Behavioral/Systems/Cognitive

Stress-Related Methylation of the Catechol-*O*-Methyltransferase Val^{158} Allele Predicts Human Prefrontal Cognition and Activity

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DNA methylation at CpG dinucleotides is associated with gene silencing, stress, and memory. The catechol-*O*-methyltransferase (*COMT*) Val^{158} allele in rs4680 is associated with differential enzyme activity, stress responsivity, and prefrontal activity during working memory (WM), and it creates a CpG dinucleotide. We report that methylation of the Val^{158} allele measured from peripheral blood mononuclear cells (PBMCs) of Val/Val humans is associated negatively with lifetime stress and positively with WM performance; it interacts with stress to modulate prefrontal activity during WM, such that greater stress and lower methylation are related to reduced cortical efficiency; and it is inversely related to mRNA expression and protein levels, potentially explaining the *in vivo* effects. Finally, methylation of *COMT* in prefrontal cortex and that in PBMCs of rats are correlated. The relationship of methylation of the *COMT* Val^{158} allele with stress, gene expression, WM performance, and related brain activity suggests that stress-related methylation is associated with silencing of the gene, which partially compensates the physiological role of the high-activity Val allele in prefrontal cognition and activity. Moreover, these results demonstrate how stress-related DNA methylation of specific functional alleles impacts directly on human brain physiology beyond sequence variation.

Introduction

Single nucleotide polymorphisms (SNPs) can create or abolish CpG dinucleotides, which constitute a site for DNA methylation, an epigenetic mechanism for regulation of gene expression. Earlier studies in rodents have indicated that DNA methylation is sensitive to environmental stressful exposures early in development and later in life (Jirtle and Skinner, 2007; Miller and Sweatt, 2007; Szyf et al., 2008; McGowan et al., 2009; Murgatroyd et al., 2009; Miller et al., 2010). Moreover, other studies in mice have reported that memory formation and maintenance are sensitive to epigenetic programming by early life stress and, more specifically, to DNA methylation of specific neurons (Miller and Sweatt, 2007; Murgatroyd et al., 2009; Miller et al., 2010). Experiments in human monozygotic twins have suggested a relationship between dynamic epigenetic mechanisms and environment (Fraga et al., 2005; Wong et al., 2010). However, to date, there is no evidence in humans of the relationship among stress, DNA methylation, and brain activity during memory.

In humans, prefrontal dopamine (DA) and stress are tightly related with working memory (WM) behavior and related prefrontal cortex (PFC) activity, suggesting that lower DA and greater stress are associated with reduced prefrontal efficiency (Hains and Arnsten, 2008). Catechol-*O*-methyltransferase (COMT), a key enzyme for inactivation of prefrontal DA (Gogos et al., 1998), contains a SNP (rs4680, G→A, Val→Met), in which the ancestral Val allele is associated with greater enzyme activity, blunted stress responses, and greater prefrontal activity during WM, that is, reduced efficiency (Egan et al., 2001; Chen et al., 2004; Papaleo et al., 2008; van Winkel et al., 2008; Mier et al., 2010; Walder et al., 2010). Notably, rs4680 in *COMT* exon 4 also abolishes a CpG site so that each Val allele has one CpG site and the Met allele has none.

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The objective of the present study was to evaluate the relationship among life stress, *COMT* methylation at the rs4680 site (C2) measured in peripheral blood mononuclear cells (PBMCs), WM performance, and related prefrontal activity measured by functional magnetic resonance imaging (fMRI) in healthy humans. Moreover, we addressed the effects of DNA methylation on *COMT* expression in PBMCs to determine a molecular mechanism for the *in vivo* findings. Finally, we addressed in a sample of rats the question of whether *COMT* methylation patterns in PBMCs and in brain are correlated.

Materials and Methods

Subjects. Healthy subjects ($n = 84$; 32 males; mean age ± SD, 25.90 ± 5.58 years) entered the study. All participants were unrelated, and all were Caucasian; all provided written informed consent. Protocols and procedures were approved by the local Institutional Review Board. Subjects underwent the Structured Clinical Interview for *DSM-IV* to exclude any Axis I psychiatric disorder. None had a history of significant drug or alcohol abuse (no active drug use in the past 6 months), head trauma with loss of consciousness, or significant medical illness.

Methylation analysis and genotyping. DNA was extracted from PBMCs using standard procedures and bisulfite treated as described previously (Bollati et al., 2007). Methylation analysis was performed with pyrosequencing (primer sequences available on request) and focused on CpG methylation sites at *COMT* rs4680 and within the gene promoter. A consensus *LINE-1* sequence was also analyzed to estimate global DNA methylation (Bollati et al., 2007). In the *COMT* promoter, we analyzed methylation of three CpGs, two of which had been already identified as differentially methylated (Murphy et al., 2005; Dempster et al., 2006; Mill et al., 2006). Methylation was expressed as percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines (%5mC) (Tost and Gut, 2007). SNP rs4680 was genotyped using the pyrosequencing assay designed to interrogate percentage of methylation in this region. In addition, genotypes were double-checked by DNA direct sequencing (primer sequences available on request).

Stress assessment. Because DNA methylation is sensitive to early and later environmental stimuli (Jirtle and Skinner, 2007; McGowan et al., 2009; Murgatroyd et al., 2009; Miller et al., 2010), we set out to determine a score of lifetime exposure to stress capturing both early and later stressors. To determine this score, we used obstetric complications (OCs) and stressful life events (SLEs) questionnaires as follows. On the basis of interviews administered to mothers using a standard questionnaire developed from other published reports (McIntosh et al., 2002), OCs are referred to as "somatic complications and conditions occurring during pregnancy, labor-delivery and the neonatal period" experienced as an offspring with special focus on the CNS (McNeil et al., 1994) and were determined to obtain a measure of early life stress of the subjects. OC data were rated using the McNeil–Sjöström scale for obstetric complications (McNeil et al., 1994). Consistent with earlier studies (Preti et al., 2000; Verdoux et al., 2002), we used this scale to assign each subject a binary OC score equal to 0 or 1, depending, respectively, on absence or presence of at least one serious OC (severity score ≥ 4 on a scale of 1–6). SLEs were assessed with the aid of a life-history calendar (Caspi et al., 2003). The number of stressful events was normalized to age to obtain an SLE score, which was then converted to a binary measure so that it could be summed to the OC score. Finally, SLE and OC scores were summed to obtain a categorical lifetime stress score, equal to 0 (lower stress), 1, or 2 (greater stress).

WM task. During fMRI, all subjects completed a blocked paradigm of the *n*-back task (Bertolino et al., 2010). Briefly, *n*-back refers to how far back in the sequence of stimuli the subject has to recall. The stimuli consisted of numbers (1–4) shown in random sequence and displayed at the points of a diamond-shaped box. There was a visually paced motor task that also served as a non-memory-guided control condition (0-back) that simply required subjects to identify the stimulus currently seen. In the WM condition, the task required recollection of a stimulus seen two stimuli previously (2-back) while continuing to encode additionally incoming stimuli.

fMRI data acquisition. Blood oxygen level-dependent (BOLD) fMRI was performed on a GE Signa 3T scanner (General Electric), equipped with a standard quadrature head coil as previously described (Bertolino et al., 2010). A gradient-echo planar imaging sequence (repetition time, 2000 ms; echo time, 28 ms; 20 interleaved axial slices; thickness, 4 mm; gap, 1 mm; voxel size, 3.75 mm (isotropic); flip angle, 90°; field of view, 24 cm; matrix, 64×64) was used to acquire 120 volumes while subjects performed the WM task. The first four scans were discarded to allow for a T1 equilibration effect.

fMRI data analysis. Analysis of the fMRI data was completed using statistical parametric mapping (SPM5; http://www.fil.ion.ucl.ac.uk/spm). All preprocessing was performed as described previously (Bertolino et al., 2010). A boxcar model convolved with the hemodynamic response function at each voxel was generated. In the first-level analysis, linear contrasts were computed, producing a *t* statistical map at each voxel for the 2-back condition, assuming the 0-back condition as a baseline. All individual contrast images were entered in a second-level random-effects analysis. A one-sample *t* test ($p < 0.005$; $k = 5$), performed on the whole group, was used to assess the functional network involved in performance of the WM task. A multiple regression model, entering percentage of methylation of *COMT* C2 and lifetime stress score as predictors, was used to evaluate the main effects as well as the interaction between C2 methylation and stress in Val/Val subjects. Because of our strong hypothesis about prefrontal behavior and activity, the statistical threshold of $p < 0.05$ after familywise error (FWE) small-volume correction for multiple comparisons was used to identify significant responses in bilateral PFC, using three 10 mm radius spheres centered on coordinates published in previous WM studies (Wager and Smith, 2003; Habeck et al., 2005; Tan et al., 2007). Activation outside these regions of interest was FWE whole-brain corrected ($p < 0.05$; $k = 5$). Similar analyses were also performed for the other genotype groups and for the whole sample when evaluating the potential association with methylation of the *COMT* promoter or of *LINE-1*. For anatomical localization, we converted statistical maxima of activation to conform to the standard space of Talairach and Tournoux.

COMT *m-RNA levels by quantitative reverse transcription-PCR.* We analyzed *COMT* mRNA expression in 46 healthy individuals (14 Val/Val, 18 Val/Met, 14 Met/Met). MB-*COMT* (long transcript) and total-*COMT* (both long and short transcripts) mRNA levels were assessed using the comparative C_T method with β-actin as reference (control) gene (Gao et al., 2010) (primers available on request). Real-time quantitative PCR was performed using ABI PRISM 7700 Sequence Detection System in combination with continuous SYBR Green detection (Applied Biosystems).

Western blotting. We further evaluated the relationship between C2 methylation and *COMT* expression (both MB- and S-COMT proteins) in 21 Val-carrier healthy individuals (7 Val/Val, 14 Val/Met), given that only the Val allele has a CpG dinucleotide at C2. PBMCs were isolated as previously described (Castri et al., 2007). Aliquots (2 μl) of samples were used for the protein determination by Bio-Rad Protein Assay kit (Bio-Rad); 80 μg of total proteins were separated by SDS-PAGE, using 15% polyacrylamide gels, blotted onto nitrocellulose and probed overnight using commercial anti-*COMT* antibody (1:7000, Millipore Bioscience Research Reagents, Cat. AB5873). Membranes were then washed and reprobed with anti-tubulin (1:5000, Millipore, Cat. 05-661) to verify equal protein loading. Antibody against COMT was able to recognize both S- and MB-COMT. Blots were then incubated in horseradish peroxidase-conjugated secondary antibodies and target proteins visualized by ECL detection (Pierce), followed by quantification by Quantity One software (Bio-Rad). Optical density values were normalized to tubulin for variation in loading and transfer.

Methylation analysis in rat lymphocytes and PFC. Adult male Wistar rats (Charles River) of 4 months of age, weighing 491 ± 11 g, were housed two per cage in a temperature-controlled room at 21 ± 1°C, with 50 ± 5% of humidity and a 12 h light–dark period (lights on 7:00 A.M. to 7:00 P.M.). Food (Standard Diet 4RF21; Charles River) and tap water were provided *ad libitum*. Animals were killed by decapitation between 9:00 and 11:00 A.M., trunk blood (5 ml) was collected in tubes containing EDTA (2 mg/ml blood), brains were removed from the skull, and the PFC was rapidly dissected using a standard procedure (Gearhart et al., 2006), frozen on dry ice, and kept at −80°C until processed. Similarly,

the hippocampus and striatum were also dissected from the same rats as control regions. Both anatomical regions are sensitive to stress like the PFC, but they have a different relationship with rs4680 genotype in response to several cognitive paradigms (Smolka et al., 2005; Bertolino et al., 2006; Yacubian et al., 2007; Krugel et al., 2009). Consistent with these functional imaging data, both regions differentially express *COMT* when compared with PFC, thus potentially implicating different mechanisms for gene expression (Sesack et al., 1998; Matsumoto et al., 2003). Analysis of rat *LINE-1* and *COMT* methylation was performed with pyrosequencing (primer sequences available on request).

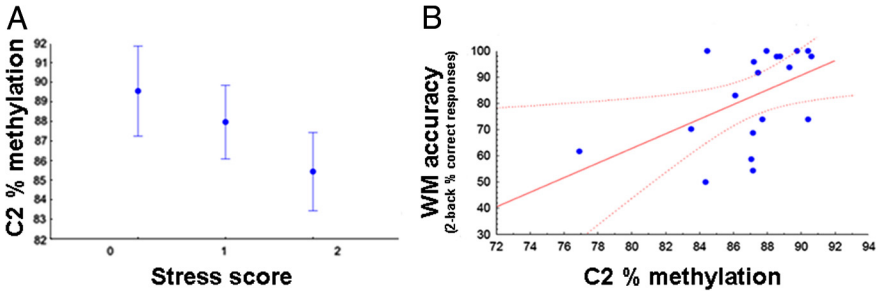

Figure 1. C2 methylation in Val/Val subjects: relationship with stress and WM behavioral performance. ***A***, Mean ± 0.95 confidence interval of the effect of stress on methylation of *COMT* C2 rs4680 in Val/Val homozygotes—subjects with greater stress scores have reduced methylation. ***B***, Scatter plot of the correlation between methylation of *COMT* C2 rs4680 and WM accuracy—Val/Val subjects with greater methylation have greater accuracy (see Results for statistics).

Results

The relationship among *COMT* methylation, rs4680 genotype, and stress

As expected, methylation of the C2 rs4680 site was affected by rs4680 genotype (ANOVA: $F_{(2,81)} = 7336.8$, $p < 0.0001$; Val/Val > Val/Met > Met/Met) given that only the Val allele has a CpG site in this position. Therefore, separate analyses were conducted in the three genotype groups. ANOVA demonstrated that Val/Val subjects with greater stress scores have significantly reduced methylation in C2 compared with subjects with lower scores ($F_{(2,20)} = 4.1$, $p = 0.03$; *post hoc* with Fisher's least significant difference: "2" < "0," $p = 0.01$; "2" < "1," $p = 0.06$) (Fig. 1*A*). Consistent with earlier studies (Dempster et al., 2006), we also found an effect of rs4680 genotype on methylation of the *COMT* promoter sites, indicating that subjects carrying two Met alleles have greater methylation than Val/Met subjects, who, in turn, have greater methylation than Val/Val subjects (ANOVA: $F_{(2,81)} = 11.2$, $p < 0.01$; data not shown). However, no significant relationship was found between methylation of sites in the *COMT* promoter or in *LINE-1* and stress in the whole sample (ANOVAs: F values ≤ 0.1, p values > 0.9) and in any genotype group (ANOVAs: F values < 2.0, p values > 0.1).

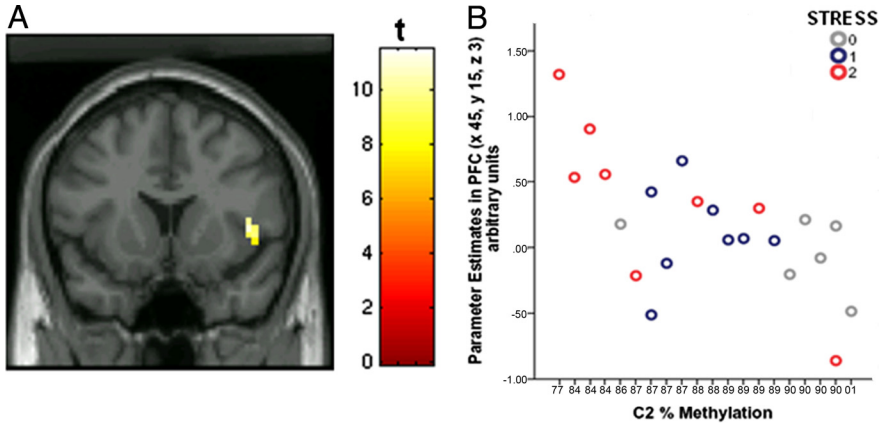

Figure 2. Interaction between C2 methylation, stress scores, and prefrontal activity during WM in Val/Val subjects. ***A***, Coronal section of the interaction between stress scores and C2 methylation on BOLD fMRI response in PFC. ***B***, Scatter plot of the interaction showing that subjects with greater stress scores and lower methylation have greater activity in PFC, i.e., less efficiency (see Results for statistics).

The relationship among *COMT* methylation, stress, and prefrontal cognition and activity

As expected given that DNA methylation may reduce gene expression, C2 methylation in Val/Val subjects was positively correlated with 2-back WM accuracy ($n = 19$, Spearman's $\rho = 0.59$, $p = 0.007$), with greater methylation predicting greater percentage of correct responses (Fig. 1*B*). No statistically significant relationship was found between WM accuracy and stress ($F_{(2,16)} = 2.1$, $p > 0.1$). Consistent with earlier fMRI studies demonstrating an inverse relationship between Val alleles and PFC activity during WM, multiple regression in SPM5 demonstrated the following in Val/Val subjects: a negative correlation between C2 methylation and bilateral PFC activity during WM [$x = -38$, $y = 23$, $z = 9$, Brodmann area 45/13 (BA45/13), $k = 11$, $Z = 3.38$, $p_{\text{FWE-corrected}} = 0.01$; $x = 45$, $y = 15$, $z = 3$, BA47, $k = 7$, $Z = 3.54$, $p_{\text{FWE-corrected}} = 0.009$]; a positive correlation between stress scores and activity in left PFC ($x = -45$, $y = 48$, $z = -3$, BA10/47, $k = 6$, $Z = 2.9$, $p_{\text{FWE-corrected}} = 0.04$); and an interaction between C2 methylation and stress scores on bilateral prefrontal activity during WM, suggesting that greater stress and lower methylation are related to greater activity (i.e., inefficient; $x = -45$, $y = 48$, $z = -3$, BA10/47, $k = 11$, $Z = 3.26$, $p_{\text{FWE-corrected}} = 0.02$; $x = 49$, $y = 11$, $z = 6$, BA44/45, $k = 8$, $Z = 3.18$, $p_{\text{FWE-corrected}} = 0.03$) (Fig. 2). On the other hand, no significant relationship was found between methylation of sites in the *COMT* promoter or in *LINE-1* and stress, WM performance, and related PFC activity in any other genotype group or in the whole sample (p values > 0.1). Lack of a correlation among *COMT* promoter methylation, stress, and prefrontal cognition or activity suggests that methylation of the rs4680 CpG site and of the promoter respond to different mechanisms, at least in the context of the relationship with stress and WM. Finally, no significant relationship between activity in other brain regions, including parietal cortex, and methylation of *COMT* or stress was evident at the statistical threshold identified *a priori* (data not shown).

The relationship between methylation of Val[158] and *COMT* expression

To determine a molecular mechanism for the *in vivo* findings, we evaluated the effects of methylation on *COMT* expression in PBMCs by quantitative real-time reverse transcription-PCR and Western blotting analysis. As predicted, C2 methylation in Val/Val subjects was inversely correlated with MB-*COMT* (the isoform more expressed in brain) mRNA expression ($n = 14$, $\rho = -0.57$, $p = 0.03$) (Fig. 3) and with levels of MB-COMT protein

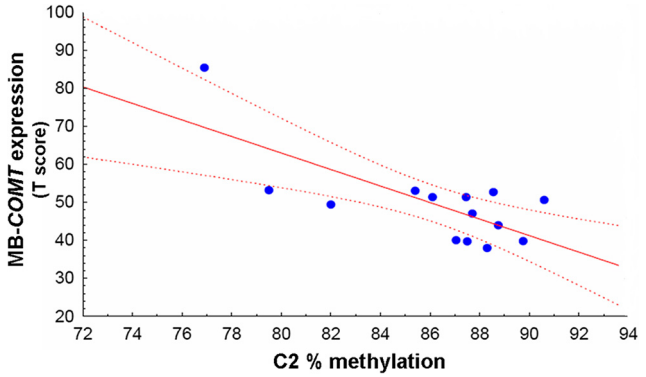

Figure 3. Scatter plot of the correlation between C2 methylation and *t* scores of MB-*COMT* expression levels (transformed $-\Delta\Delta$Ct) in PBMCs of Val/Val subjects; greater methylation is correlated with lower expression (see Results for statistics).

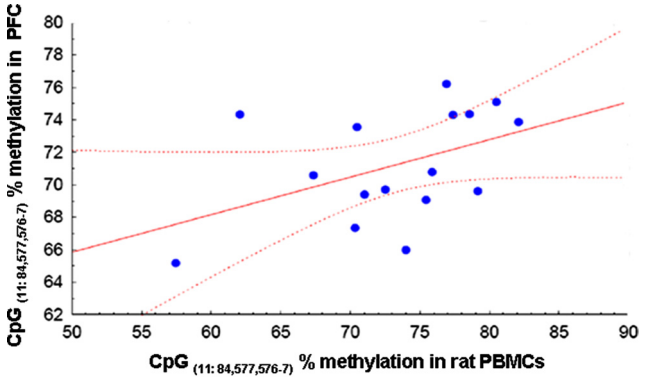

Figure 4. Scatter plot of the correlation between methylation of *COMT* in PBMCs and in PFC of a group of 16 rats—methylation analysis on a CpG (chromosome 11: 84,577,576-7) in the *COMT* region (Leu[151]) corresponding to the region where the human rs4680 site is found shows a direct correlation between methylation in PBMCs and in PFC (see Results for statistics).

($n = 7$, $\rho = -0.78$, $p = 0.03$), whereas no relationship was found with S-COMT protein levels ($n = 7$, $\rho = -0.14$, $p = 0.7$), further suggesting the specificity of this relationship. In Val/Met subjects, we found no relationship between C2 methylation and *COMT* mRNA or protein levels (p values > 0.1).

Correlation of *COMT* methylation in brain and PBMCs

Because at present it is impossible to determine methylation in the brain of living healthy humans and we have evaluated DNA methylation from PBMCs, we addressed in a sample of rats the question of whether *COMT* methylation patterns in PBMCs and in brain are correlated. Moreover, because rats do not have the Val[158]Met mutation, we analyzed methylation on a CpG (location: chromosome 11: 84,577,576-7) in the *COMT* region (Leu[151]) corresponding to the region where the human rs4680 site is found. This analysis revealed a direct correlation between methylation in PBMCs and in PFC ($n = 16$, $\rho = 0.49$, $p = 0.05$), indicating that *COMT* methylation in PBMCs can be used as a proxy for *COMT* methylation in PFC (Fig. 4), as also suggested by others (Murphy et al., 2005). However, methylation in PBMCs was not significantly correlated with methylation in hippocampus ($n = 16$, $\rho = 0.33$, $p = 0.2$) or in striatum ($n = 15$, $\rho = -0.13$, $p = 0.6$). Finally, in the same rats, *LINE-1* methylation in PBMCs and PFC were not significantly correlated, further suggesting a relatively specific relationship for *COMT*.

Discussion

This study demonstrates for the first time in living humans that environment-related methylation of alleles within functional SNPs is an important mechanism for regulation of gene expression and behavior-related brain activity. Earlier studies have indicated that DNA methylation, prefrontal DA levels, and prefrontal activity are modulated by stress (Arnsten and Goldman-Rakic, 1998; Pani et al., 2000; Arnsten, 2009). Moreover, a series of earlier studies have demonstrated that *COMT* is a key factor in prefrontal DA inactivation (Gogos et al., 1998). Furthermore, the enzyme containing the Val[158] allele has higher activity (Lotta et al., 1995), presumably leading to lower prefrontal DA. Consistently, the Val allele has been repeatedly associated with inefficient prefrontal activity, that is, greater activity for a given level of behavioral performance (Egan et al., 2001; Bertolino et al., 2004; Mier et al., 2010). Other studies have also indicated that the Val[158] allele is a protective factor against stress, suggesting specific behavioral correlates of pleiotropic effects (Mattay et al., 2003; Papaleo et al., 2008; van Winkel et al., 2008; Walder et al., 2010). Consistent with this earlier body of work, here we have demonstrated that methylation of the Val allele in homozygous subjects is sensitive to stress; is correlated with WM behavioral performance; and interacts with stress, modulating PFC responses during WM performance such that greater stress and lower methylation are correlated with more inefficient prefrontal activity. These *in vivo* results are consistent with the role of DNA methylation in gene silencing and alternative transcription, even when outside of CpG islands (Jirtle and Skinner, 2007; Szyf et al., 2008; Irizarry et al., 2009; Maunakea et al., 2010). In fact, methylation of the Val allele in rs4680 is inversely related to MB-*COMT* expression and protein levels in Val/Val subjects, suggesting that silencing of the gene partially compensates the physiological role of the high-activity Val allele in prefrontal DA inactivation. This interpretation is also consistent with previous findings indicating that Val behaves as a high-activity allele when found on a high genetic expression background, but not otherwise (Meyer-Lindenberg et al., 2006; Nackley et al., 2006). In other words, the *in vivo* effects on memory performance and brain activity of the high-activity ancestral allele can be partially compensated by greater methylation, which is related to reduced expression of the *COMT* isoform (MB-) mainly responsible for inactivation of prefrontal DA.

It is interesting to note that no significant relationship among *COMT* rs4680 methylation, stress, prefrontal cognition, and *COMT* expression was found in Val/Met subjects. These negative results suggest that C2 methylation is not sufficient to modulate *COMT* function in heterozygous subjects, probably because methylation of both alleles is necessary to modify transcription and translation. In fact, *COMT* function in heterozygote subjects is associated with expression and activity of both alleles (Chen et al., 2004). The presence of one Met allele in heterozygotes may compensate for the effects related to methylation of the Val allele on expression and/or activity of *COMT*, preventing demonstration of a significant correlation. Furthermore, we cannot exclude the possibility that other CpGs modulate function of the chromosome containing a Met allele and might be sensitive to stress. For this reason, future studies of *COMT* methylation in Val/Met subjects should take into account the possibility of identifying and characterizing other CpGs present on chromosomes carrying the Met allele in addition to C2, which is present only on the Val allele.

The mechanisms correlating methylation of the Val allele in rs4680 with *COMT* transcription are not known. However, some

speculations are possible. Cytosine methylation is most commonly associated with transcriptional repression mediated by direct interference with binding of transcription factors and/or through recruitment of methylated DNA-binding proteins and repressive complexes (Meaney and Ferguson-Smith, 2010). Consistently, bioinformatic tools to perform gene regulation analyses and search of complex regulatory patterns (http://www.genomatix.de/) suggest that rs4680 (G→A, Val→Met) leads to loss of a binding site for AHRARNT.01, a transcription factor belonging to the AHR/ARNT heterodimers and AHR-related factors family. Therefore, methylation of rs4680 CpG (C2) on the Val allele might affect gene expression through interference with binding of this transcription factor, potentially involved in several functions, including response to environmental toxicants and diverse developmental processes, such as neuronal differentiation (Oesch-Bartlomowicz et al., 2005; Sartor et al., 2009). Interestingly, AHR agonists at the lowest effective dose have been reported to induce an increase in MB-*COMT* expression in rat brain cortex (Desaulniers et al., 2005), and the induction of gene expression by AHR/ARNT has been associated with modification of specific chromatin marks (Schnekenburger et al., 2007). Consistent with these speculations and with earlier studies demonstrating that intragenic methylation has a major role in regulating alternative promoters within gene bodies (Maunakea et al., 2010), SNP rs4680 also seems to be located within a *COMT* alternative promoter (name: GXP_2244264; locus: COMT/GXL_744584; position: chromosome 22: 19950722–19951322, 601 bp; transcripts: ENST0000428707). Until further experiments are specifically performed, these contentions about the mechanisms by which methylation of rs4680 is correlated with *COMT* expression must be considered speculative.

Some limitations of our study concerning the assessment of stress and methylation have to be acknowledged. To evaluate the potential relationship between stress and methylation, we used a categorical lifetime stress score. This is indeed a crude measure of stress that does not consider all nuances of experiencing stress. The main reason why we used this crude measure of stress is that methylation is subject to environmental modifications that can take place both early and later in the life of an individual. Despite its simplistic nature, our measure does take into account early and later events that can be correlated with methylation. Moreover, further analyses separating OCs and SLEs also indicate that both scores correlate with methylation in Val homozygotes (results available on request). Despite all limitations intrinsic to such an oversimplification of measuring stress, our results suggest that the relationship with methylation has behavioral, physiological, and molecular implications.

Another limitation is that we have evaluated DNA methylation from PBMCs rather than directly in the brain, which is at present impossible in living healthy humans. Because epigenetic profiles can differ across tissues and cell types, this is an unavoidable limitation of our study. However, we believe that this limitation is tempered by our experiments in rats demonstrating that *COMT* methylation patterns in PBMCs and in PFC are correlated, suggesting that *COMT* methylation in PBMCs can be used as a proxy for *COMT* methylation in PFC. These results are also consistent with another report (Murphy et al., 2005). On the other hand, methylation in PBMCs did not correlate with methylation in hippocampus or striatum. Albeit weak, the correlation in the hippocampus was in the same direction as in PFC. In the striatum, where expression of *COMT* is very low, there was no relationship whatsoever; if anything, it was in the opposite direction. We believe that these further data suggest that other factors in addition to methylation of this CpG may be involved in regulation of MB-COMT expression in hippocampus and striatum. In other words, *COMT* methylation may have an important role in gene expression in PBMCs and in PFC, less so in other brain regions where it is expressed. Consistently, Li et al. (2010) have recently demonstrated in mice that B2 SINE, a transposon in the 3′ UTR, is an important source of variation in *COMT* expression in the hippocampus and striatum but not in PFC, suggesting that specific transpositions can alter mRNA isoform use and modulate behavior.

Even if we cannot exclude rs4680 methylation as being at least in part stochastic or inherited, our results suggest that it might mediate the relationship between stress and WM prefrontal activity in Val/Val subjects, thus providing a molecular mechanism for this gene–environment interaction. It is also plausible that rs4680 methylation could mediate other gene–environment interactions involving *COMT* (Caspi et al., 2005; Kolassa et al., 2010). More generally, our findings indicate that better understanding of the complex interplay among genome, epigenome, and environment can be obtained by studying methylation of CpG sites created or abolished by SNPs or mutations, which during evolution may originate themselves from "mutagenic" CpG methylation (Holliday and Grigg, 1993; Zemach et al., 2010). In fact, DNA methylation is sensitive to environment, and methylated cytosines (5-methylcytosine) can undergo spontaneous deamination to thymine, causing G/C to A/T transition and thus creating genetic variants and abolishing CpG sites that may be involved in regulation of gene expression. At the same time, these genetic variants creating or abolishing a CpG site could alter response to environmental factors that might act via methylation.

In conclusion, our findings suggest that genetic variation at *COMT* rs4680 and stress interact with and through methylation on prefrontal activity, thus demonstrating how epigenetics may modulate the effect of genetic variation and create a two-way bridge between genes and environment.

Notes

Supplemental material (primer sequences and supplemental results) is available at http://www.psichiat.uniba.it/png/ursinijneurosci2011SM.pdf. This material has not been peer reviewed.

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