Allelic mRNA expression of X-linked monoamine oxidase a (MAOA) in human brain: dissection of epigenetic and genetic factors

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A pVNTR repeat polymorphism located in the promoter region of the X-linked MAOA gene has been associated with mental disorders. To explore the effect of polymorphisms and epigenetic factors on mRNA expression, we have measured allelic expression imbalance (AEI) in female human brain tissue, employing two frequent marker single nucleotide polymorphisms (SNPs) in exon 8 (T890G) and exon 14 (C1409T) of MAOA. This approach compares one allele against the other in the same subject. AEI ratios ranged from 0.3 to 4 in prefrontal cortex, demonstrating the presence of strong cis-acting factors in mRNA expression. Analysis of CpG methylation in the MAOA promoter region revealed substantial methylation in females but not in males. MAOA methylation ratios for the three- and four-repeat pVNTR alleles of MAOA did not correlate with X-chromosome inactivation ratios, determined at the X-linked androgen receptor locus, suggesting an alternative process of dosage compensation in females. The extent of allelic MAOA methylation was highly variable and correlated with AEI (R² = 0.5 and 0.7 at two CpG loci), indicating that CpG methylation regulates gene expression. Genetic factors appeared also to contribute to the AEI ratios. Genotyping of 13 MAOA polymorphisms in female subjects showed strong association with a haplotype block spanning from the pVNTR to the marker SNP. Therefore, allelic mRNA expression is affected by genetic and epigenetic events, both with the potential to modulate biogenic amine tone in the CNS.

INTRODUCTION

Genetic predisposition to mental disorders appears to involve multiple genes, but a causative relationship has been difficult to establish. Extensive studies on suspected disease susceptibility genes have focussed on functional polymorphisms that change the encoded amino acid sequence. However, polymorphisms in regulatory regions, or those affecting mRNA processing, also affect clinical phenotypes. Recent genome-wide surveys suggest that these cis-acting polymorphisms might account for much of human phenotypic diversity (1–4). Yet, a systematic analysis of the prevalence and impact of cis-acting regulatory polymorphisms has yet to be performed for most susceptibility genes implicated in mental disorders.

This study focuses on monoamine oxidase A (MAOA), a candidate gene implicated in multiple CNS disorders, most notably drug abuse (5–7), aggression (8–11), antisocial behavior (12), anxiety (13), attention deficit hyperactivity disorder (14–16), anorexia nervosa (17,18), bipolar disorder (19–21) and Alzheimer’s disease (22). Monoamine oxidases catalyze the oxidation of biogenic amines and are the target of a class of antidepressant drugs. A repeat polymorphism in the promoter region of MAOA (pVNTR) has been extensively studied in vitro and in clinical association studies (5,6,10,19,23,24). The four-repeat pVNTR yielded higher expression levels of a reporter gene than the three repeats, in a heterologous in vitro system (23). On the basis of this result, the pVNTR of MAOA has been a marker for numerous association studies, suggesting a link to increased susceptibility to impulsivity and early abuse experiences in males (5,10), whereas other studies have failed to demonstrate significant associations with various disorders (25–31). The three-repeat pVNTR variant also influences aggressive behavior in Rhesus

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monkeys (11). Moreover, MAOA knockout mice display offensive aggressive behavior in males (32). Yet, the functional relevance of the pVNTR and its contribution to overall genetic diversity of MAOA in the CNS has yet to be demonstrated.

Epigenetic factors provide an alternative mode of gene regulation. MAOA is located on the X-chromosome at Xp11.3, adjacent to the MAOB gene. The MAOA gene spans at least 90.6 kb from the promoter to the 3'-untranslated region (Fig. 1). Although one commonly assumes that in each cell one X-chromosome is randomly inactivated in females (33), unequal X-inactivation or selection of one active X-chromosome over the other in somatic cells has been observed (34–36). Earlier reports had suggested that MAOA is subject to X-chromosome inactivation in humans (33). However, a recent survey of X-inactivation found that MAOA ranks among the 15% of X-linked genes that escape inactivation (37). Therefore, the contribution of X-inactivation or other epigenetic factors to regulation of MAOA remain unexplored.

This study addresses the question how genetic and epigenetic processes interact to regulate MAOA gene expression using human autopsies from brain tissues. We address this question by quantitatively measuring the relative amounts of mRNA generated from each of the two alleles in female subjects, using two marker single nucleotide polymorphisms (SNPs) in the transcribed region. An allelic expression imbalance (AEI) indicates the presence of cis-acting factors in gene regulation and/or mRNA processing. Analysis of AEI has been successfully applied in several recent studies, including those involving brain tissues (2,4,38–40). AEI results provide a quantitative measure of the allelic differences in each individual, one allele serving as the control for the other, while canceling out any trans-acting factors. It also enables scanning a gene for functional polymorphisms, using AEI as a phenotype as previously demonstrated for MDR1 and OPRM1 (39,41). This is the first study to exploit AEI as a quantitative phenotype for dissecting the contribution of genetic and epigenetic factors to interindividual variability.

Measuring allelic mRNA expression compares one allele against the other in a relevant autopsy target tissue of the same individual—females in the case of X-linked genes. Allelic expression ratios appear to represent a more robust phenotypic marker than absolute mRNA levels, which can fluctuate strongly because of trans-acting factors and post-mortem decay. To survey diverse MAOA alleles that may be enriched in disease, we have included control subjects and those previously diagnosed with schizophrenia and bipolar disorder. We have analyzed autopsy brain samples from 105 individuals (36 females and 69 males) previously diagnosed with bipolar disorder or schizophrenia (35 each) and 35 controls obtained from the Stanley Foundation. Although the number of female subjects in this study was sufficient for detection and evaluation of cis-acting factors by pairwise allele comparisons, the size of the cohorts was not designed to permit a robust association analysis in a case–control study design. Brain tissues were taken from prefrontal cortex and in four cases from three other brain regions as well. All samples were genotyped for 13 common polymorphisms, two of which served as marker SNPs in the transcribed region for analyzing AEI. In addition, we measured total mRNA levels in all samples. To account for epigenetic effects, we determined CpG island methylation in the MAOA promoter region in two loci, in comparison to X-inactivation measured at the X-linked androgen receptor locus. In the present study, the male samples served to assign unambiguous haplotypes and to compare CpG methylation between males and females. The results reveal epigenetic gene regulation by CpG methylation in the MAOA promoter region in females (but not in males) representing a possible dosage compensation mechanism that does not correlate with X-inactivation. After accounting for epigenetic factors, one or more cis-acting polymorphisms also affect allelic mRNA levels. The functional variant locates to an MAOA haplotype region spanning from the pVNTR in the promoter to the 3' end of MAOA.
RESULTS

Genotype and haplotype analysis of MAOA

We genotyped 13 polymorphisms, spanning the MAOA gene (Fig. 1) in 105 samples (69 males, 36 females) from the Stanley foundation brain collection, including the promoter variable nucleotide tandem repeat (pVNTR). Allele frequencies, linkage and other information about each polymorphism (shown in Supplementary Material, Table S1) are consistent with previous results (42). We identified 14 unambiguous haplotypes in the males, carrying only a single X-chromosome. Through the use of an estimation maximization algorithm to assess haplotypes and their frequencies including males and females, we identified 10 additional haplotypes for a total of 23. Haplotype information is depicted in Supplementary Material, Table S2. The haplotype block extends at least 115 kb upstream from the MAOA locus (42). Downstream of MAOA, the haplotype block ends ~10 kb from the 3’ end. Pair-wise linkage disequilibrium (LD) results (Supplementary Material, Table S1) are consistent with these data. A haplotype block of six abundant (>30% allele frequency) SNPs in very high LD spreads over the 3’ portion of MAOA. This includes three high frequency SNPs in transcribed regions (exons 8 and 14 and 3’-UTR), from which we have selected the exons 8 and 14 SNPs as markers for AEI assays. These two marker SNPs are linked to each other in all but one individual. In the majority of samples with a four-repeat pVNTR, the four repeat is linked to the major alleles, and the three repeat to the minor ones, of the two indicator SNPs in exons 8 and 14, with four notable exceptions. The latter are important for assessing those gene regions that might contribute to AEI.

Allele-specific mRNA analysis

We next measured the ratios of MAOA genomic DNA alleles in comparison to the corresponding allelic mRNA ratios, in prefrontal cortex samples. Any significant difference in these ratios documents the presence of AEI, and hence cis-acting factors determining mRNA levels. From the available genotype data, we have first selected the synonymous C/T SNP (C1409T) in exon 14 (rs1801291) as a marker for the AEI analysis. Among 36 female DNA samples from the Stanley Foundation brain collection, for which mRNA from the prefrontal cortex was available, 17 samples were heterozygous for the marker SNP and therefore suitable for AEI analysis. This enables evaluation of functional differences for 34 chromosomes. These included six controls, seven bipolar patients and five schizophrenic patients. The genomic DNA ratios varied within a narrow range and were normalized to 1.0 (SD = 0.03) (Table 1, column II), showing the excellent reproducibility of the DNA ratio analysis, even in extracted brain autopsy samples. Figure 2 shows a plot of the mRNA C/T ratios derived from measurements of each allele and normalized to a genomic ratio of 1. The intra-sample error of the mRNA C/T ratios derived from measurements of each allele was 0.33 ± 0.05 to 4.2 ± 0.1, revealing a substantial expression imbalance of an order likely to have physiological relevance. Three female subjects were heterozygous for the marker SNP but homozygous for the pVNTR (Table 1, columns VII and VIII). Nevertheless, these samples displayed significant AEI values, indicating that the pVNTR is not affecting these ratios but other or additional factors are present. These data were validated by repeating the AEI analysis with a second synonymous SNP in exon 8 (rs6323) yielding similar results as shown in Figure 3 (Pearson correlation = 0.98). The high correlation between the two independent AEI assays validates the allelic ratio analysis. Furthermore, we obtained AEI data for an additional sample heterozygous for rs6323 (ST451) which is homozygous for rs1801291, but yet displays significant AEI (AEI ratio = 1.6 ± 0.3). In this case, rs1801291 cannot be the cause of the AEI ratio.

To explore the possibility of tissue-specific differences in allelic expression, we analyzed allele-specific mRNA ratios from different brain regions: cerebellum, occipital lobe and parietal lobe from four individuals from the Stanley Foundation collection. Samples ST255 and ST381 had the highest C/T ratios (4.2 ± 0.1 and 4.0 ± 0.1, respectively), whereas samples ST380 and ST392 had the lowest ratios (0.33 ± 0.05 and 0.77 ± 0.02, respectively). As shown in Figure 4, there is some variability in the allelic mRNA expression ratios from tissue to tissue in the same individual that could be due to tissue-specific factors or sample quality. However, the overall trend in different tissues across individuals remains the same. The two individuals with high C/T ratios in prefrontal cortex maintained consistently high C/T ratios in the other brain regions, and the two individuals with low C/T ratios in prefrontal cortex also had lower C/T ratios in other brain regions.

MAOA methylation of a promoter CpG island in comparison to X-chromosome inactivation measured at the androgen receptor locus

We next performed a set of experiments to assess methylation in the CpG island located within the promoter region of MAOA, in comparison to X-inactivation. We determined allelic methylation ratios of the three- and four-repeat alleles of MAOA in females, using the methylation-sensitive restriction enzymes HhaI and Smal. A first assay relied on simultaneous amplification of three- and four-repeat alleles of the pVNTR, after digestion with Smal, yielding a set of allelic methylation ratios for 15 females for each three- and four-repeat alleles. CpG methylation prevents digestion, revealing undigested three- and four-repeat amplicons. Ratios of three-repeat over four-repeat methylation (Table 1, column V) varied over a 10-fold range. X-inactivation ratios were obtained by measuring CpG island methylation in the polyallelic promoter region of the androgen receptor (36). Methylation at the androgen receptor locus has been shown to correlate with inactivation of the X-chromosome (43).

The Figure 5A depicts a comparison between the allelic methylation ratios of the androgen receptor gene, a measure of unequal X-inactivation, against the MAOA allelic mRNA expression ratios, determined with HhaI. The low concordance (Pearson correlation $r = 0.29$, $P = 0.50$), combined with previous findings that MAOA escapes X-inactivation (37),
Table 1. Allele-specific measurements of mRNA, \textit{MAO}A locus methylation ratios using \textit{SmaI}, X-inactivation and diplotypes for 17 female samples that are heterozygous for the marker SNP

<table>
<thead>
<tr>
<th>(A)</th>
<th>Disease profile (I)</th>
<th>Genomic DNA C/T ratio (II)</th>
<th>mRNA C/T ratio ( n = 3 ) (III)</th>
<th>X-inactivation ratio (IV)</th>
<th>Methylation ratio of three/four repeat (V)</th>
<th>mRNA C/T ratio divided by methylation ratio (VI)</th>
<th>Haplotype 1 (VII)</th>
<th>Haplotype 2 (VIII)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST119</td>
<td>BP</td>
<td>0.99</td>
<td>2.7 ± 0.0</td>
<td>2.7 ± 0.3</td>
<td>1.5 ± 0.4</td>
<td>1.8</td>
<td>A,4, A,A, A,A, A,A</td>
<td>A,3, B,B, A,B, B,B, B,A</td>
</tr>
<tr>
<td>ST192</td>
<td>BP</td>
<td>1.04</td>
<td>2.3 ± 0.1</td>
<td>2.3 ± 0.3</td>
<td>0.9 ± 0.1</td>
<td>2.7</td>
<td>A,4, A,A, A,A, A,A</td>
<td>A,3, B,B, A,B, B,B, B,A</td>
</tr>
<tr>
<td>ST193</td>
<td>C</td>
<td>1.06</td>
<td>2.9 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>2.0 ± 0.2</td>
<td>1.4</td>
<td>A,4, A,A, A,A, A,A</td>
<td>A,3, B,B, A,B, B,B, B,A</td>
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<tr>
<td>ST199</td>
<td>S</td>
<td>1.01</td>
<td>2.6 ± 0.1</td>
<td>1.5 ± 0.0</td>
<td>1.4 ± 0.1</td>
<td>1.9</td>
<td>A,4, A,A, A,A, A,A</td>
<td>A,3, B,B, A,B, B,B, B,A</td>
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<tr>
<td>ST204</td>
<td>BP</td>
<td>0.98</td>
<td>3.0 ± 0.1</td>
<td>na</td>
<td>1.5 ± 0.3</td>
<td>2.0</td>
<td>A,4, A,A, A,A, A,A</td>
<td>B,3, B,B, A,B, B,B, B,A</td>
</tr>
<tr>
<td>ST255</td>
<td>C</td>
<td>0.99</td>
<td>4.2 ± 0.1</td>
<td>1.6 ± 0.0</td>
<td>nd</td>
<td>-</td>
<td>A,4, A,A, A,A, A,A</td>
<td>B,3, B,B, A,B, B,B, B,A</td>
</tr>
<tr>
<td>ST302</td>
<td>BP</td>
<td>1.03</td>
<td>2.7 ± 0.1</td>
<td>1.6 ± 0.0</td>
<td>1.9 ± 0.2</td>
<td>1.4</td>
<td>A,4, A,A, A,A, A,A</td>
<td>B,3, B,B, A,B, B,B, B,A</td>
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<tr>
<td>ST328</td>
<td>C</td>
<td>0.99</td>
<td>1.3 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>1.9</td>
<td>A,4, A,A, A,A, A,A</td>
<td>B,3, B,B, A,B, B,B, B,A</td>
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<tr>
<td>ST357</td>
<td>BP</td>
<td>1.00</td>
<td>2.3 ± 0.0</td>
<td>1.5 ± 0.2</td>
<td>na</td>
<td>-</td>
<td>A,3, B,A, A,A,A,A,B,B</td>
<td>B,3, B,B, A,B, B,B, B,A</td>
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<tr>
<td>ST369</td>
<td>S</td>
<td>0.95</td>
<td>1.8 ± 0.0</td>
<td>2.9 ± 0.4</td>
<td>0.8 ± 0.1</td>
<td>2.2</td>
<td>A,4, A,A, A,A,A,A,A,A</td>
<td>B,3, B,B, A,B, B,B, B,A</td>
</tr>
<tr>
<td>ST380</td>
<td>C</td>
<td>0.96</td>
<td>0.3 ± 0.1</td>
<td>na</td>
<td>0.2 ± 0.0</td>
<td>1.8</td>
<td>A,4, A,A, A,A,A,A,A,A</td>
<td>B,3, B,B, A,B, B,B, B,A</td>
</tr>
<tr>
<td>ST381</td>
<td>BP</td>
<td>0.96</td>
<td>4.0 ± 0.1</td>
<td>3.4 ± 0.3</td>
<td>na</td>
<td>-</td>
<td>A,4, A,A, A,A,A,A,A,A</td>
<td>A,4, A,B,B,B,B,B,B,B</td>
</tr>
<tr>
<td>ST392</td>
<td>S</td>
<td>1.05</td>
<td>0.8 ± 0.0</td>
<td>nd</td>
<td>nd</td>
<td>-</td>
<td>A,4, A,A, A,A,A,A,A,A</td>
<td>A,3, B,B, A,B,B,B,B,B</td>
</tr>
<tr>
<td>ST404</td>
<td>S</td>
<td>1.00</td>
<td>2.1 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>0.8 ± 0.0</td>
<td>2.5</td>
<td>A,4, A,A, A,A,A,A,A,A</td>
<td>A,3, B,B, A,B,B,B,B,B</td>
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<tr>
<td>ST449</td>
<td>C</td>
<td>0.96</td>
<td>1.4 ± 0.1</td>
<td>1.6 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>1.0</td>
<td>A,4, A,A, A,A,A,A,A,A</td>
<td>A,3, B,B, A,B,B,B,B,B</td>
</tr>
<tr>
<td>ST450</td>
<td>BP</td>
<td>0.96</td>
<td>2.0 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>na</td>
<td>-</td>
<td>A,4, A,A, A,A,A,A,A,A</td>
<td>A,4, A,B,B,B,B,B,B,B</td>
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<tr>
<td>ST476</td>
<td>C</td>
<td>0.96</td>
<td>3.0 ± 0.1</td>
<td>1.5 ± 0.3</td>
<td>1.6 ± 0.1</td>
<td>1.8</td>
<td>A,4, A,A, A,A,A,A,A,A,B</td>
<td>B,3, B,B, A,B,B,B,B,B</td>
</tr>
<tr>
<td>ST451</td>
<td>S</td>
<td>0.95</td>
<td>1.6 ± 0.3</td>
<td>nd</td>
<td>nd</td>
<td>-</td>
<td>A,4, A,A, A,A,A,A,B,B,A</td>
<td>B,3, B,B, A,B,B,B,B,B</td>
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<td>Average</td>
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<td></td>
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<tr>
<td>SD</td>
<td></td>
<td></td>
<td>0.99</td>
<td>2.3</td>
<td>2.9</td>
<td>1.2</td>
<td>1.9</td>
<td></td>
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<table>
<thead>
<tr>
<th>(B)</th>
<th>C/T ratio versus X-inactivation ratio</th>
<th>C/T ratio versus methylation ratio</th>
<th>Methylation ratio versus X- inactivation ratio</th>
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</thead>
<tbody>
<tr>
<td>Correlation</td>
<td>0.23</td>
<td>0.83</td>
<td>-0.30</td>
</tr>
<tr>
<td>(R^2)</td>
<td>0.08</td>
<td>0.69</td>
<td>0.09</td>
</tr>
<tr>
<td>Significance (two-tailed)</td>
<td>0.50</td>
<td>0.0008</td>
<td>0.39</td>
</tr>
</tbody>
</table>

(A) Column I: disease profile of individual sample. C, control; BP, bipolar; S, schizophrenia. Column II: allelic DNA ratios, normalized to 1.0. Column III: allele-specific mRNA ratios (mean = 2.3, SD = 1.0, \(n = 3\)) of \textit{MAO}A in 17 prefrontal cortex samples heterozygous for the marker SNP in exon 14 (except for Sample 451, which is heterozygous only for rs6323 in exon 8), normalized with DNA ratios. Average mRNA ratios and standard deviations are based on three independent measurements. Column IV: X-chromosome inactivation ratios measured as methylation at the androgen receptor gene locus using a polymorphic promoter repeat (ratio is high/low methylated allele. Phasing between the androgen receptor and \textit{MAO}A on the X-chromosome is unknown. na, homozygous for the repeat polymorphism; nd, the reaction was not done or failed. Column V: methylated \textit{MAO}A DNA expressed as a ratio of three over four repeat (results with \textit{SmaI}). Column VI: allelic C/T ratios adjusted for methylation by dividing C/T ratios by allele specific three/four-repeat methylation ratios. Only samples that were heterozygous at both polymorphisms were adjusted. Columns VII and VIII: haplotype assignments for the two alleles. Samples are heterozygous for the indicator SNP used here (in bold). ‘A’ denotes the major allele and ‘B’ the minor presented in order as shown in Figure 1. Three and four designate \(pVNTR\) repeats (no five repeats present). (Asterisk) Homozygous for the \(pVNTR\) and heterozygous for marker SNP.

(B) Pairwise Pearson correlations and \(P\)-values calculated for a three way comparison of \textit{MAO}A expression ratios (A, column III), X-inactivation ratios (A, column IV) and methylated \textit{MAO}A DNA ratios for the two alleles (three and four repeat) (column V).
indicates that the observed AEI of MAOA is independent of unequal X-inactivation. Allelic methylation ratios obtained with SmaI also correlated poorly with X-inactivation ($r = 0.05$).

**MAOA CpG methylation in comparison to AEI**

We next compared allele-specific CpG methylation with AEI ratios in samples heterozygous for both the marker SNPs and the pVNTR. Heterozygosity in the latter was needed because the methylation assay exploited the three and four repeats to distinguish between alleles. If CpG methylation of the MAOA promoter affects transcription, differences in methylation between alleles should result in AEI ratios distinct from unity. Indeed, when the MAOA three/four-repeat methylation ratios are plotted against the AEI ratios (Fig. 5B), a robust correlation is revealed (Pearson correlation $r = 0.83$, $P = 0.0008$ for SmaI; Pearson correlation $r = 0.73$, $P = 0.004$ for HhaI). This result indicates that methylation affects transcription and could account for 50–70% of the observed AEI ratios; however, other factors appear also to play a role and likely involve genetic polymorphisms.

The pVNTR itself contains CpG islands and is contiguous with the main CpG island of the MAOA promoter. Therefore, relative allelic methylation could have varied with the number of repeats in the pVNTR. Allele-specific methylation ratios in six females heterozygous for the pVNTR and homozygous for the marker SNP were determined from percent methylation of each allele (indicated by asterisk in Table 2). These ratios were distributed randomly and failed to correlate with the pVNTR genotype (data not shown). Therefore, CpG methylation appears to be independent of pVNTR genotype.

Measuring the ratio of methylated alleles does not provide information on the overall extent of methylation at the MAOA locus. Therefore, we analyzed the fraction of CpG methylation for each MAOA allele separately, using a newly developed method with HhaI (Table 2A). Of 12 male tissues analyzed, no significant methylation was detectable at the MAOA locus (data not shown). In contrast, the 35 female samples analyzed displayed variable levels of total methylation of both alleles, ranging from 2% to near complete methylation (mean of the two alleles). This is incompatible with a mechanism of X-inactivation, where one would expect a mean of 50% methylation between the two alleles in female tissues. Analysis of females heterozygous for the three- and four-repeat pVNTR again revealed variable extent of methylation for each of the two alleles.

**Association of MAOA genotypes with AEI**

Because promoter methylation cannot fully account for the observed AEI ratios, we used the allelic expression ratios shown in Table 1 as the phenotype to scan the MAOA gene locus for regions containing associated polymorphisms. If cis-acting polymorphisms contribute to the measured
AEI ratios, in addition to epigenetic factors, significant correlations should be detectable. For this analysis, it is helpful to know the phasing of each SNP and the pVNTR with the marker SNPs. Phasing between two polymorphisms can be ambiguous. For MAOA, however, accurate assignment of the haplotypes (inferred from all male and female samples) enabled us to relate allelic expression ratios (at the marker SNP) directly to the corresponding haplotypes for each of the female samples assayed for AEI (Table 1, columns VII and VIII). On the basis of these results, we conducted a single locus association test between SNP genotype and allelic expression in the female samples (Fig. 6). Alleles of each SNP were sorted according to whether they were found on the high or low expressing allele in the allele-specific mRNA analysis. The significance of the contribution of each SNP towards the high or low phenotype was determined. Four SNPs: rs6323 (exon 8), rs2205718, rs979606 and rs979605 were significantly associated with expression level, with Bonferroni corrected $P$-values less than 0.001. rs1801291 (the marker in exon 14) and rs3027407 (3'UTR) had $P$, 0.01, whereas the pVNTR and rs909525 were less strongly but still significantly associated with AEI (Bonferroni corrected $P < 0.05$). These significant associations indicate that genetic factors also contribute to the observed AEI, by affecting mRNA expression levels.

The highly significant association of the block of four SNPs between the exon 14 marker SNP and pVNTR strongly suggests that this region harbors a genetic variant contributing to AEI; however, unequal allelic methylation could confound this interpretation. The three samples homozygous for the pVNTR showing significant AEI (Table 1) support the notion that the functional SNP is placed elsewhere. However, these three samples did show some degree of overall methylation (Table 2); therefore, we cannot entirely exclude the possibility that these three AEI ratios were generated by unequal CpG methylation between the two alleles, in each case favoring expression from the major allele. We were unable to measure this because the allele-specific methylation assay depends on heterozygosity in the pVNTR.

The quantitative nature of AEI ratios as an immediate phenotype enables an estimate of the relative contributions of a genetic polymorphism to the observed AEI, compared to epigenetic factors in females. Assuming that MAOA methylation inhibits or interferes with transcription, we can account for the contribution of allelic methylation before linking the AEI to any underlying polymorphisms. Table 1 (column VI) shows the allelic mRNA ratios adjusted for methylation. Adjusted values were derived by dividing the mRNA C/T ratios by the methylation three/four-repeat ratios, approximating the contribution of a causative polymorphism toward AEI. The adjusted ratios indicate that the C allele is expressed ~1.9-fold higher than the T allele. This result strongly indicates that both epigenetic and genetic cis-acting factors are operative. Remarkably, methylation appears to account for at least one of the two samples with low C/T AEI ratios (Table 1).

**Relationship of overall mRNA levels with CpG methylation and genotype**

We measured total MAOA mRNA levels (relative to β-actin mRNA as the control) in all 105 samples, males and females.
Overall expression of MAOA mRNA was high, but fluctuated over a broad range. For males, the range in arbitrary units was 0.06–10.8 and for females 0.06–4.5 while cycle thresholds for β-actin mRNA varied much less (ΔΔCt < three cycles or 8-fold). However, no association was detectable between MAOA mRNA levels and genotype and/or CpG methylation status (Supplementary Material, Fig. S1). Possibly, any genetic influence (estimated from the AEI data to be ΔΔCt < 1.9-fold) on overall mRNA levels was too small relative to the large mRNA variability to yield significant associations. In contrast, some samples were nearly fully methylated in the promoter region. If CpG methylation would have completely suppressed transcription, we would expect a robust correlation between methylation and mRNA levels. As this was not the case ($R^2 = 0.06$), we propose that CpG methylation of the MAOA promoter does not abolish but modulates transcription, detectable only with the more sensitive AEI ratio measurements. Therefore, the large variation of mRNA levels was caused by trans-acting factors or postmortem degradation or both.

**Relationship of allelic measurements, total methylation and mRNA levels with disease**

This study was designed to compare functions of MAOA alleles within each individual. When sorting the sample populations into controls, bipolar and schizophrenia patients, we observed no statistically significant relationships between AEI, X-inactivation, MAOA locus methylation ratios and overall methylation in females with disease status of the samples ($P = 0.83, P = 0.09, P = 0.84$ and $P = 0.21$).
respectively for patients versus controls; \( t \)-test). The subject with the lowest AEI value (ST380, Table 1) was from the control group, indicating that the extreme AEI values are not directly linked to disease status. Similarly, we did not detect significant differences between RNA levels and patient status, as assayed by \( t \)-test in males and females separately (\( P = 0.65 \) for females, \( P = 0.73 \) for males). There also was no significant association between AEI, X-inactivation or \( MAOA \) locus methylation and other available demographic data such as history of smoking, alcohol or antipsychotic drug use, as assayed by recursive partitioning (HelixTree) (data not shown). These correlations, as well as genotype/haplotype associations, will be addressed in a study involving a larger sample cohort.

**In vitro analysis of transfected \( MAOA \) cDNA variants**

Whereas in vitro reporter gene assays suggest that the \( pVNTR \) affects \( MAOA \) expression (23), our genotype-AEI association analysis favored a region in the 3’ portion of \( MAOA \). Because the marker SNP itself (rs1801291) and two other SNPs (rs6323 and rs3027407) located in the cDNA are highly linked to AEI, those loci were promising candidates. We prepared human \( MAOA \) cDNA constructs containing the three SNPs together, as well as each SNP individually, and transfected each one along with the wild-type allele construct in cultured Chinese hamster ovary cells (hamster \( MAOA \) DNA and mRNA did not interfere with our assays). mRNA expression peaked at \( \sim 10 \) h after transfection and then declined over the next 2–3 days (Fig. 7). Mutant cDNA constructs were cotransfected with wild-type \( MAOA \) under the promoter of the vector, and plasmid DNA ratios and mRNA (after conversion to cDNA) ratios were determined at various time points (8, 24 and 48 h). DNA ratios remained constant and allelic mRNA ratios similarly did not deviate from DNA ratios at all time points measured (AEI: 8 h, 1.0 ± 0.0; 24 h, 1.0 ± 0.0; 48 h, 1.1 ± 0.1). Thus, AEI analysis failed to detect a difference in expression between any of the constructs and wild-type, suggesting that none of the three SNPs has an effect on \( MAOA \) expression from cDNA plasmids.

**DISCUSSION**

We have dissected the genetic and epigenetic mechanisms involved in the regulation of allelic expression of \( MAOA \), an X-linked gene implicated in multiple CNS disorders. This was performed on autopsy samples from prefrontal cortex, a brain region implicated in schizophrenia. The location of \( MAOA \) on the X-chromosome simplifies the haplotype analysis, because males have only a single X-chromosome. The haplotypes identified in this sample cohort are consistent with recently published or publicly available haplotype results (42,44). To identify \( cis \)-acting factors modulating gene expression and mRNA processing, we have measured allelic expression of \( MAOA \) mRNA in human brain tissues. The analysis of mRNA levels in autopsy tissues, in particular brain, has been problematic, whereas measuring allelic mRNA ratios appears to be more robust, under the assumption that each allele degrade at the same rate postmortem. (We cannot exclude with certainty that this is not the case; however, the functional correlations with methylation and genotype indicate that measured AEI ratios reflect \( in \) \( vivo \) events.)

The assay procedure was optimized and validated to yield precise and accurate results, building on experience with previous studies of AEI ratios in several genes expressed in brain tissues, such as \( hOPRM1 \) and \( hPEPT2 \) (39,45). Use of two marker SNPs provided an independent estimate of allelic mRNA ratios. The excellent agreement between the AEI ratios measured by the two assays supports the accuracy of the results. The precision with which AEI ratios can be measured enabled us in the present study to dissect epigenetic and genetic factors in mRNA expression and provide estimates of their relative contributions. This sets the stage for understanding the role of each in mental disorders.

The results demonstrate the presence of significant allele-specific differences (up to 4-fold) in mRNA expression of \( MAOA \) in females heterozygous for a marker SNP, with ratios ranging from 0.3 to 4.2. This range of AEI ratios suggested the likely presence of more than one \( cis \)-acting factor. Because a majority of ratios were \( > 1 \) (15/17), indicating a greater expression from the main wild-type variant, at
least one factor has to be preferentially associated with one allele over the other. With methylation apparently occurring at random between the two pVNTR alleles in females (determined in females homozygous for the marker SNPs), we surmise that a polymorphism (in strong LD with the marker SNPs) accounts for the bias of AEI ratios $>1$.

The main purpose of this study was to dissect epigenetic and genetic effects by comparing one allele against the other in a target tissue, which can provide important insight into the potential and limits of genetic association between MAOA and mental disorders. Because samples used in this study included only DNA and RNA extract (Stanley Foundation), we were unable to determine the relationship between genotype or methylation status and MAOA protein or enzyme activity. Moreover, in the present study, the cohort sizes were too small for association analysis between diseased subjects and controls. To address the biological relevance of differences in allelic expression, a future study reflecting MAOA protein activity will rely on whole tissue samples obtained from a sufficiently large population cohort of patients and controls.

Allelic mRNA expression can be affected by differences in regulatory factors or mRNA processing and epigenetic events between different tissues. We acknowledge that the DNA and RNA extracts obtained from defined brain regions contain many types of neurons and glia, so that the measured AEI ratios represent only an average for the region. Similar allelic expression ratios in various brain regions from the same individual indicated that variation between brain regions are small compared to inter-individual differences. A 4-fold difference in gene expression between alleles likely has physiological relevance. However, measured overall mRNA levels were too variable to permit linkage studies, whereas allelic expression ratios are robust because one allele serves as the control for the other in a target tissue. Therefore, the present study focuses on the mechanisms underlying differential expression from the two X-chromosomes in females.

CpG island methylation of MAOA and relationship to X-inactivation and AEI

We considered the possibility that promoter methylation could have contributed to the AEI observed for MAOA. In females, regulation of $>80\%$ of genes on the X-chromosome is commonly dominated by X-inactivation. Unequal X-inactivation could potentially cause AEI, which can remain constant between various tissues in the same individual (35). Unequal X-inactivation can occur by numerous mechanisms and is a common phenomenon; if present, a majority of X-linked genes would show AEI. However, recent studies indicate that both MAOA (37) and MAOB, positioned adjacent to MAOA, escape X-inactivation (46). To test this further, we measured X-inactivation ratios of 15 female samples using the androgen receptor locus (36). The androgen receptor X-inactivation ratios varied considerably between samples, as expected from previous results (35). If MAOA were to undergo methylation as part of X-inactivation, and CpG methylation would interfere with MAOA expression, we would expect the androgen receptor X-inactivation ratios to correlate with the AEI ratios of MAOA. However, these two events were not significantly correlated with each other. These results argue against skewed X-inactivation as a contributor to AEI of MAOA.

We next explored the possibility of methylation at the MAOA locus independent of, or not directly related to, X-inactivation. Gene silencing and imprinting by CpG island methylation play a general role in regulating gene expression (47,48) and, moreover, can result in allelic differences in transcription. We have measured allele-specific methylation, using methylation-sensitive restriction enzymes at two sites (Smal and HhaI), determining either allelic methylation ratios only or additionally the overall extent of methylation for both alleles. CpG methylation of MAOA occurs exclusively in females, where it ranged from 2\% to near complete methylation. Lack of correlation between the degree of MAOA methylation and overall mRNA expression in our RNA samples appears to be a result of a rather large variability in mRNA levels. Because MAOA mRNA levels are robustly expressed, even in samples with high methylation, any effect of CpG island methylation can only be partial at the most.

We also found that promoter methylation varied greatly between the three- and four-repeat alleles in the same individual. Because allele-specific methylation is significantly correlated with allele-specific mRNA expression (correlation with AEI ratios $R = 0.7 \sim 0.8$), these findings support the hypothesis that MAOA promoter methylation modulates transcription. MAOA promoter methylation could therefore represent a mechanism of partial dosage compensation independent of X-chromosome inactivation, which, however, is highly variable among individuals. How this affects biogenic amine metabolism in vivo remains to be studied, in conjunction with protein analyses.

Dissection of epigenetic and genetic factors

We next considered whether the proposed AEI effect of a cis-acting polymorphism can be distinguished from epigenetic effects. Assuming that methylation reduces transcription, dividing the measured AEI ratios with the methylation ratios should yield a rough estimate of the effect exerted only by cis-acting polymorphism(s). The mean of the adjusted AEI ratios did not differ substantially from the mean of the measured AEI ratios, consistent with the notion that methylation is random between alleles. More importantly, the adjusted AEI ratios fell in a more narrow range ($2.3 \sim 1.0$ before and $1.9 \pm 0.5$ after the adjustment) (Table 1), suggesting that this ratio reflects a potential cis-acting polymorphism more accurately. Remarkably, allelic methylation differences appeared to account for the sample with the lowest AEI ratios (0.33), owing to high methylation of the main wild-type allele. Because ratios are not linearly related to expression activity and moreover methylation appears to modulate rather than abolish transcription, these estimates are only approximations. Nevertheless, these results taken together support a contribution from genetic factors of $\sim 2$-fold in regulating MAOA expression, which is superimposed on variable changes afforded by CpG methylation. We conclude that both genetic and epigenetic factors contribute to nearly similar extents to variable mRNA expression in females. However, in males, methylation was not observed.
and only genetic factors could play a role. Genetic association studies need to reflect these relationships, with clear differences in gene regulation between males and females. This is consistent with large sex differences in susceptibility and presentation of mental disorders (49). The impact of these regulatory events on overall MAOA protein function and clinical relevance will be studied separately.

Search for the functional polymorphism(s)

Previous in vitro studies have associated the four-repeat pVNTR with higher levels of transcription than the three repeats (23). Accurate inference of the haplotypes enabled us to relate AEI ratios directly to specific alleles of the pVNTR and all SNPs in female samples heterogeneous for the marker SNPs. In most samples, the main wild-type marker allele was linked to the four repeats, which was associated with higher mRNA expression than the three repeats in a majority of samples. This result is consistent with previous in vitro data that the four-repeat pVNTR causes higher expression than the three repeat (23). However, these in vitro results do not assure that the pVNTR has the same influence in human brain tissues. Moreover, the pVNTR is in strong LD over a large region of the MAOA gene locus, raising the question which domain contains the functional polymorphism. The pVNTR cannot account for all of our observed allelic expression ratios, because the presence of marked AEI in three tissues homozygous for the pVNTR resulted in considerably stronger association of the AEI phenotype to the 3' region containing both marker SNPs, including several completely linked SNPs in the same haplotype block (Fig. 6). However, this analysis is confounded by allelic methylation differences. Because we were unable thus far to measure allele-specific CpG methylation in samples homozygous for the pVNTR, the contribution of methylation in these samples could not be evaluated in this study. Nevertheless, the results favor the presence of a functional polymorphism in the marker SNP region. In contrast, the data are also consistent with the presence of more than one functional polymorphism, including the pVNTR.

Because of the strong association of AEI with the haplotype block containing the marker SNPs, we tested the two marker SNPs and one SNP in the 3'-UTR (rs1801291, rs6323 and rs3027407), all located in the cDNA, in cell culture. In this approach, one cotransfects equal amounts of wild-type and variant cDNA in an expression vector, followed by AEI analysis of the plasmid DNA and respective mRNA at different time points. The transfection conditions effectively remove epigenetic factors from playing a role. Lack of any detectable AEI for the 3 SNPs, either tested alone or linked together in the same vector, demonstrated that none of the tested SNPs was functional when analyzed in the context of intronless cDNA constructs. Although this excludes a mechanism involving mature mRNA processing and turnover, as we have observed for OPRM1 and MDR1 (41), we cannot exclude possible effects occurring at the level premature hRNA (maturation and splicing). Moreover, there are other highly linked intronic SNPs in the haplotype block that could be contributing to AEI but cannot be studied with this cDNA approach. A larger cohort of heterozygous samples and further in vitro studies will be required to determine the identity of the functional cis-acting factor.

The results of this study have implications for future clinical genetic association studies, providing evidence for the presence of genetic factors affecting mRNA expression in human brain tissues, which is further modulated by CpG island methylation in female subjects. The use of the pVNTR alone in clinical studies may not accurately represent the true genetic variability in a subject cohort. The finding that promoter methylation affects allelic MAOA transcription and varies considerably between females indicates that epigenetic factors also play a significant role in modulating biogenic amine tone in the CNS of female subjects, and hence mental activity and disorders. However, we have detected no methylation in male brain tissues. This study is the first to use AEI analysis for dissecting genetic and epigenetic factors in human brain tissue. However, it remains to be determined how differences in allelic expression of MAOA mRNA affect the functional expression of the enzyme in vivo. Future work will address the biological and clinical significance of these findings.

MATERIALS AND METHODS

Description of the DNA, mRNA and tissue samples

Postmortem brain tissue, mRNA and DNA was donated by The Stanley Medical Research Institute’s brain collection. We obtained genomic DNA and total mRNA extracted from the prefrontal cortex of 105 individuals previously diagnosed with bipolar disorder or schizophrenia (35 each) and 35 controls. Extracted RNA is from Brodmann’s area 46 (dorsolateral prefrontal cortex). Additional brain tissue from four of the individuals analyzed above was obtained from the following regions: cerebellum, parietal lobe and occipital lobe. Average postmortem interval for these samples was 32.9 ± 16.0 h. Additional demographic data available for these samples included age, sex, cause of death and history of smoking, alcohol use and lifetime use of antipsychotic medication.

DNA genotyping using GC clamp and differential melting curve analysis

A total of 13 polymorphisms were genotyped spanning the MAOA gene (Fig. 1 and Supplementary Material, Table S3). Ten SNPs were genotyped by allele-specific PCR, with primer for one allele containing a GC-rich sequence at the 5' end. Allele discrimination was achieved with melting curve analysis (50). Primers for each SNP are listed in Supplementary Material, Table S3.

DNA genotyping of the variable nucleotide tandem repeat (pVNTR)

PCR amplification of the promoter VNTR followed a protocol from Sabol et al. (23), with modifications. Primers are listed in Supplementary Material, Table S3. The forward primer was labeled with a fluorescent dye for analysis of the PCR product on an Applied Biosystems 3730 sequence sequencer,
separating three, four and five repeats (3.5 repeats were not encountered and the five-repeat allele was found in two male subjects and thus not analyzed for AEI). PCR cycling conditions were as follows: 1 min at 95 °C, 1 min at 62 °C, 1 min at 72 °C for 35 cycles.

**Haplotype analysis**

One hundred and five samples were genotyped. Sixty-nine male samples (containing only one MAOA allele) allowed unambiguous assignments of haplotypes. For the female subjects, haplotypes and their frequencies were assigned by an estimation maximization algorithm [HelixTree Golden Helix software package (51)]. Together with haplotype information from males, this provided unambiguous assignments in essentially all female cases. Importantly, haplotype assignments in female enabled the linkage of the pVNTR (three and four repeats) with the two alleles of the marker SNPs.

cDNA synthesis

cDNA was synthesized from 105 prefrontal cortex mRNA samples. Approximately 1 μg total RNA was digested with 2 U of DNase I in appropriate buffer for 20 min at 37 °C. Enzyme was inactivated with DNA Free slurry (Ambion). RNA was transferred to new tubes containing 1 μl 10 mm dNTP, 1 μl 0.5 mg/ml oligo-dT and 0.5 μl 2 μM gene specific primers (MAOA SNaPshot and β-actin) (Supplementary Material, Table S3). In addition to oligo-dT, gene-specific primers for cDNA synthesis targeting a region just downstream of the marker SNP was found to enhance significantly the cDNA yield for the region of interest as fragmentation of mRNA renders oligo-dT priming less effective. Incubation was at 65 °C for 5 min. Four microliters of 5× first-strand synthesis buffer (Invitrogen), 4 μl RNase-free water and 1 μl RNase inhibitor were added to each reaction and incubated at 42 °C for 2 min. One milliliter of SuperScript II (Invitrogen) was added to reaction and incubated at 42 °C for 50 min.

**Statistical analyses**

Alleles from females heterozygous for the indicator SNP were sorted into two groups based on whether they were on the high or low expressing allele. HelixTree software was used to test the significance of each genotype to its presence on the high or low expressing allele. Expression (high or low) was used as the dependent variable in a tree analysis, and a two-loci P-value plot was created for every possible combination of SNPs. The single locus associations included in the analysis were taken for the plot (Fig. 6). The recursive partitioning function of the HelixTree software package was used to test for significant associations of AEI results with patient demographics. Student’ t-tests were performed to assay for significant differences in AEI, X-inactivation and MAOA locus methylation ratios, as well as total MAOA locus methylation and MAOA mRNA expression levels when samples were sorted by disease profile or case versus control.

**LD analysis**

Pair-wise LD was determined for each combination of SNP pairs using HelixTree software (Golden Helix, Inc., Bozeman, MT, USA). D’, an alternative measure of LD, (51) is calculated by the HelixTree software and included in Supplementary Material, Table S1. Because there is no mechanism in the HelixTree software to recognize hemizygous genotypes, to accurately determine allele frequencies, correct LD and haplotype frequencies, male genotype data were treated as homozygous (instead of hemizygous), whereas female genotype data were doubled (to account for doubling of male data).

**Determination of allelic ratios of DNA and mRNA using a marker SNP**

Allele-specific mRNA analysis was performed after PCR amplification of DNA and cDNA, using a primer extension assay based on SNaPshot™ (Applied Biosystems, Foster City, CA, USA), as described elsewhere (45). The marker SNPs we employed were a synonymous C/T SNP in exon 14 (rs1801291) and a synonymous T/G SNP in exon 8 (rs6323). Use of two marker SNPs yielded two independent assay procedures for comparison and validation. Primers used are listed in Supplementary Material, Table S3. For each sample, DNA ratios were measured in duplicate, and the mean (± SD) calculated across all samples, assuming that the allelic ratio is unity in female subjects. No single sample deviated by more than 3 standard deviations from the mean. Messenger RNA allelic ratios were measured at least three times for each sample, enabling an assessment for each individual sample, whether or not the cDNA ratios deviated from DNA ratios. We also performed a standard curve experiment for marker SNP rs1801291, using plasmid DNA at the following mixtures: 30, 40, 50, 60 and 70% of one allele relative to the other, at three dilutions (1:0, 1:2 and 1:4). R² for the three dilutions against the standard ranged from 0.98 to 0.97.

**Plasmid construction**

The MAOA cDNA clone in the vector pCMV6-XL4 was obtained from Origene®, containing the major allele at all three SNP positions. We constructed four variants by site-directed mutagenesis, containing each SNP variant singly or in combination of all of them. Overlapping primers containing the SNP allele were designed for each SNP (rs6323, rs1801291 and rs3027407).

| rs6323 Mut F GAGAGAAACCCGTTAATTTCGGCGGCTTCCATGGGAGCTG |
| rs6323 Mut R CAGCTTCCATTGGAAGCGTGAATTAACCTGTTCTCTC |
| rs1801291 Mut F CCGAGAAAGATATGCGGTACAAAGAAATAGGGC |
| rs1801291 Mut R CGTCTTGGATCCAGGTTCAGGACATGACTAAACAGG |
| rs3027407 Mut FGACTTTATTTGAGACTATCAAACAGGAAGAAATAGGGC |
| rs3027407 Mut RGCCCTATTTCTCTCTGTGTGATACTTCAACAAATAACAGTC |
Stratagene Quick Change® kit was employed to create each SNP. About 50 μl PCR reactions containing primers, reaction buffer, dNTP mix, 50 ng of template and Pfu enzyme were cycled 18 times with the following parameters: 95°C for 50 s, 60°C for 50 s, 68°C for 9 min. The reaction was treated with DpnI (20 U) at 37°C for 1 h. Reactions were transformed into competent XL10 Gold (Stratagene). Plasmids were purified from colonies and sequenced to identify site-directed mutations.

Cell culture and transient transfection
CHO-K1 cells were cultured in F-12 nutrient medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO2. Twenty-four hours before transfection, cells were seeded into six or 12-well dishes. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer’s protocol, using equal amounts of the plasmid carrying the wild-type cDNA and of the variant cDNA. Cells were collected 8, 24 and 48 h after treatment. For plasmid DNA preparation, cells were trypsinized and collected. Plasmid DNA was prepared using a Qiagen DNA mini-prep kit. For RNA preparation, cells were lysed with TRIzol reagent and prepared as described above. MAOA plasmid and mRNA were analyzed by SnPshot after conversion to cDNA. Control assays without transfection yielded no detectable amounts of hamster genomic DNA and mRNA.

X-chromosome inactivation assay
The method employed to measure the X-chromosome inactivation ratio was modified from a previously described procedure (36). Gene-specific primers (Supplementary Material, Table S3) were used to amplify a polymorphic region of the androgen receptor gene in genomic DNA. The DNA was either untreated or had previously been digested overnight with HhaI methyl-sensitive restriction endonuclease. The forward primer was labeled with a fluorescent dye so that the products could be visualized on an ABI 3730 analyzer. PCR conditions were as follows: 30 cycles of 94°C for 1 min, 68°C for 1 min and 72°C for 1 min. Peak areas for both amplification products from each sample were determined and ratios were calculated. Each sample was assayed three times.

MAOA methylation assay I
The method used to measure differential methylation at the MAOA locus was modified from an X-inactivation protocol by Hendriks et al. (52), but instead of amplifying a polymorphic dinucleotide region in intron 1, the pVNTR was amplified. Genomic DNA was digested overnight at 30°C with 5 U of SmaI in appropriate buffer. The forward, fluorescently labeled primer used to genotype the pVNTR (Supplementary Material, Table S3) was combined with a reverse primer (Supplementary Material, Table S3) just downstream of a SmaI site shown to be methylated (52). PCR conditions were as follows: 1 min at 95°C, 1 min at 62°C, 2 min at 72°C for 35 cycles. Eight milliliters of each PCR reaction was digested with 10 U of SsrI enzyme overnight at 37°C, in appropriate buffer, to short the PCR product so that it would be visible by capillary electrophoresis.

MAOA methylation assay II
This method quantitates both total and allelic MAOA methylation. First is divided into two equal samples, each consisting of 20–120 ng of DNA in 1× HhaI digestion buffer. One sample is digested with 10 U of the methylation sensitive restriction enzyme (HhaI). Digested and undigested DNA were tagged with two different address primers in a quantitative pre-amplification step, using a common 3’ MAOA reverse primer (downstream of the HhaI site) and two distinct forward primers. Each forward primer targets the same MAOA sequence located upstream of the pVNTR, but is tagged with a different address at its 5’ end. Specific, quantitative pre-amplification was achieved using 5 nM primer concentrations for eight cycles of denaturation at 95°C for 30 s, then annealing/extension at 60°C for 2 h. After separate PCR pre-amplifications, equal volumes of the two samples are mixed. One microliter of the combined pre-amplified DNA is then competitively amplified for 25 PCR cycles using 300 nM concentrations of the common 3’ primer and FAM or HEX fluorescently labeled 5’ primers targeting the tag sequence in the forward primer (representing cut and uncut DNA). Using the ABI 3730 instrument, a portion of the resulting PCR product was analyzed by capillary electrophoresis. One obtains two fluorescent peaks which are proportional to the amount of amplifiable HhaI-pretreated and untreated DNA. The ratio of the peaks is used to calculate the percent methylation. For samples heterozygous for the pVNTR, two sets of peaks were obtained representing cut and uncut DNA from the three- and four-repeat alleles (in female carriers), whereas for all other samples (including males), we determine the level of overall methylation in percent.

To achieve equal PCR amplification efficiency for both alleles, we tested several combinations of address primers to arrive at the optimal pair for MAOA. To construct standard curves, pooled uncut DNA was divided into portions and carried through the entire procedure without HhaI digestion of any one sample. FAM and HEX peak areas were obtained from the ABI 3730 and the ratios plotted, yielding a linear standard curve ($r > 0.99$).

Quantitative mRNA analysis by RT–PCR
PCR was performed on cDNA samples using SYBR green dye on an ABI 7000 sequence detection system (Applied Biosystems). PCR (21 μl) was performed in standard 96-well plates with heat-activated Taq DNA polymerase and SYBR Green. SYBR green fluorescence was measured after each cycle. After full amplification, the fluorescence intensity of the PCR product was measured from 60–92°C at a temperature gradient of 0.2°C/min to control for spurious amplification with different melting curves. Forward and reverse primers listed in Supplementary Material, Table S3 were used to amplify MAOA and β-actin transcripts. Each reaction was replicated once. Cycle thresholds ($C_T$), at which an increase in reporter fluorescence above a baseline signal is
detected, were determined with ABI 7000 SDS software. Replicate cycle thresholds were averaged, and MAOA expression levels in arbitrary units were calculated by subtracting the \( \beta\)-actin \( C_t \) from the MAOA \( C_t \) to get a \( \Delta C_t \). Arbitrary units for each sample = 100/\( \Delta C_t \).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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**Conflict of Interest statement.** None declared.

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