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MAOA EXPRESSION PREDICTS VULNERABILITY FOR ALCOHOL USE

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Abstract

The role of the monoamines dopamine (DA) and serotonin (5HT) and the monoamine-metabolizing enzyme monoamine oxidase A (*MAOA*) have been repeatedly implicated in studies of alcohol use and dependence. Genetic investigations of *MAOA* have yielded conflicting associations between a common polymorphism (*MAOA-LPR*) and risk for alcohol abuse. The present study provides direct comparison of tissue-specific *MAOA* expression and the level of alcohol consumption. We analyzed rhesus macaque *MAOA (*rh*MAOA)* expression in blood from males before and after 12-months of alcohol self-administration. In addition, nucleus accumbens core (NAc core) and cerebrospinal fluid (CSF) were collected from alcohol-access and control (no alcohol access) subjects at the 12-month time point for comparison. The rh*MAOA* expression level in the blood of alcohol-naïve subjects was negatively correlated with subsequent alcohol

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AUTHORS CONTRIBUTION

BF, SRJ, KAG and RCJ were responsible for the study concept and design. KAG provided the animal samples. CH performed the tissue necropsies. SG provided the alcohol and water intake data. RCJ performed DNA, RNA isolation, real-time PCR analysis and bisulfite sequencing. RL performed the initial bisulfite pyrosequencing analysis. LJW performed the informatics analysis of the bisulfite data. BP contributed statistical analysis. RCJ, JL, LJW, RL and BF assisted with data analysis and interpretation of findings. RCJ and BF drafted the manuscript. SRJ, RL, GW, KAG and BP provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved the final version for publication.

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All of the authors declare they do not have conflicts of interest.

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consumption level. The mRNA expression was independent of rh*MAOA-LPR* genotype and global promoter methylation. After 12 months of alcohol use, blood rh*MAOA* expression had decreased in an alcohol dose-dependent manner. Also after 12 months, rh*MAOA* expression in the NAc core was significantly lower in the heavy drinkers, as compared to control subjects. The CSF measured higher levels of DA and lower DOPAC/DA ratios amongst the heavy drinkers at the same time point. These results provide novel evidence that blood *MAOA* expression predicts alcohol consumption and that heavy alcohol use is linked to low *MAOA* expression in both the blood and NAc core. Together, the findings suggest a mechanistic link between dampened *MAOA* expression, elevated DA and alcohol abuse.

Keywords

alcohol; dopamine; *MAOA*; *MAOA-LPR*; methylation; nucleus accumbens; non-human primate

INTRODUCTION

Sustained alcohol use can lead to abuse and dependence, collectively known as alcohol use disorders (AUDs), and ultimately to life-threatening medical, psychological and social consequences[1]. Risk for hazardous drinking has been ascribed to an array of genetic[2, 3] and environmental factors[4, 5], while the transition to dependence involves neuronal adaptations to alcohol exposure that evolve over time[6, 7]. These adaptations translate to behavioral changes that persist long after alcohol abstinence, contributing to the high rates of relapse. Ethanol-induced release of serotonin (5HT) and dopamine (DA) in the nucleus accumbens (NAc)[8, 9] and ventral tegmental area of the mesoaccumbens reward pathway have been implicated in the mechanisms underlying development of alcohol dependence[10–12] and relapse[13].

The monoamine oxidase A (MAOA) enzyme metabolizes the monoamines 5HT, DA and norepinephrine, and therefore its activity has direct implications on the function of the reward system. A promoter-linked length polymorphism upstream of the *MAOA* gene, *MAOA-LPR*[14], has been widely associated with numerous neuropsychiatric disorders, including alcoholism[15–18]. It has been suggested that the *MAOA-LPR* promoter alleles may modulate gene expression[14], and correspondingly, the downstream levels of these key neurotransmitters. In addition, Caspi and colleagues[19] highlighted the critical role of the environment when they reported that an association between the low activity *MAOA-LPR* allele with impulsive behaviors was contingent upon exposure to developmental stressors. However, years of research have yielded conflicting results as to the specific *MAOA-LPR* alleles that contribute to risk for psychiatric disorders[20–22] and have also reinforced the possibility of an interaction between genotype and adverse life history[20–23]. Furthermore, recent studies directly exploring MAOA abundance have detected a lack of association between the *MAOA-LPR* genotype and postmortem[24] or *in vivo* brain MAOA protein levels[25, 26]. These findings suggest that *MAOA-LPR* genotype has little effect in modulating brain MAOA levels or that its effect is dependent on other factors.

Epigenetic regulatory mechanisms enable the adaptive and precise modulation of gene expression. Among them, DNA methylation involves the addition of a methyl group to the cytosine residue within CpG dinucleotides. Hypermethylation in regulatory regions can attenuate gene expression, while DNA methylation within the gene-body has been linked to tissue- and cell-specific gene regulation[27]. Several studies have identified DNA methylation as a potential molecular link between adverse life history events and risk for neuropsychiatric disorders, including addiction[28–33]. A study of *MAOA* promoter methylation in women identified an association between methylation level and alcohol dependence[16]. In addition, recent studies in healthy males demonstrated that variability in *MAOA* methylation detected in the blood was correlated with MAOA protein activity in the brain indicating that blood-based methylation levels may serve as a useful biomarker for neural MAOA measures[25, 26].

The discovery of human genetic and epigenetic liabilities influencing risk for alcohol dependence relies on the use of retrospective and self-reported data, which may be biased or incorrect. Furthermore, significant findings obtained from retrospective studies of alcoholic and healthy populations are limited in their ability to distinguish preexisting conditions from alcohol-induced effects. These limitations can be addressed by using animal models for the study of long-term alcohol self-administration, providing highly accurate alcohol consumption data and the ability to collect blood before and after alcohol use. A well-established model of oral alcohol self-administration in macaques demonstrates a wide range of voluntary alcohol consumption[34–36] and recapitulates a variety of alcohol seeking patterns observed among humans. In addition, macaques have the advantage of sharing similar genetic and epigenetic composition, anatomy, physiology, and endocrine characteristics with humans[37, 38]. Some functionally similar gene polymorphisms, including *MAOA-LPR,* are also shared in both species. As with humans, specific rhesus *MAOA-LPR* alleles have been associated with high levels of alcohol consumption[37]. Thus the NHP alcohol self-administration model provides a relevant and unique opportunity to investigate the genetic, epigenetic and environmental factors contributing to the vulnerability, progression and pathogenesis of AUDs[39].

In this study we test the hypothesis that rhesus *MAOA* (rh*MAOA*) expression level in the blood of alcohol-naïve subjects is a predictive biomarker of risk for alcohol abuse. We investigated potential genetic and epigenetic mechanisms that might contribute to the initial population variance in gene expression. We then determined the within-individual changes in blood *rhMAOA* expression and DNA methylation following chronic alcohol use. To explore the association between neural rh*MAOA* expression levels and alcohol consumption we compared the mRNA levels in the NAc core of control subjects (no-alcohol access) and animals that consumed alcohol for 12 months. Finally, we considered how the NAc core rh*MAOA* expression relates to monoamine levels by measuring DA, 5HT and their metabolites in the cerebrospinal fluid (CSF) after 12 months of alcohol self-administration, as the CSF temporarily stores these monoamines and their metabolites[40].

MATERIALS AND METHODS

Subjects

Male rhesus macaques (n=38, *Macaca mulatta*) differing slightly in age, late adolescents and young adults (3.0–5.0 and 5.1–7.0 years at the start of the 12 month open access period, respectively; n=19 per age group), were included in this study. All of the monkeys were born and reared at the Oregon National Primate Research Center (ONPRC) with their mothers until 2–3 years of age. All subjects were initially selected to minimize relatedness. The average kinship coefficient of all subjects in the study was 0.003. Ten of the animals were assigned as controls. The control subjects were selected by matching on age and weight, and then kinship. The mean kinship coefficient did not exceed 0.003 in either the control or ethanol access groups. Controls were housed in the same housing rooms as the experimental subjects and underwent the same training for awake blood draws, medical check-ups and MRI imaging. Controls were also placed on the same diet, timing and order of experimental phases and had equal experience with the research technicians. However, control monkeys did not receive access to alcohol. Instead, at baseline, controls were yoked to a future ethanol monkey based on weight, and received a quantity of 10% maltose-dextrin solution matched in calories to the previous day's intake of their yoked ethanol monkey. Monkeys were individually housed and ethanol self-administration was induced using schedule-induced polydipsia as previously described[34, 35]. During the following 12 months, subjects had open access to 4% alcohol and water (ethanol subjects) or water only (control subjects). The average g/kg body weight per day (g/kg/day) was based upon all ethanol or water consumed during the 12 months of open access. For the ethanol self-administration data, technicians were not blind to the condition (ethanol or control) of the monkeys, however the intake data were collected and recorded in an automated fashion by computer and analyzed by individuals who did not interact with or know the drinking status of the monkeys. All of the animal procedures used in this study were approved by the ONPRC IACUC and were performed in accordance with the NIH and the National Resource Council's *Guide for the Care and Use of Laboratory Animals.*

Genomic DNA isolation

Blood was collected from awake monkeys[34] prior to (0 months) and after 12 months of open alcohol access. Genomic DNA (gDNA) was extracted using the 5 PRIME ArchivePure DNA purification kit (Thermo Scientific, Pittsburgh, PA) following the manufacturer's protocol.

After 12 months of open access to alcohol or water, a detailed necropsy protocol was used to systematically collect tissues from all subjects[41]. Animals were sedated (ketamine 15mg/kg), CSF was collected, and then the animals were brought into a surgical plane of anesthesia with intravenous administration of sodium pentobarbital (30–50 mg/kg, i.v.). Following extraction, the entire brain was blocked or sectioned into slides that were fresh frozen and stored at −80° C. The NAc core was later dissected from frozen brain slices. The typical postmortem interval for brain extractions was < 5 minutes. Genomic DNA and RNA were extracted using the All Prep DNA/RNA/miRNA Universal kit (Qiagen Sciences Inc, Germantown, MD) following the manufacturer's instructions.

Genotyping

The rh*MAOA-LPR* fragment size analysis was performed as previously described[42]. The rh*MAOA-LPR* primers (Supplementary Table 1) generated 206 bp, 224 bp and 242 bp products for the 5, 6 and 7 copy number alleles, respectively (Life Technologies, Carlsbad, CA). Since the rh*MAOA* gene is located on the X chromosome, there was only a single copy of rh*MAOA* detected in each of the male subjects.

Bisulfite sequencing

Correction for bisulfite-converted PCR bias was carried out as described by Moskalev et al.[43]. Methylated and unmethylated human gDNAs (Zymo Research, Irvine, CA) were bisulfite-converted using the EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA), according to the manufacturer's instructions. The bisulfite converted DNAs were combined to create a series of methylation rate standards ranging from 100% to 0% methylated. gDNA (500ng) from NHP blood and NAc core was bisulfite converted following the same protocol. Primers were designed to amplify a 223 bp region of the *MAOA* promoter in both humans and rhesus macaques (Supplementary Table 1). Amplification was carried out using a C1000 Thermal Cycler (Bio-Rad, Hercules, CA) with 20 ng of bisulfite-treated DNA per PCR reaction. The amplification conditions were as follows: Phase 1: 10 cycles of 94°C for 30sec., 68°C for 1min., with a touchdown decrease of 1°C per cycle. Phase 2: 28 cycles of 94°C for 30sec. and 58°C for 45sec. Sequencing libraries were prepared using the NETflex DNA Sequencing Kit (BIOO Scientific, Austin, TX) according to manufacturer's instructions. The libraries were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and were normalized to 2nM with 10 mM Tris-HCl. The libraries were then pooled and sequenced on a MiSeq (Illumina, Inc. San Diego, CA) by the Molecular & Cellular Biology Core, (ONPRC, Beaverton, OR) to generate 250base, paired-end reads. The reads were trimmed using Trim Galore[44] and aligned to the reference genome using Bismark[45]. M-bias plots were then generated[46], and reads were trimmed further as needed. Bismark alignment data was converted to CpG methylation rate using the Bismark methylation extractor[45] and custom scripts.

To assess the linearity of the PCR amplification we plotted the observed versus expected methylation rates obtained from the methylated DNA standard dilution series[43], producing a good linear fit (R^2=0.98).

RNA isolation and reverse transcription

RNA was extracted from blood collected in PAXgene Blood RNA tubes (PreAnalytix GmbH, Qiagen, Germantown, MD) and stored at −80°C. Total RNA was extracted using the PAXgene Blood mRNA Kit (PreAnalytix GmbH, Qiagen Sciences Inc, Germantown, MD) following the manufacturer's protocol.

The RNA from the NAc core was extracted using the All Prep DNA/RNA/miRNA Universal kit (Qiagen Sciences, Inc., Germantown, MD) following the manufacturer's instructions.

RNA quantity and quality was evaluated on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). SuperScript III First-strand Synthesis System (Life Technologies,

Carlsbad, CA) was used to reverse-transcribe 500 ng of each RNA sample following the manufacturer's instructions.

Relative quantitative real-time PCR (qRT-PCR)

The *β-Actin* gene was selected as the endogenous control since it was equally expressed in all sample types. Standard curves for each gene (rh*β-Actin* and rh*MAOA*), were prepared using serial dilutions of cDNA (from 25 ng to 0.125 ng). Real-time PCR was performed using a QuantStudio 12K Flex (Life Technologies, Carlsbad, CA) with the TaqMan Gene Expression Kit. Each reaction (10μl) contained 5μl of the 2x TaqMan Gene Expression Master Mix (Life Technologies, Carlsbad, CA) and 1μl (12.5ng) of cDNA. For rh*β-Actin*, we used 300nM amplification primers with 250nM of rh*β-Actin* probe. For rh*MAOA*, 0.5μl of the custom 20x TaqMan Gene Expression reagents (Supplementary Table 2) was added. The reactions were incubated at 50°C for 2 min., and 95°C for 10 min., followed by 40 cycles of 15 sec at 95°C and 1 min. at 60°C. The QuantStudio 12K Flex system software was used to calculate Ct values. Data from the dilution series were used to generate standard curves for both the rh*β-Actin* control and target. Target values were normalized using the endogenous control. Sample 10086 was used as the calibrator to calculate the fold change in expression of other samples. The normalized quotient at 12 months was divided by the quotient at 0 months to determine the fold change. For each experimental sample, the amplification curve for rh*β-Actin* and rh*MAOA* fell within the detectable range of the control titration curves. Two sets of triplicate assays were performed for each sample, and two investigators reviewed the results.

CSF content of monoamines and metabolites

The NAc core was unavailable to analyze for tissue content of monoamines and their metabolites. As the NAc core is one of the main targets of DA and 5HT within the brain, and the CSF acts as a temporary reservoir of monoamines and their metabolites[40], we used the CSF to investigate the effects of long-term (12 months) alcohol consumption on monoamine levels in the brain. CSF samples were collected at the same time point as the NAc core tissue, after 12 months of open access to alcohol (ethanol subjects) or water only (control subjects). No CSF sample was available from early time points for comparison.

The CSF content of monoamines and their metabolites was measured as previously described[47] with minor modifications. Briefly, the CSF was centrifuged and the supernatants were analyzed for DA, 5HT and their metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5HIAA) using high-performance liquid chromatography (HPLC) coupled to electrochemical detection at +220 mV (ESA Inc., Chelmsford, MA) and separated on a Luna 100 × 3.0 mm C_{18} 3 μm HPLC column (Phenomenex, Torrance, CA). The mobile phase consisted of 50 mM citric acid, 90 mM sodium dihydrogen phosphate, 1.7–2.0 mM 1-octanesulfonic acid, 50 μM EDTA, 10–12% acetonitrile and 0.3% triethylamine in a volume of 1 L (pH 3.0). Analytes were quantified using Chromeleon v 6.8 software (eDAQ Inc, Colorado Spring, CO) and a calibration curve. Samples were assayed in triplicate and were reviewed by two investigators.

Statistical Analysis

The exploratory nature of the present study limited our ability to estimate a priori the sample size needed to detect the effects investigated in this study. Therefore, we used both a longitudinal study design (blood) and a group comparison based on alcohol consumption level (NAc core, CSF) to maximize the opportunity to detect significant effects. Not all tissues were available from all study subjects at all-time points, and thus the number of subjects per tissue comparison varies slightly. Only animals that had at least two tissues available (blood, NAc or CSF) were included in this study. The maximum number of samples available was used for each analysis.

The Shapiro-Wilk test (appropriate for small sample sizes) was used to assess the normality of the continuous variables (Ave. Ethanol intake, global CpG methylation (Blood 0m, 12m, NAc core 12m), mRNA expression levels (Blood 0m, 12m, NAc core 12m), DA, DOPAC, HVA, 5HT, 5HIAA, DOPAC/DA, HVA/DA, 5HIAA/5HT). All of them followed a normal distribution.

Repeated measures of ANOVA was used to compare longitudinal changes in the relative rh*MAOA* expression in blood. The ethanol group (LMD, HD) was used as between group factor, and the time point (0m, 12m) as within group factor. Unstructured covariance was assumed to be a within subject covariance/correlation structure. Tukey-Kramer correction for the multiple comparisons was used to correct the overall type I error rate. In addition, the mRNA expression change between 0 and 12 months was compared using a contrast test.

One-way ANOVA was used compare the difference in the NAc core mRNA relative expression and the 5HIAA/5HT between controls and ethanol drinkers (LMD and HD). Prior to applying one-way ANOVA, Levene's test was used to test homogeneous variance assumption for parametric methods (NAc core mRNA: p=0.1492; 5HIAA/5HT: p=0.3911). Because of the heterogeneous variance difference between controls and ethanol drinkers (LMD and HD; p=0.0014, Levene's test for equal variance) we used the nonparametric Kruskal-Wallis test to assess the DOPAC/DA levels between controls and low-moderate alcohol drinkers (LMD) and heavy alcohol drinkers (HD). Bonferroni correction for the multiple comparisons was used to correct the overall type I error rate.

The unpaired Student's t-test for independent samples was used to compare the NAc core rh*MAOA* expression levels between controls and ethanol drinkers.

Pearson correlation analysis was used to explore associations between rh*MAOA* mRNA (blood and NAc core), global average methylation (blood and NAc core), average ethanol or water (g/kg/day) consumption and CSF neurotransmitter levels.

A multiple regression model with stepwise selection was used to test whether the inclusion of other independent variables (age (continuous), *MAOA-LPR* genotype (discrete)) along with regional methylation values improved the accuracy in predicting values for the dependent variables (rh*MAOA* mRNA levels, average daily ethanol intake).

All analyses were performed using IBM SPSS Statistics 22 (New York, NY) and SAS 9.4 (Cary, NC) with two sided p-values <0.05.

RESULTS

Ethanol drinking patterns

As previously described[36], subjects are classified as HD if they consume at least 3 g/kg/day for 20% or more of the open-access days; they are defined as LMD if they do not meet the 20% day threshold (Supplementary Figure 1A). The overall average g/kg/day for heavy drinking subjects ranges from 2.25 g/kg/day to 4.4g/kg/day (Supplementary Figure 1B). Both groups showed distinct ethanol consumption patterns over the 12 months of open access. The LMD group consumed an average of 1.5 g/kg/day during the first month of open access, and increased intake modestly over the 12-month period to 2.25 g/kg/day. In contrast the HD males began at a higher consumption rate at an average of 2.04 g/kg/day, and increased to 3.42 g/kg/day by the end of the study (Supplementary Figure 2). To evaluate whether the range of drinking behaviors reflect general differences in thirst, we compared water and ethanol intake levels. The results demonstrate that ethanol consumption was independent of water consumption level ($r=0.105$, $p=0.633$, $n=23$; Supplementary Figure 3A).

Blood rh*MAOA* expression levels before and after alcohol consumption

To determine the relationship between *rhMAOA* expression and risk for hazardous alcohol use, we first evaluated *rhMAOA* expression in the blood of alcohol naïve subjects, and then recorded the amount of alcohol they subsequently consumed over 12 months of open access to alcohol. There was a significant negative association between pre-alcohol blood rh*MAOA* mRNA level and the average amount of ethanol they later consumed ($r= -0.589$, $p=0.016$, $n=14$; Figure 1A). Interestingly, the predictive potential of the rh*MAOA* mRNA levels was observed as early as the 1st month of ethanol access ($r= -0.586$, $p=0.017$; Supplementary Table 3) and was stable after 7 months.

The rh*MAOA* mRNA levels were not associated with the amount of water consumed ($r= -0.076$, $p=0.778$, $n=16$; Supplementary Figure 3B). In addition, the rh*MAOA* expression and the average alcohol consumption levels were not associated with the age of the subjects ($mRNA_{0m}$: $p(Age)=0.5042$, $Alcohol_{12m}$: $p(Age)=0.2416$; multiple regression with stepwise selection).

To determine if *rhMAOA* expression changed following chronic alcohol use, blood from the same subjects was collected after the 12-month open access period. A significant reduction in the rh*MAOA* mRNA was detected after alcohol use in both groups of ethanol drinkers (LMD: Adjusted $p<0.0001$, HD: Adjusted $p=0.0200$; repeated measures ANOVA with Tukey-Kramer correction for the multiple comparisons; Figure 1C). In addition, the decrease in rh*MAOA* expression was negatively associated with the amount of alcohol consumed ($r= -0.679$, $p=0.008$, $n=14$; Figure 1B). Lighter drinkers that initially had higher levels of mRNA experienced a greater decrease in gene expression (LMD: −0.049 vs HD: −0.019, $p=0.0015$, repeated measures ANOVA with contrast test), and consequently, all subjects measured similar expression levels after 12 months of alcohol use (LMD: 0.973 vs HD 0.984: Adjusted $p=0.6095$, repeated measures ANOVA with Tukey-Kramer correction for the multiple comparisons).

rh*MAOA* expression in the nucleus accumbens core after chronic alcohol use

Based on the significant relationship between rh*MAOA* expression in the blood and alcohol intake, we next investigated rh*MAOA* expression in the NAc core, a central component in the reward system. This neural tissue is only accessible at the terminal time point, and thus comparisons were made between rh*MAOA* gene expression in control, LMD and HD subjects following 12 months of open access to alcohol or water. There was a significant negative association between the level of rh*MAOA* expression and the amount of ethanol consumed ($r= -0.637$, $p=0.014$, $n=14$; Figure 2A), even after accounting for age ($p(Age)=0.3122$; multiple regression with stepwise selection). The rh*MAOA* expression levels were significantly lower in alcohol drinkers as compared to the alcohol naïve control subjects ($p=0.023$, $n_{control}= 10$, $n_{ethanol}= 14$; independent t-test). Interestingly, our results indicate that these low levels of expression were driven by the HD subjects ($p=0.008$, $n_{LMD}=7$, $n_{HD}=7$; one-way ANOVA with Bonferroni correction for multiple comparisons, Figure 2B), while there was no significant difference in expression between HD and LMD groups ($p=0.122$; one-way ANOVA with Bonferroni correction for multiple comparisons, Figure 2B).

Effect of the LPR genotype and promoter methylation on rh*MAOA* expression and ethanol use

To investigate potential mechanisms regulating rh*MAOA* expression we considered the contribution of both rh*MAOA-LPR* genotype and the methylation status within the promoter. Both the 7 copy (“low” activity) and 5–6 copy (“high” activity) alleles were represented with similar frequency ($n_{LPR\_7}= 12$, $n_{LPR\_5/6}= 16$) in the study. The rh*MAOA-LPR* genotype was not associated with blood and NAc core rh*MAOA* expression level ($Blood_{0m}$: $p=0.3559$; $Blood_{12m}$: $p=0.3198$; NAc core: $p=0.0505$; multiple regression with stepwise selection).

Recent evidence suggested that the CpG methylation levels of a human *MAOA* promoter region may contribute to the gene regulation[26], therefore we focused on the orthologous region of the CGI_1060 rh*MAOA* promoter in rhesus macaques. Eleven of the 12 CpGs within the region are conserved in humans and rhesus macaques (Supplementary Figure 4). The relative NHP blood methylation levels were very similar to that reported for humans[26], while they were lower in the NAc core (Supplementary Figure 4). Neither the global methylation levels of the rh*MAOA* promoter in the blood or NAc core were significantly associated with ethanol consumption level (Supplementary Table 4). The methylation rate in blood and NAc core was also not associated with mRNA expression in the corresponding tissues (Supplementary Table 4).

Monoamine levels in the cerebrospinal fluid after long-term ethanol consumption

Given the central role of MAOA in catalyzing monoamine metabolism, we considered whether low rh*MAOA* expression was also correlated with elevated monoamines. Thus monoamines and their metabolites were measured in CSF collected in the rhesus macaques after 12 months of open alcohol or water access. CSF was not collected at earlier time points. A significant positive correlation was detected between the average ethanol consumption and the levels of DA ($r=0.557$, $p=0.038$, $n=14$; Figure 3A). The metabolic rate

of DA, measured by the DOPAC/DA ratio, was correlated with the amount of alcohol consumed (r= −0.670, p=0.012, n= 13; Supplementary Table 5) and the NAc core rh*MAOA* expression level (r=0.722, p=0.043, n=8, Supplementary Table 5). Thus, the more alcohol consumed, the less rh*MAOA* mRNA expression, and the less DA was metabolized into DOPAC. In addition, the amount of ethanol consumed established a clear separation between the DOPAC/DA levels (Adjusted $p_{(LMD\ vs\ HD)}$= 0.004, Kruskal-Wallis with Bonferroni correction for multiple comparisons; Figure 3B). Interestingly, the DOPAC/DA levels and the DA levels in the control subjects spanned the full range of values observed in the alcohol drinkers (Figure 3B and Supplementary Figure 5, respectively). These results suggest the pre-alcohol existence of variability in DA levels within the population.

Contrary to the DA behavior, there was a negative correlation between the average ethanol consumption and the levels of 5HT (r= −0.708, p=0.033, n=9; Figure 3C) and a positive correlation between the ethanol and 5HIAA levels (r=0.514, p=0.042, n=16; Supplementary Table 5). There were no significant differences in the 5HT metabolic rate (5HIAA/5HT) among ethanol groups (p=0.05, one-way ANOVA with Bonferroni correction for multiple comparisons; Figure 3D).

DISCUSSION

The historic link between MAOA and risk for neuropsychiatric disorders, and the variability in MAOA activity recently described in humans[25, 26] led us to investigate its potential contribution to hazardous alcohol use. Our results identified, for the first time, a predictive relationship between blood rh*MAOA* mRNA level in alcohol-naïve individuals and the subsequent amount of alcohol consumed over the course of a 12-month self-administration study. Individuals that had lower levels of rh*MAOA* expression in their blood prior to alcohol access went on to drink larger amounts over the next 12 months. Moreover, the chronic alcohol consumption caused a significant reduction in rh*MAOA* expression in blood that was alcohol-dose dependent, with a smaller change in expression measured in the heavier drinkers. The apparent rh*MAOA* expression plateau reached by both low and heavy drinking subjects after long-term alcohol might reflect a minimum, or a “floor effect”, since lower expression levels could have been detected. Taken together, these findings suggest that rh*MAOA* levels in alcohol-naïve individuals may be an informative biomarker to identify risk for alcohol abuse[48, 49]. However, long-term alcohol consumption effectively establishes similar expression levels among all subjects. Additional studies are needed to understand how rapidly rh*MAOA* expression changes with alcohol use, and whether mRNA levels rebound during periods of abstinence.

It is notable that the rh*MAOA* expression levels detected in the blood prior to alcohol exposure are similar to the expression patterns measured in the NAc core after 12 months of alcohol use; rh*MAOA* levels in the NAc core are lowest among the heaviest alcohol drinkers. Although we do not know the pre-alcohol levels of rh*MAOA* mRNA in the NAc core we speculate that, as in blood, the pre-alcohol rh*MAOA* expression levels were lower amongst the individuals that subsequently consumed the most alcohol. Supporting this hypothesis, the rh*MAOA* expression levels measured in the control subjects spanned the full range of expression values detected in the LMD and some of the HD subjects (Figure 2B). As with

blood, the NAc rh*MAOA* expression may also decrease in response to chronic alcohol use. Consistent with that notion, some of the HD subjects have lower mRNA levels than those detected in the controls.

It is known that alcohol produces its rewarding/reinforcing effects by increasing extracellular DA in the NAc[50]. The low levels of rh*MAOA* mRNA present in the NAc of heavy drinking subjects are in agreement with reduced DA metabolism contributing to the maintenance of high DA levels. Accordingly, the low levels of MAOA may enhance the sensitivity of the NAc to the reinforcing effects of alcohol[51]. Thus, in alcohol vulnerable subjects alcohol encounters the perfect molecular scenario to mount a potent reward response, promoting alcohol dependence. In contrast, subjects with higher amounts of MAOA (LMD) are able to regulate the surge of alcohol-induced DA and trigger a moderate reinforcement response (Figure 4). A recent study showed increases in DA release in response to alcohol occurring preferentially in human social-drinkers at risk for alcohol dependence (based on family-history of alcohol use disorders)[52]. The authors suggest that this response reflects a neurobiological vulnerability that alters behavioral responses toward alcohol and other rewards in high-risk individuals[52]. The range of DA detected in control subjects provides evidence of preexisting variability in the levels of DA in the population. In addition, our finding of variability in rh*MAOA* expression levels in alcohol-naïve subjects suggests a molecular mechanism contributing to the initial variability in DA levels and alcohol-vulnerability (Figure 4).

In contrast to DA, the CSF level of 5HT was negatively correlated with the amount of alcohol consumed. Our results are in agreement with previous reports of decreased extracellular 5HT in alcoholic individuals[53, 54]. In addition, our finding suggests that the net balance of 5HT amongst heavy drinkers may be more prominently influenced by factors other than MAOA, such as tissue-specific decreases in 5HT biosynthesis[55], decreased serotonin transporter density[56, 57] or degeneration of serotonergic fibers[58]. We did not detect a significant difference in 5HT metabolic rate between low and heavy alcoholic subjects, perhaps due to the limited number of LMD subjects for which both 5HT and 5HIAA levels were measurable. In that light, it would be interesting to evaluate additional subjects in future studies.

Our study also provides the first direct analysis of *MAOA-LPR* genotype and *in vivo* gene expression level. Although gene-reporter studies of the *MAOA-LPR* alleles in cell culture detected a relationship between *MAOA-LPR* genotype and gene expression[14, 59–61], our *in vivo* studies do not support those conclusions. While it is possible that *MAOA-LPR* genotype influences *MAOA* expression in tissues other than blood and brain, our studies are consistent with recent reports finding a lack of genotype association[25, 26].

Although we did not detect a significant association between rh*MAOA* promoter methylation and gene expression level in the blood or NAc core, we cannot exclude the possibility that DNA methylation in other regulatory regions contribute to *MAOA* transcriptional regulation. It will also be of interest to determine how CpG methylation varies among female subjects. A previous report that promoter methylation was associated with alcohol dependence in females[16] and that *MAOA-LPR* genotype was only associated

with increased alcoholism among women that also had a history of abuse[62] suggests that epigenetic regulation could be a contributing factor in females.

In summary, the results obtained in this study provide novel evidence for the role of rh*MAOA* in alcohol vulnerability and dependence. First, we show that blood rh*MAOA* expression level in alcohol-naïve subjects predicts risk for alcohol consumption. In addition, our results support the proposition that rh*MAOA* is responsive to environmental stressors, such as chronic alcohol exposure. Finally, our findings suggest that a decrease in rh*MAOA* expression, and an ensuing increase in DA metabolism, may provide a feed-forward mechanism linking this key enzyme with the alcohol addiction process. Future studies will be needed to clarify whether the alcohol-associated changes in rh*MAOA* expression are reversed during periods of abstinence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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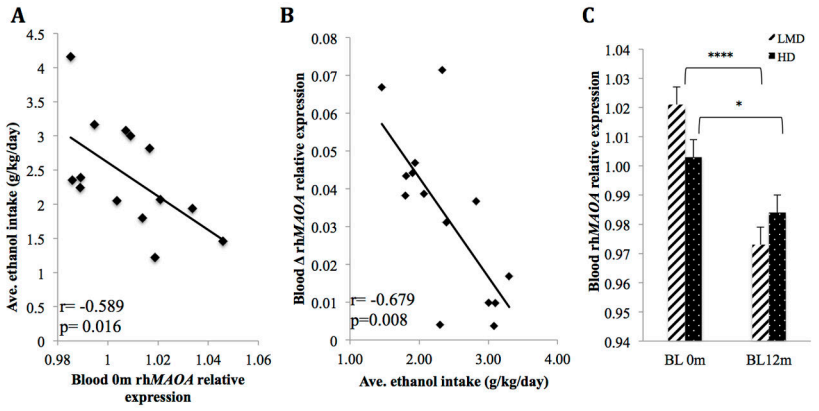

Figure 1.

rh*MAOA* (Monoamine oxidase A) mRNA expression rates in blood before and after 12 months of alcohol consumption. **A)** Correlation analysis of the average daily amount of ethanol (g/kg/day) consumed over 12 months of self-administration and the blood mRNA levels prior to ethanol exposure. **B)** Correlation analysis of the average daily amount of ethanol (g/kg/day) consumed over 12 months of self-administration and the difference in mRNA levels before and after ethanol exposure. **C)** Comparison of the total average rh*MAOA* expression at 0 and 12 months of alcohol use between low-moderate drinkers (LMD) and heavy drinkers (HD). Whiskers represent standard error of means. Repeated measures of ANOVA with Tukey-Kramer correction for multiple comparisons, **** Adjusted $p<0.0001$; * Adjusted $p<0.05$.

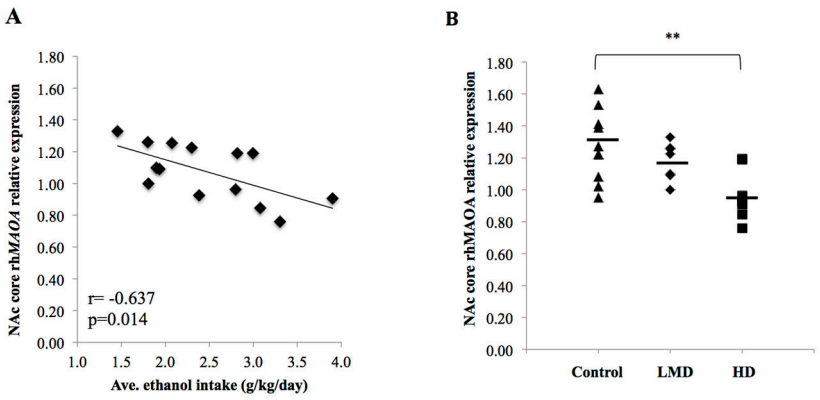

Figure 2.

rh*MAOA* (Monoamine oxidase A) mRNA expression rates in the nucleus accumbens (NAc) core after 12 months of alcohol consumption. **A)** Correlation analysis of the average daily amount of ethanol (g/kg/day) consumed over 12 months of self-administration and NAc core mRNA level after ethanol exposure. **B)** Comparison of the average mRNA level between control subjects, low-moderate drinkers (LMD) and heavy drinkers (HD). Middle line represents the mean. One-way ANOVA with Bonferroni correction for multiple comparisons; ** $p<0.01$.

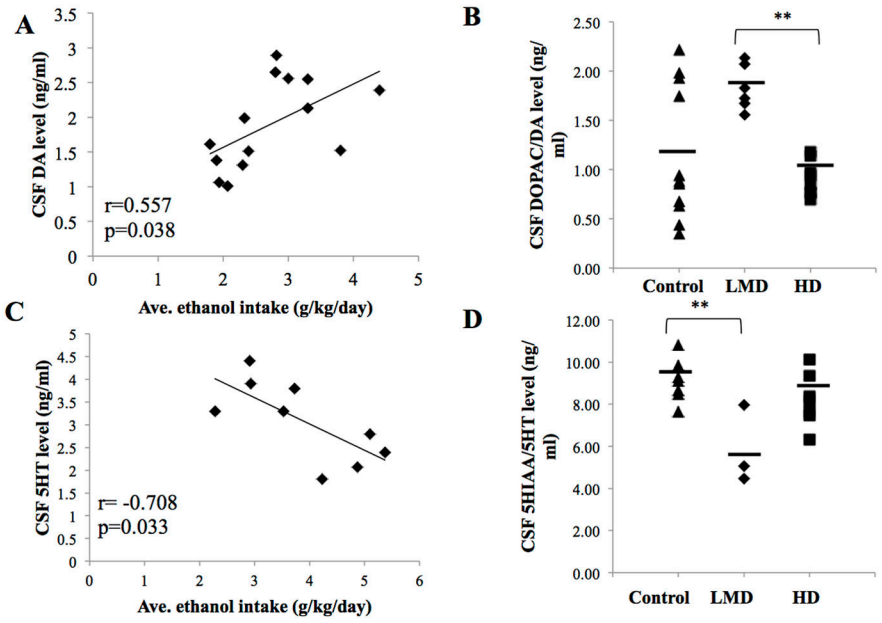

Figure 3.

The CSF levels of monoamines after 12 months of alcohol consumption. **A)** Correlation analysis of the average dopamine (DA) measured in the CSF versus average g/kg/day ethanol consumed during 12 months of self-administration. **B)** Distribution of the DA metabolic activity measured in CSF in control subjects, low-moderate drinkers (LMD) and heavy drinkers (HD). Middle line represents the mean. Kruskal-Wallis test, with Bonferroni correction for multiple comparisons; ** $p<0.01$. **C)** Correlation analysis of the average serotonin (5HT) measured in the CSF versus average g/kg/day ethanol consumed during 12 months of self-administration. **D)** Distribution of 5HT metabolic activity measured in CSF in control subjects, LMD and HD. Middle line represents the mean. One-way ANOVA with Bonferroni correction for multiple comparisons. ** $p<0.01$.

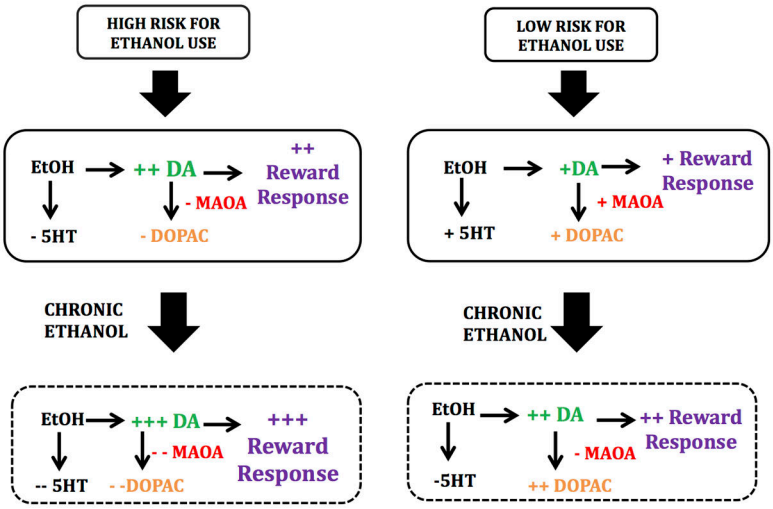

Figure 4.

Model for the contributing role of *MAOA* to alcohol vulnerability (alcohol naive) and dependence (chronic alcohol). Subjects with increased vulnerability for heavy alcohol consumption initially have lower levels of *MAOA* and higher levels of dopamine (DA). Following chronic alcohol consumption, *MAOA* levels decrease further, resulting in further elevation of DA and a more potent reward response. In contrast, individuals with lower risk for alcohol consumption, initially have higher levels of *MAOA*, and following chronic alcohol consumption, have a more modest increase in DA.