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FKBP5 Moderates Alcohol Withdrawal Severity: Human Genetic Association and Functional Validation in Knockout Mice

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Alcohol withdrawal is associated with hypothalamic–pituitary–adrenal (HPA) axis dysfunction. The *FKBP5* gene codes for a co-chaperone, FK506-binding protein 5, that exerts negative feedback on HPA axis function. This study aimed to examine the effects of single-nucleotide polymorphisms (SNPs) of the *FKBP5* gene in humans and the effect of *Fkbp5* gene deletion in mice on alcohol withdrawal severity. We genotyped six *FKBP5* SNPs (rs3800373, rs9296158, rs3777747, rs9380524, rs1360780, and rs9470080) in 399 alcohol-dependent inpatients with alcohol consumption 48 h before admission and recorded scores from the Clinical Institute Withdrawal Assessment-Alcohol revised (CIWA-Ar). *Fkbp5* gene knockout (KO) and wild-type (WT) mice were assessed for alcohol withdrawal using handling-induced convulsions (HICs) following both acute and chronic alcohol exposure. We found the minor alleles of rs3800373 (G), rs9296158 (A), rs1360780 (T), and rs9470080 (T) were significantly associated with lower CIWA-Ar scores whereas the minor alleles of rs3777747 (G) and rs9380524 (A) were associated with higher scores. The haplotype-based analyses also showed an association with alcohol withdrawal severity. *Fkbp5* KO mice showed significantly greater HICs during withdrawal from chronic alcohol exposure compared with WT controls. This study is the first to show a genetic effect of *FKBP5* on the severity of alcohol withdrawal syndrome. In mice, the absence of the *Fkbp5* gene enhances sensitivity to alcohol withdrawal. We suggest that *FKBP5* variants may trigger different adaptive changes in HPA axis regulation during alcohol withdrawal with concomitant effects on withdrawal severity.
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INTRODUCTION

The alcohol withdrawal syndrome (AWS) is precipitated by cessation of excessive alcohol consumption, and is characterized by symptoms of central nervous system hyperexcitability, autonomic hyperactivity, and tremor. These symptoms follow a characteristic temporal course, typically emerging within the first 24–48 h of abstinence and resolving within 1 week. Although neither necessary nor sufficient for a diagnosis of alcohol dependence (AD; hereafter equated with alcoholism), the AWS has recently received renewed research interest because of its potential role in long-term neuroadaptations that occur with repeated cycles of alcohol intoxication and withdrawal (Heilig *et al*, 2010). An understanding of genetic factors that moderate the severity of the AWS may therefore contribute to an improved understanding of neuroadaptive processes that contribute to the progressive course of alcoholism.

The intensity and time course of AWS varies between individuals, consistent with the possibility of underlying genetic influences, but inheritance of alcohol withdrawal severity has not been directly demonstrated in humans. Interestingly, a recent twin analysis indicated that the presence of withdrawal symptoms loads on a genetic factor that is also associated with the criterion of continued use despite problems (Kendler *et al*, 2012). In mice, alcohol withdrawal severity is moderately heritable (Metten and Crabbe, 2005). Nevertheless, genes influencing alcohol withdrawal severity are largely unknown, except for the multiple PDZ-binding domain protein (MPDZ), which may modulate withdrawal severity both in mice (Shirley *et al*,

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2004) and humans (Karpyak *et al*, 2009). Existing reports on the association of vulnerability genes with acute withdrawal severity have largely focused on specific severe phenotypes such as delirium tremens (DTs), seizures, or hallucinations (Okubo *et al*, 2003; Limosin *et al*, 2004; Rujescu *et al*, 2005; Tadic *et al*, 2005; Preuss *et al*, 2006; van Munster *et al*, 2007; Karpyak *et al*, 2010; Du *et al*, 2011), associations with high or low number of withdrawal symptoms (Schumann *et al*, 2003), or phenotypes categorized as mild or severe AWS (Schmidt and Sander, 2000; Koehnke *et al*, 2002; Schumann *et al*, 2003; Wernicke *et al*, 2003). However, the symptom profile of AWS runs on a continuum with substantial individual variation, and little is known about the potential genetic components that influence the severity of AWS in individuals who do not experience DTs or withdrawal seizures.

Hypothalamic–pituitary–adrenal (HPA) axis function involving glucocorticoid, central glucocorticoid receptors (GR), and corticotropin-releasing factor is dysregulated following chronic alcohol exposure. Alterations in HPA axis responsivity contribute to the emergence of withdrawal symptoms, which in turn influence the risk of relapse (Adinoff *et al*, 1998, 2005; Becker, 2012). Alcohol-dependent patients have increased cortisol levels despite blunted adrenocortical sensitivity during withdrawal (Costa *et al*, 1996; Adinoff *et al*, 1998; Esel *et al*, 2001) and the cortisol levels may be associated with relapse vulnerability (Stephens and Wand, 2012). Animal studies also have shown a considerable increase in both circulating and brain corticosterone levels following alcohol withdrawal (Rose *et al*, 2010) and elevated blood corticosterone levels could enhance alcohol withdrawal severity (Roberts *et al*, 1994) through GR activation (Sharrett-Field *et al*, 2013). These effects may be of interest beyond the acute withdrawal period, as recent evidence suggests a role for GR-mediated plasticity in escalation of voluntary alcohol consumption in rats (Vendruscolo *et al*, 2012).

Genetic factors that regulate GR signaling may contribute to individual differences in HPA axis responses. FK506-binding protein 5 (FKBP5) is a co-chaperone of heat-shock protein 90 in an inactive GR complex. Once cortisol is bound to GR, FKBP5 is exchanged with other co-chaperones, whereupon the GR complex can translocate to the nucleus and bind the DNA. The binding of FKBP5 to the GR complex causes lower affinity for cortisol and less efficient nuclear translocation of GR (Wochnik *et al*, 2005). Therefore, *FKBP5* gene variants may influence GR sensitivity to cortisol and the efficiency of negative feedback on the HPA axis (Binder, 2009). Accordingly, variation in the *FKBP5* gene has been associated with reduced dexamethasone suppression (Binder *et al*, 2008), incomplete normalization of stress-elicited cortisol secretion (Ising *et al*, 2008), and response to antidepressants and recurrence of depressive episodes (Binder *et al*, 2004). *FKBP5* variation has also been shown to interact with childhood trauma exposure to increase the risk for posttraumatic stress disorder (PTSD) (Binder *et al*, 2008; Xie *et al*, 2010) as well as suicidal behavior (Roy *et al*, 2010). Furthermore, genotype-specific *FKBP5* DNA methylation may mediate these gene–childhood trauma interactions (Klengel *et al*, 2013). More importantly, a recent study has reported differential expression of the *FKBP5* gene in postmortem brains of alcoholics (McClintick *et al*, 2013), suggesting a role for this gene in the pathophysiology of AD. Here, we examined whether genetic variation at the *FKBP5* locus moderates the severity of alcohol withdrawal in alcohol-dependent patients who had consumed their last drink within 48 h of admission to an inpatient unit. To functionally validate our human association findings, we assessed the withdrawal response following acute and chronic alcohol exposure in mice with deletion of the *Fkbp5* gene.

MATERIALS AND METHODS

Human Association Study

Participants. Participants who met the *Diagnostic and Statistical Manual for Mental Disorders, 4th Edition, Text-revised* (*DSM-IV-TR*) criteria for AD were consecutively recruited from September 2005 to March 2013 at the National Institute on Alcohol Abuse and Alcoholism (NIAAA) Inpatient Unit at the National Institutes of Health (NIH) Clinical Center where they were voluntarily admitted to a 28-day inpatient treatment protocol. Subjects had consumed their last alcoholic drink within 48 h of admission, were literate in English, and were not suffering from active psychotic symptoms or cognitive impairment. Informed consent was obtained in accordance with the Declaration of Helsinki and the NIH Combined Neuro-Science Institutional review board.

Clinical assessment. The *Structured Clinical Interview for* DSM-IV *Axis I Disorders* (*SCID*) (First *et al*, 1996) was used for the diagnostic assessment of alcohol and other substance use disorders. Severity of AD was assessed using the Alcohol Dependence Scale (Skinner and Horn, 1984), and alcohol consumption during the preceding 3 months was assessed using the timeline follow-back (TLFB). Smoking intensity parameters included pack-years and Fagerstrom test of nicotine dependence score. The baseline measurement of depression and anxiety symptoms on the second day of admission were based on the Comprehensive Psychopathological Rating Scale (CPRS) (Asberg *et al*, 1978). Withdrawal intensity was evaluated using the Clinical Institute Withdrawal Assessment-Alcohol revised (CIWA-Ar) Scale approximately every 2–4 h while awake for the first 3–5 days after admission. Participants did not receive any prescription medications other than benzodiazepines (BZDs; diazepam, oxazepam, or lorazepam), which were given based on clinical decisions. We analyzed the maximum and average CIWA-Ar scores during withdrawal, as well as total BZD usage, which was converted to diazepam equivalent doses where necessary.

Genotyping. With the exception of rs1360780, genotyping was performed at the NIAAA Laboratory of Neurogenetics on the Illumina OmniExpress BeadChip (Illumina, San Diego, CA), which includes >700 000 single-nucleotide polymorphisms (SNPs). The average genotype reproducibility was 0.99994. Linkage disequilibrium (LD), that is, the non-random association of alleles at two or more loci, of the *FKBP5* gene, is generally high in most populations including Caucasians and African Americans (Binder, 2009). In this study, the *FKBP5* SNPs were selected based on those that

have been implicated in a previous study (Roy *et al*, 2010) and could be captured from the available genetic information in the existing Illumina data set for *FKBP5*, including rs3800373, rs9296158, rs3777747, rs9380524, and rs9470080. An additional SNP not included in the Illumina Omni-Express array, rs1360780, was also genotyped as the functionality of this variant in the negative feedback of GR regulation has been investigated and characterized (Binder *et al*, 2004; Ising *et al*, 2008; Roy *et al*, 2010; Xie *et al*, 2010; Zannas and Binder, 2014). Rs1360780 was genotyped using the assay-on-demand (assay ID: C___8852038_10) from Applied Biosystems (Foster City, CA). The allele was discriminated by post-PCR plate read on ABI PRISM 7900HT Sequence Detector System (Applied Biosystems). Data were processed using SDS 2.4 software (Applied Biosystems) at the Laboratory of Clinical and Translational Studies, NIAAA, NIH. All six SNPs were in Hardy–Weinberg equilibrium (HWE) and had minor allele frequencies $>5\%$.

Assessment of population stratification using ancestry informative markers. Ancestry informative markers (AIMs, $n=2500$) were extracted from the Illumina array to calculate ancestral proportions for all study participants (missing data for five subjects). Using methods described previously for an AIMs panel including 186 markers (Hodgkinson *et al*, 2008), which were not available for our data set, the ancestry assessment identified six ethnic factors (Africa, Europe, Asia, Far East Asia, Oceania, and Americas). For subjects who self-reported as Caucasian or white, the mean European factor score was 0.94 (median, 0.96) and the mean African factor score was 0.013 (median, 0.001). For subjects who self-reported as black, the mean African factor score was 0.74 (median, 0.77) and the mean European factor score was 0.23 (median, 0.20).

Mouse Experiments

Subjects. Alcohol-naive adult male and female *Fkbp5* knockout (KO) and litter mate wild-type (WT) mice were used. These mice were generated through mating of heterozygous Fkbp5 mice as described in the previous report (Yong *et al*, 2007). KO mice used for this experiment have been backcrossed with C57BL/6J for four generations. On the first day of each experiment, mice were between 120 and 294 days old. During experiments, mice age matched for genotype were group-housed in polycarbonate cages ($29.2\times19.0\times12.7$ cm) with aspen wood shavings in groups of 2–4 per cage (experiment 1: acute alcohol exposure) or singly housed with nestlets, shredded paper, and chew toys (experiment 2: chronic alcohol exposure). Ambient room temperature was maintained at $21\pm2\,^{\circ}$C. Mice in experiment 1 had free access to food (Rodent Lab Diet 5001, Purina Mills, St Louis, MO) and water in the home cage at all times, except when testing procedures took place. Mice in experiment 2 had free access to a nutritionally complete liquid diet (Rodent Liquid Diet Lieber-DeCarli '82 Shake and Pour, Bio-Serv, Frenchtown, NJ), combined with up to 6% alcohol or calorie-matched maltodextrin, and water in the home cage at all times, except when testing procedures took place. Experimental procedures were conducted during the light phase of a 12:12 light:dark cycle (lights off at 1900 hours).

All experimental procedures were approved by the Purdue Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Alcohol treatment. Alcohol (95% v/v) was diluted to 20% (v/v) with 0.9% physiological saline and injected IP at a dose of 4.0 g/kg (25.2 ml/kg; experiment 1) or diluted to 2, 4.5, or 6% (v/v) in the nutritionally complete liquid diet (experiment 2).

Procedures. Handling-induced convulsion (HIC) procedures consisted of two baseline assessments conducted 20 min apart and immediately before alcohol/saline injections (experiment 1) or the introduction of alcohol into the liquid diet (experiment 2). HICs during withdrawal were assessed hourly beginning at 2 h after injection or removal of diet through hour 12 (6 measurements) and again at 24 and 25 h in withdrawal. Mice were gently and quickly lifted by the tail and spun 180°; the resultant convulsions were rated on a scale of severity ranging from 0 to 7. The HICs technique and assessment scale were adapted from published methods of Crabbe and colleagues (e.g., Metten and Crabbe, 1994). Experimenters were blinded to genotype and treatment (alcohol *vs* control).

Baseline HICs were assessed at 0700 hours, immediately before the IP injections of alcohol or saline (experiment 1) or 48 h after the introduction of plain liquid diet and immediately before introduction of alcohol into the diet (experiment 2). HICs were then assessed hourly at 2–12, and again at 24 and 25 h following acute injection or following removal of the 6% alcohol liquid diet.

For experiment 1, 48 *Fkbp5* KO (24 males and 24 females) and 43 WT (19 males and 24 females) mice were randomly assigned to receive acute exposure to either alcohol or saline; 5 Fkbp5 KO (2 males and 3 females) and 4 WT (0 males and 4 females) mice had previously undergone a similar HICs assessment procedure in which they had only received saline. For experiment 2, 8 *Fkbp5* (3 males and 5 females) and 10 WT (4 males and 6 females) from experiment 1 were exposed to an alcohol liquid diet. Of these mice, approximately half had received alcohol injections and half had received saline injections previously, during experiment 1. At least 4 months had passed since the end of experiment 1 and the start of experiment 2. For experiment 2, mice were gradually introduced to increasing percentages of alcohol over the course of 7 days, receiving 0, 2, and 4.5% alcohol diet for 2 days each until the final concentration of 6% was reached on day 7. The 6% alcohol liquid diet was then supplied for 14 days, until day 21 when it was replaced with a calorie-matched maltodextrin liquid diet at 0700 hour for withdrawal assessment.

Statistical Analysis

We used the HaploView software program version 4.2 to assess HWE, LD (using the D′ and r^2 index), allelic/haplotype frequencies, and haplotype blocks, the latter of which were defined by the default D′/LOD method (Barrett

et al, 2005). The association analyses were conducted by linear regression using PLINK version 1.06 (Purcell, http://pngu.mgh.harvard.edu/~purcell/plink/) to calculate the effect size (beta coefficient) and significance level. The association of SNPs with withdrawal severity (maximum as well as average CIWA-Ar scores during withdrawal) was examined based on the most significant *p*-value among additive, allelic, dominant, and recessive model assumptions. The direction of the regression coefficient represents the effect of each extra minor allele. All models were also adjusted for age, gender, AIMs scores, pack-years of smoking, Fagerstrom scores, comorbidity with other substance dependence or not, use of other substances within the past 30 days, and the CPRS measures of depression and anxiety from day 2, which may affect the alcohol withdrawal symptoms but have also been shown to be associated with *FKBP5* variants (Binder *et al*, 2004, 2008, 2009; Xie *et al*, 2010). As BZD administration may also influence the withdrawal severity, we further adjusted the results for BZD usage (receiving BZD or not as well as total diazepam equivalent dose). To correct for multiple testing, we used the Benjamini–Hochberg method (Benjamini and Hochberg, 1995) to control for the false discovery rate (FDR). For haplotype-based analysis, the FDR represents a permutated *p*-value adjusted for testing multiple haplotypes (5000 permutations). In this study, we controlled the FDR at $q^* = 0.05$ and assumed *p*-values were independently distributed under null hypotheses. A *p*-value < 0.05 after multiple testing correction was considered to be statistically significant.

For mouse behavior analyses, HICs scores (baseline and withdrawal) were averaged based on scores from two independent raters with an historic inter-rater reliability of $>90\%$. Baseline scores of KO and WT subjects were not significantly different; however, area under the curve (AUC) for all withdrawal time points (2–12, 24, and 25 h) was corrected with reference to the baseline score by subtracting each subject's average baseline score from each of their eight scores measured during the withdrawal time course (negative values were converted to a zero) (Metten and Crabbe, 1994). HICs data (AUC) were not normally distributed and so the non-parametric Kruskal–Wallis test was used for analysis. Probability values < 0.05 were considered to be significant.

RESULTS

Demographic Data

Demographic and clinical characteristics of the 399 subjects (274 males and 125 females; mean age: 41.7 ± 9.9) are shown in Table 1.

Table 1 Demographic and Clinical Characteristics of 399 Patients with Alcohol Dependence

Sex	
Male/female, *N* (%)	274/125 (68.7/31.3)
Age, mean (SD), years	41.7 (9.9)
Race, N *(%)*	
EA	230 (57.6)
AA	136 (34.1)
Others	33 (8.3)
Number of years of alcohol use, mean (SD)	22.211 (10.15)
Number of years of intoxicating alcohol use, mean (SD)	14.59 (9.3)
Alcohol dependence score, mean (SD)	21.80 (8.06)
Timeline follow-back, 90 days, mean (SD)	
Total drinks	1164.12 (751.53)
Drinking days, days	74.30 (20.45)
Average drinks/day, drinks	15.23 (8.23)
Heavy drinking days, days	70.32 (23.88)
Fagerstrom score, mean (SD)	2.93 (2.73)
Cigarette pack-years, mean (SD)	12.42 (15.60)
With any diagnosis of other substance dependence, *N* (%)[a]	140 (35.1)
Other substance use within the past 30 days, *N* (%)	234 (58.7)
Maximum scores on CIWA-Ar	9.43 (6.52)
Average scores on CIWA-Ar	6.48 (5.21)
Laboratory values	
Albumin (U/l)	4.10 (0.36)
GGT (g/dl)	182.65 (313.87)
AST (U/l)	68.96 (71.84)
ALT (U/l)	63.09 (54.08)
ALP (U/l)	80.19 (27.12)
CDT (%)	0.09 (0.09)
Receiving BZD treatment, yes/no, N *(%)*	197 (49.4)/202 (50.6)
Diazepam equivalence dose (mg)[b]	83.26 ± 110.37

Abbreviations: AA, African Americans; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BZD, benzodiazepine; CDT, carbohydrate-deficient transferring; CIWA-Ar, Clinical Institute Withdrawal Assessment-Alcohol revised; EA, European Americans; GGT, gamma-glutamyltransferase.
[a]Other substances include amphetamine, cocaine, cannabis, hallucinogen, inhalant, phencyclidine, sedatives, or opioids.
[b]Diazepam equivalent doses were logarithmically transformed for analysis.

Single Marker Association

The genotype information for each SNP is shown in Table 2. All six SNPs were significantly associated with both maximum (Table 3) and average (Supplementary Table 1) CIWA-Ar scores. Negative beta coefficients (rs3800373, rs9296158, rs1360780, and rs9470080) in the regression models indicate that individuals carrying the minor allele of these markers have lower CIWA-Ar scores, while positive coefficients (rs3777747 and rs9380524) indicate higher CIWA-Ar scores. All associations remained significant after adjusting for the potential covariates, receiving BZD or not, and total BZD dose. Even after further adjustment by AD severity scores and drinking variables from the TLFB data, all associations remained significant. When we tested the associations in European Americans only, we found rs3800373, rs9296158, rs1360780, and rs9470080 were significantly associated with withdrawal severity (see Supplementary Table 3).

Table 2 The Genetic Information for *FKBP5* Gene SNPs

SNP	Chromosome position	Function	Alleles 1/2	Genotype distributions (11/12/22)	MAF	HWE
rs3800373	35 650 454	3′UTR	G/T	44/166/184	0.3223	0.49
rs9296158	35 675 060	Intron 5	A/G	51/165/182	0.3354	0.18
rs3777747	35 686 980	Intron 5	G/A	83/195/119	0.4547	0.84
rs9380524	35 697 048	Intron 3	A/C	1/64/332	0.0831	0.50
rs1360780	35 715 549	Intron 2	T/C	46/163/187	0.3220	0.25
rs9470080	35 754 413	Intron 1	T/C	54/162/174	0.3462	0.12

Abbreviations: HWE, *p*-values of the Hardy–Weinberg equilibrium; MAF, minor allele frequency.
Alleles 1 and 2 refer to minor and major allele, respectively. Genotype distributions are shown as the counts of three genotypes, 11/12/22.

Table 3 Associations between *FKBP5* Gene SNPs and Alcohol Withdrawal Severity Measured by CIWA-Ar

			CIWA-Ar scores[a]								
			No adjustment			Adjusted for covariates[b], receiving BZD or not, and total BZD dose[c]			Adjusted for covariates[b], receiving BZD or not, total BZD dose[c], and drinking variables[d]		
FKBP5 SNPs	Alleles 1/2	Mean CIWA-Ar scores for each genotype (11/12/22)	Beta	*p*-Value	FDR	Beta	*p*-Value	FDR	Beta	*p*-Value	FDR
rs3800373	G/T	7.2/9.0/10.3	−1.49	0.002^{A}	0.003*	−1.62	0.002^{L}	0.003*	−1.57	0.002^{L}	0.004*
rs9296158	A/G	7.0/9.2/10.3	−1.49	0.002^{A}	0.003*	−1.61	0.001^{L}	0.003*	−1.57	0.002^{L}	0.004*
rs3777747	G/A	10.2/9.8/8.3	1.61	0.02^{D}	0.02*	1.19	0.02^{L}	0.02*	1.31	0.007^{L}	0.009*
rs9380524	A/C	7.0/11.4/9.1	2.20	0.01^{D}	0.02*	1.98	0.03^{L}	0.03*	2.17	0.02^{L}	0.02*
rs1360780	T/C	6.9/9.1//10.3	−1.55	0.001^{A}	0.003*	−1.59	0.002^{L}	0.003*	−1.54	0.002^{L}	0.004*
rs9470080	T/C	6.8/9.4/10.4	−1.61	0.0006^{A}	0.003*	−1.66	0.0008^{L}	0.003*	−1.62	0.001^{L}	0.004*

Abbreviations: ADS, Alcohol Dependence Scale; AIMs, ancestry informative markers; BZD, benzodiazepine; CIWA-Ar, Clinical Institute Withdrawal Assessment-Alcohol revised; CPRS, Comprehensive Psychopathological Rating Scale; FDR, false discovery rate; TLFB, timeline follow-back.
Alleles 1 and 2 refer to minor and major allele, respectively.
P-value represents the minimal *p*-value from four models, including additive (A), allelic (L), dominant (D), and recessive (R) models.
FDR was calculated using Benjamini and Hochberg's method (1995) for multiple testing corrections.
[a]The maximum scores on CIWA-Ar during withdrawal were analyzed.
[b]The potential covariates used for adjustment include age, gender, AIMs, smoking variables (Fagerstrom score and cigarette pack-years), other drug use variables (comorbidity with other drug dependence and drug use within the past 30 days), baseline depression and anxiety scores on CPRS.
[c]Total BZD dose are counted by total diazepam equivalence dose.
[d]Drinking variables include ADS scores and TLFB data (TLFB measurement of alcohol consumption variables during the preceding 3 months including total drinks, number of drinking days, average drinks per day, and heavy drinking days).
*$p < 0.05$.

LD Structure

Figure 1 shows that the six SNPs that span *FKBP5* are in strong LD. There are four haplotypes with frequency >5% accounting for 94.5% of the haplotype diversity. We compared the structures of LD across the 150-kb region containing the *FKBP5* gene using genotype data from the CEU and ASW HapMap samples and found the LD structure across this region showed high similarity between the two populations.

Haplotype-Based Association

We examined the association of haplotypes based on the selected SNPs with overall maximum (Table 4) and average (Supplementary Table 2) withdrawal severity. Haplotype GAACTT (frequency = 0.30) and the complementary haplotype TGGACC (frequency = 0.08) were associated with lower and higher withdrawal severity, respectively. These results were consistent with what we found using the individual markers, that is, haplotype GAACTT was composed of alleles on each maker that were associated with lower CIWA-Ar scores, whereas TGGACC included alleles that were associated with higher CIWA-Ar scores (see Table 3). Associations for the two haplotypes, however, were no longer significant after adjustment for covariates. In the subsample of European Americans, the significant association between haplotype GAACTT and alcohol withdrawal severity also disappeared after covariate adjustment (Supplementary Table 4). When we examined haplotypes composed of rs3800373, rs9296158, rs1360780, and rs9470080 (see Supplementary Figure 1 for LD structure of these four SNPs), which have been previously

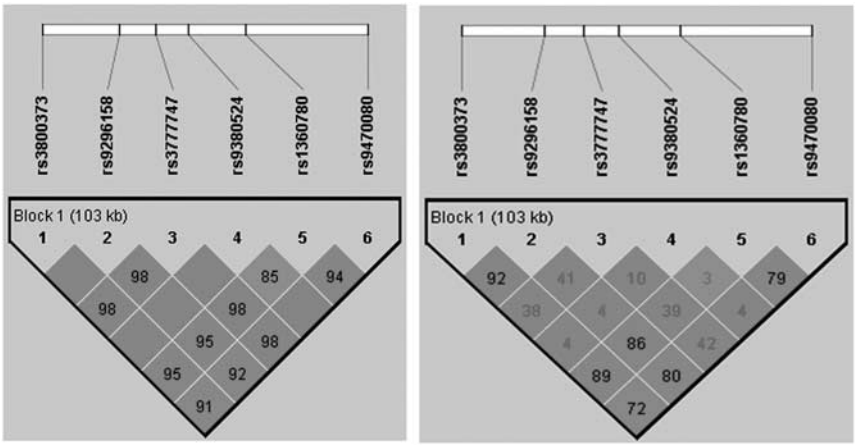

Figure 1 The haplotype structure of six *FKBP5* gene SNPs. The numbers in the squares refer to pairwise LD measured as D′ (left) and r^2 (right).

Table 4 Haplotype-Based Association of *FKBP5* Gene SNPs with Alcohol Withdrawal Severity Measured by CIWA-Ar

		CIWA-Ar scores[a]								
		No adjustment			Adjusted for covariates[b], receiving BZD or not, and total BZD dose[c]			Adjusted for covariates[b], receiving BZD or not, total BZD dose[c], and drinking variables[d]		
Haplotype	Frequency	Beta	*p*-Value	FDR	Beta	*p*-Value	FDR	Beta	*p*-Value	FDR
FKBP5 gene (rs3800373–rs9296158–rs3777747–rs9380524–rs1360780–rs9470080)										
GAACTT	0.2990	−1.49	0.002	0.007*	−0.54	0.14	0.42	−0.30	0.40	0.82
TGGACC	0.0806	2.23	0.01	0.04*	0.71	0.28	0.68	0.74	0.26	0.63
TGGCCC	0.3680	0.53	0.27	0.66	0.20	0.57	0.95	0.32	0.36	0.78
TGACCC	0.1830	0.70	0.21	0.57	0.40	0.34	0.75	0.12	0.78	0.99

Abbreviations: ADS, Alcohol Dependence Scale; AIMs, ancestry informative markers; BZD, benzodiazepine; CIWA-Ar, Clinical Institute Withdrawal Assessment-Alcohol revised; CPRS, Comprehensive Psychopathological Rating Scale; FDR, false discovery rate; TLFB, timeline follow-back.
Results were based on haplotype-based association tests; only haplotypes with frequency >0.05 were considered.
FDR represents a permutated *p*-value adjusted for testing multiple haplotypes (5000 permutations).
[a]The maximum scores on CIWA-Ar during withdrawal were analyzed.
[b]The potential covariates used for adjustment include age, gender, AIMs, smoking variables (Fagerstrom score and cigarette pack-years), other drug use variables (comorbidity with other drug dependence and drug use within the past 30 days), baseline depression and anxiety scores on CPRS.
[c]Total BZD dose are counted by total diazepam equivalence dose.
[d]Drinking variables include ADS scores and TLFB data (TLFB measurement of alcohol consumption variables during the preceding 3 months including total drinks, number of drinking days, average drinks per day, and heavy drinking days).
*$p<0.05$.

reported to be potentially functional and associated with several measures of psychopathology (Binder *et al*, 2004, 2008; Ising *et al*, 2008; Roy *et al*, 2010; Xie *et al*, 2010), we found that haplotype GATT and the complementary TGCC were positively and negatively associated with withdrawal severity, respectively (Supplementary Table 5); however, these results also did not remain after adjustment for covariates. These findings suggest that haplotypes consisting of minor alleles are linked to a lower severity of alcohol withdrawal.

HICs in *Fkbp5* KO and WT Mice following Acute Alcohol Exposure

Analysis of treatment group AUC within each genotype using the Kruskal–Wallis non-parametric test showed a main effect of treatment group in KO mice ($H(1)=4.859$, $p=0.028$; alcohol > saline) but not in WT mice (Figure 2).

HICs *Fkbp5* KO and WT Mice following Chronic Alcohol Exposure

Analysis of HICs AUC using the Kruskal–Wallis non-parametric test showed a main effect of genotype ($H(1)=4.381$, $p=0.036$; KO > WT) (Figure 3).

DISCUSSION

We found that *FKBP5* gene SNPs (rs3800373, rs9296158, rs3777747, rs9380524, rs1360780, and rs9470080), both

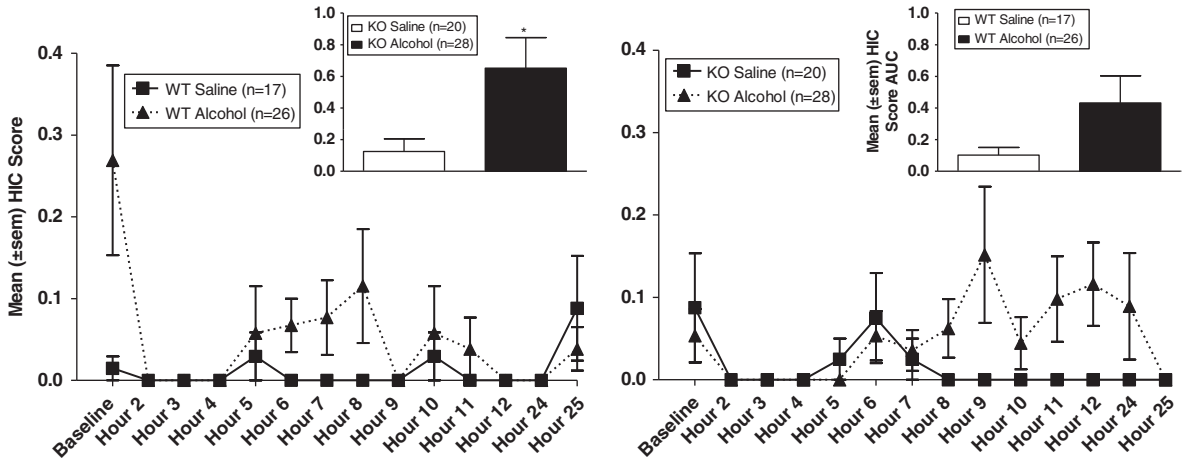

Figure 2 Mean (±SEM) HICs at each time point (baseline, 2–12, 24, and 25 h) following acute injection of either alcohol or saline. Inset: mean (±SEM) AUC (corrected for baseline) for HICs across the withdrawal measurement time course. $*p < 0.05$; alcohol > saline.

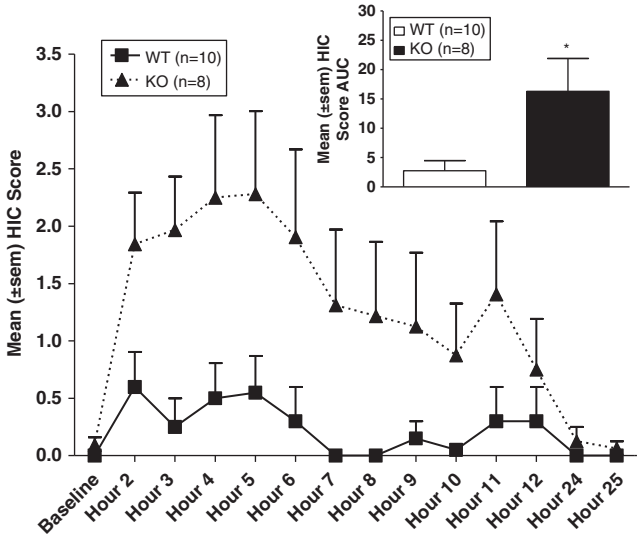

Figure 3 Mean (±SEM) HICs at each time point (baseline, 2–12, 24, and 25 h) following removal of alcohol diet. Inset: mean (±SEM) AUC (corrected for baseline) for HICs across the withdrawal measurement time course. $*p < 0.05$, KO > WT.

individually and in a haplotype-based analysis, influence the severity of alcohol withdrawal severity. To our knowledge, this is the first study to investigate the potential genetic factors influencing severity of the overall AWS, as previous studies of predisposing characteristics for severe alcohol withdrawal were focused on DT, seizures, or hallucinations. As human genetic association studies are fraught with numerous potential confounds and frequently fail to replicate, we validated our findings using genetically modified mice. We found converging evidence in a KO mouse model that suggests an important role for the *Fkbp5* gene in modulating alcohol withdrawal severity. KO mice showed greater alcohol withdrawal-induced HICs following chronic alcohol exposure compared with WT mice.

The SNPs in the *FKBP5* gene with possible functional effects (eg, rs1360780) have been suggested to serve as 'intronic enhancers,' which are intronic SNPs with a binding site for transcriptional factors such as GR (Binder, 2009). Upon cortisol binding, a direct GR-mediated upregulation of FKBP5 expression via the intronic response elements (Vermeer *et al*, 2003) will act as part of an intracellular ultra-short negative feedback loop for GR activity, conferring a state of GR resistance and affecting HPA axis responsivity. Several *FKBP5* gene variants have been demonstrated to be functionally involved in the regulation of HPA axis responsivity. For example, *in vitro* exposure of cells to glucocorticoids induces an increase in *FKBP5* mRNA, with genotypes composed of rs1360780 and rs3800373 minor alleles being the highest expressing variants (Vermeer *et al*, 2003). In humans, healthy adults homozygous for these alleles show a greater and more prolonged cortisol response during the Trier Social Stress Test compared with those who have complementary genotypes (Ising *et al*, 2008). These SNPs, as well as rs9296158 and rs9470080, have been associated with a higher FKBP5 expression and a stronger induction of *FKBP5* mRNA by cortisol (Binder *et al*, 2004; Tatro *et al*, 2010), and subsequently have been correlated with depression (Binder *et al*, 2004; Tatro *et al*, 2010) and suicide (Brent *et al*, 2010; Perez-Ortiz *et al*, 2013). They also affect cortisol responses and result in diminished dexamethasone suppression (Binder *et al*, 2008). In addition, the minor alleles of rs9470080 and rs1360780 are associated with total cortisol secretion during the day expressed as the AUC ($cortisol_{AUC}$) (Velders *et al*, 2011). Finally, a haplotype of the above-listed SNPs has been associated with suicidal attempts (Roy *et al*, 2010) or aggressive behavior (Bevilacqua *et al*, 2012) among individuals with childhood trauma.

The functional association between *FKBP5* gene variants and HPA axis regulation appears to be altered, for example, the high expression alleles may instead be associated with increased GR sensitivity, in the presence of disease where feedback inhibition has been impaired along with activation of the HPA axis (Binder *et al*, 2004; Bevilacqua *et al*, 2012). Such is the case in subjects with depression or PTSD, in whom the high expression alleles are associated with greater glucocorticoid sensitivity, compared with controls where they are associated with increased glucocorticoid resistance

(Binder *et al*, 2004, 2008; Bevilacqua *et al*, 2012). Binder *et al* (2004, 2009) suggest that the high expression alleles of the *FKBP5* gene may counteract depression-related HPA axis hyperactivity via compensatory mechanisms, allowing a faster restoration of normal HPA axis function and a more rapid response to antidepressants in depressed individuals. It is possible that similar mechanisms may also have a role in the reduced AWS severity we observed in minor allele carriers of the *FKBP5* variants. Given that AWS also produces a pattern of HPA axis dysregulation, and a normalization of HPA axis responsivity has been associated with resolution of AWS (Costa *et al*, 1996; Adinoff *et al*, 1998, 2005; Esel *et al*, 2001) individuals with higher expression alleles (eg, rs3800373, rs92962158, rs1360780, and rs9470080) may have better compensatory adaptive mechanisms to resist AWS, and thus manifest lower CIWA-Ar scores. Although the *in vitro* function and biological impact of rs3777747 and rs9380524 has not been studied yet, we postulate that the minor alleles of these two SNPs (G and A allele, respectively) are not high expression alleles favoring compensatory mechanisms, because of the observation that the minor allele carriers manifest higher CIWA-Ar scores, suggesting a poorer adaptive response to HPA activation. Our haplotype analyses also showed a consistent pattern of association between minor alleles of each SNP and withdrawal severity, that is, two complementary haplotypes involving the SNPs (GAACTT and TGGACC) were associated with lower and higher CIWA-Ar scores, respectively, although the association disappeared after adjustment for covariates. In sum, our finding adds to existing evidence that genetic factors contribute to the central neuroadaptation to alcohol withdrawal-induced impairment of HPA axis regulation in humans. Our findings of greater withdrawal after chronic alcohol exposure in KO mice are in agreement with the notion that FKBP5 function affects alcohol withdrawal severity.

Findings from this study should be interpreted in light of several limitations. Previous investigations of the genetic component of alcohol withdrawal-related phenotypes have typically classified subjects as with or without a specific history of DTs, hallucinations, and seizures (Schmidt and Sander, 2000; Koehnke *et al*, 2002; Okubo *et al*, 2003; Schumann *et al*, 2003; Wernicke *et al*, 2003; Limosin *et al*, 2004; Rujescu *et al*, 2005; Tadic *et al*, 2005; Preuss *et al*, 2006; van Munster *et al*, 2007; Karpyak *et al*, 2010; Du *et al*, 2011). It is important to note that the withdrawal severity in our patients was generally mild to moderate, and as a result our findings cannot be extended to patients exhibiting severe forms of withdrawal. Similarly, the HIC measure in mice assesses physical withdrawal signs that vary on a spectrum of mild to severe (convulsion-like signs are rated on a scale of 0–7) and the degree of withdrawal severity observed in this study was also mild to moderate in magnitude. Moreover, HIC measurement is but one way to assess physical withdrawal severity. As a result, additional detailed methods to assess withdrawal severity as well as the inclusion of more severe forms of withdrawal phenotypes should be considered. Furthermore, HIC measurement can only be used in mice. There is likely an incomplete overlap of the mechanisms regulating the withdrawal phenotypes measured between mice and humans. Second, it is not clear whether the associations between *FKBP5* SNPs and withdrawal severity may be attributed to other comorbid psychiatric disorders, such as major depression or PTSD. In addition, childhood trauma has been shown to interact with *FKBP5* gene variation to influence the stress response (Binder *et al*, 2008; Roy *et al*, 2010; Xie *et al*, 2010; Bevilacqua *et al*, 2012) and is considered to be a confounding variable in HPA axis dysregulation in alcohol-dependent patients (Schafer *et al*, 2010). Therefore, we were unable to exclude the contribution from stressful life events to our results. Third, the haplotype-based findings may be biased, in that the SNPs in our study were selected from those that have been published previously rather than based on independent criteria for tag SNP selection across the entire gene locus. We do not know whether the results would be different after examination of additional SNPs. Fourth, we did not include heterozygous animals that may provide insight into the recessive and dominant characteristics of *Fkbp5* gene and the pertinent underlying mechanisms. Fifth, data on cortisol/coticosterone levels or HPA axis reactivity were not available for our human subjects or for the mice. Therefore, we are not able to directly assess potential moderating effects of *FKBP5* SNPs on HPA axis dysregulation associated with AWS. We believe direct endocrine measures including CRH levels, which is also strongly implicated in the pathogenesis of AD (Becker, 2012), are needed in future studies to elucidate the roles of FKBP5 in regulating HPA axis activity, GR sensitivity, and the feedback on CRH. Sixth, it should be noted that there are other pathways, other than regulation of the GR, by which FKBP5 may influence the alcohol withdrawal phenomenon. For instance, FKBP5 has direct anxiogenic effects when overexpressed in the amygdala (Attwood *et al*, 2011). Moreover, FKBP5 and indirect GR sensitivity could feedback on CRH levels, which may be directly involved in the mediation of AWS (Sommer *et al*, 2008). Last but not least, while the Fkbp KO mouse experiments support the role of *FKBP5* gene involvement in alcohol withdrawal, they do not explicitly model human genetic variation. In addition, possible compensatory changes that occur during development may have been overlooked in this study. The utilization of different types of genetic animal models such as a congenic mouse model to explore the role of genetic variation at genetic loci is warranted. Despite these limitations, our findings suggest that a potential role for *FKBP5* gene in modulating alcohol withdrawal severity is an important avenue of research that warrants further study, in particular, mechanistic exploration of the functional significance of the SNP variants and their association with the alcohol withdrawal phenotypes studied here.

In conclusion, we show that the *FKBP5* gene in mice and *FKBP5* genetic variants in humans are significantly associated with alcohol withdrawal severity. These differences in withdrawal severity outcome may be attributable to variation in the ability of these variants to regulate FKBP5 expression (Binder *et al*, 2004, 2009) and ensuing HPA axis responsivity. Further studies are needed to replicate and verify these tentative allelic associations.

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The authors declare no conflict of interest.

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