OXFORD

International Journal of Neuropsychopharmacology, (2016) 19(9): 1–8

doi:10.1093/ijnp/pyw033
Advance Access Publication: April 12, 2016
Research Article

RESEARCH ARTICLE

Association of Superoxide Dismutase 2 (*SOD2*) Genotype with Gray Matter Volume Shrinkage in Chronic Alcohol Users: Replication and Further Evaluation of an Addiction Gene Panel

Miri Gitik, PhD; Vibhuti Srivastava, PhD; Colin A. Hodgkinson, PhD; Pei-Hong Shen, MSc; David Goldman, MD; Dieter J. Meyerhoff, PhD

Laboratory of Neurogenetics, National Institute on Alcohol Abuse and Alcoholism, NIH, Rockville, Maryland (Drs Gitik, Srivastava, and Hodgkinson, Ms Shen, and Dr Goldman); Department of Radiology and Biomedical Imaging, University of California, San Francisco, California (Dr Meyerhoff); Center for Imaging of Neurodegenerative Diseases, Veterans Administration Medical Center, San Francisco, California (Dr Meyerhoff).

Current address (V.S.): Molecular Genetic Technology Program, School of Health Professions, MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030.

M.G. and V.S. contributed equally to the article.

Correspondence: Dieter J. Meyerhoff, PhD, DVA Medical Center, Center for Imaging of Neurodegenerative Diseases CIND, School of Medicine UCSF, 4150 Clement Street #114M, San Francisco, CA 94121 (dieter.meyerhoff@ucsf.edu).

Abstract

Background: Reduction in brain volume, especially gray matter volume, has been shown to be one of the many deleterious effects of prolonged alcohol consumption. High variance in the degree of gray matter tissue shrinkage among alcohol-dependent individuals and a previous neuroimaging genetics report suggest the involvement of environmental and/or genetic factors, such as superoxide dismutase 2 (*SOD2*). Identification of such underlying factors will help in the clinical management of alcohol dependence.
Methods: We analyzed quantitative magnetic resonance imaging and genotype data from 103 alcohol users, including both light drinkers and treatment-seeking alcohol-dependent individuals. Genotyping was performed using a custom gene array that included genes selected from 8 pathways relevant to chronic alcohol-related brain volume loss.
Results: We replicated a significant association of a functional *SOD2* single nucleotide polymorphism with normalized gray matter volume, which had been reported previously in an independent smaller sample of alcohol-dependent individuals. The *SOD2*-related genetic protection was observed only at the cohort's lower drinking range. Additional associations between normalized gray matter volume and other candidate genes such as alcohol dehydrogenase gene cluster (*ADH*), *GCLC*, *NOS3*, and *SYT1* were observed across the entire sample but did not survive corrections for multiple comparisons.
Conclusion: Converging independent evidence for a *SOD2* gene association with gray matter volume shrinkage in chronic alcohol users suggests that *SOD2* genetic variants predict differential brain volume loss mediated by free radicals. This study

Received: November 30, 2015; **Revised:** March 28, 2016; **Accepted:** April 1, 2016

Published by Oxford University Press on behalf of CINP 2016. This work is written by (a) US Government employee(s) and is in the public domain in the US.

also provides the first catalog of genetic variations relevant to gray matter loss in chronic alcohol users. The identified gene-brain structure relationships are functionally pertinent and merit replication.

Keywords: Alcohol use disorder, gray matter volume, superoxide dismutase 2 (SOD2), neuroimaging, genetics

Introduction

Alcohol use disorders are associated with neurodegenerative processes (de la Monte and Kril, 2014), and brain imaging has been used to demonstrate the adverse effects of chronic alcohol consumption on brain structure and morphometry (Buhler and Mann, 2011). One of the most consistent findings is a reduction of gray matter (GM) and white matter (WM) volumes, which results in whole brain volume shrinkage in individuals with alcohol use disorders compared with age-matched light- or non-drinking controls. Such atrophy, measured by quantitative in vivo magnetic resonance imaging (MRI), has been related to wide-ranging deficits in cognitive and motor functions (e.g., Sullivan et al., 2010). The exact mechanisms by which chronic alcohol consumption produces these brain effects are under intense investigation, and several molecular pathways have been implicated via in vitro and in vivo model systems and through the study of postmortem human brains (for review, see e.g., Mukherjee, 2013).

One such injury mechanism is oxidative stress from free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), induced by alcohol and its metabolic byproducts. The excessive alcohol-associated production of free radical species shifts the delicate balance required for normal cellular function between the amount of free radicals (such as superoxide anion and peroxynitrite) and antioxidant molecules/enzymes (such as glutathione, catalase, and superoxide dismutase [*SOD*]) that exist in the body to reduce these active radicals and counterbalance their damaging effects on cellular tissue. The excess in free radicals and a potentially associated failure of antioxidant mechanisms to quickly and efficiently remove ROS/RNS cause oxidative/nitrosative cellular stress. This leads to increased oxidation of DNA, proteins, and lipids; disruption of cellular and mitochondrial membranes; and eventually to damaged neurons and apoptotic or necrotic cell death (Kumar et al., 2013; Reddy et al., 2013). On a macromolecular scale, such damage is reflected qualitatively in atrophy and quantitatively in brain tissue volume reductions seen on brain MRI of individuals with alcohol use disorders.

The brain is particularly susceptible to oxidative stress due to its relatively high content of polyunsaturated fatty acids, low concentration of antioxidant enzymes, and high level of oxygen metabolism (Sun and Sun, 2001). Cellular ROS and RNS production from alcohol metabolism and chronic smoking lead to diminished neuronal viability (Haorah et al., 2008, Crews and Nixon, 2009; Hack et al., 2009; Durazzo et al., 2010; Alfonso-Loeches and Guerri, 2011). An in vivo rat study showed increased mitochondrial lipid peroxidation and protein oxidation with decreased neurocortical expression of *SOD2*, a mitochondrial enzyme of the SOD family (Reddy et al., 2013). Peripheral *SOD2* activity was also found downregulated in individuals with alcohol dependence (Huang et al., 2009a, 2009b). These results and others strengthen the link between insufficient antioxidant activity and chronic alcohol-associated neurodegeneration and atrophy.

Alterations in brain structure potentially related to neurodegenerative processes have been observed by in vivo neuroimaging in the majority of studies of treatment-naïve and treatment-seeking alcohol-dependent individuals. A common finding is shrinkage of the cortical GM, detected with large inter-individual variation both in the severity of effects and the magnitude of recovery of neurobiological abnormalities with abstinence from alcohol (Cui et al., 2015). This inter-individual heterogeneity points to variation in vulnerability of the brain to the detrimental effects of chronic alcohol consumption or in the brain's ability to recover; such an inter-individual variation may be attributed to an interplay of genetic and environmental factors.

Previously, we identified in 76 treatment-seeking alcohol-dependent individuals an association between greater GM shrinkage and a *SOD2* homozygous diplotype (rs10370TT-rs4880GG), containing a functional missense variant (Ala16Val, rs4880) (Srivastava et al., 2010). In this report, we set out to replicate the results of a brain structural intermediate phenotype in a larger dataset of 103 alcohol-drinking individuals and to identify additional potential genetic variants influencing chronic alcohol-induced morphological brain alteration in select genes associated with oxidation and neuronal function. To achieve our goals, we used a custom genotyping array (Hodgkinson et al., 2008), an approach that is more cost effective than whole genome sequencing and more relevant and focused than genome-wide association study platforms, yet more powerful and efficient than genotyping individual candidate single nucleotide polymorphisms (SNPs). This approach enabled the first comprehensive cataloging of relevant genetic variations of chronic alcohol-induced brain shrinkage, with the inclusion of Ancestry Informative Markers (AIMs) as a method to correct for confounding effects of population admixture. The custom array included SNPs from 92 loci/genes belonging to/representing 8 different pathways relevant to chronic alcohol-induced neuropathology.

Materials and Methods

Participants

All alcohol-dependent participants were recruited from the VA Medical Center Substance Abuse Day Hospital and the Kaiser Permanente Chemical Dependence Recovery Program outpatient clinics in San Francisco; non-alcohol-dependent light-drinking controls were recruited from the Bay Area community. Participants provided written informed consent before engaging in study procedures, which conformed to the Declaration of Helsinki and were approved by the University of California San Francisco and the VA Medical Center. The alcohol-dependent individuals were studied by 1.5-Tesla brain imaging at an average of 32 ± 11 days of abstinence from alcohol and other substances except tobacco. Primary inclusion criteria were current DSM-IV diagnosis of alcohol dependence, fluency in English, consumption of >150 alcohol-containing drinks/month (1 alcoholic drink equivalent = 13.6 g pure alcohol) for at least 8 years before enrollment for men and consumption of >80 drinks/mo for at least 6 years before enrollment for women. All participants completed the Structured Clinical Interview for DSM-IV-Axis I Disorders, Patient Edition, Version 2.0, and standardized questionnaires that assessed average number of alcoholic drinks/

month consumed over lifetime and lifetime alcohol consumption (LAC) via a lifetime drinking history interview as well as the level of nicotine dependence via the Fagerstrom Tolerance Test for Nicotine Dependence, including the total number of cigarettes smoked per day and lifetime years of smoking. Primary exclusion criteria for all participants are fully detailed elsewhere (Durazzo et al., 2004). In brief, all participants were free of psychiatric, neurological, physical, and medical conditions known or suspected to influence brain morphology, with the exceptions of hepatitis C, hypertension, and Type-2 diabetes; these medical conditions are highly prevalent in those with alcohol use disorders (Mertens et al., 2005; Moss et al., 2010), and excluding patients with these conditions would have resulted in a biased sample. For alcohol-dependent individuals, current or past unipolar mood disorders (e.g., major depression, substance-induced mood disorder) were not exclusionary given the high comorbidity with substance dependence (Mertens et al., 2003; Durazzo et al., 2010). The alcohol-dependent participants were monitored for abstinence while in treatment, and they were screened for recent alcohol and illicit substances at the time of brain imaging to assure abstinence. All study participants provided a blood sample for extracting genomic DNA at the clinical laboratories of the VA.

From a total of 103 individuals enrolled, 93 participants were selected for the study proper. Ten individuals were excluded from the final analysis due to non-European or African American ancestry. A total of 71 were European (76%) and 22 were African American (24%) as determined by self-report and confirmed by AIMs. The median LAC across the entire study sample was 839 kg, and for further analysis participants were divided into "low" and "high" drinking groups who had consumed alcohol below and above this median split, respectively. The average age of the low drinkers was 48.5±10.8 and 54.3±6.6 for the high drinkers. The average Fagerstrom total score was 4.8±2.0 for the 21 smokers among the low drinkers and 5.7±1.7 for the 33 smokers among the high drinkers, indicating a moderate degree of dependence on tobacco products, predominantly cigarettes. Both age and Fagerstrom total score were significantly different between groups ($P < .003$) (Table 1). Of the final study group, 15 were light-drinking controls (including 3 cigarette smokers) and 70 were recovering alcohol-dependent individuals at about 1 month of abstinence (including 46 smokers) and 8 were 1-month-abstinent alcohol-dependent individuals who also were dependent on at least one stimulant, primarily cocaine (including 5 smokers).

MRI Data Acquisition and Processing

Brain MRI was performed on a 1.5-Tesla system (Vision, Siemens Medical Systems, Iselin, NJ) at the San Francisco VA Medical Center as part of a larger study to investigate the effects of abstinence from abused substances on brain morphometry and neurocognition. We applied a T1-weighted magnetization prepared rapid acquisition gradient echo sequence (TR/TI/TE = 9/300/4 ms, 1 x 1 mm^2 in-plane resolution, 1.5-mm slabs) oriented orthogonal to the long axis of the hippocampus and an oblique-axial T2-weighted spin-echo sequence (TR/TE = 2500/80 ms, 1 x 1 mm^2 in-plane resolution, 3-mm slice thickness) oriented along an imaginary line connecting the anterior and posterior commissures as seen on a mid-sagittal scout image.

Tissue intensity-based segmentation of cortical and subcortical GM, WM, and cerebrospinal fluid (CSF) from T1-weighted images was carried out with the semi-utomated expectation-maximization segmentation method (Van Leemput et al., 1999). This method assigns a probability of GM, WM, and CSF to each MRI voxel based on T1-weighted tissue intensity (see Mon et al., 2013 for details). The T2-weighted images were used to remove nonbrain tissue voxels from the coregistered segmented images. Absolute volumes for total cortical GM and WM as well as CSF are reported in mL. Intracranial volume (ICV) was calculated as the sum of total tissue and CSF volumes. To account for individual variability in ICV, absolute GM and WM tissue volumes for each participant were standardized to the participant's ICV, resulting in GM ratio (GMR).

Genetic Analyses

The genetic analyses were performed at the Laboratory of Neurogenetics of the National Institute on Alcohol Abuse and Alcoholism. The custom array we used included a total of 768 SNPs from 84 candidate genes (including SOD2) and 8 nonannotated loci based on the platform of Illumina Golden gate array (Illumina) (supplementary Table 1). A sequence of 5 kb up- and 1 kb downstream of the candidate gene was searched (NCBI SNP build 37.1), and tagging SNPs were identified based on the criteria below (details in Hodgkinson et al., 2008). Haplotype tagging SNPs for African and European populations that captured haplotypes with a frequency of at least 0.006 were selected using a double classification tree search algorithm. Based on the haplotype block structure, large genes with complex haplotype structures were split into 2 or 3 regions and each region was tagged separately. Nonsynonymous SNPs, SNPs within 10 bp of intron/exon splice sites, were specifically included by forcing these SNPs into the panels used for haplotype tagging. Of these 768 SNPs, 142 SNPs were specifically included as AIMs. These SNPs are a subset of AIMs panel originally described in Enoch et al. (2006). The minimum distance between AIM SNPs was 80 kb, and pairwise reference allele frequency (RFA) was at least 0.75 (Haploview version 4.2), with a 10-fold difference between RFA1 and RFA2. AIMs

Table 1. Characteristics of Full Study Sample and of Subgroups with LAC below and above Median Split of 839 kg (mean ± SD)

	Full Study Sample	Low Drinkers (LAC ≤ 839kg)	High Drinkers (LAC > 839 kg)
n (females)	93 (7)	46 (6)	47 (1)
Age (y)[a]	51.4 ± 9.3	48.5 ± 10.8	54.3 ± 6.6
(LAC) (kg)	1032 ± 927	376 ± 247	1711 ± 874
Fagerstrom score (FTND)[b]	5.3 ± 1.9 (n = 54)	4.8 ± 2.0 (n = 21)	5.7 ± 1.7 (n = 33)
Total GMV (mL)	574.7 ± 52.9	582.1 ± 49.7	567.0 ± 54.9
GMR = GMV/intracranial volume)[c]	0.379 ± 0.016	0.386 ± 0.016	0.372 ± 0.013
Total WM volume (mL)	402.0 ± 43.9	406.8 ± 49.7	397.2 ± 45.1
WM ratio (= WM volume/intracranial volume)[b]	0.265 ± 0.015	0.260 ± 0.015	0.269 ± 0.014

Abbreviations: FTND, Fagerstrom Test for nicotine dependence; GM, gray matter; GMR, gray matter ratio; GMV, gray matter volume; LAC, lifetime alcohol consumption; WM, white matter.

Low vs high *t* test: [a]$P = .003$, [b]$P = .002$, [c]$P < .001$.

data were analyzed using Structure 2.1 to generate population assignments for all individuals (Pritchard et al., 2000).

Genotyping was performed according to Illumina GoldenGate genotyping protocols for Sentrix BeadChip Array Universal-32 BeadChip (768 Bead Types V8a). A total of 500 ng of genomic DNA was used per assay. All pre-PCR processing was performed using a TECAN liquid handling robot running Illumina protocols. Arrays were imaged using an Illumina Beadstation GX500 and the data analyzed using GenCall v5.2.0 and GTS Reports software v (Illumina). Genotype clusters were manually adjusted to correct for the variation arising from DNA integrity. A score of zero was assigned for those loci, for which 3 distinct clusters could not be resolved. SNPs with a Gencall score <0.25, genotyping rate <90%, and minor allele frequency <0.05 were excluded. Genotyping accuracy for this array in our laboratory was determined empirically and independent of this particular sample by duplicate genotyping of 10% of samples selected randomly, and the error rate was determined at <0.05%.

For validation purposes, the SOD2 functional SNP rs4880 in all study participants was additionally regenotyped by TaqMan assay (C___8709053_10, Applied Biosystems, Foster City, CA) according to the manufacturer's protocol, and the genotype end point was determined using the ABI 7900HT Sequence Detection System. Genotyping accuracy was determined empirically by duplicate genotyping of 25% of the samples selected randomly. The error rate was <0.005 and the completion rate was >0.95. No genotyping discrepancies between the 2 platforms were identified.

Linkage disequilibrium was calculated for each gene using haploview version 4.2. Only 1 SNP was included in regression analyses from a pair showing a high correlation (pairwise r^2 value >0.8). All markers were tested for Hardy-Weinberg Equilibrium.

Statistical Analyses

Statistical tests were performed using the JMP10 (SAS Institute, Cary, NC). Normality of distribution of brain volume measures was assessed by Shapiro–Wilk W-test and Welch ANOVA testing was used for group comparison. If either of the assumption of ANOVA was violated, a less powerful, nonparametric Wilcoxon rank sums test (Kruskal–Wallis H-test) was used. Mean values were calculated for normally distributed variables. An individual's GMR was calculated by dividing an individual's total GM volume in mL by his/her ICV in mL. Univariate analyses (ANOVA or chi square) determined the independent effects of age, LAC, ethnicity, smoking status, or gender on GMR.

The standard least square method was used to construct linear regression models with GMR as the dependent variable and the following initial independent predictors: gene-specific independent SNPs/diplotype, LAC, age, gender, FTND, duration of abstinence, and African and European ancestry scores. If a variable did not significantly contribute to the model (i.e., significance level was $P > .1$), the variable was eliminated from the model in an iterative process. Tukey's HSD analysis was used as a posterior test. To determine whether LAC affects the ability to detect associations between genetic variations and GMR (as seen previously, Srivastava, Buzas et al., 2010), a median split on the LAC measure was applied to the entire study sample (at 839 kg), which yielded high and low drinking groups drinking above and below this median, respectively. The main analyses were repeated for these subgroups. For all analyses, haplotypes were constructed and diplotypes were estimated using PHASE version 2.0.2 (Stephens et al., 2001). To estimate the error rate stemming from multiple testing, the false discovery rate was assessed (Benjamini and Hochberg 1995). $P < .05$ was considered statistically significant.

A Manhattan plot was constructed using Haploview version 4.2. Ingenuity Pathway Analysis identified enrichment in "diseases and disorders" and "molecular and cellular functions" and investigated known direct or indirect molecular interactions between significantly associated genes (www.ingenuity.com).

Results

Refinement of the Sample by Measured Ancestry

In the initial sample set of 103 participants, AIMs assessment (Enoch et al., 2006) identified 6 ethnic factors. Ten participants showed no clear European or African ethnicity, so they were excluded from further analyses. This exclusion was based on our previous study (Srivastava et al., 2010) that overwhelmingly enrolled individuals of African and European descent, and it also served to reduce the noise in regression modeling by reducing the number of required independent predictors. The final group of 93 participants was divided into low and high drinkers, based on the median LAC of the entire study group (Tables 1 and 2). The Fagerstrom score was different between low drinker (21 smokers and 25 nonsmokers) and high drinker (33 smokers and 14 nonsmokers) groups ($P = .002$), but no significant effect of smoking status on GMR was observed in regression analyses. The low drinking group (LAC < 839 kg) showed an average GMR of 0.386 ± 0.016, whereas the high drinking group had an average GMR of 0.372 ± 0.013, a significant difference of about 4% ($P < .001$). The corresponding WM ratio was about 3% lower in the high vs low drinking group ($P = .002$), but it had no significant effect in the whole model analyses. Within the low drinker group, linear regression analyses identified inverse effects of age ($P < .001$) and LAC ($P = .006$) on GMR.

Genetic Analyses

A total of 768 SNPs from 92 candidate genes and loci (supplementary Table 1) were genotyped for each sample in the whole data set from 93 individuals. Fifty-one SNPs were excluded from final analyses because of low heterozygosity and 11 were excluded because of unreliable clustering. Therefore, 706 SNPs were used in final regression analyses as possible predictors of GMR. No significant effects of any of these SNPs on GMR in the full sample were found.

SOD2 Association Analysis

Haplotypes were constructed with *SOD2* SNPs rs5746136 (tag SNP in present sample set) and rs4880 (Ala16Val); 9 diplotypes were inferred based on these haplotypes. Three of these diplotypes were Ala16/Ala16 homozygous (H1-H3, H3-H3, and H1-H1), a frequency similar to that previously observed (Srivastava et al., 2010). Mean GMR and LAC for low and high drinking groups across all *SOD2* diplotypes are presented in Table 3. A linear regression analysis in the low drinking group (GMR as dependent variable; *SOD2* diplotypes, age, gender, FTND, LAC, duration of abstinence, ancestry scores for both European and African ancestry as independent predictors) showed significant association of *SOD2* diplotype with GMR ($P = .009$), in addition to the significant relationships of GMR to LAC, age, described above. This whole model accounted for 70% of variance of GMR in the low drinking group (Table 2). Tukeys posthoc analysis revealed that the Ala16/Ala16 -homozygous diplotype was significantly ($P < .009$) associated with the lowest GMR in the low drinking group but showed no significant association among the higher than median drinking group (Table 3).

Table 2. Effects of Independent Predictors on GMR (GMR = GM/ICV) in Low and High Drinking Groups

	Low Drinkers		High Drinkers	
Variables	F-ratio	P value	F-ratio	P value
Age	32.9	<.0001	7.3	0.01
LAC (kg)	8.5	0.006	0.1	0.72
European ethnicity (AIM scores)	9	0.005	0.2	0.68
SOD2 diplotype	3.1	0.009	0.5	0.84
Fagerstorm score (FTND)	0.4	0.5	3.5	0.07
Full model F/R^2	7.3/0.7	<.0001	1.4/0.3	0.2

Abbreviations: AIM, Ancestry Informative Marker; FTND, Fagerstrom Test for nicotine dependence; GMR, gray matter ratio; LAC, Lifetime alcohol consumption.

Table 3: Mean gray matter ratio (GMR = GM/ICV) and lifetime alcohol consumption (LAC) for 'low' and high' drinking groups across all *SOD2* diplotypes (mean ± standard deviation)

	'Low' drinkers		'High' drinkers	
Diplotype	GMR	LAC [kg]	GMR	LAC [kg]
H1-H1*	0.382±0.013 (n=3)	190±127	0.363±0.01 (n=5)	1553±344.3
H1-H2	0.394±0.012 (n=11)	345±214.5	0.383±0.009 (n=8)	1715±536
H1-H3*	0.371±0.013 (n=2)	72±42.1	0.380±0.008 (n=6)	1906±1504
H2-H2	0.392±0.023 (n=5)	260±268	0.372±0.014 (n=5)	1623±470.5
H2-H3	0.383±0.013 (n=12)	420±239	0.373±0.012 (n=17)	1592±494.9
H2-H4	0.381±0.002 (n=2)	448±243	0.380±0.006 (n=2)	1223±103.3
H3-H3*	0.382±0.01 (n=6)	290±77	-	-
H3-H4	0.373±0.02 (n=3)	533±107	0.374 (n=1)	2420
H4-H4	0.382±0.003 (n=2)	700±50	0.383±0.02 (n=3)	2501±155.09

[+] From haplotypes constructed with *SOD2* SNPs rs5746136-rs4880 (ala16val)
*Ala homozygous diplotypes

Global Gene-Wise Regression Analyses

Of the 67 genes, 8 showed significant associations in analyses of the entire sample, with GMR as dependent variable and gene-specific independent SNPs, LAC, age, gender, smoking status, duration of abstinence, and African and European ancestry scores as independent predictors. SNPs rs2213041 from ADH1A-B-C cluster, rs2494731 from *AKT1*, rs1042389 from *CYP2B6*, rs534957 from *GCLC*, rs2364725 from NFE2L2, rs3918188 and rs7830 from *NOS3*, rs4593985 from *SLC1A2*, and rs17293059 from *SYT1* genes were associated with GMR (Table 4). Of these 8 nominally significant associations, 4 genes were also significant predictors of GMR in the low drinking group (rs2213041, rs534957, rs7830, and rs17293059). None of the associations in the entire sample or in the low drinking group survived correction for multiple testing. The genotypes showing the lowest average GMR are presented in Table 4 and a graphical representation of all the uncorrected *P* values is presented in the Manhattan plot of Figure 1.

Discussion

In this large-scale hypothesis-driven genetic analysis of chronic alcohol-associated brain volume loss, we replicated a previous association between GM shrinkage and *SOD2* genotype in individuals with relatively low LAC levels (Srivastava et al., 2010). We also identified several novel associations between our GM volume measure and specific genes on a custom addiction array, which, however, did not survive rigorous multiple comparison correction. Candidate genes included in the array were those previously found to influence substance addiction and generally belonged to diverse yet biologically interconnected metabolic pathways. The genes are shown in supplementary Table 1 and can broadly be divided into 2 groups: one directly affecting the extent of oxidative challenge to enzymes and proteins involved in alcohol metabolism, ROS/RNS clearance, and antioxidant molecule production and another group that regulates neuronal function (neurotransmitter signaling and neurotrophic factor coding genes) and pro/antiapoptotic factors.

The most notable result of this study is the finding that the *SOD2* diplotype (rs5746136CC-rs4880GG) containing the functional missense variant (Ala16Val, rs4880) was differentially associated with GMR in alcohol-drinking individuals, a replication of a previous finding in an independent cohort of alcohol-dependent individuals (Srivastava et al., 2010). The Ala16 low activity allele was found to be a risk factor for GM loss in individuals in the low drinking group (below the cohort median of 839 kg LAC) but not among the alcohol-dependent individuals in the high drinking group. This indicates that genetic protection from this specific gene may be ineffective at levels of alcohol consumption that exceed 839 kg LAC, corresponding to approximately 8 standard alcoholic drinks/d for a period of 20 years. Evidence for the involvement of the *SOD2* genotype in chronic alcohol-associated GM shrinkage in 2 independent cohorts with relatively low LAC strongly suggests that the Ala16Val is a key genetic variant in alcohol-mediated brain tissue loss that conveys genetic protection only at relatively low cumulative drinking levels.

Due to the combination of African American and European origin subjects in this study, we evaluated the frequencies of the SOD2 haplotypes (rs4880- rs5746136) between African and European populations using a public database (HapMap v3) and found no significant frequency difference between them. In particular, the frequency of the Ala/Ala risk haplotype is numerically identical between these 2 diverse populations (supplementary Table 2).

In addition to the critical *SOD2* functional polymorphism, associations of other genes on the addiction array to GM volume in alcohol users were observed. Genetic variations from *ADH*, *GCLC*, *NOS3*, and *SYT1* were significant predictors of GMR among individuals with lower than the median LAC, which was 839 kg in this cohort. These genes are in some way or another part of oxidative stress pathways (Figure 2). To probe the extent and nature of molecular interactions between these genes, we carried out an interaction analysis using the "connect" function in IPA. Different molecular interactions emerged among the candidates. Most of the relationships were direct (represented by solid line/arrow in Figure 2), where 2 molecules made physical contact with each other such as during a phosphorylation event. The remainder were indirect interactions (represented by broken line/arrow), which did not involve physical contact between the 2 molecules, such as signaling events; some other genes functioned in tandem without molecular interactions, such as *ADH* and *CYP2B6*.

The genes encoding alcohol dehydrogenases (*ADH1 A-B-C*) showed no evidence of direct or indirect interaction with the other genes identified to relate to alcohol-associated GM loss in this study. *ADH1A*, *1B*, and *1C* code for specific subunits and are collectively grouped into class I ADH. Class I ADH is abundant in liver and is responsible for first pass metabolism of alcohol (Crabb et al., 2004) directly controlling the amount

Table 4: Other genetic variations nominally predicting the gray matter ratio (GMR) in the context of alcohol exposure in the entire study sample (mean ± standard deviation)

Gene/Gene cluster	Associated SNP	R	F-ratio	p-value	Genotype with lowest GMR
ADH1A-B-C	rs2213041 * (intergenic)	0.83	11.15	<0.0001	A A = 0.377 ± 0.010 (n=5)
AKT1	rs2494731 (intron)	0.70	3.73	0.028	C C = 0.366 ± 0.012 (n=5)
CYP2B6	rs1042389 (UTR-3')	0.73	3.98	0.020	T T = 0.377 ± 0.017 (n=56)
GCLC	rs534957 * (intron)	0.77	6.24	0.003	G G = 0.378 ± 0.010 (n=6)
NFE2L2	rs2364725 (5' to gene)	0.70	3.75	0.030	A A = 0.375 ± 0.016 (n=29)
NOS3	rs3918188 (intron)	0.73	4.96	0.010	C C = 0.377 ± 0.018 (n=36)
	rs7830 * (intron)	0.71	4.30	0.017	G G = 0.378 ± 0.016 (n=42)
SLC1A2	rs4593985 (intron)	0.83	4.53	0.016	C C = 0.377 ± 0.015 (n=50)
SYT1	rs17293059 * (intron)	0.76	3.42	0.038	G G = 0.366 ± 0.019 (n=2)

* Also significant predictor of GMR in 'low' drinking group alone

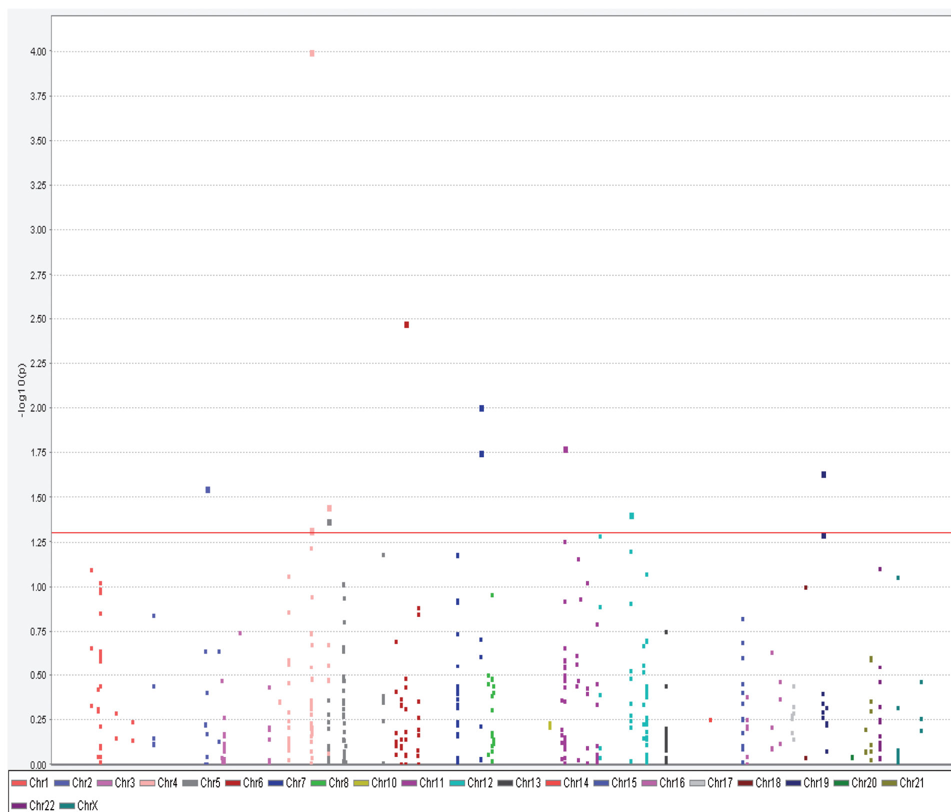
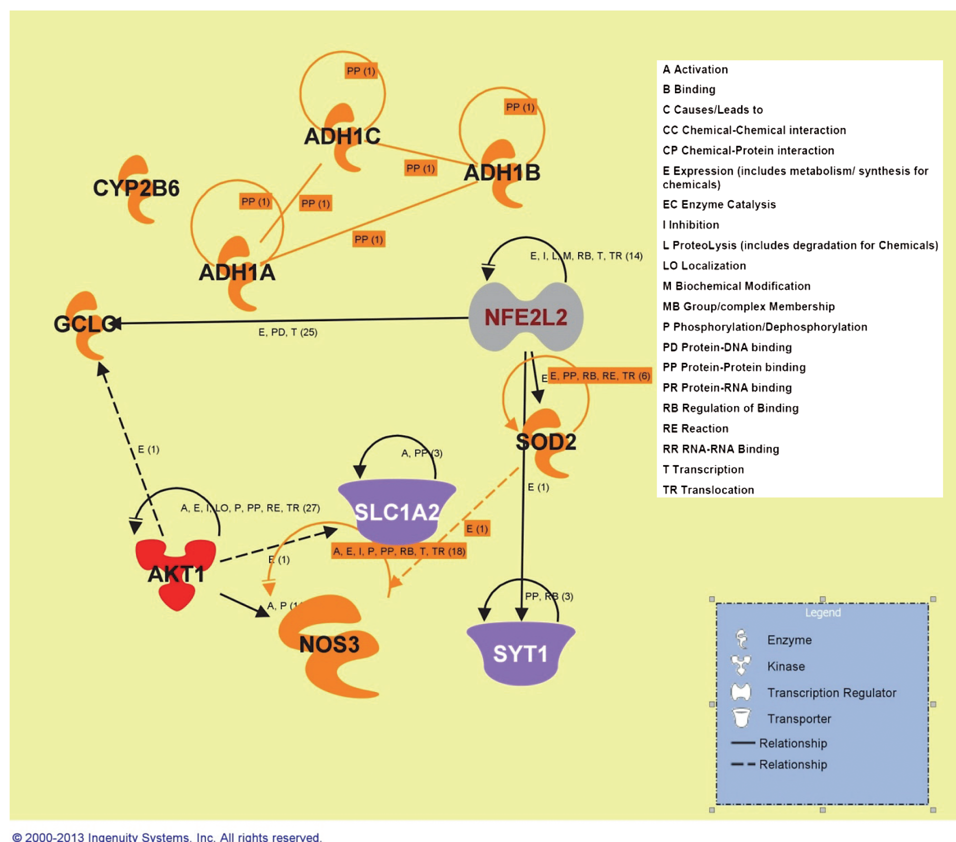


Figure 1. Manhattan plot of all the *P* values obtained in the study (the *P* values are derived from linear regression models from each gene); red line represents uncorrected significant threshold *P* value; y-axis represents log transformed *P* value; X-axis represents chromosomes.

of alcohol in systemic circulation and availability to the target organs. Thus, based on the efficiency of class I ADH, individuals with a comparable range of LAC may have varying levels of ROS load, which, coupled with differential efficiencies of the oxidative stress system, leads to largely varying degrees of GM tissue loss.

GCLC (Glutamate-cysteine ligase or γ-glutamylcysteine synthetase) is the first rate-limiting enzyme of glutathione synthesis. Functional mutations in *GCLC* can reduce the synthesis of this all-important antioxidant in the brain and thereby increase oxidative tissue damage. NOS3 (nitric oxide synthase 3) or eNOS (endothelial NOS) is responsible for the production of NO, which has antioxidant properties as it, among other functions, reduces superoxide anion formation by inducing the expression of SOD. SYT1 (Synaptotagmin-1) is a member of the Synaptotagmin family involved in regulating neurotransmitter release. Crucial for normal



© 2000-2013 Ingenuity Systems, Inc. All rights reserved.

Figure 2. Molecular interactions between genes significantly associated with gray matter ratio (GMR).

neuronal synaptic transmission, it has been shown recently to play a role in alcohol preference models (Barbier et al., 2015).

This study provides a focused and contextual analysis of genetic variation affecting a specialized phenotype with a small but functionally relevant effect size. The select polymorphisms on our custom array tagged maximum genetic variation per locus, which maximized the coverage and minimized noise. All the gene specific associations were adjusted for confounding effects of age, gender, smoking status, level of alcohol consumption, and ethnicity. However, none of the observed *P* values were significant following FDR correction. The effect sizes of genes on an intermediate phenotype are expected to be larger than observed in our study (Hodgkinson et al., 2010). A retrospective power calculation assuming an average of 5 independent SNPs per gene per regression model estimated a sample size of 847 and 122 individuals to achieve a statistical power of 80% for small and medium effect sizes, respectively. Our sample size was marginally short of medium effect size. Thus, in all probability this is the reason why the phenotype of alcohol-induced GM loss shows a small effect size. Collecting a large sample number for such phenotypes is a limitation at clinical and practical levels that can be overcome by pooling and harmonizing data from different studies/laboratories. Nonetheless, and considering that genetic association signals have been rarely replicated, replication of a previously reported association of *SOD2* Ala16Val polymorphism with GMR in an independent sample is the highlight of this study. Furthermore, we provide the first cataloging of genetic variations relevant to GM tissue loss associated with chronic alcohol consumption. It is important to note that no significant genetic associations were observed in patients who chronically consumed large amounts of alcohol (>8 standard alcoholic drinks/d or a mean of 16 alcoholic drinks/d for 20 years), signifying that genetic protection from a specific *SOD2* SNP is ineffective at very high levels of alcohol intake. Nevertheless, the reported associations in the low drinking group are informative, functionally pertinent, and merit follow-up.

Acknowledgments

We thank the staff at the San Francisco VA Medical Center Substance Abuse Day Hospital and the Kaiser Permanente Chemical Dependency Recovery Program in San Francisco for their valuable assistance in recruiting participants. Our gratitude also goes to the study participants, who alone made this research possible. We also thank Dr. Yuan Qiaoping and Goli Yamini for their advice and contribution in generation and analysis of the custom genotyping array.

This work was supported by grants from the National Institutes of Health (AA10788 and DA025202 to D.J.M.) administered by the Northern California Institute for Research and Education, and by the use of resources and facilities at the San Francisco Veterans Administration Medical Center and also by the Intramural Research Program of the National Institute on Alcohol Abuse and Alcoholism, NIH.

Statement of Interest

None.

References

Alfonso-Loeches S, Guerri C (2011) Molecular and behavioral aspects of the actions of alcohol on the adult and developing brain. Crit Rev Clin Lab Sci 48:19–47.

Barbier E, Tapocik JD, Juergens N, Pitcairn C, Borich A, Schank JR, Sun H, Schuebel K, Zhou Z, Yuan Q, Vendruscolo LF, Goldman D, Heilig M (2015) DNA methylation in the medial prefrontal cortex regulates alcohol-induced behavior and plasticity. J Neurosci 35:6153–6164.

Buhler M, Mann K (2011) Alcohol and the human brain: a systematic review of different neuroimaging methods. Alcohol Clin Exp Res 35:1771–1793.

Crabb DW, Matsumoto M, Chang D, You M (2004) Overview of the role of alcohol dehydrogenase and aldehyde dehydrogenase and their variants in the genesis of alcohol-related pathology. Proc Nutr Soc 63:49–63.

Crews FT, Nixon K (2009) Mechanisms of neurodegeneration and regeneration in alcoholism. Alcohol Alcohol 44:115–127.

Cui C, Noronha A, Warren KR, Koob GF, Sinha R, Thakkar M, Matochik J, Crews FT, Chandler LJ, Pfefferbaum A, Becker HC, Lovinger D, Everitt BJ, Egli M, Mandyam CD, Fein G, Potenza MN, Harris RA, Grant KA, Roberto M, Meyerhoff DJ, Sullivan EV (2015) Brain pathways to recovery from alcohol dependence. Alcohol 49:435–452.

de la Monte SM, Kril JJ (2014) Human alcohol-related neuropathology. Acta Neuropathol 127:71–90.

Durazzo TC, Gazdzinski S, Banys P, Meyerhoff DJ (2004) Cigarette smoking exacerbates chronic alcohol-induced brain damage: a preliminary metabolite imaging study. Alcohol Clin Exp Res 28:1849–1860.

Durazzo TC, Meyerhoff DJ, Nixon SJ (2010) Chronic cigarette smoking: implications for neurocognition and brain neurobiology. Int J Environ Res Public Health 7:3760–3791.

Enoch MA, Shen PH, Xu K, Hodgkinson C, Goldman D (2006) Using ancestry-informative markers to define populations and detect population stratification. J Psychopharmacol 20:19–26.

Hack KE, Koopman-Esseboom C, Derks JB, Elias SG, de Kleine MJ, Baerts W, Go AT, Schaap AH, van der Hoeven MA, Eggink AJ, Sollie KM, Weisglas-Kuperus N, GH AV (2009) Long-term neurodevelopmental outcome of monochorionic and matched dichorionic twins. PLoS One 4:e6815.

Haorah J, Ramirez SH, Floreani N, Gorantla S, Morsey B, Persidsky Y (2008) Mechanism of alcohol-induced oxidative stress and neuronal injury. Free Radic Biol Med 45:1542–1550.

Hodgkinson CA, Yuan Q, Xu K, Shen PH, Heinz E, Lobos EA, Binder EB, Cubells J, Ehlers CL, Gelernter J, Mann J, Riley B, Roy A, Tabakoff B, Todd RD, Zhou Z, Goldman D (2008) Addictions biology: haplotype-based analysis for 130 candidate genes on a single array. Alcohol Alcohol 43:505–515.

Hodgkinson CA, Enoch MA, Srivastava V, Cummins-Oman JS, Ferrier C, Iarikova P, Sankararaman S, Yamini G, Yuan Q, Zhou Z, Albaugh B, White KV, Shen PH, Goldman D (2010) Genome-wide association identifies candidate genes that influence the human electroencephalogram. Proc Natl Acad Sci U S A 107:8695–8700.

Huang MC, Chen CC, Peng FC, Tang SH, Chen CH (2009a) The correlation between early alcohol withdrawal severity and oxidative stress in patients with alcohol dependence. Prog Neuropsychopharmacol Biol Psychiatry 33:66–69.

Huang MC, Chen CH, Peng FC, Tang SH, Chen CC (2009b) Alterations in oxidative stress status during early alcohol withdrawal in alcoholic patients. J Formos Med Assoc 108:560–569.

Kumar A, LaVoie HA, DiPette DJ, Singh US (2013) Ethanol neurotoxicity in the developing cerebellum: underlying mechanisms and implications. Brain Sci 3:941–963.

Mertens JR, Lu YW, Parthasarathy S, Moore C, Weisner CM (2003) Medical and psychiatric conditions of alcohol and drug treatment patients in an HMO: comparison with matched controls. Arch Intern Med 163:2511–2517.

Mertens JR, Weisner C, Ray GT, Fireman B, Walsh K (2005) Hazardous drinkers and drug users in HMO primary care: prevalence, medical conditions, and costs. Alcohol Clin Exp Res 29:989–998.

Mon A, Durazzo TC, Gazdzinski S, Hutchison KE, Pennington D, Meyerhoff DJ (2013) Brain-derived neurotrophic factor genotype is associated with brain gray and white matter tissue volumes recovery in abstinent alcohol-dependent individuals. Genes Brain Behav 12:98–107.

Moss HB, Chen CM, Yi HY (2010) Prospective follow-up of empirically derived Alcohol Dependence subtypes in wave 2 of the National Epidemiologic Survey on Alcohol And Related Conditions (NESARC): recovery status, alcohol use disorders and diagnostic criteria, alcohol consumption behavior, health status, and treatment seeking. Alcohol Clin Exp Res 34:1073–1083.

Mukherjee S (2013) Alcoholism and its effects on the central nervous system. Curr Neurovasc Res 10:256–262.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959.

Reddy VD, Padmavathi P, Kavitha G, Saradamma B, Varadacharyulu N (2013) Alcohol-induced oxidative/nitrosative stress alters brain mitochondrial membrane properties. Mol Cell Biochem 375:39–47.

Srivastava V, Buzas B, Momenan R, Oroszi G, Pulay AJ, Enoch MA, Hommer DW, Goldman D (2010) Association of SOD2, a mitochondrial antioxidant enzyme, with gray matter volume shrinkage in alcoholics. Neuropsychopharmacology 35:1120–1128.

Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 68:978–989.

Sullivan EV, Harris RA, Pfefferbaum A (2010) Alcohol's effects on brain and behavior. Alcohol Res Health 33:127–143.

Sun AY, Sun GY (2001) Ethanol and oxidative mechanisms in the brain. J Biomed Sci 8:37–43.

Van Leemput K, Maes F, Vandermeulen D, Suetens P (1999) Automated model-based bias field correction of MR images of the brain. IEEE Trans Med Imaging 18:885–896.