples [71], supporting the role of the ER stress pathway in bipolar disorder.

More recently, Matigian et al. [72] performed a gene expression analysis in three pairs of monozygotic twins discordant for bipolar disorder. These researchers suggested that the WNT pathway is altered in bipolar disorder. While they did not demonstrate the down-regulation of XBP1 and HSPA5 in these three pairs of monozygotic twins discordant for bipolar disorder, they did show the down-regulation of ECGF1 and ATF5 [72]. ATF5 may also be related to the ER stress pathway and interact with DISC1, the most established causative gene for schizophrenia and mood disorders. However, our single nucleotide polymorphism (SNP) analysis did not support the association of ATF5 with bipolar disorder [73].

We postulated that the observed differences in gene expression between twins might be caused by a difference in DNA methylation status [74]. Although it has been reported that differences in DNA methylation were observed between monozygotic twins discordant for schizophrenia [75–77], there have been no such studies in bipolar disorder. The DNA methylation status of XBP1 did not differ between twins [68]; therefore, we began a comprehensive search for genes showing differential DNA methylation patterns between discordant twins. To this end, we employed a molecular biological technique developed by Ushijima and colleagues [78], called MS-RDA (methylation-sensitive representational difference analysis), which was initially developed to search for genes differentially methylated in cancer tissue. This method, which consists of digestion by methylation-sensitive restriction enzyme and subsequent subtraction, is able to selectively amplify differentially methylated genomic regions. This method had been used for the detection of differentially methylated genes from a pair of genomic DNAs obtained from one individual, one sampled from cancer tissue and the other from neighboring normal tissue. These DNAs had the same genomic sequences but different DNA methylation statuses. This method cannot be used for a case control analysis because DNA sequence differences complicate the results. However, this method can be used for the analysis of monozygotic twins having the same genomic sequences. Applying this method to a pair of monozygotic twins discordant for bipolar disorder, we isolated ten DNA fragments derived from CpG islands or putative promoters [79]. Among these ten fragments, DNA methylation differences in four regions was confirmed by bisulfite sequencing between the bipolar twin and control co-twin. Fraga et al. [80] reported that DNA methylation differences between monozygotic twins increase with age. Consequently, the differences in DNA methylation in discordant twins may not always be related to the pathophysiology of the illness.

To test the pathophysiological significance of DNA methylation, Kuratomi et al. [79] performed a case control analysis using Pyrosequencing and found an altered DNA methylation status of spermine synthase (SMS) in female patients with bipolar disorder. However, DNA methylation had increased in the bipolar patients, while it had decreased in the bipolar twin. On the other hand, this case control analysis also found a decreased methylation status of PPIEL (peptidylprolyl isomerase E-like) in the affected twin and patients with bipolar II disorder. The DNA methylation status of PPIEL was significantly correlated with its mRNA expression level ($R = -0.81$) and also with the DNA methylation levels in peripheral leukocytes ($R = 0.41$). Because PPIEL is a primate-specific gene, further analysis is not easy. However, we found that mRNA levels of PPIEL were lower in the frontal cortex and hippocampus and highest in the substantia nigra and pituitary gland [79]. These findings suggest that this gene may be involved in dopaminergic neurotransmission and/or neuroendocrine systems. Although it is not known whether the reduced DNA methylation status of PPIEL is a causative factor for bipolar II disorder or the result of the disease, it may in some way be related to the pathophysiology of the illness.

Discussion

As summarized above, epigenetic studies of bipolar disorder have just begun. The results of clinical genetic studies carried out to date suggest the role of genomic imprinting, but no study has yet been reported that directly tests this hypothesis. Pharmacological studies imply that pharmacological manipulation of DNA methylation status might alter mood states, but this has also not been tested experimentally in humans. There have been several studies recently that directly examine the DNA methylation status in samples obtained from patients.

In the case of schizophrenia, the results of DNA methylation analyses of RELN (reelin gene) in postmortem brains are not concordant. Two groups reported increased methylation in schizophrenia [81, 82], but two groups did

not [83, 84]. This discrepancy could be caused by methodological problems, such as the process of sodium bisulfite treatment, PCR bias, or cloning bias. Several studies used the methylation-specific PCR method, which has been successfully used for other applications, such as the diagnosis of imprinting [85] or the detection of hypermethylation in cancer [86]. Although methylation-specific PCR would be useful to analyze such "all-or-none" phenomena, it might not be ideal for the quantitative analysis of DNA methylation in the study of psychiatric illness. For the quantitative analysis of a large number of clinical samples, Pyrosequencing analysis of bisulfite-treated DNA [66, 79] or real-time PCR quantification of DNA digested by a methylation-sensitive restriction enzyme [83] would, in practical terms be useful approaches. Although none of these studies have used serially diluted standard samples to calibrate the DNA methylation levels, such approach may also be useful to obtain reliable and reproducible results in future studies.

The nature of the tissue used for DNA methylation analysis is also critical. In the case of lymphoblastoid cell lines, the DNA methylation status can be potentially affected by transformation by the Epstein-Barr virus; consequently, results in lymphoblastoid cells should be interpreted with caution. In the case of DNA methylation analysis in the brain, tissue heterogeneity might potentially affect the results [87] because brain tissue contains many cell types, including neurons and glia. The ideal approach would be to separate specific cell types in the brain, but the amount of DNA required for DNA methylation analysis currently hampers such approaches. Further technical development is necessary for future studies.

Conclusion

Epigenetic studies focusing on mood disorder are very recent developments. The results of several initial findings, however, seem quite promising; such as alterations in DNA methylation of the GR gene associated with maternal care and stress vulnerability, altered histone modification associated with antidepressive treatments, altered methylation status of COMT in the brains of patients with bipolar disorder and altered DNA methylation status of PPIEL in lymphoblastoid cells of patients with bipolar disorder. Further studies are needed to clarify the biological basis and pathophysiological significance of these findings.

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ORIGINAL ARTICLE

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Tissue specificity of methylation and expression of human genes coding for neuropeptides and their receptors, and of a human endogenous retrovirus K family

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Abstract The purpose of the present study was to understand the tissue specificity of DNA methylation and the relationship between methylation and expression of genes with essential roles in neurodevelopment and brain function. We chose dopamine receptor genes (*DRD1* and *DRD2*), *NCAM*, and *COMT* as examples of genes with CpG islands around the promoter region, and serotonin receptor genes (*HTR2A* and *HTR3A*), *HCRT*, and *DRD3* as genes without CpG islands. Methylation states were investigated in fetal brain, fetal liver, placenta, and in adult peripheral leukocytes from three individuals by Southern blot and bisulfite-modified DNA sequencing. A repetitive sequence, human endogenous retrovirus (HERV)-K was also examined. All genes examined were almost completely unmethylated in brains. The genes with CpG islands were unmethylated regardless of their expression state. In contrast, genes without CpG islands showed various methylation patterns, which did not necessarily reflect the transcriptional activity of the genes. Most HERV-K loci were methylated, but some loci showed relatively low methylation in the placenta and liver. Interestingly, we found inter-individual differences in methylation levels in *HTR2A* and *HCRT* in the placenta and in some loci of HERV-K in the placenta and liver. The sample with the lowest methylation levels in the two unique genes showed higher methylation of HERV-K loci than the other samples. These results provide detailed information about the methylation states of the genes analyzed and evidence for inter-individual variations in methylation in both unique and repetitive sequences.

Keywords Tissue specificity · DNA methylation · Gene expression · Dopamine and serotonin receptors · Human endogenous retrovirus

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Introduction

Epigenetic modifications are involved in a wide range of normal and pathological cellular phenomena (Jones and Laird 1999; Jaenisch and Bird 2003). Cytosine methylation in CpG and some CpNpG sequences is the sole epigenetic modification of DNA with known biological functions. One of these functions is gene regulation through the construction of heterochromatin (Razin 1998). Unusual hypermethylation has often been observed in tumors in the promoter regions of tumor suppressor genes (Jones and Laird 1999).

Aberrant epigenetic modifications occur not only in somatic cells, resulting in tumors, but also in some inherited diseases (Bickmore and van der Maarel 2003). Mental dysfunction is a major symptom in most inherited diseases with aberrations in epigenetic modifications. The neurodevelopmental disorder Rett syndrome is caused by mutations in a methyl-CpG-binding protein, MeCP2, and the characteristic clinical features partially resemble those observed in autism and schizophrenia (Robertson and Wolffe 2000; Shahbazian and Zoghbi 2002). Rett syndrome is a typical example of the implications of epigenetic modifications such as DNA methylation, and suggests that accurate fine-tuning of gene expression by epigenetic mechanisms is essential for normal brain function.

Studies on the role of DNA methylation in the pathogenesis of psychiatric disorders have increased in recent

years but are still few in number, which may be due to the unique features of DNA methylation as well as the technical challenges involved in correlating methylation with particular pathologies. DNA methylation undergoes a genome-wide erasure and re-establishment during embryogenesis and shows tissue-specific patterns, as does gene expression (Hsieh 2000). It is evident that two major psychiatric disorders, schizophrenia and bipolar disorder, are inherited illnesses, and it is also true that both disorders are greatly influenced by environmental conditions (Abdolmaleky et al. 2005). Although seemingly paradoxical, DNA methylation could explain both the ambiguous inheritance and the roles of environmental factors in the etiology of these disorders. There is increasing evidence that some epigenetic signals may exhibit partial meiotic stability and can be transmitted from one generation to the next (Roemer et al. 1997; Morgan et al. 1999; Sutherland et al. 2000; Rakyan et al. 2002). Similarly, mitotic transmission of DNA methylation patterns demonstrates partial stability, but such patterns can be changed by hormones, nutritional factors, aging, or stochastic events in the cell (Wolff et al. 1998; Ahuja and Issa 2000; Thomassin et al. 2001).

In the present study, we examined the DNA methylation status of eight genes that are actively expressed in the brain: the dopamine receptors *DRD1*, *DRD2*, and *DRD3*, catechol-O-methyltransferase (*COMT*), neural cell adhesion molecule (*NCAM*), the 5-hydroxytryptamine receptors *HTR2A* and *HTR3A*, and hypocretin (*HCRT*; also called orexin). *DRD1*, *DRD2*, *COMT*, and *NCAM* harbor CpG islands around the promoter regions and the other four genes do not. In addition to these eight single copy genes, we also examined methylation states in the 5′ long terminal repeat (LTR) of the human endogenous retrovirus (HERV)-K family in order to compare patterns between unique sequences and repetitive sequences, as well as to examine possible inter-individual differences.

Materials and methods

DNA and RNA

In the present study, we used DNA and RNA extracted from human fetal whole brains, fetal livers, placentas, and from peripheral blood leukocytes (PBL) of healthy volunteers. We prepared DNA using the standard proteinase K/phenol method (Sambrook et al. 1989). RNA from tissues other than PBL was extracted by the guanidine/CsCl method (Sambrook et al. 1989). RNA from PBL was isolated using ISOGEN (Nippon Gene, Japan) according to the manufacturer's instructions. DNA and RNA from fetal tissues and placentas were all from samples obtained in 1997. The fetuses (and placentas) were artificially aborted at 16 (Sample a), 19 (Sample b), and 21 (Sample c) weeks of gestation. Detailed information on the fetuses was not available.

Methylation analyses

We examined the methylation states of the eight single copy genes by Southern blot hybridization and using the sodium bisulfite-modified DNA sequencing method. HERV-K methylation was examined by Southern blot hybridization alone. Using the Grail 1.3 program (http://www.compbio.ornl.gov/Grail-1.3/), we confirmed that the sequences to be analyzed satisfied the established definition of a CpG island. DNA was first digested with appropriate non-methylsensitive restriction enzymes to yield a distinct band(s) in the methylation analysis by Southern blot hybridization. The restriction digests were then digested with methylsensitive restriction enzymes (10 U/μg DNA), separated on 0.8% agarose gels, and transferred to Hybond-N^+ membrane (Amersham Biosciences, Piscataway, NJ). Probes for the Southern blot hybridization were amplified by PCR, cloned into the pGEM-T easy vector (Promega, Madison, WI), and confirmed by sequencing. Probe DNA was labeled with [α-^{32}P]dCTP using a Megaprime DNA Labeling kit (Amersham Biosciences). Primer sequences and temperatures for the final wash of the hybridized membranes are shown in Table 1. The blots were hybridized in 6×SSC/0.5% SDS at 65°C overnight and were sequentially washed in 2×SSC/0.1% SDS at 55°C and in 0.1×SSC/0.1% SDS at the appropriate temperatures (Table 1).

Sodium bisulfite treatment was carried out as described (Grunau et al. 2001), with minor modifications. The bisulfite-treated DNA was amplified by PCR using the following conditions: denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, annealing temperature (see Table 2) for 1 min, 72°C for 1 min, and a final elongation at 72°C for 5 min with a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA). Nested PCR was performed for 25 cycles. The primer sequences and sizes and positions of the regions analyzed are shown in Table 2. The primer sequences were designed using a program for predicting modified sequences (Singal and Grimes 2001). The PCR products were cloned into the pGEM-T easy vector (Promega), and ten clones each were sequenced using a BigDye sequencing kit (Applied Biosystems). In the case of the CpG island genes, we performed the methylation analysis using the bisulfite method only for fetal brain and adult PBL from one fetus and one person, respectively.

Reverse transcription-PCR

RNA used for RT-PCR was pre-treated twice with 10 to 30 U RNase-free DNase I (Roche, Mannheim, Germany) for 40 min at 37°C. DNase I-treated total RNA (5 μg) was reverse-transcribed with random primers and the M-MLV reverse transcriptase (Invitrogen, La Jolla, CA) for 1 h at 37°C in a 20 μl reaction mix. The reverse transcription products were diluted 6- to 25-fold and 2 μl used for PCR (18–36 cycles) in a total volume of

Table 1 Southern blot analysis: primers for probe generation and wash conditions. *COMT* Catechol-O-methyltransferase, *DRD1* dopamine receptor D1, *DRD2* dopamine receptor D2, *NCAM* neural cell adhesion molecule, *HTR2A* 5-hydroxytryptamine receptor 2A, *HCRT* hypocretin, *DRD3* dopamine receptor D3, *HTR3A* 5-hydroxytryptamine receptor 3A, *HERV-K* human endogenous retrovirus K

Gene	Sense primer 5′–3′	Antisense primer 5′–3′	Washing temperature
COMT	tctggtcggatctaggggagtct	gggctgggtgccttgtctaa	55°C
DRD1	agtggcattgttgttggctctga	ccctccggtgcctgtgct	56°C
DRD2	gaccaaggcgggacaccaat	ctgaaccgtccccctctcca	59°C
NCAM	cccgctttctccacccctactttt	gccgttgctgggtgttgtttggata	52°C
HTR2A	agggaggaaagttgtctgctaat	ggggctggatttttgtcttc	57°C
HCRT	attgggcttggttttggcttctta	gcgctggcctgggacaat	59°C
DRD3	caaagccctgaggaaatggt	caaataaatgagtgccgagtaaga	52°C
HTR3A	tcgccttgctcctccccacac	gggaaaggacaaaaaggagag	55°C
HERV-K	gacttaatcctacggcaccaccta	agccctatttcttcggacctgttc	57°C

Table 2 A brief summary of PCR conditions and locations analyzed by the bisulfite method. *S* Sense-strand, *A* antisense-strand. Gene symbols as in Table 1

Gene	PCR	Primer 5′–3′	Length (location)[a]	Accession no.[b]	Annealing temperature
COMT	First	S: tttagttttttatttgggaaggg	290 bp		52°C
		A: acaaccctaactaccccaaaaac	−239 to +51	NM_000754	
	Second	S: tttttgagtaagattagattaagaggt	43384–43673	AC000090.3	53°C
		A: acaaccctaactaccccaaaaac			
DRD1	First	S: gttaggggttgattttaagagg	396 bp		54°C
		A: aaacactcccaaaactaatcaccta	−305 to +91	NM_000794	
	Second	S: tgagttttgtttttaggggatttaa	111587–111982	AC091393.3	52°C
		A: accttcaaccctacaaaacaaaac			
DRD2	First	S: gtygtagagttgtttagttttagtgt	239 bp		58°C
		A: crcacaaacttctaatcctaacct	−45 to +194	NM_000795	
	Second	S: gggtygggagttagggat	111269–111507	AP002840.3	55°C
		A: crcacaaacttctaatcctaacct			
NCAM	First	S: ggaaggttgggtagtaggag	335 bp		55°C
		A: cctaaaaacaaacaattaccaaac	−284 to +51	NM_181351	
	Second	S: gaaattttagttttttagggag	95902–96236	AP000802.5	52°C
		A: atttttacaaaattatttcctacc			
HTR2A (upstream)	First	S: tatyatattatgytggtggaagat	220 bp		46°C
		A: aaatacatccartrttaatcccata	−741 to −521	NM_000621	
	Second	S: tggtggaagatyaagaagagggga	45919–46138	AL160397.17	57°C
		A: acaactttcctccctrraaattctcatt			
HTR2A (downstream)	First	S: ttgattgtatgttattttaataatattgttgttaa	356 bp		52°C
		A: ttaatataccacactctataacactaaaactaata	−304 to +52	NM_000621	
	Second	S: tgttattttaataatattgttgttaaattagtatt	45346–45701	AL160397.17	51°C
		A: cacactctataacactaaaactaatatacatactat			
HCRT	First	S: ttttttatgaaggaagaagg	410 bp		50°C
		A: ccaaaaaccttaaaactatc	−399 to +11	NM_001524	
	Second	S: gattgttgttggttgtttta	57183–57592	AC099811.7	57°C
		A: tatcaattataacccactcc			
DRD3	First	S: ggtaattaaattgaggaaggtgagag	283 bp		55°C
		A: ctaacaacaaacatacccaaacaaaac	−210 to +73	NM_000796	
	Second	S: aaaattaagattaaaaagagtattgaggag	5952–6234	AC093010.7	53°C
		A: ctacttttccaacttccctattaaacc			
HTR3A	First	S: tttttggggaaatatggttaagt	390 bp		52°C
		A: atcaaccaaatcctactacttcc	−176 to +214	NM_000869	
	Second	S: agtttttttggttgaatgggtgga	25226–25615	AP000908.4	55°C
		A: tctacttaccttctccctatacc			

[a]Locations relative to cDNA and genomic sequences/genes are indicated in the rows corresponding to the first and second rounds of PCR, respectively

[b]GenBank accession numbers of cDNA and genomic sequences are described, with NM numbers indicating cDNA and other numbers the genomic sequences of genes

20 μl. The PCR products were separated by electrophoresis using a 6.0% polyacrylamide gel. The intensity of the PCR products was measured using Science Lab Image Gauge software (Fujifilm, Japan). The primers and PCR conditions used for the RT-PCR assays are shown in Table 3.

Table 3 Primers and conditions for semi-quantitative RT-PCR. Gene symbols as in Table 1. Ex1a is the authentic first exon of HTR3A and Ex1b may be a novel exon (see Fig. 4 and text)

Gene	Sense primer 5′–3′	Antisense primer 5′–3′	Annealing temperature	Cycles
COMT	agctcagaggagaccccagac	tgggctgcaggatgaactcgt	58°C	23, 26, 29
DRD1	tgcccccagcgaagtccacat	tcctgggcctctgctctgcta	62°C	28, 31, 34
DRD2	cgctgcagaccaccaccaact	gtcgatgctgatggcacacaa	58°C	25, 28, 31
NCAM	gcccaggtgcagtttgatgaa	ctgatctcacccagccctttg	58°C	20, 23, 26
HTR2A	tgctgctgggtttccttgtca	tctggagttgaagcggctgtg	58°C	27, 30, 33
HCRT	cggctaccccaccctgag	tcgtagaggcggcaagag	58°C	28, 31, 34
DRD3	tgcaggagccgaagtggtaaa	atgagcgcgcagtaggagagg	58°C	28,31,34
HTR3A	ggtgtgcgccccgtgagg	ccgtggggatggacaact	58°C	28, 31, 34
HTR3A (Ex1a)	cttgctcctccccacact	ccgtggggatggacaact	60°C	30, 33, 36
HTR3A (Ex1b)	tgctcttccaagccagat	ccgtggggatggacaact	58°C	30, 33, 36
GAPDH	gaaggtgaaggtcggagtc	gaagatggtgatgggatttc	60°C	18, 21, 24

Results

Methylation states of genes with or without CpG islands and of HERV-K loci

We chose four genes, *DRD1* (Minowa et al. 1993), *DRD2* (Samad et al. 1997), *COMT* (Tenhunen et al. 1994), and *NCAM* (Hirsch et al. 1991) that harbor CpG islands in the regulatory or flanking regions of the gene (CpG island genes) and four genes, *DRD3* (D'Souza et al. 2001), *HTR2A* (Zhu et al. 1995), *HTR3A* (Bedford et al. 1998), and *HCRT* (Waleh et al. 2001), that do not have CpG islands in the corresponding regions (non-CpG island genes). All eight genes are predominantly and abundantly expressed in brain. In addition, methylation of the HERV-K family, one of the HERV families classified as human retrotransposons, was examined (Löwer et al. 1996).

We first examined the methylation states of the 5′-flanking regions of the genes by Southern blot hybridization using methylation-sensitive restriction enzymes. The CpG island genes all showed completely cleaved patterns with the exception of *COMT* in one of the PBL samples (Fig. 1). The partial methylation pattern of *COMT* in PBL Sample c is most likely the result of incomplete digestion due to insufficient purity of the sample, as well as the enzymatic characteristics of *Sac*II. A similar partial methylation pattern in *DRD1* was observed in some PBL samples (data not shown) using *Sac*II, even though the bisulfite method did not detect any methylated CpG within the *Sac*II recognition sequence. We confirmed by bisufite-modified DNA sequencing that all, or almost all, cytosine residues in the CpG sequences analyzed were unmethylated, as were cytosine residues in non-CpG sequences (Fig. 1).

In contrast, Southern blot hybridization of the non-CpG island genes showed a variety of methylation patterns, depending on the tissue and the gene analyzed (Figs. 2, 3). One of the prominent trends is the methylation status in brain: methylation was very low or absent in all four genes in each of the three individuals. Placental DNA showed the next lowest levels of methylation in the non-CpG island genes, with the exception of *HCRT*, while liver DNA revealed a trend towards hypermethylation. PBL also showed hypermethylated patterns of *HCRT* and *HTR3A*. *DRD3* was unexpectedly extremely hypomethylated in all tissues and individuals examined. Interestingly, differences in the degree of methylation between individuals were observed in *HTR2A* and *HCRT* in the placental samples: the methylation levels of *HTR2A* and *HCRT* were lower in Sample a than in Samples b and c (Fig. 2). Although the Southern blot method can reveal the methylation status only of the CpG sequence within a recognition sequence of a methyl-sensitive restriction enzyme, the results obtained with the bisulfite method were largely consistent with the results of Southern blot hybridization. The inter-individual differences in methylation in *HTR2A* and *HCRT* were also detected by the bisulfite method.

In addition to the single copy genes, we analyzed the methylation states of a repetitive sequence region of HERV-K by Southern blot hybridization. When the CpG within the *Sma*I recognition sequence in the 5′ LTR of HERV-K is unmethylated, a *Pst* I–*Sma* I fragment of approximately 1.8 kb is produced from multiple HERV-K loci (Fig. 4, top panel). The blot hybridized with a probe prepared from the *gag* region yielded numerous signals with various sizes, among which the signal at 1.8 kb was most intense in the *Pst* I plus *Xma* I-cleaved lanes, indicated by a black arrowhead in the middle panel in Fig. 4. In contrast, the signal indicated by an open arrowhead at approximately 4.3 kb in size disappeared in those lanes. Therefore, the 1.8 kb fragment results in part from the 4.3 kb fragment. When cutting with *Pst* I and *Sma* I, a methyl-sensitive isoschizomer of *Xma* I, this inverse relation was clearly recognized in the placental samples. We measured the intensities of the 1.8 and 4.3 kb signals and normalized them relative to an internal control (not shown). Inter-individual differences in intensities were observed in both signals in the placental samples, and an inverse relation between the 1.8 and 4.3 kb signals was evident (Fig. 4, bottom panel). Although the inter-individual

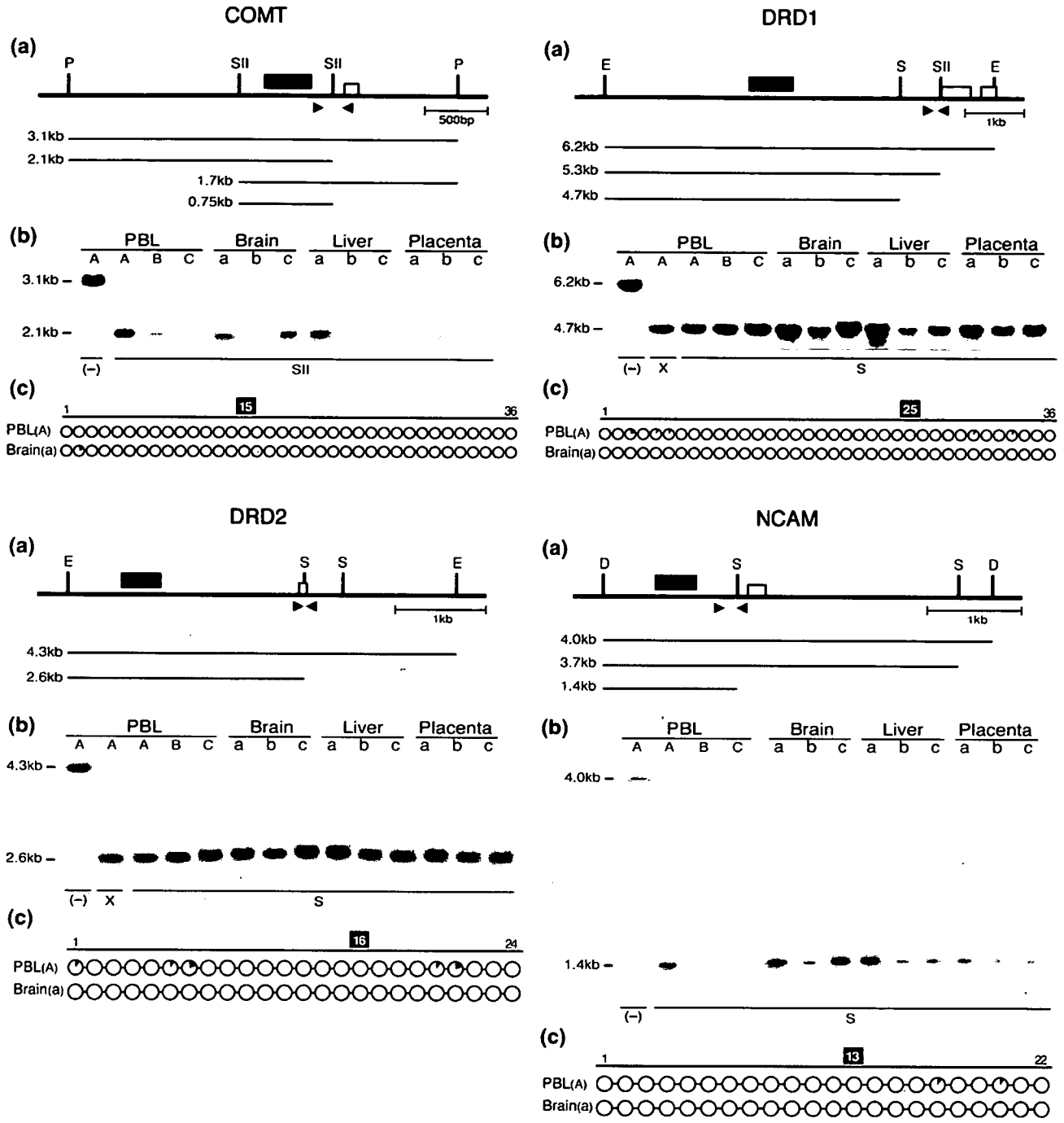

Fig. 1 Methylation analyses of genes with CpG islands. The three panels (**a–c**) presented for each gene show the following. **a** Schematic of the region analyzed where *filled boxes* indicate probes for Southern blot analysis, *open boxes* indicate the first exon of the gene, and *arrowheads* indicate positions of the primer sets for bisulfite-modified DNA sequencing. Lines under the restriction maps are possible fragments generated by restriction digestion. **b** The results of Southern blot analysis. *Letters* above the lanes indicate samples from different individuals. Samples *a*, *b*, and *c* were obtained from fetal tissues and placentas with gestational ages of 16, 19, and 21 weeks, respectively. **c** Schematic of methylation states of all cytosines in the CpG sequence as analyzed by the bisulfite method. Each nucleotide position is represented by a circle summarizing the results of ten clones analyzed. *Black sectors* indicates the percentage of methylated cytosine. Each number on the *line* indicates the position of the CpG region analyzed. The number in the *black box* indicates the position of the cytosine (in CpG) in the recognition sequences for methyl-sensitive restriction enzymes. P *Pst*I, S II *Sac*II, E *Eco*RI, S *Sma*I, D *Dra*I, *COMT* catechol-O-methyltransferase, *DRD1* dopamine receptor D1, *DRD2* dopamine receptor D2, *NCAM* neural cell adhesion molecule, *PBL* peripheral blood leukocytes

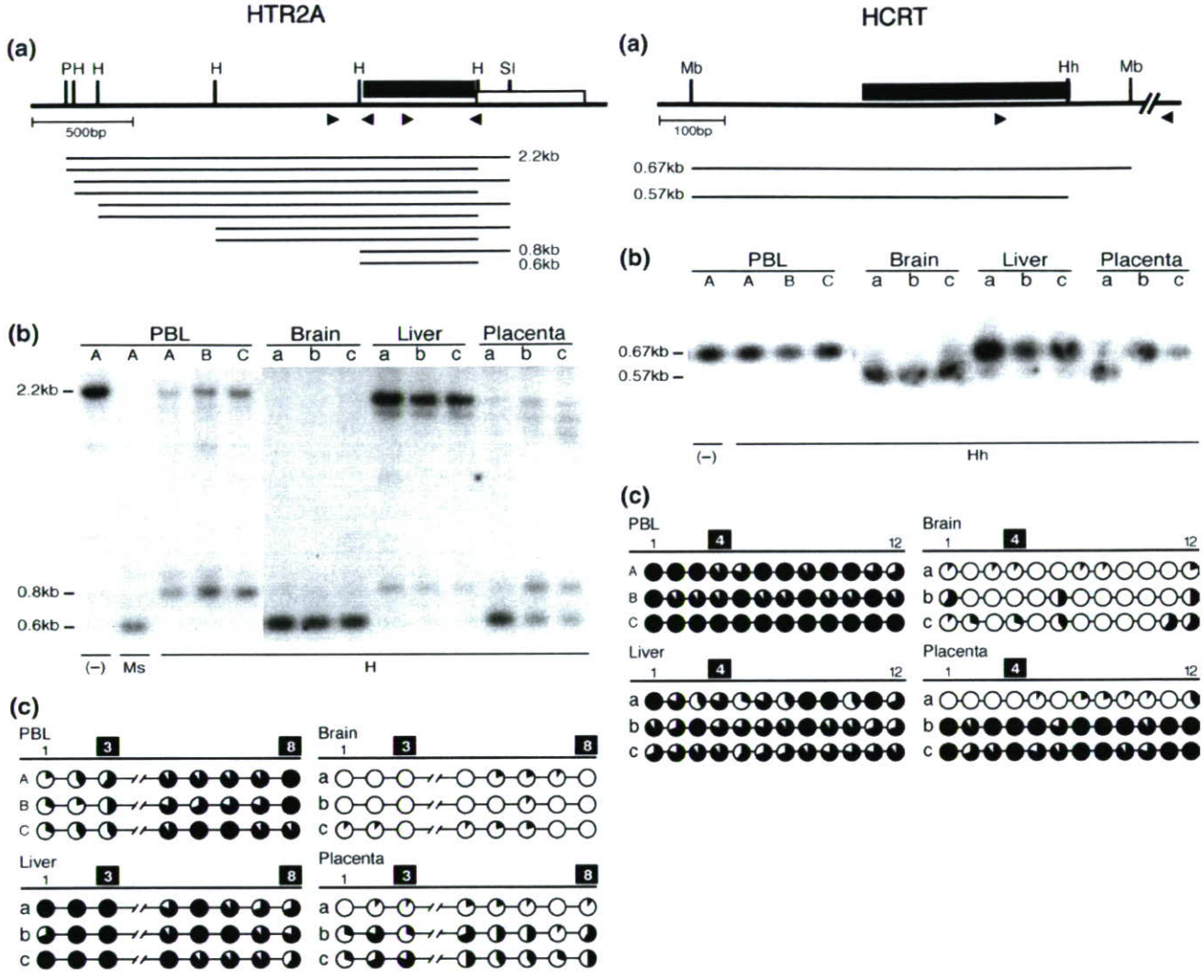

Fig. 2 Methylation analyses of genes without CpG islands. Description of panels **a**–**c** for each gene and *symbols* as in Fig. 1. P *Pst*I, H *Hpa*II, S I *Sac*I, Mb *Mbo*I, Hh *Hha*I, *HTR2A* 5-hydroxytryptamine receptor 2A, *HCRT* hypocretin (also called orexin)

differences in intensity were not detected in the 1.8 kb signal in the liver samples, possibly due to overall weak signal intensity, differences could be clearly seen in the 4.3 kb signal intensity, and the trend was similar to that in the placental samples. Sample a was most strongly methylated and Sample c most hypomethylated in both the placenta and liver.

Expression analysis by semi-quantitative RT-PCR

To examine the relationship between DNA methylation and gene expression, we estimated steady state levels of mRNA transcribed from each of the eight genes by semi-quantitative RT-PCR. The amount of RT product used as a template for PCR was adjusted relative to the internal control glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*). Primer pairs were located in exons of the corresponding genes.

Among the four genes with CpG islands, all but *COMT* were exclusively or predominantly expressed in the brain (Fig. 5). *COMT* was actively expressed in all of the tissues examined, consistent with the results of Tenhunen et al. (1994). The PCR products of *DRD1* and *DRD2* were detected in placental samples three to six cycles later than in brain samples. The *NCAM* PCR product was barely detected in the other tissues after the maximum number of cycles. Thus, the transcriptional activity of the three CpG island genes, *DRD1*, *DRD2*, and *NCAM*, was much lower in the liver, placenta, and in PBL than in the brain, and these same genes showed little or no methylation in their promoter regions.

The non-CpG island genes were also expressed most abundantly in the brain among the four tissues examined (Fig. 5). *HTR2A* was expressed in the other three tissues, albeit at very low levels. *HCRT* and *DRD3* showed low levels of expression in the placenta and PBL, respectively. Unlike these genes, *HTR3A* was expressed

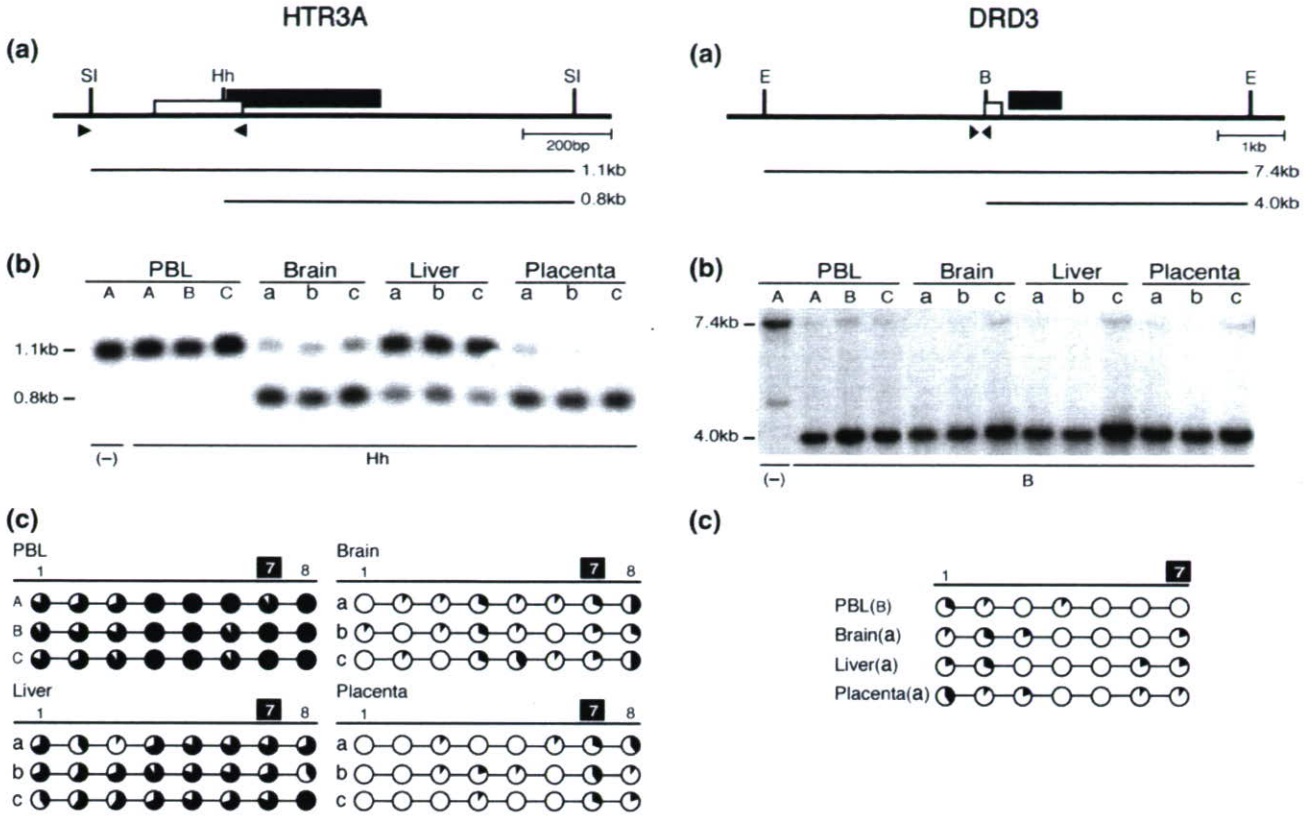

Fig. 3 Methylation analyses of genes without CpG islands. *Open boxes* indicate the first exons of the genes; the first exon of *HTR3A* corresponds to Ex1a described in Fig. 6. Description of panels **a**–**c** for each gene and *symbols* as in Fig. 1. *B* Methyl-sensitive restriction enzyme *Bst*BI, *HTR3A* 5-hydroxytryptamine receptor 3A, *DRD3* dopamine receptor D3

in the liver and PBL at levels similar to that found in brain, even though the gene was heavily methylated in both tissues. The discrepancy between the expression and methylation of *HTR3A* prompted a database search in which we found an EST (GenBank accession number, BG341613) from primary B cells from tonsils that was located between the first and second exons of *HTR3A* (Ex1b in Fig. 6). Using new primers designed from Ex1b and a downstream exon produced a PCR product of the expected size in the liver and PBL, but no product was detected in the brain. In contrast, another primer from Ex1a yielded a PCR product detected in the brain, and in the liver at very low levels (Fig. 6). These data suggest that *HTR3A* may have at least two promoters: an upstream promoter used in the brain and a downstream one used in PBL and liver.

Discussion

In spite of the general recognition that DNA methylation plays an important role in biological functions, studies implicating methylation in the pathogenesis of inherited diseases, especially psychiatric disorders, are still very limited. Here, we investigated methylation states in or near the promoter regions of eight single copy genes and one type of repetitive sequence, HERV-K, by the bisufite and/or Southern blot method(s).

The results obtained by the two methods were largely consistent. Each method has some inherent limitations, for example, artifactual partial digestion in the Southern blot method (Fig. 1, *COMT* in the PBL Sample c) and biased amplification and cloning in the bisulfite method. The Southern blot method was useful to identify the presence of biases and had the additional advantage of providing a visual representation of methylation states spanning several kilobases. Nevertheless, both methods are time-consuming and laborious, limiting the extent of the analysis. Genome-wide analyses of DNA methylation need more robust methods, such as that demonstrated in a recent study (Weber et al. 2005).

Half of the eight single copy genes had CpG islands in the 5′ flanking regions (CpG island genes), and these CpG sequences were unmethylated in the four tissues examined. Among the four CpG island genes, all but *COMT* were predominantly expressed in the brain. *COMT* was abundantly expressed in all four tissues examined. Thus, the cytosines in the CpG islands were protected from methylation, regardless of the transcriptional state of the gene, which is consistent with the general recognition of hypomethylation in CpG islands

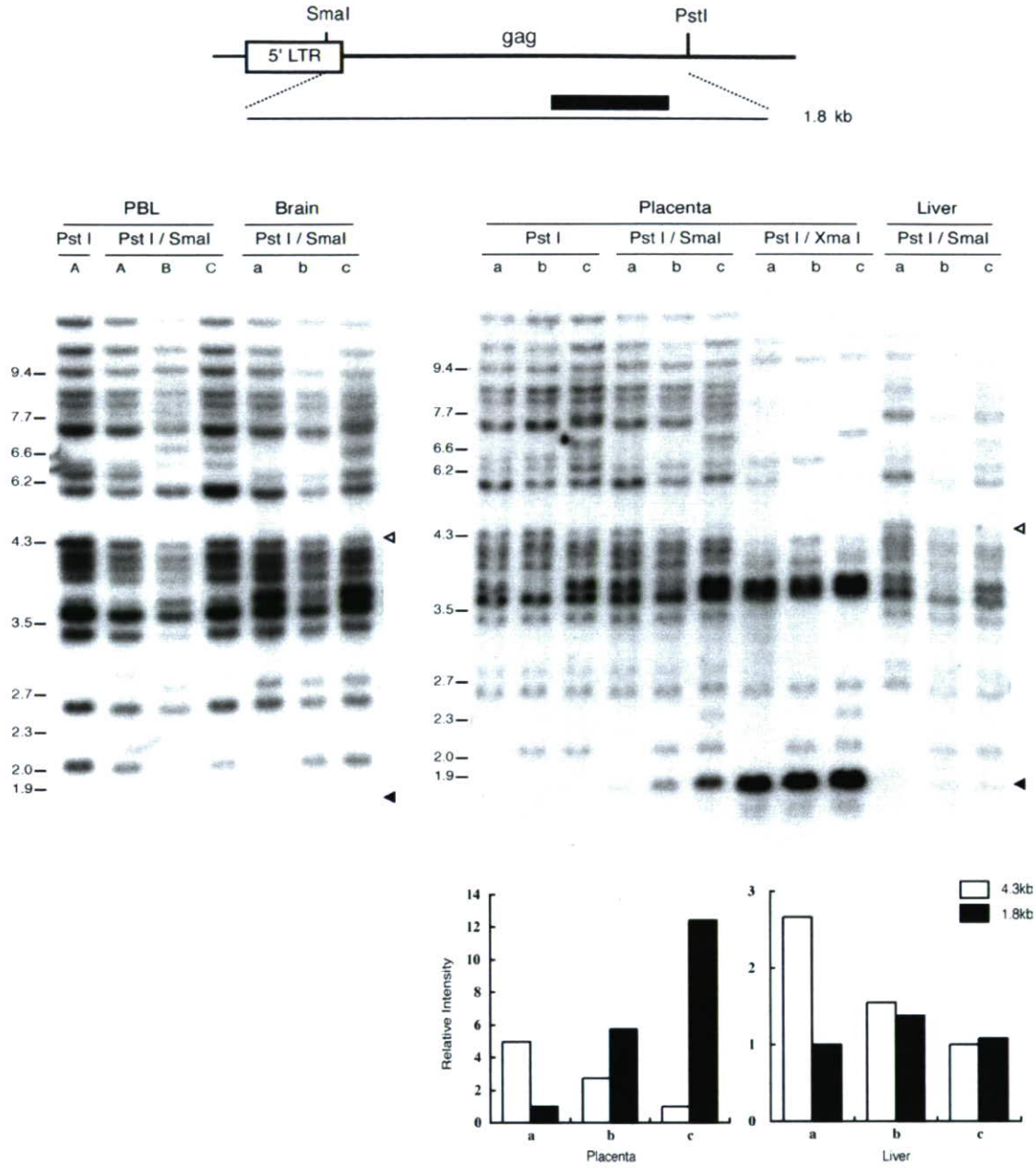

Fig. 4 Methylation analysis of human endogenous retrovirus (HERV)-K. Part of a typical HERV-K (5′ LTR and gag region) is shown at the top. The flanking sequence is depicted by a *thin line*. A *black bar* indicates the location of the probe used for Southern blot hybridization. The middle panels show the Southern hybridization results. *Letters* above the lanes indicate samples from different individuals. Two bands that showed inter-individual differences in signal intensity are indicated by *open* and *filled triangles*. The intensities of these two signals were quantified by the Science Lab Image Gauge software (Fujifilm, Japan) and normalized relative to an internal control (not shown). The bottom panel shows the normalized intensities for each band

except for either allele of the X chromosome genes in females and imprinted genes (Yoder et al. 1997).

The non-CpG island genes revealed a variety of methylation patterns that were different between tissues, genes, and even between individuals (Fig. 2, placental Samples a versus b and c in *HTR2A* and *HCRT*). The only common feature was hypomethylation in the brain samples in all four non-CpG island genes. In general, methylation in regulatory regions of a gene suppresses the transcriptional activity of that gene. However, *HTR3A* was heavily methylated in the liver and PBL samples, and was actively transcribed in these tissues. We subsequently identified an EST whose sequence was located between the first and second exons of *HTR3A*. RT-PCR with a primer located in the authentic exon 1 (Ex1a in Fig. 6) and a common primer amplified a

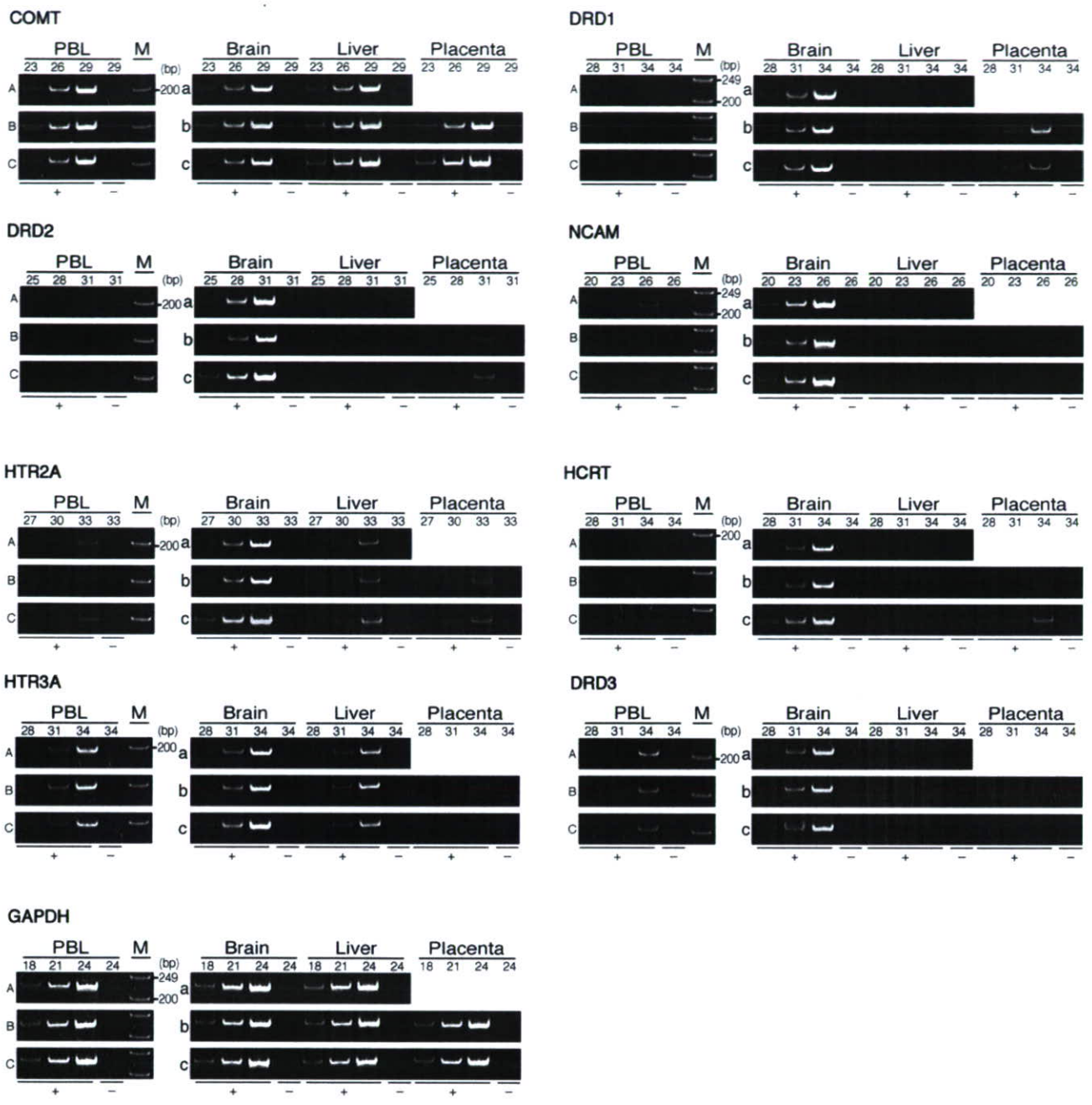

Fig. 5 Semi-quantitative analysis of the expression of the eight single copy genes. The amount of cDNA (reverse transcription products) used for PCR was adjusted relative to that of an internal control gene, glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*). Equal volumes of PCR reaction mixes at the cycles indicated by the numbers above the lanes were fractionated in 4% polyacrylamide gels and stained with ethidium bromide. RNA from placental Sample **a** was not available. Gene symbols as in Fig. 1

product from brain cDNA, but much less abundantly from liver samples, and nothing was detected after the maximum number of cycles in the PBL and placenta samples. In contrast, RT-PCR with a novel exon-specific primer (Ex1b in Fig. 6) and the common primer did not produce a PCR product from brain, while amplification was clearly observed in the liver and PBL samples. Thus, *HTR3A* may be transcribed from a brain-specific promoter in the brain and from a different promoter in PBL and liver. *HTR2A* may also possess tissue-specific promoters, although *HTR2A* expression in the liver and PBL was much lower than in brain. Bunzel et al. (1998) reported polymorphic imprinting in expression of *HTR2A* in the human adult brain. The methylation pattern in the PBL, albeit not in the brain, seemed consistent with a differentially methylated region as

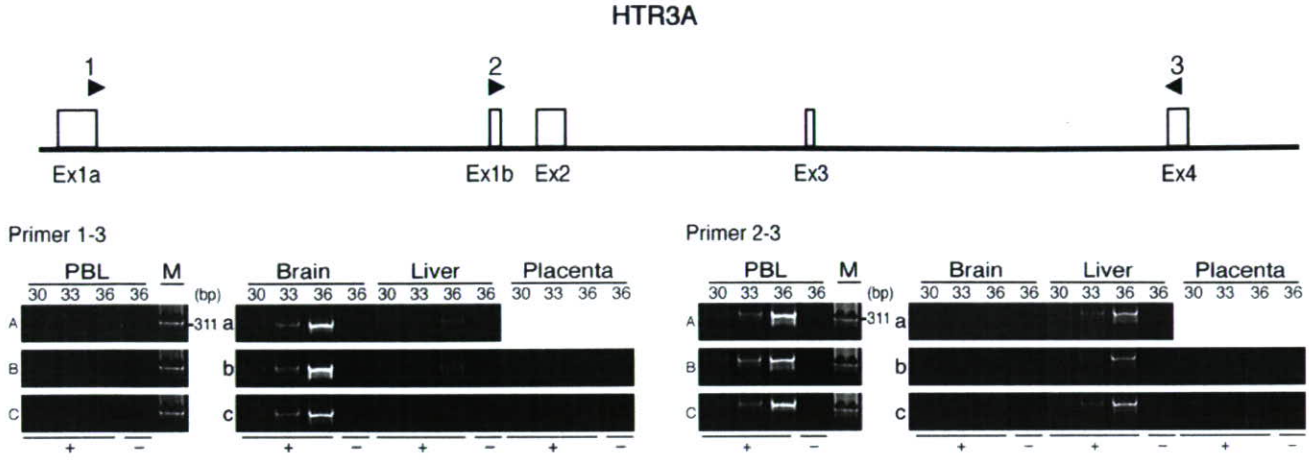

Fig. 6 HTR3A transcription from a brain-specific promoter and a novel putative promoter. *Upper panel HTR3A* gene structure. The *boxes* on the line indicate the relative size and location of the exons. The transcript-specific primers 1 and 2, and the common primer 3 are shown schematically. Ex1a is the first exon of *HTR3A* and Ex1b is the alternative exon 1 that is possibly transcribed from another promoter. *Lower panel* Tissue specificity of the transcripts generated with Primers 1 and 3 or from Primers 2 and 3. *Numbers* above the lanes indicate the number of amplification cycles and + or − below the lanes indicates whether reverse transcriptase was included in the reverse transcription reaction followed by the PCR reaction. *Letters* indicate samples from different individuals

found in imprinted genes (Robertson 2005). The methylation states in *DRD3* were also inconsistent with the expression status. Despite lacking the CpG island, *DRD3* was not methylated in the liver and placenta, where the *DRD3* transcript was not detected. Thus, our data suggest that for non-CpG island genes 5′-flanking regions are not methylated in any tissues in which the genes are actively expressed, but the converse is not necessarily true.

We are interested in the presence of methylation differences between individuals. In this study, we found inter-individual differences in methylation in two genes, *HTR2A* and *HCRT*, in the placental samples. Sample a showed much lower methylation in both these genes than in Samples b and c. We were unable to determine the methylation-expression relationship of *HTR2A* and *HCRT* because RNA was not available from Sample a.

To examine multiple loci and the relationship between methylation in single copy genes and repetitive sequences, we examined the HERV-K methylation status and identified two signals that showed inter-individual differences in signal intensity in placental and liver samples. The two signals possibly consist of multiple loci, with the larger one having a *Sma* I recognition sequence (CCCGGG) and the smaller one resulting from *Sma* I digestion of this locus. The inter-individual differences were most distinctive in the placental samples, with methylation highest in Sample a, moderate in Sample b, and lowest in Sample c, which is the reverse of the trend seen in *HTR2A* and *HCRT*. In addition, this order was the same as found in the liver samples. The reasons for inter-individual methylation differences remain to be ascertained, but here we could exclude a developmental effect on DNA methylation because we found opposite trends in different genes and our previous study revealed that methylation levels undergo global changes in the developing placenta (Fuke et al. 2004).

To date, the most widely used methods for DNA methylation analysis are Southern blot hybridization in combination with methyl-sensitive restriction enzymes and bisulfite-modified DNA sequencing. Both methods have intrinsic weaknesses, an artifactual partial methylation in the former and biased amplification and cloning in the latter. In our present study on DNA methylation, these methods were complementary and provided very reliable basic data on methylation status within or near the promoter region of genes whose activities are essential to development and brain function. We additionally determined the relationship between methylation and expression of these genes. Furthermore, we identified inter-individual differences in DNA methylation, which may be helpful in elucidating methylation mechanisms. It is especially worth noting that the difference in HERV-K methylation levels between individuals clearly observed in the placenta was also detected in the liver. This finding may support our approach and hypothesis to explore the possible implications of epigenetics in the molecular etiology of psychiatric disorders (Nakamura et al. 2003; Fuke et al. 2004, and unpublished data). Recently, a genome-wide methylation analysis was performed using a novel combination of existing methods (Weber et al. 2005), and this approach will contribute to rapid progress in understanding the etiological roles of DNA methylation in the pathogenesis of common diseases, especially psychiatric disorders.

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Genetic and Environmental Etiology of Effortful Control

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We examined whether effortful control (EC), a temperament proposed by Rothbart and Bates (1998), has genetically coherent structure. A self-report measure of EC was administered to 450 Japanese twins (151 males and 299 females, ages 17 to 32 years) including 152 monozygotic and 73 dizygotic pairs. Univariate genetic analysis revealed that AE model fit best for the total EC as well as its subscales. The heritability estimate for total EC was 49%, and the estimates for subscales ranged between 32% and 45%. Multivariate genetic analysis revealed that the subscales of EC were genetically correlated to a high degree and environmentally correlated to a moderate degree. These results suggest that EC has substantial genetic basis and genetically coherent structure, supporting the validity of the construct. The implications to molecular genetic study and study of psychopathology were discussed.

Since Ebstein et al. (1996) and Benjamin et al. (1996) reported the association between human personality and a genetic polymorphism, many researchers have been trying to investigate the molecular genetic basis of personality. Ebstein et al. (1996) and Benjamin et al. (1996) observed that individual differences in novelty seeking (Cloninger et al., 1993) were associated with a polymorphism of dopamine receptor D4 (DRD4). Further, Lesch et al. (1996) reported that harm avoidance (Cloninger et al., 1993) was associated with a polymorphism of the serotonin transporter long promoter region (5-HTTLPR). Increasing attempts to replicate the relationships yielded inconsistent results: some studies were successful (Murakami et al., 1999; Ono et al., 1997; Strobel et al., 1999; Tomitaka et al., 1999), whereas others were not (Gelernter et al., 1997; Kumakiri et al., 1999; Mitsuyasu et al., 2001). Finally, recent meta-analysis concluded that such associations were negligible (Kluger et al., 2002; Munafo et al., 2003; Schinka et al., 2002).

Several reasons contribute to the difficulty in obtaining the molecular genetic basis of personality. The first reason is small effect size of a single polymorphism. Even in studies that revealed a significant association, variance of a single polymorphism is typically 2% to 3%. This is because many polymorphisms exert influence on complex psychological traits like personality. Therefore, a typical sample size of 100 to 200 participants is sometimes not sufficient to detect such a small effect size. The second reason is sampling bias. For example, many studies use a psychiatric population as a sample, but having psychopathology may distort the subjects' self-report on personality, thereby obscuring the association between genes and personality. In addition, some associations between genes and personality may be specific to a certain age group, gender and culture. The third reason is the heterogeneity of the personality scale used. For example, anxious personality may be associated with the 5-HTTLPR polymorphism when measured with the Neuroticism scale of the Revised NEO Personality Inventory (NEO-PI-R; Costa & McCrae, 1992) but not when measured with the Harm Avoidance scale of the Temperament and Character Inventory (TCI; Cloninger et al., 1993). In fact, this conclusion was reached in a recent meta-analysis (Schinka et al., 2004; Sen et al., 2004). The reason why some scales are more related with certain polymorphisms may be due to (1) slight difference in the content they measure; or (2) low reliability, particularly low internal consistency. Given the high reliability of scales included in standard personality questionnaires such as NEO-PI-R and TCI, it is more plausible to consider that differences in the content, rather than reliability, are responsible for the inconsistent pattern of results.

However, the problem for association studies is that reliability is computed by phenotypic correlations among items or subscales, and not by the

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