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Ethanol-Induced *Htr3a* Promoter Methylation Changes in Mouse Blood and Brain

Jacqueline M. Barker, **Yuqi Zhang**, **Fan Wang**, **Jane R. Taylor**, and **Huiping Zhang**
Department of Psychiatry (JMB, FW, JRT, HZ), Yale University School of Medicine, New Haven, Connecticut; Interdepartmental Neuroscience Program (JMB), Yale University School of Medicine, New Haven, Connecticut; Department of Biology (YZ), Trinity College of Arts & Sciences, Duke University, Durham, North Carolina; and VA Connecticut Healthcare System (FW, HZ), West Haven, Connecticut.

Abstract

Background—Abnormal DNA methylation has been observed in promoter regions of a number of genes in human alcoholics. It is unclear whether DNA methylation changes in alcoholics result directly from alcohol consumption or predated the occurrence of alcohol abuse or dependence and whether altered DNA methylation influences gene expression.

Methods—We investigated ethanol (EtOH)-induced DNA methylation changes in mouse serotonin receptor 3a gene (*Htr3a*). A 5-day drinking-in-the-dark paradigm was applied to 28 male outbred CD-1 mice (15 EtOH-drinking and 13 water-drinking). The Sequenom MassARRAY approach was used to quantify methylation levels of 8 CpGs around *Htr3a* transcription start site in trunk blood and 9 brain regions (dorsomedial prefrontal cortex [DMPFC], ventromedial prefrontal cortex, ventral tegmental area, dorsolateral striatum, dorsomedial striatum [DMSTR], ventral striatum, amygdala, hippocampus [HIPPO], and cerebellum). DNA methylation differences between the 2 groups of mice (EtOH- and water-drinking) were analyzed using multivariate analysis of covariance with consideration of EtOH consumption amount. Expression levels of *Htr3a* in the DMSTR were measured by real-time PCR in 14 EtOH-drinking and 14 water-drinking male CD-1 mice.

Results—EtOH drinking increased methylation levels of specific *Htr3a* promoter CpGs in mouse blood (CpG−27: $p = 0.028$; CpG+54: $p = 0.044$) and HIPPO (CpG+151: $p = 0.012$) but reduced methylation levels of specific *Htr3a* promoter CpGs in mouse DMSTR (CpG−96: $p = 0.020$; CpG −27: $p = 0.035$) and DMPFC (CpG+138: $p = 0.011$; CpG+151: $p = 0.040$). Nevertheless, methylation levels of *Htr3a* promoter CpGs in 6 other brain regions were not significantly altered by EtOH consumption. Additionally, the expression level of *Htr3a* in the DMSTR was 1.43-fold higher in alcohol-drinking mice than in water-drinking mice ($p = 0.044$).

Conclusions—Our findings indicate that alcohol consumption may induce tissue-specific DNA methylation changes and further suggest that *Htr3a* promoter methylation levels may be reversely correlated with *Htr3a* expression levels in specific brain regions such as DMSTR.

Reprint requests: Dr. Huiping Zhang, Department of Psychiatry, Yale University School of Medicine, VA Medical Center/116A2, 950 Campbell Avenue, West Haven, CT 06516; Tel.: 203-932-5711 ext. 5245; Fax: 203-937-4741; huiping.zhang@yale.edu; Dr. Jane R. Taylor, Department of Psychiatry, Connecticut Mental Health Center, Yale University School of Medicine, 34 Park Street, New Haven, CT 06508; Tel.: 203-974-7727; Fax: 203-974-7724; jane.taylor@yale.edu.

Keywords

Mouse Serotonin Receptor 3a Gene; DNA Methylation; Gene Expression; Ethanol Drinking-in-the-Dark

The serotonergic system in the brain plays important roles in alcohol consumption and abuse. Serotonergic neurotransmission is mainly mediated by serotonin receptors. Among the 7 known serotonin (5-HT) receptor subfamilies (5-$HT_{1–7}$), only the 5-HT_3 receptor directly gates an ion channel. Ethanol (EtOH) has been shown to directly interact with the 5-HT_3 receptor and potentiate the action of 5-HT at the 5-HT_3 receptor in a dose-dependent manner (Zhou et al., 1998). This effect can be blocked by administration of the specific 5-HT_3 receptor antagonist troposetron (Lovinger and Zhou, 1998). Ondansetron, another 5-HT_3 receptor antagonist, has been reported to be efficacious in reducing heavy drinking and increasing abstinence in alcoholics (Johnson et al., 2000, 2011).

The 5-HT_3 receptor is composed of 2 major subunits: 5-HT_{3A} and 5-HT_{3B}. 5-HT_{3A} is the obligatory subunit; only 5-HT_{3A} can form receptors independently, and it is required for the formation of functional receptors (Maricq et al., 1991). Importantly, the 5-HT_3 receptor is expressed throughout the mesolimbic system, including the nucleus accumbens and the ventral tegmental area (VTA) that are known to be important for the formation of addictive behaviors (Mylecharane, 1996). Based on these data, it appears that abnormal transcription of the serotonin receptor 3A gene (*HTR3A*), resulting either from genetic polymorphisms or promoter DNA methylation alterations, could influence an individual's vulnerability to alcohol dependence.

There is a growing body of evidence demonstrates that epigenetic modifications (such as DNA methylation and histone modification that alter DNA accessibility and chromatin structure) are associated with alcohol dependence. Promoter DNA hypermethylation has been identified in a number of genes in peripheral blood of human alcoholics. These genes include the α-synuclein gene (*SNCA*) (Bonsch et al., 2005), the monoamine oxidase A gene (*MAOA*) (Philibert et al., 2008a), the serotonin transporter gene (*SLC6A4*) (Philibert et al., 2008b), the *N*-methyl-D-aspartate receptor subunit 2B gene (*NR2B* or *GRIN2B*) (Biermann et al., 2009), and the proopiomelanocortin gene (*POMC*) (Muschler et al., 2010). Our recent study also demonstrated increased DNA methylation in the promoter region of *HTR3A* in peripheral blood of human alcoholics (Zhang et al., in press). There is also evidence that epigenetic processes, including DNA methylation, could influence gene expression at the level of transcription (Gibney and Nolan, 2010). Therefore, altered methylation and subsequent expression of *HTR3A* may significantly influence vulnerability to AD by impacting serotonin signaling at the 5-HT_3 receptor.

Although the above-mentioned human studies are instrumental in examining the role of DNA methylation in alcohol dependence, the investigation of epigenetic changes in peripheral blood of human alcoholics has 2 major limitations. First, these experiments do not enable us to assess whether alcohol dependence-associated DNA methylation changes directly resulted from chronic alcohol consumption or whether these differences reflect preexisting changes that predispose individuals to alcoholism. It is already known that environmental factors such as early-life stress and malnutrition (that may be abnormal in alcoholic subjects) can also alter DNA methylation patterns (Alegria-Torres et al., 2011). Moreover, DNA methylation changes in alcoholic subjects may be caused by co-occurring drug use or comorbid psychiatric illness. Second, the above-cited studies used genomic DNA extracted from peripheral blood (or lymphoblastoid cell lines derived from peripheral blood lymphocytes) for determination of DNA methylation. It is as yet unclear whether

DNA methylation patterns in peripheral blood are representative of brain DNA methylation and/or whether any effects of alcohol use impact peripheral blood and brain DNA methylation patterns in a homogeneous manner. Because these questions are not readily answerable using human studies, alternative models for addressing these issues are needed.

To explore the causal role of EtOH exposure in DNA methylation and to assess whether EtOH-induced DNA methylation changes are consistent in peripheral blood and in the brain, we used a mouse model of subacute EtOH drinking. Specifically, we used a mouse drinking-in-the-dark (DID) paradigm (Rhodes et al., 2007), which has been shown to produce high levels of EtOH consumption while minimizing environmental stress to the animals, to investigate whether EtOH drinking directly produced hypermethylation of *HTR3A* promoter region, as recently seen in the peripheral blood of human alcoholics (Zhang et al., in press). DNA methylation was assessed in 9 different brain regions (dorsomedial prefrontal cortex [DMPFC], ventromedial prefrontal cortex [VMPFC], VTA, dorsolateral striatum [DLSTR], dorsomedial striatum [DMSTR], ventral striatum [VSTR], amygdala [AMY], hippocampus [HIPPO], and cerebellum [CBL]) that were selected based on their role in the reward pathway and their role in supporting the behavioral changes that are thought to underlie alcoholism (Everitt and Robbins, 2005; Oscar-Berman and Marinkovic, 2007). Additionally, expression levels of mouse *Htr3a* in specific brain regions were compared between alcohol- and water-drinking mice. This study made it possible to investigate (i) whether EtOH consumption led to altered DNA methylation in the promoter region of mouse *Htr3a*, (ii) whether EtOH drinking induced differential DNA methylation in mouse blood and different brain regions, and (iii) whether *Htr3a* promoter methylation changes are related to gene expression levels.

MATERIALS AND METHODS

Mouse DID Experiments

We adopted a 5-day DID paradigm based on the method developed by Rhodes and colleagues (2007) to examine EtOH-induced DNA methylation changes in mouse blood and brain. Subjects were 28 male CD-1 mice housed in the Yale University/Connecticut Mental Health Center mouse vivarium (9 to 11 weeks of age). All behavioral procedures were approved by the Yale University Institutional Animal Care & Use Committee. These mice were allowed to acclimate for 1 week with ad libitum access to food and water after which mice were split into 2 groups (15 EtOH-exposed and 13 water-drinking). Mice were singly housed and allowed ad libitum access to food and water. The light cycle was from 7:00 AM to 7:00 PM and the dark cycle from 7:00 PM to 7:00 AM. On Experimental Days 1, 2, and 3, mice were weighed at about 6:30 PM, 30 minutes before the end of the light cycle. Three hours after the beginning of the dark cycle (10:00 PM), water bottles in home cages were replaced with bottles containing 20% EtOH in tap water. Control mice received distinct bottles containing only tap water. These bottles remained in place for 2 hours (removed at 12:00 AM). The experimental procedures on Days 4 and 5 were the same as those for Days 1, 2, and 3, except the EtOH- and tap water-containing bottles remained in place for 4 hours (removed at 2:00 AM). EtOH consumption was assessed daily; average daily EtOH consumption was 6.32 g of EtOH per kg body weight (SEM = 0.60). Twenty-four hours after the initiation of the last drinking bout, mice were sacrificed via rapid decapitation. Trunk blood was collected, and tissues from 9 different brain regions, the DMPFC, the VMPFC, the VTA, the DLSTR, the DMSTR, the VSTR, the AMY, the HIPPO, and the CBL, were dissected.

Quantification of Mouse *Htr3a* Promoter Methylation Levels

Methylation levels of CpGs in mouse *Htr3a* promoter region were determined by the Sequenom MassARRAY EpiTYPER method (Sequenom, San Diego, CA). First, mouse

blood genomic DNA was extracted using the PAXgene Blood DNA Kit (PreAnalytiX, Hombrechtikon, Switzerland), and mouse brain tissue genomic DNA was extracted using the QIAamp DNA Micro Kit (QIAGEN, Valencia, CA). The extracted DNA was then treated with the bisulfite reagent included in the EZ DNA Methylation Kit (Zymo Research, Orange, CA). Next, an amplicon covering 8 mouse *Htr3a* promoter CpGs (located from 96 bp upstream of the transcription start site [TSS] to 151 bp downstream of the TSS) (Fig. 1) was generated by the polymerase chain reaction (PCR) using a pair of tagged primers (Forward: aggaagagagGGGGTTGTTAGTATGGAAAGGAATA; Reverse: cagtaatacgactcactatagggagaaggctCCTAACATCTACCAATAAACTCCCC). The reverse primer was tagged with the T7-promoter sequence for in vitro transcription. A touchdown PCR using the FastStart Taq DNA Polymerase (Roche, Mannheim, Germany) was performed, including 3 cycles of 95°C 30 s/66°C 15 s/72°C 1 min, 3 cycles of 95°C 30 s/64°C 15 s/72°C 1 min, 3 cycles of 95°C 30 s/62°C 15 s/72°C 1 min, and 37 cycles of 95°C 30 s/60°C 15 s/72° C 1 min. After treatment with alkaline phosphatase ExoSAP-IT (Affymetrix, Santa Clara, CA), the PCR products were transcribed, cleaved by RNase A at specific bases (U or C), and spotted on a 384-pad SpectroCHIP (Sequenom) followed by spectral acquisition on a MassARRAY Analyzer. The methylation calls were performed by the EpiTyper software v1.0 (Sequenom), which generates quantitative results (methyl CpG/total CpG) for each CpG site. To monitor both bisulfite conversion efficiency and accuracy of methylation detection, the CpG methylated NIH 3T3 mouse genomic DNA sample (New England Biolabs, Ipswich, MA) was taken as a positive control. The negative control DNA sample (unmethylated) was generated by whole genome amplification of the CpG methylated NIH 3T3 mouse genomic DNA sample to remove methyl groups at CpG sites using reagents in the REPLI-g Kit (QIAGEN).

Quantification of Mouse *Htr3a* Expression Levels

Mouse *Htr3a* expression levels in brain DMSTR were quantified by real-time PCR. Total RNA was extracted from about 1 mg of the DMSTR tissue sample dissected from the brain of a new cohort of 14 EtOH- and 14 water-drinking male outbred CD-1 mice (the experimental procedure was the same as described earlier) using the AllPrep DNA/RNA Mini kit (QIAGEN). About 100 ng of total RNA was obtained from each tissue sample and then reverse-transcribed to cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). The relative expression level of mouse *Htr3a* was measured using TaqMan Gene Expression Assays (target gene *Htr3a*:Mm00442874_m1; reference gene *Rn18s*: Mm03928990_g1) (Applied Biosystems), and the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guideline was followed (Bustin et al., 2009). Selection of *Rn18s* as the reference gene for *Htr3a* was based on published studies (Chu et al., 2009). PCR mixture contained 10 μl of 2× TaqMan Gene Expression Master Mix, 1 μl of 20× TaqMan Gene Expression Assay (*Htr3a* Mm00442874_m1 or *Rn18s* Mm03928990_g1), 3 μl of cDNA template, and 6 μl of RNase-free water. A standard 2-step real-time PCR was performed in the 7900HT Fast Real-Time PCR System (Applied Biosystems). The cycling conditions were: 50°C for 2 minutes, then 95°C for 10 minutes, followed by 40 two-step cycles at 95°C for 15 seconds and 62°C for 1 minute. Each reaction was performed in triplicate. The threshold cycle (C_T) of the target gene (*Htr3a*) and the reference gene (*Rn18s*) for each sample was analyzed using programs SDS (version 2.3) and RQ Manager (version 1.3) (Applied Biosystems). The relative expression level (ΔC_T) of the target gene *Htr3a* was normalized to that of the reference gene *Rn18s* ($\Delta C_T = C_{T\ Htr3a} - C_{T\ Rn18s}$).

Statistical Analysis

Methylation differences of 8 *Htr3a* promoter CpGs in the blood and 9 different brain regions between the 2 groups of mice (EtOH-exposed and water-drinking) were analyzed using the

multivariate analysis of covariance (MANCOVA) program incorporated in the SPSS 17.0 software (SPSS Inc., Chicago, IL) with consideration of EtOH consumption amount as a covariate. Besides individual CpG analysis, mean methylation levels of the 8 *Htr3a* promoter CpGs in the blood and 9 different brain regions were also compared between the 2 groups of mice (EtOH- and water-drinking) by MANCOVA. To analyze *Htr3a* expression differences between EtOH- and water-drinking mice, an unpaired *t*-test was used to compare 2 group means. Additionally, the relative quantity (RQ) of *Htr3a* expression in EtOH-drinking mice in comparison with water-drinking mice was calculated by formula: RQ = $2^{-\Delta\Delta C_T}$ ($\Delta\Delta C_T = \Delta C_{T,\text{ EtOH-drinking group}} - \Delta C_{T,\text{ water-drinking group}}$).

RESULTS

Htr3a Methylation Changes Associated with EtOH Consumption

Consistent with our findings in human subjects (Zhang et al., in press), EtOH exposure significantly increased methylation levels of 2 CpGs in mouse blood (CpG−27: $p = 0.028$; CpG+54: $p = 0.044$) and 1 CpG in mouse HIPPO (CpG+ 151: $p = 0.012$, not corrected for multiple testing; Fig. 2). However, EtOH-exposed mice had significantly lower methylation levels of 4 CpGs in either the DMSTR (CpG−96, $p = 0.020$; CpG−27, $p = 0.035$) or the DMPFC (CpG+138, $p = 0.011$; CpG+151, $p = 0.040$) than control mice (not corrected for multiple testing). Additionally, EtOH drinking did not induce significant DNA methylation changes in 6 other mouse brain regions (VTA, VSTR, DLSTR, VMFPC, AMY, and CBL) (Fig. 3). The mean methylation level of these 8 *Htr3a* promoter CpGs was significantly higher in the blood of EtOH-drinking mice than in the blood of water-drinking mice ($p = 0.029$); nevertheless, the mean methylation level of these 8 *Htr3a* CpGs was nonsignificantly lower in the DMPFC of EtOH-exposed mice than control mice ($p = 0.074$) (Fig. 4). Eight other mouse brain regions did not show significant differences between EtOH-exposed and water-drinking mice in mean methylation levels of the 8 *Htr3a* promoter CpGs. Mean methylation levels of these 8 *Htr3a* promoter CpGs also varied dramatically in different brain regions (Fig. 4).

Htr3a Expression Changes Associated with EtOH Consumption

As 2 *Htr3a* promoter CpGs (CpG−96 and CpG−27) showed hypomethylation in brain DMSTR of EtOH-drinking mice (Fig. 2), we further explored whether *Htr3a* expression in brain DMSTR was altered using a new set of mouse DMSTR tissue samples. Through real-time PCR assays and the comparative C_T method, *Htr3a* expression in the DMSTR was found to be 1.43-fold higher in alcohol-drinking mice than in water-drinking mice (unpaired *t*-test: $p = 0.088$ [2-tailed] or 0.044 [1-tailed]; RQ = 1.43).

DISCUSSION

Although alcohol dependence may result from neuroadaptation to chronic alcohol consumption involving changes in the expression of multiple genes (Melendez et al., 2012), the precise biological mechanisms are not well known. Our data indicate that subacute alcohol consumption may possibly lead to epigenetic changes (e.g., DNA methylation), potentially impacting gene transcription. Because investigation of DNA methylation levels in human subjects does not allow assessment of the causal role of alcohol consumption on epigenetic changes, or comparison between DNA methylation in peripheral blood and brain, we used a subacute mouse drinking model to estimate the validity of EtOH-induced *HTR3A* promoter methylation changes in the peripheral blood of human alcoholics and deduce the effects of chronic alcohol consumption on *HTR3A* methylation and expression in human brain.

First, we observed that the methylation levels of 2 CpG sites (CpG−27 and CpG+54) in the mouse gene *Htr3a* promoter region and the mean methylation level of the 8 *Htr3a* promoter CpGs were significantly higher in the blood of EtOH-exposed mice when compared to that of water-drinking mice (Figs 2 and 4). This result is consistent with our recent finding that an *HTR3A* promoter CpG was hypermethylated in the peripheral blood of alcohol dependent human subjects (Zhang et al., in press). These data indicate that chronic EtOH consumption can causally alter *HTR3A* promoter methylation status and produce the hypermethylation observed in the peripheral blood of human alcoholics. Nevertheless, this conclusion should be made with caution, as the mouse *Htr3a* promoter DNA sequence analyzed in this study was not homologous to the human *HTR3A* promoter DNA sequence and, of course, the subacute EtOH consumption in the DID model does not recapitulate consumption patterns in human alcoholics.

Additionally, we used the mouse model to investigate EtOH-induced methylation changes of mouse *Htr3a* promoter CpGs in 9 different brain regions to model DNA methylation changes caused by chronic EtOH consumption in the human brain (which is not easily accessible) and to assess whether peripheral changes are indicative of DNA methylation levels in the brain. Here, we investigated EtOH-induced DNA methylation changes in mouse prefrontal cortex, striatum, VTA, AMY, and HIPPO, all of which play important roles in reward and motivational processes because of their involvement in dopaminergic and glutamatergic neurotransmission and thus mediate reinforcing effects of alcohol or drugs of abuse (Jentsch and Taylor, 1999; Lobo and Nestler, 2011; Pascual et al., 2009). The tissue from the CBL, which mediates many of alcohol's effects on motor control, was also collected. As shown in Fig. 2, EtOH drinking resulted in either hypermethylation (in the HIPPO) or hypomethylation (in the DMPFC and the DMSTR) of certain CpGs in mouse *Htr3a* promoter region. The altered methylation of *HTR3A* promoter region caused by alcohol consumption may have a significant effect on *HTR3A* transcription and ultimately influence vulnerability to alcohol dependence by impacting serotonin signaling at the 5-HT_3 receptor.

Several brain regions (including CBL) did not show significantly altered DNA methylation levels of *Htr3a* in EtOH-exposed mice. These findings suggest that EtOH-induced DNA methylation changes are tissue specific and that gene expression in certain brain regions may be more greatly impacted by EtOH treatment than other brain regions. Moreover, the baseline mean methylation level of 8 mouse *Htr3a* promoter CpGs in the 9 mouse brain regions varied from about 10% (in CBL) to about 50% (in the VSTR) (Fig. 4). This result suggests a native tissue-specific DNA methylation pattern in the brain of mice that were not exposed to EtOH. It may also help explain the tissue-specific gene expression phenomenon in human and animal brains, given the intimate correlation between gene expression levels and gene promoter methylation levels.

Additionally, EtOH consumption-associated *Htra3* expression changes were examined in mouse brain DMSTR tissue samples. As 2 CpGs (CpG−96 and CpG−27) in the promoter of *Htr3a* showed hypomethylation in brain DMSTR of EtOH-drinking mice, we hypothesized that *Htr3a* expression in the DMSTR would be increased as previous studies have demonstrated an inverse correlation between methylation and gene expression levels in alcoholic subjects (Bleich et al., 2006; Bonsch et al., 2006). Expression levels of *Htr3a* in the DMSTR were measured by real-time PCR and compared in a separate cohort of 14 EtOH-drinking and 14 water-drinking male CD-1 mice using the comparative C_T method. *Htr3a* expression level in the DMSTR was found to be 1.43-fold higher in alcohol-drinking mice than in water-drinking mice ($p = 0.044$). Our findings further suggest that promoter methylation levels may be reversely correlated with gene expression in specific brain regions such as the DMSTR. Brain tissues in which methylation changes were not in *Htr3a*

promoter region were not analyzed as these methylation changes are not expected to impact expression levels.

Importantly, the use of the 5-day DID approach in CD-1 mice allows mice to drink a maximum amount of EtOH in a nonstressful environment (Rhodes et al., 2007), therefore reducing an impact of stress on DNA methylation levels and enabling specific detection of EtOH-induced DNA methylation changes in mouse blood and brain. Moreover, CD-1 mice were drawn from a large breeding population that has accumulated many recombination events. The CD-1 genome displays similar patterns of linkage disequilibrium and heterogeneity as those of wild-caught mice (Agrawal et al., 2010). Hence, the observations in CD-1 mice are applicable to a broad range of genetic studies. It is important to consider that EtOH-induced DNA methylation alterations could be species specific. Although a mouse model has been used to study cocaine-induced histone epigenetic modifications (but not DNA methylation) in the brain (Maze et al., 2011), there are no such published studies using an animal model to investigate EtOH-induced DNA or histone epigenetic alterations in the brain. It is unknown whether EtOH-induced DNA methylation changes in the outbred CD-1 mice appropriately correlate to that in human alcoholics. It would be useful to confirm these findings with additional models. In addition, although these data indicate that a 5-day EtOH treatment is sufficient to induce detectable DNA methylation changes, a longitudinal study to measure EtOH-induced DNA methylation changes at different time points may provide additional information about the effects of chronic EtOH consumption on alterations in DNA methylation, and the effects of EtOH on *Htr3a* in particular.

Taken together, we employed a mouse model to validate changes in DNA methylation seen in human alcoholics (Zhang et al., in press). Importantly, our data indicate a causal relationship between EtOH consumption and changes in DNA methylation and gene expression, which has not been possible to investigate in human populations. In addition, our data indicate a lack of a simple relationship between peripheral blood DNA methylation levels with those seen in the brain, although hypermethylation of *HTR3A* in peripheral blood could potentially serve as a biomarker reflecting the situation in specific brain regions such as the HIPPO. This information is critical for the investigation of epigenetic changes in the population and highlights the importance of using animal models to validate work with human subjects. The findings from this study contribute to our understanding of the epigenetic mechanisms that underlie the development of alcohol dependence and may help in the effort to identify strategies to modify DNA methylation patterns of genes to prevent or treat alcohol dependence.

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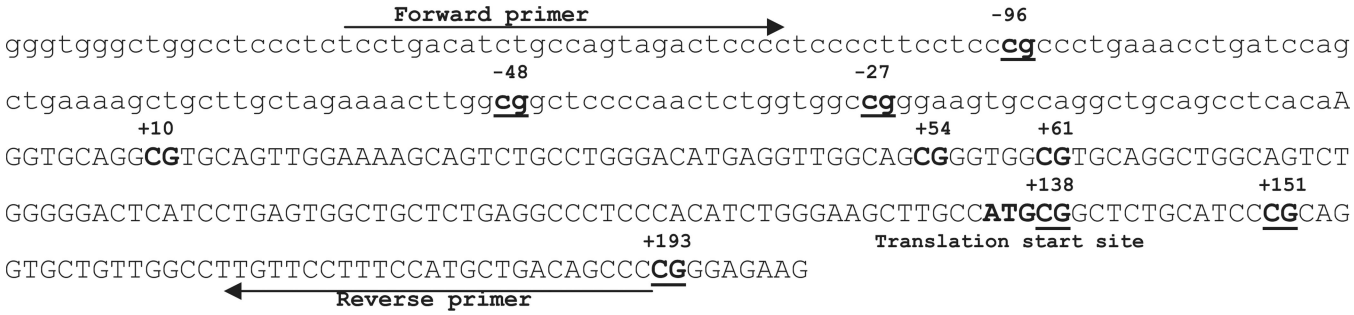

Fig. 1.

CpGs examined in the promoter region of mouse serotonin receptor 3a gene (*Htr3a*). Methylation levels of 8 *Htr3a* CpGs (CpG−96, CpG−48, CpG−27, CpG+10, CpG+54, CpG +61, CpG+138, and CpG+151) were measured by the Sequenom MassARRAY EpiTYPER method. Lower case letters in the DNA sequence: bases in *Htr3a* promoter region; capital letters in the DNA sequence: bases in *Htr3a* exon 1.

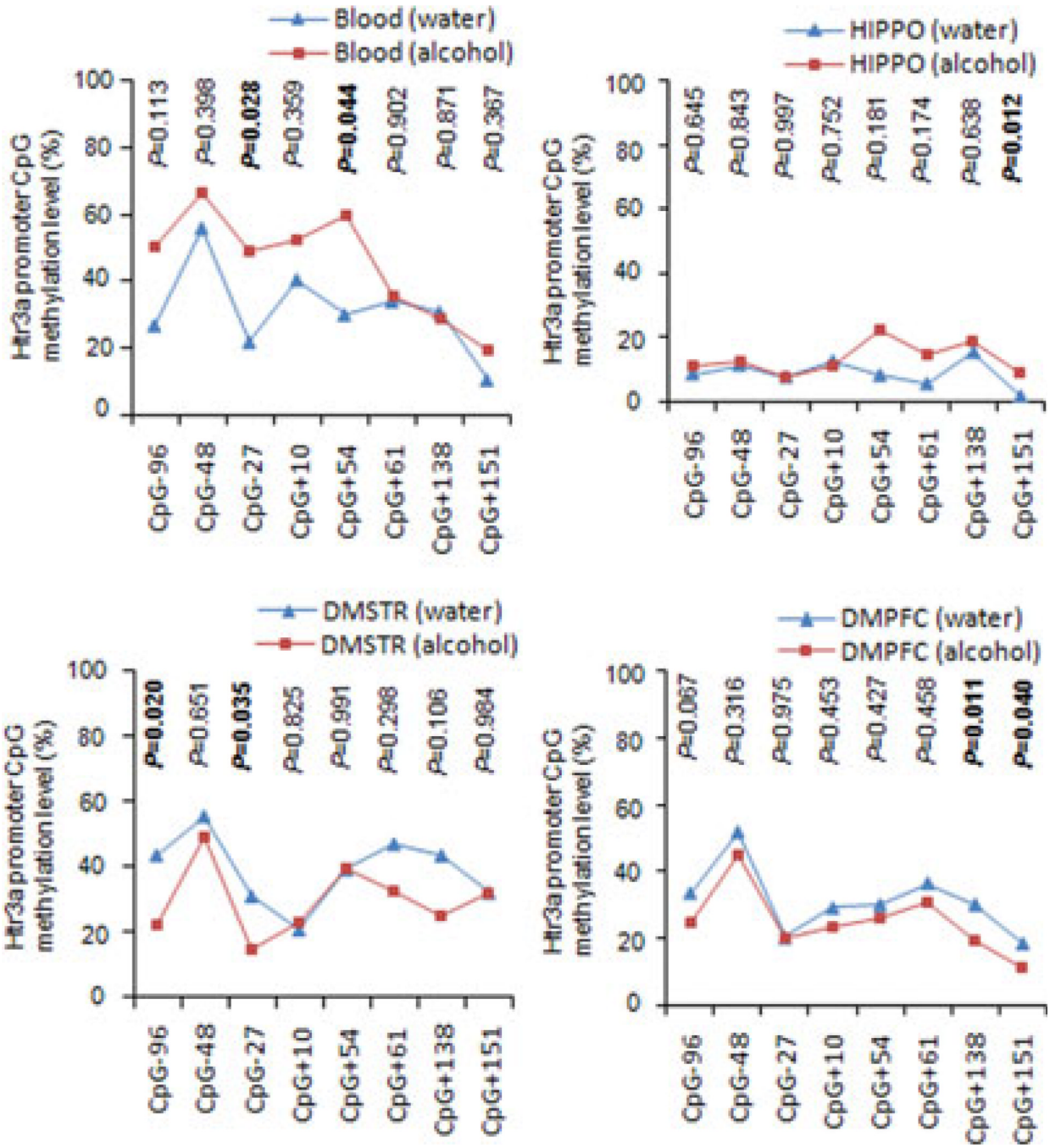

Fig. 2.

Ethanol (EtOH)-induced *Htr3a* DNA methylation changes in mouse blood and 3 brain regions. EtOH-induced DNA methylation changes were observed in mouse blood and 3 brain regions (the hippocampus [HIPPO], the dorsomedial striatum [DMSTR], and the dorsomedial prefrontal cortex [DMPFC]). Bold *p*-values indicate significant differences ($p < 0.05$).

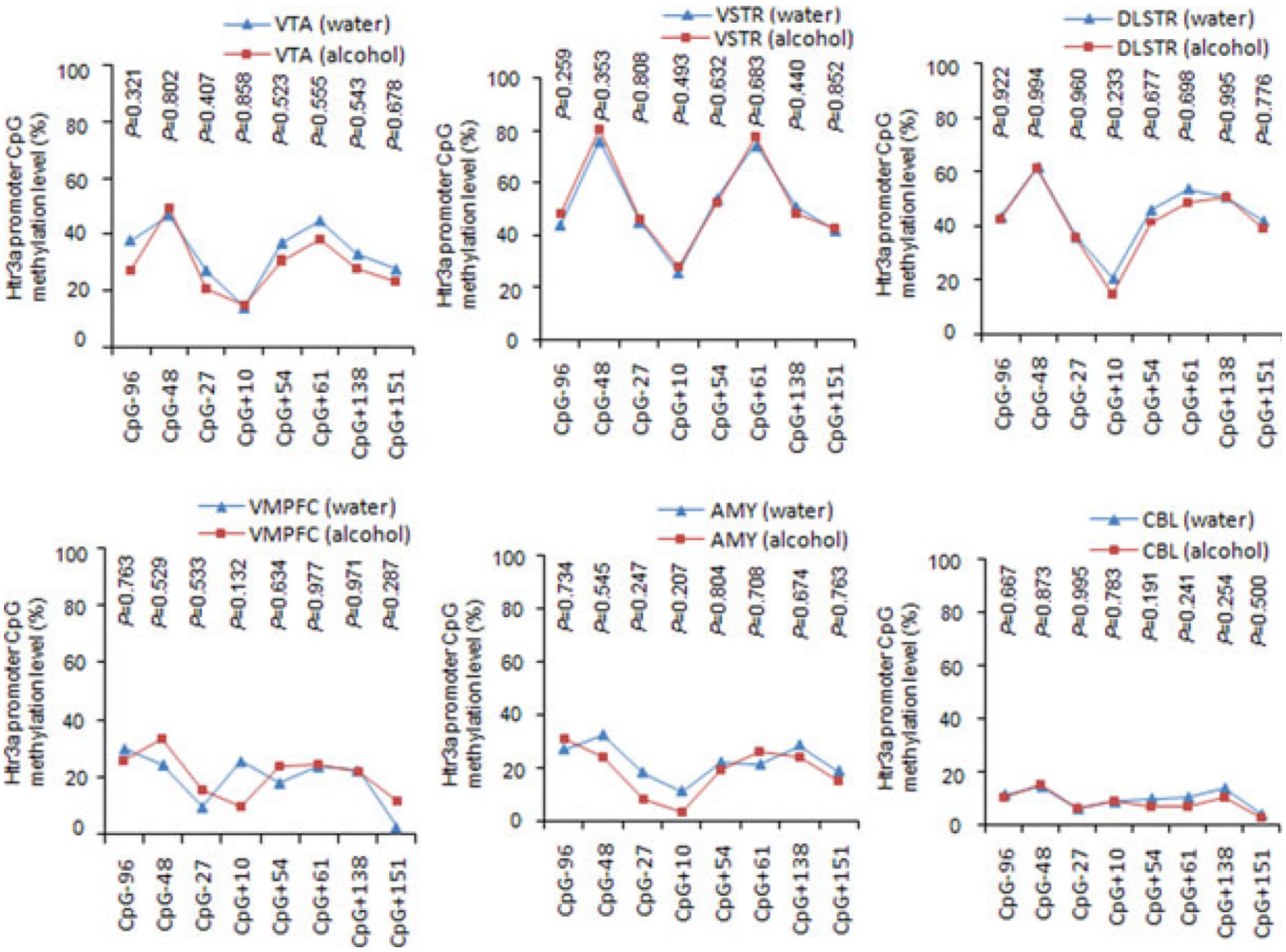

Fig. 3.

Ethanol (EtOH)-induced *Htr3a* DNA methylation changes in 6 mouse brain regions. DNA methylation levels of 8 *Htr3* promoter CpGs were not found to be significantly altered by EtOH consumption in 6 mouse brain regions (the ventral tegmental area [VTA], the ventral striatum [VSTR], the dorsolateral striatum [DLSTR], the ventromedial prefrontal cortex [VMPFC], the amygdala [AMY], and the cerebellum[CBL]).

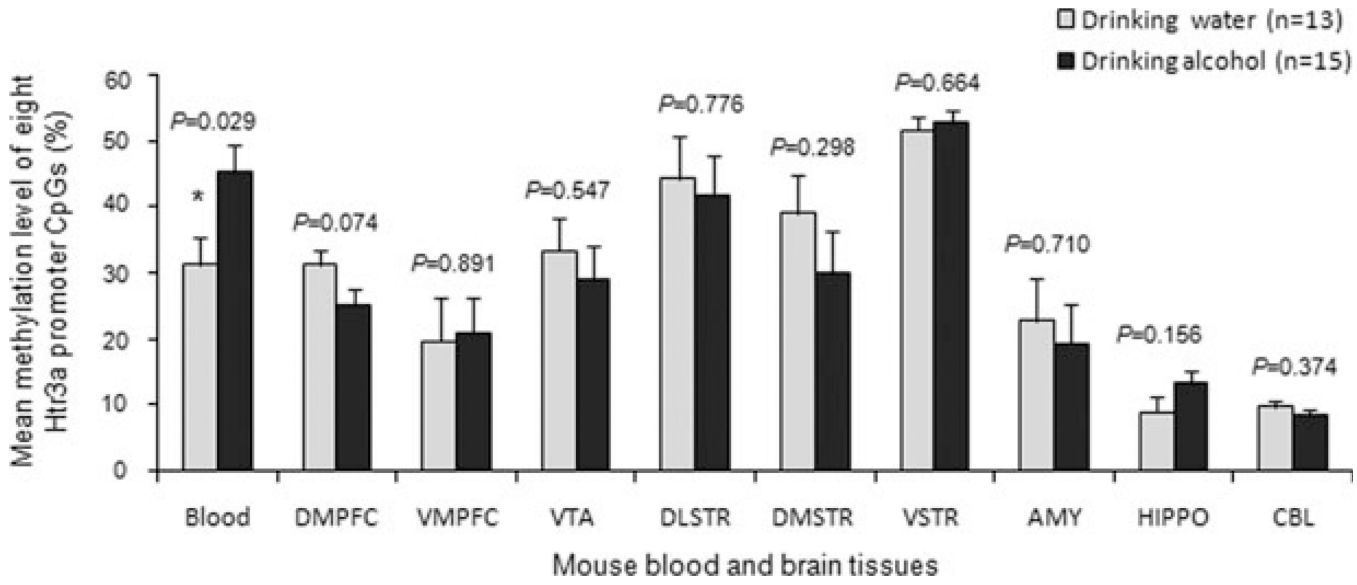

Fig. 4.

Differences of mean methylation levels of 8 *Htr3a* promoter CpGs between ethanol (EtOH)-exposed and water-drinking mice. EtOH consumption led to a significantly increased mean methylation level of 8 *Htr3a* promoter CpGs in mouse blood but not in 9 mouse brain regions. *Significant difference ($p < 0.05$). DMPFC, dorsomedial prefrontal cortex; VMPFC, ventromedial prefrontal cortex; VTA, ventral tegmental area; DLSTR, dorsolateral striatum; DMSTR, dorsomedial striatum; VSTR, ventral striatum; AMY, amygdala; HIPPO, hippocampus; CBL, cerebellum.