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# The role of TACR1 genotypes in craving, depression, and alcohol dependence.

Sara Keelan Blaine

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**THE ROLE OF TACR1 GENOTYPES IN CRAVING,  
DEPRESSION, AND ALCOHOL DEPENDENCE**

**BY**

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**BA, PSYCHOLOGY, CORNELL UNIVERSITY 2006**

**THESIS**

Submitted in Partial Fulfillment of the  
Requirements for the Degree of

**Masters of Arts  
Psychology**

The University of New Mexico  
Albuquerque, New Mexico

**May, 2011**

## DEDICATION

To Eric Claus, PhD, the scientist who taught me everything I know about fMRI, and the friend who never stopped believing in me.

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by

Sara K. Blaine

*B.A., Psychology, Cornell University, 2006*

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## ABSTRACT

Given that stress and major depression are putative causal factors in alcohol consumption, the exploration of the genes and the associated neurobiological mechanisms that influence the relationship between stress, depression, and alcohol dependence (AD) is a first step toward the development of novel medications for AD. The tachykinin receptor 1 (TACR1) gene is a promising candidate gene, showing an association with stress-related behaviors (Thorsell et al., 2010), major depression (Kramer et al., 2004), and AD treatment outcome (George et al., 2008). The purpose of the current study was to determine if TACR1 single nucleotide polymorphisms (SNPs) are associated with (1) blood oxygen level dependent (BOLD) activation in response to alcohol cues, (2) DSM-IV-TR AD symptom count, (3) DSM-IV-TR depression diagnoses, and (4) DSM-IV-TR AD symptom count in a large, publicly available dataset. To address these questions, the current study examined relationships between neural responses during a craving task in 326 individuals with alcohol use disorders and SNPs within the TACR1 gene. Of the 70 SNPs tested, rs3771863 was predictive of AD symptom count and BOLD activation in response to alcohol cues, regardless of major depression status, as well as AD symptom count in the SAGE dataset. Additionally, rs3771810 and

rs12477553 also predicted BOLD activation in response to alcohol cues, but the relationship between these SNPs and AD symptom count was moderated by major depression status. Finally, rs1106855 is a SNP associated with BOLD activation that should be explored for possible functional significance due to its location within a stop codon. The exploration of TACR1 receptor antagonism as a form of AD treatment should be further examined in less heterogeneous samples, as it might be most effective for those with primary or secondary alcohol dependence in addition to a diagnosis of major depression.

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## Introduction

Current pharmacological and psychosocial treatments for alcohol dependence (AD) are only modestly effective; naltrexone, 12 step facilitation, motivational enhancement approaches, and cognitive behavioral therapies have led to 12 month abstinence rates between 17 to 35% (Miller et al., 2001). These results speak to the large variability of biological and psychosocial mechanisms involved in AD. Many of the most important mechanisms in the development of AD are heritable susceptibility factors, which contribute 50-60% of the disease risk (Dick et al., 2006). These genetic factors interact with environmental factors to produce and maintain the disease state, although studies to date have only explained 2-3% of the genetic variance in diagnosis (Dick et al., 2006; Goldman et al., 2006).

Nonetheless, researchers are now exploring the idea of personalized medicine; that is, patients with AD could potentially be matched to various treatments based on genetic profiles that have been linked to the functional neurobiological mechanisms which maintain AD. The tachykinin receptor 1 (TACR1) gene has shown an association with an affective neuroimaging phenotype (Gilman and Hommer, 2010) as well as AD treatment outcome (George et al., 2008). Because the TACR1 receptor is also mechanistically related to negative affect and depression (Heilig et al., 2007; Kramer et al., 2004), which are putative causal factors of AD, the TACR1 gene should be considered as a potential point of pharmacological intervention for AD.

### *Candidate gene associations.*

To date, many genetic studies of AD have been based on candidate gene approaches. That is, single genes were chosen based on theoretical connections to AD.

When the function of a gene is known, candidate gene studies can facilitate the development of mechanistic hypotheses. Such studies have identified variations in several chromosomal regions, including regions of chromosomes 1,2,4,7, and 11, which are related to intermediate phenotypes associated with AD (Dick et al., 2006). Genes that have been implicated in AD involve synaptic plasticity, growth regulation, cation transport, lipid signaling, gene regulation and cell adhesion (Treutlein et al., 2009).

While some genes have been replicated in multiple studies, replication at the level of single nucleotide polymorphisms (SNPs) is difficult because one must control for the number of statistical tests performed, as a single gene can contain thousands of SNPs. Furthermore, many findings linking genes to diagnostic phenotypes have not been replicated, despite large sample sizes, because the effects of single genes on neurological activity are much like that of tributaries of a river which come together to influence downstream effects, (i.e., neurotransmission, long term potentiation, etc; see Cannon & Keller, 2006). The development of brain based intermediate phenotypes could allow for discovery of gene-neurobiology associations, in addition to gene-phenotype associations.

*The BOLD response is a powerful intermediate phenotype for candidate gene studies*

The current study used Blood Oxygen Level Dependent (BOLD) response to alcohol cues as an intermediate phenotype to explore an association between a novel candidate gene and AD. Functional neuroimaging enables an evaluation of the association between changes at the molecular level (due to genetic variation) and changes in *in vivo* brain function, as measured by the BOLD response. In AD research, the primary advantage of neuroimaging is that it is an objective indicator of the AD

phenotype, as compared to subjective indicators, such as self-report measures and clinician diagnoses.

Alcohol interacts with many different areas of the brain, especially those associated with reward. Once a person is addicted, craving for alcohol is related to ventral striatal activation (Heinz et al., 2009; Kalivas & Volkow, 2005). The ventral tegmental area also facilitates drug intake (Volkow et al., 2006). Alcohol associated cues are processed in the anterior cingulate, hippocampus, amygdala and medial prefrontal cortex (Kalivas & O'Brien, 2007; Heinz et al., 2009). The insula is an area associated with both conscious and visceral aspects of craving (Brody et al., 2002) and the maintenance of addiction (Koob & Volkow, 2009; Naqvi et al., 2007). The rewarding effects of alcohol have also been linked to dopamine release in the nucleus accumbens (Heinz et al., 2009; Kalivas, Volkow, & Seamans, 2005). Thus, one would expect to see BOLD activation to alcohol cues in these areas.

Studies have shown that these brain areas are also associated with risk factors and behavioral indices of AD. For example, fMRI studies have shown that alcohol, marijuana, and polydrug cues elicit activation in the right insula, left anterior caudate and left prefrontal cortex in college students at risk for alcohol dependence (Bates et al., 2010). Additionally, increased activity in the right insula in response to drug cues predicts relapse to methamphetamine use (Paulus et al., 2007). Furthermore, people with substance use disorders have been shown to have abnormal processing of reward predictions. For example, prediction errors in those with AD correlate with phasic dopamine release in the ventral striatum. Park and colleagues (2010) therefore suggest

that the connectivity of the frontostriatal regions during the performance of prediction errors is different between those with and without AD.

Several studies have associated brain-based phenotypes (BOLD activation) for alcohol dependence with specific candidate genes (e.g., CNR1, OPRM1, DRD4; Filbey et al., 2008; Hutchison et al., 2008). For example, GABRA2 genotypes correlate with larger activation in the medial prefrontal cortex to alcohol odors as opposed to appetitive controls- an effect that is moderated by family history of alcoholism (Karaken et al., 2010). Additionally, the low expressing allele of the 5HTT promoter is associated with heightened amygdala response to emotional cues, a risk factor for alcohol dependence (Rao et al., 2007).

Moreover, recent studies have linked candidate genes to treatment outcomes. For example, individuals with a single base pair substitution in the OPRM1 gene respond to a naltrexone regimen better than those carrying the more common allele (Anton et al., 2008); that is, those with the rare alleles have fewer drinking days and drink less during drinking episodes. In addition, studies with other medications (e.g., topiramate, olanzapine) have found that genetic variables predict treatment outcomes (e.g., Hutchison et al., 2006; Hutchison, 2008; Seniveratne et al., 2009).

*TACR1 is a promising candidate gene associated with AD.*

Recently, Seneviratne and colleagues (2009) linked one SNP of the TACR1 gene (rs6715729) and two haplotypes (formed by combinations of rs6715729-rs735668-rs6741029) to susceptibility for AD in Caucasians (odds ratio for first haplotype 1.89 (95% CI= 1.16, 3.11) and 11.31(95% CI= 3.62, 32.35 for the second haplotype). The TACR1 gene encodes neurokinin 1 (NK1) receptors. The gene resides on the 2p11

region of chromosome 2 and is approximately 115kB in length and contains 5 exons. It is transcribed into 4 variants (two found in the human brain) via alternative promoter usage, differential slicing or both (Seneviratne et al., 2009). A peptide known as substance P (SP) is the endogenous ligand that binds to the TACR1 receptor, a G protein coupled receptor found in both the central and peripheral nervous systems. SP is an 11-amino acid peptide belonging to the neurokinin family, whose other members include substance K (neurokinin 2) and neuromedin K (neurokinin 3). In the human brain, TACR1 receptors are the predominantly expressed subtype of neurokinin receptors (Rigby et al., 2005).

Seniveratne and colleagues (2009) postulated that the SNPs that were significantly associated with AD could affect SP signal transduction by altering receptor density through alternative splicing of TACR1 messenger ribonucleic acid (mRNA).

#### *Preclinical Studies of TACR1 and alcohol.*

In preclinical studies performed by George and colleagues (2008), mice genetically deficient in TACR1 receptors showed a marked decrease in voluntary alcohol consumption and an increased sensitivity to the sedative effects of alcohol relative to wild type mice. This effect can be mimicked in wild type mice by administering a TACR1 antagonist (Thorsell et al., 2010). These mice have altered reward systems, which are normalized by the administration of a TACR1 receptor antagonist (Yan et al., 2009). Intracerebral infusion of Substance P into the amygdala also reduces alcohol consumption in both wild type rats and “anxious” rats (June et al., 2009). Furthermore, the effect of TACR1 genotypes on alcohol consumption has been

shown to be via a direct regulation of alcohol consumption, rather than a developmental effect on NK1 function or structure (Thorsell et al., 2010).

*TACR1 association with BOLD activation.*

Gilman and Hommer (2008) showed that fMRI responses to affective stimuli also suggested beneficial effects of the TACR1 receptor antagonist. Placebo-treated alcoholics showed robust responses to negative affective images, while alcoholics who received LY 686017 (a TACR1 antagonist) demonstrated less activation in the insula and medial temporal gyrus in response to negative images. Additionally, alcoholics who received the TACR1 receptor antagonist showed greater activation to positive International Affective Picture System (IAPS) images, suggesting a shift in the emotional valence of positive and negative stimuli. This is an important finding because greater activation to positive stimuli is predictive of lower alcohol consumption in the first six months after detoxification (Heinz et al., 2007).

*TACR1 potential for AD treatment.*

George and colleagues (2008) demonstrated the clinical efficacy of a TACR1 antagonist for AD. In a randomized controlled experimental study, they treated recently detoxified alcoholic in-patients with a TACR1 receptor antagonist, LY 686017, (n = 25) or placebo (n = 25). The TACR1 receptor antagonist suppressed spontaneous alcohol cravings, improved overall well-being, blunted cravings induced by the Trier social stress test, and attenuated concomitant cortisol responses.

Because George and colleagues (2008) demonstrated that TACR1 antagonists represent a promising treatment for AD, it is important to determine whether

polymorphisms in the gene for the TACR1 receptor may be associated with differential treatment response.

*TACR1 is associated with stress, an important factor in the development and maintenance of AD.*

Studies of TACR1 receptor antagonists suggest a major role of the SP-TACR1 receptor system in stress-related behaviors (Sommer et al., 2008). The expression of SP and TACR1 receptors has been mapped to neural circuits involving the caudate, putamen, amygdala, striatum, hippocampus and other components of stress-response circuitry (Duffy, 2004; Hietala et al., 2005). Within these areas, the concentration of SP increases in response to noxious or aversive stimulation. More directly, both in experimental animals and humans, exposure to stressors has been shown to facilitate a release of SP in the amygdala, while selective blockade of TACR1 receptors inhibits the associated behavioral stress responses (Duric &McCarson, 2005; Ebner et al., 2004; Furmark et al., 2005; Kramer et al., 2004; Michelgard et al., 2007).

TACR1 activation by SP interacts with other neurotransmitters such as 5HT, GABA, DA, Glu, ACh, and NE (Ebner, Rupniak, Saria, & Singewald, 2004; Levesque et al., 2007), which have been implicated in addictive processes. Substance P modulates the release of GABA in the substantia nigra and interacts with the mu opioid receptor (OPRM1) in modulating nociceptive transmission (Pinto et al., 2008). Norepinephrine release in the hippocampus increases TACR1 receptor activity, which in turn inhibits serotonin reuptake in the brain (Gobbi & Blier, 2005; Gobbi et al., 2007; Haddjeri & Blier 2008).

*TACR1 is associated with negative affect and depression, additional factors involved in the development and maintenance AD.*

*Preclinical studies support NK1 antagonism as an antidepressant.*

Both pharmacological blockade and genetic deletion of TACR1 receptors decrease emotionality and depression-related behaviors in animal models (Frisch et al., 2010). When depression is operationalized physiologically, TACR1 antagonism reduces separation-induced vocalizations in guinea pigs and lowers glucocorticoid secretion by rats during the forced swim test and mice during tail suspension paradigms (Mclean, 2005). Furthermore, in a tree shrew model of depression, increases in hippocampal neurogenesis and volume, comparable to those due to SSRI administration, can be obtained by administering a TACR1 antagonist (Czeh et al., 2001). This finding has been replicated in TACR1 knockout mice: they respond to TACR1 antagonism the way wild type mice respond to antidepressants (van der Hart et al., 2002).

*Clinical studies show mixed results regarding TACR1 antagonism for depression.*

While three studies have shown efficacy of TACR1 antagonists in treating depression at the same level as fluoxetine and better than placebo (Herpfer and Lied, 2005; Kramer et al., 2004; Ranga and Krishnan, 2002), others have found it does not perform better than placebo (Rupniak et al., 1999). It has been suggested that a subpopulation of severely depressed patients respond to NK1 antagonism because they have higher initial levels of Substance P than nonresponders (Bondy et al., 2003; Murtra et al., 2000). Higher levels of SP correspond with high stress reactivity, a risk factor for depression (Ebner et al., 2009; Frisch et al., 2010).

In sum, the TACR1 receptor is mechanistically related to stress, depression, and addiction. The connection between TACR1, stress, and depression is important because stress, negative affect, and depression influence the course of AD.

*Stress contributes to the development and maintenance of AD.*

Drinking to cope with stress has been qualitatively distinguished from drinking for positive reinforcement, but the role of stress in the development of alcohol dependence is not clear. It is known that negative affect is predictive of adolescent substance use (Ohannessian & Hesselbrock, 2008). Additionally, drinking to cope motives mediate the relationship between generalized anxiety and heavy drinking in college students (Goldsmith et al., 2009). Preclinical and clinical studies also show a connection between anxiety and propensities to self-administer alcohol (Ciccocioppo et al., 2006).

More extensive information is available on the role of stress in relapse. There is a general consensus among researchers that stress does play a role in relapse, but the exact mechanisms remain unknown (Heilig et al., 2010). Elevated stress reactivity in early withdrawal from alcohol and cocaine predicts increased craving and shorter time to relapse (Sinha, 2008; Sinha et al., 2009). Furthermore, stress and negative affect are often cited by AD patients as significant causes for relapse to alcoholic drinking many months after treatment (McKay et al., 1995).

This stress response in AD patients is largely due to changes in cellular metabolism in the extended amygdala (including the bed nucleus of the stria terminalis and the nucleus accumbens) due to alcohol consumption the extended amygdala is responsible for the emotional component of pain processing (Koob & Volkow, 2010). In AD individuals, a specific stress related dysregulation is maintained by alcoholic

drinking; this dysregulation develops via neuroadaptations in stress pathways and reward circuits in the brain over the course of repeated cycles of abstinence and relapse (Heilig & Koob, 2007; Holgter et al., 2000; Koob et al., 2004; Sinha, 2007). Negative affect and withdrawal therefore begin to function as conditioned stimuli capable of eliciting craving (Stewart et al., 1984). Post acute withdrawal, which can last more than a year, is associated with negative affect, attenuated pleasure from natural rewards, dysphoria, malaise, irritability, etc. (George et al., 2008; Gilman and Hommer, 2008; Heilig et al., 2010; Heinz et al., 2007; Koob, 2009). As a result of post-acute withdrawal symptoms, craving and drug seeking behavior is reinstated in order to achieve short term homeostasis.

Each time alcohol is used in response to stress or drug cues, the connection between that cue and affective relief is further solidified through long-term potentiation in the striatum (De Filippo et al., 2009). The ventral striatum connects motivational aspects of salient environmental and interoceptive stimuli with motor reactions (Wrase et al., 2007). Goldstein and colleagues (2009) have hypothesized that the switch from voluntary drug use to habitual use might be related to changes in the neural representation of craving moving from prefrontal regions to the striatum. This change could lead to compromised insight into behavior and an unconscious drive to drink. As described earlier, the TACR1 receptor is highly expressed in the ventral striatum and throughout the extended amygdala; hence it may play a role in developing and maintaining alcoholic drinking in response to stress.

*A bidirectional relationship also exists between MD and AD.*

A review of 35 articles discussing the rates of alcohol abuse among depressed patients showed that 16% of depressed patients had current alcohol problems, as opposed to 7% of the general population (Sullivan et al., 2005). Of inpatients receiving treatment for alcohol dependence, 25% meet DSM-IV-TR criteria for depression. Furthermore, higher depression rates exist among binge drinkers than among non-heavy drinkers (16%, 11% respectively; Levola et al., 2011; Manninen et al., 2006). Three theoretical etiologies for comorbid AD and depression exist: (1) depressed people self medicate with alcohol, (2) alcohol induces depressive symptoms, and (3) shared biological and environmental risk factors cause both disorders (Kendler et al., 1993). Most studies support the self-medication hypothesis, with alcohol dependence developing secondarily to a depression diagnosis, especially for those high in neurosis or loneliness and among single people (Boschloo et al., 2011).

#### *Study aims and hypotheses*

In summary, stress and major depression are putative causal factors in the development of and relapses to alcohol dependence. Understanding how genetic variations and the associated neurobiological mechanisms influence the relationship between stress and/or depression and alcohol is an important step toward developing treatments for AD. The current research combined the study of a candidate gene (TACR1) and a neurobiological phenotype for AD. It was hypothesized that significant clusters within the incentive regions of the brain, such as the insula, striatum, and amygdala, and the control regions of the brain, such as the prefrontal cortex, would be associated with genotypic variation in the TACR1 gene. It was also hypothesized that

the same SNPs that are significantly associated with BOLD response would also be significantly associated with AD symptom count both in our sample and in an independent sample: the Study of Addictions: Genetics and Environment Genome Wide Association study (SAGE GWAS). Additionally, the relationship between SNPs and symptom count was hypothesized to be mediated by brain activation in the incentive regions of the brain and moderated by lifetime and current major depression.

## Methods

### *Participants*

*Sample characteristics.* Three hundred twenty-six heavy drinking individuals (100 females, 30.9%) were recruited from a large metropolitan area in the southwest region of the United States. The sample consisted of a racially diverse group, with 48.1% identifying themselves as White, 27.8% Latino, 4.7%, Native American, 0.6% Asian, 0.6% Native Hawaiian/Pacific Islander, 1.9% Black, and 16.3% Mixed. The average age of participants was 31.86 ( $SD = 9.72$ ). The average number of drinks per drinking day in the last month was 7.35 ( $SD = 4.16$ ) and the mean Alcohol Dependence Score was 13.47 ( $SD = 8.17$ ).

*Recruitment and Screening.* Participants were recruited from the greater Albuquerque area by newspaper advertisements, personal contact, internet advertisements, and Mind Research Network sponsored outreach programs. Research participation was open to treatment seeking individuals as well as individuals who were not interested in changing their alcohol use. To be considered for participation in the treatment study, participants had to meet several criteria. Treatment seeking individuals had to (a) have a primary diagnosis of alcohol dependence, (b) have been within 21 days of their last drink, (c) obtained a score less than 8 on the Clinical Institute Withdrawal Assessment for Alcohol Scale (CIWA; Sullivan, Sykora, Schneiderman, Naranjo, & Sellers, 1989), and (d) met specific criteria for alcohol use (females drink >14/week, males drink >21/week). Non-treatment seeking participants were eligible for the study if they had 5 binge drinking episodes (4 or more drinks for females, 5 or more

for males) per month for the past 3 months and had no history of treatment for alcohol problems.

### *Procedures*

*Study Protocol.* All participants received informed consent. Drinking alcohol within 24 hours of the fMRI session was expressly prohibited. Each subject was breathalyzed at the start of the session to ensure that they had not been drinking; only participants with a breath alcohol concentration of zero were allowed to participate in the fMRI session. Participants underwent the Structured Clinical Interview for DSM-IV and filled out pencil and paper measures related to personality, drinking history, drinking problems, and family history of alcohol problems prior to scanning.

*fMRI Acquisition.* All MRI data was collected on a 3T Siemens Trio (Erlangen, Germany) whole body scanner. Prior to the acquisition of anatomical scans, localizer scans were acquired. An echo-planar gradient-echo pulse sequence (TR=2000ms, TE=29, flip angle=75°) was acquired with an 8-channel head coil, and images were acquired parallel to the ventral surface of a participant's orbitofrontal cortex to reduce signal dropout and distortion in this region. Each volume acquired consisted of 33 axial slices (64x64 matrix, 3.75 x 3.75 mm<sup>2</sup>, 3.5 mm thickness, 1 mm gap).

We utilized a taste cue task previously reported to elicit BOLD response in mesocorticolimbic areas (medial prefrontal cortex, amygdala, striatum, caudate) where TACR1 is highly expressed (Filbey et al., 2007, 2008). All taste stimuli were delivered to the participants via Teflon tubing using a computer controlled delivery system as described by Frank and colleagues (2003). The alcohol stimuli used were each subject's preferred alcoholic beverage, whereas the control stimulus was kept constant

across subjects. The control stimulus (litchi juice) provided an appetitive control for the activation of the mesocorticolimbic circuitry (Berns et al., 2001). During the EPI run, there were 12 pseudorandomized alcohol and control trials (six of each). Each trial consisted of a 24-s taste delivery period, followed by a washout period to allow the liquid taste to dissipate before the next trial. The word 'TASTE' was visually presented throughout the taste period. The washout period consisted of a 16-s rest period during which the word 'REST' appeared on the screen; nothing was delivered during the rest period. The washout was followed by a 2-s urge question and a 2-s prompt screen. During the urge question, the subjects were asked to rate their current subjective urge to drink alcohol using a scale of 1 (no urge at all) to 4 (very high urge).

*Image analysis.* The first 7 volumes of each functional run were discarded to allow the magnet to reach steady state. MCFLIRT (FMRIB) was used to motion correct images within a run; each image within the run was aligned to the first volume within the run. Images were then deskulled using BET, spatially smoothed with a 8 mm full-width half-max Gaussian kernel, temporally filtered using a high-pass filter of 100 sec, and grand mean intensity normalized; all of these steps were performed using FEAT (FMRIB Expert Analysis Tool). Statistical analyses were performed using the general linear model as implemented in FEAT. Customized square waveforms representing the condition of interest (taste, urge, control, rest) and the duration of stimulus presentation were convolved with a double gamma hemodynamic response function. In addition, movement parameters estimated by MCFLIRT were used as covariates within the multiple regressions.

Time series analyses were conducted using FILM (FMRIB Improved Linear Model) with local autocorrelation estimation. Contrast maps were created by contrasting alcohol taste vs. control taste conditions, i.e. activation in response to alcohol with activation in response to the control stimulus subtracted. Contrast maps were then registered to the participant's high-resolution anatomical image and the MNI 152 brain template using FLIRT (FMRIB Linear Image Registration Tool). Individual runs were combined within participants using a fixed effects model, which determines estimates of each participant's contrast map as well as variance map. These second level analyses were then used in a third level analysis using FLAME (FMRIB Local Analysis of Mixed Effects) stage 1 only. Before computing group level statistics, all second level images were registered to the MNI template. When examining group level maps at the whole brain level, we used minimum cluster sizes of  $64 \times 2 \times 2 \text{ mm}^3$  voxels thresholded at  $z=2.32$ ,  $p<.005$ .

*Collection of Genetic Material.* Participants were instructed to generate and deliver 5 ml of saliva in to a sterile 50 ml conical centrifuge tube. The saliva sample was then placed in the refrigerator and lysis buffer was added within 24 hours. Tris-HCl, pH 8; EDTA, pH 8; SDS and NaCl were added at 100 mM, 20 mM, 0.5% and 125 mM final concentrations; respectively. The tubes were refrigerated until the DNA is extracted, usually within 48 hours. Proteinase K (0.2 mg/ml) was added and the tubes were incubated at  $65^\circ\text{C}$  for 60 minutes. An equal volume of isopropyl alcohol was then added to each tube, the contents were mixed, and the DNA was collected by centrifugation at  $3,500 \times g$  for 10 minutes. The DNA pellet was rinsed once with one ml of 50% isopropyl alcohol and allowed to air dry. For RNase treatment, 20 ug/ml RNase A and 50 U/ml

RNase T1 were added and incubated at 37°C for 30 minutes. To precipitate the DNA, two volumes of 95% ethanol was added and mixed by gentle inversion then collected by centrifugation at 3,500 xg for 15 minutes. The samples were allowed to air dry followed by re-suspension in 1 ml of 10 mM Tris-HCl, 10 mM EDTA buffer, pH 8.0, and placed in a 1.8 ml cryovial. The concentration of DNA was calculated from the absorbance at 260 nm analysis and then adjusted to a concentration of 10 ng/μL.

The DNA was then purified and quantified in preparation for fragmentation and ethanol precipitation. The DNA was re-suspended in hybridization buffer and applied to the bead chip array for an overnight incubation. The amplified and fragmented DNA samples annealed to locus-specific 50-mers (covalently linked to one of over 1,000,000 bead types) during the hybridization step. Following hybridization, the arrays were washed to eliminate unhybridized and non-specifically hybridized DNA. One bead type corresponds to each allele per SNP locus. The samples then underwent single base extension and staining followed by more washing. The arrays were allowed to dry and then scanned using the Illumina iScan system and in turn analyzed using Illumina's software for automated genotype calling.

Analysis of the scanned results was achieved using Illumina's BeadStudio software in conjunction with the BeadStudio genotyping module. BeadStudio Software is a modular analysis tool for genotyping, gene expression, and methylation applications. The data was then filtered in BeadStudio for call rate, and minor allele frequency to remove bad samples and bad SNPs. BeadStudio comes equipped with several plug-ins to generate compatible files for further analysis using third party and/or custom software packages.

## Data Analysis

The goals of the current research were (1) to examine the association between TACR1 genotypes and BOLD activation in response to alcohol cues, (2) to determine if any significant associations between SNPs and AD symptom count were mediated by BOLD activation or moderated by depression and (3) to test whether these associations replicated in an independent clinical sample. It was hypothesized that significant clusters within the incentive regions of the brain, such as the insula, striatum, and amygdala, would be associated with genotypic variation in the TACR1 gene. It was also hypothesized that the same SNPs that are significantly associated with our brain based phenotype would also be significantly associated with AD symptom count our sample and in an independent GWAS sample (SAGE). Lastly, it was hypothesized that the relationships between SNPs and AD symptom count were mediated by brain activity and/or moderated by major depression.

### *Hypothesis 1: TACR1 genotype association with BOLD response*

We included known SNPs 20,000 base pairs up and downstream from the TACR1 gene in this analysis in order to capture any potentially important SNPs in the 3' or 5' untranslated region (UTR). 808 SNPs were identified via the UCSC Genome Browser, 108 of which were represented on the Illumina 1MDuo Chip used in this analysis. The analyses were focused on the 70 of the 108 SNPs that met the following criteria: 1) each allele represented in at least 20% of the population in the Southwestern United States 2) each allele represented in at least 20 people of our sample and 3) the SNP was used in the SAGE GWAS. Each SNP was analyzed separately using an additive model (AA > Ab > bb) in FSL. For the analysis, we had two broadly defined

regions of interest, or masks, which encapsulate the incentive areas (ventral tegmental area, ventral and dorsal striatum, medial prefrontal cortex, putamen, caudate, insula, and orbitofrontal cortex) and control areas (prefrontal cortex and precentral gyri) of the brain. We had two intermediate phenotypes for the alcohol>control contrast: (1) BOLD response within the incentive mask and (2) BOLD response within the control mask. The association test for each SNP was a regression of the SNP on a measure the spatial extent of significant BOLD response in each mask and the associated p value.

The first step was to examine the association between the group variable (in this case a SNP) and the alcohol > control difference in BOLD activation at each voxel within the masks. Each voxel within the mask was tested for an association with each SNP and only those voxels that exceed a statistical threshold ( $z > 2.32$ ) were highlighted in a spatial map of the active voxels within the mask. Cluster size was defined as the largest number of spatially contiguous voxels which exceed the statistical threshold within the mask. To control for the number of voxels tested in the first step, the analysis software evaluated cluster size in relation to the number of voxels tested (e.g., 8732 in our incentive mask) and calculated a probability distribution for how likely it would be to find a contiguous cluster of a specific size by chance (5% of the time for  $p < .05$ ), given the number of voxels tested. A cluster that exceeded this corrected cluster threshold indicated that the SNP was significantly associated with the cluster at  $p < .05$ .

*Hypotheses 2: Relationship between TACR1 SNPs and AD symptom count.*

SNPs that were both previously identified as associated with psychiatric conditions and significantly associated with brain activation in response to alcohol cues were tested for a significant correlation with both current and/or lifetime AD symptom count.

*Hypothesis 3: Mediation of relationship between TACR1 SNPs and AD symptom count by BOLD activation.*

If a significant relationship between the SNP and AD symptom count was identified, it was tested to see if the association was mediated by BOLD activation using general linear modeling. BOLD activation was quantified as mean percent signal change in the incentive or control masks. AD lifetime and current symptom count was determined via the Structured Clinical Interview for the DSM-IV-TR. First, analyses were run to see if BOLD activation predicted AD symptom count. If both the SNP and BOLD signal independently predicted AD symptom count, they were both used as predictors of AD symptom count in a regression analysis. If the significant relationship between a SNP and AD symptom count became insignificant when BOLD was also used as a predictor, this was considered evidence of mediation.

*Hypothesis 4: Moderation of relationship between TACR1 SNPs and AD symptom count by major depression.*

The same significant relationships between SNPs and AD symptom count were also tested for a moderating effect of either a lifetime or current diagnosis of major depression, according to DSM-IV-TR, criteria using general linear modeling. Diagnosis of depression was determined via the Structured Clinical Interview for the DSM-IV-TR. Moderation was evident if regression of AD symptom count on the SNP in the

unaffected group was significantly different from regression of AD symptom count on the SNP in the depressed group.

*Hypothesis 5: Replication of TACR1 association with a clinical phenotype.*

The health research sample of the SAGE dataset was downloaded from dbGap to test the association between TACR1 SNPs and a clinical phenotype. We included only non-related Caucasian individuals (n=2605), as the rest of the sample was mostly African American and only 3% Hispanic. 69 TACR1 SNPs were assayed in this GWAS. In the SAGE sample, genotypes were coded log-additively (0, 1, 2 copies of the minor allele). Covariates represented sex, age, and two principal components indexing continuous variation in race/ethnicity. Correlation analyses between genotype for each SNP and number of AD symptoms was performed.

*Power Analysis and Multiple Comparisons Correction*

With a family-wise alpha level of 0.05, a sample size of n=326, and three genotype groups, we could detect an effect size of  $f=0.17$  with .80 power. Additionally, we previously corrected for multiple comparisons per voxel in the fMRI analysis by applying an ROI threshold of  $p<0.05$  to the contrast maps. The power to detect an association between three genotype groups of TACR1 SNPs and SCID symptom count in the SAGE dataset with a sample of n=2605 was therefore more than adequate. In order to avoid type 1 errors, we used a conservative approach, a Bonferroni correction, in the determination of the significance of all p-values for genetic associations between each SNP and activation and also between each SNP and symptom count. The results were also assessed for statistical significance using a False Discovery Rate correction.

## Results

The pattern of BOLD activity in response to the alcohol taste cue task replicated previous findings (Claus et al., in submission), with activation in the alcohol minus litchi contrast mainly in the mesocorticolimbic pathways where TACR1 receptors are highly expressed. These pathways include connections among the putamen, caudate, amygdala, cingulate, pallidum, and lateral orbitofrontal cortex. The sample included heavy drinkers, with average AD lifetime and current symptom counts of  $6 \pm 3$ , mean Alcohol Dependence Scale score  $18 \pm 8$ , and mean Alcohol Use Disorders Identification Test score of  $19 \pm 8$ . Twenty percent of the sample had current major depression diagnoses and one third of the sample had a lifetime diagnosis of major depression.

### *Hypothesis 1*

BOLD activation in the incentive and control masks in response to gustatory alcohol cues was predicted by 16 of the 70 SNPs in the TACR1 gene that met inclusion criteria (Table 2). All of the SNPs associated with BOLD activation are located in introns; one SNP, rs1106855, is located in a stop codon near the 3' region of the gene. It should be noted, however, that if a Bonferroni correction were to be applied to this data ( $p = 0.05/70 \text{ SNPs} \times 2 \text{ ROIs} = 0.05/140 = 0.00036$ ) only 3 of these SNPs would significantly associated with BOLD activation at a level above chance. If one were to apply a False Discovery Rate (FDR) correction for multiple tests, 7 out of the 140 tests would be expected to be significant by chance. Using an FDR correction, we can be confident that some of these associations are not due to chance alone.

### *Hypothesis 2*

SNPs previously identified in the literature that also significantly predicted BOLD activation before a Bonferroni or FDR correction (rs3771810, rs3771863, rs12477554, and rs1106855) were tested for an association with AD symptom count. Rs3771810 predicted current AD symptom ( $p=0.037$ ,  $df= 325$ ) while rs3771863 and rs12477554 predicted lifetime AD symptom count ( $p= 0.036$  and  $0.030$ , respectively,  $df=325$ ). Rs1106855 genotypes did not predict AD symptom count. However, these analyses did not pass a family wise error correction.

### *Hypothesis 3*

Because each of the SNPs previously identified in the literature predicted AD symptom count, in addition to predicting BOLD activation in our sample, a mediational analysis was performed. It was hypothesized that the relationship between the SNPs and current or lifetime AD symptom count was mediated by BOLD activation. However, brain activation in the incentive, control, or specific ROIs did not predict AD symptom count. Therefore, there is no mediation of the association between TACR1 SNPs and AD symptom count by BOLD activation, despite the fact that there is a significant association of those same TACR1 SNPs and BOLD activation in response to alcohol cues.

### *Hypothesis 4*

Since TACR1 is associated with depression, an analysis was performed to see if the relationship between the four SNPs previously reported in the literature (rs3771810, rs3771863, rs1247754) and AD symptom count was moderated by lifetime or current major depression. Rs12477554 predicted lifetime AD symptom count for those without

any lifetime history of depression ( $p < 0.036$ ,  $df = 325$ ), but not for those with a history of depression. Rs3771810 predicted current AD symptom count for those participants with current major depression ( $p < 0.033$ ,  $df = 325$ ), but not for those without current major depression. These moderating relationships are responsible for the association of AD symptom counts with the SNPs in the full sample. The relationships between rs3771863 and rs1106855 and AD symptom count was not moderated by lifetime or current MD.

#### *Hypothesis 5*

Three of 70 TACR1 SNPs predicted AD symptom count in the SAGE sample (rs10490308, rs11688000, and rs3771863) at a statistically significant level ( $p = .0054$ ,  $0.0273$ ,  $0.0335$  respectively,  $df = 69$ ) (Table 3). The correlation between rs3771863 and AD symptom count in this sample is of note because rs3771863 significantly predicted brain activation in the full sample and AD symptom count in the full sample (Figure 5). However, it should be noted that these associations do not pass an FDR correction for the number of statistical tests performed ( $0.05 \times 70 = 3.5$ ).

## Discussion

The goal of this research was to find genetic variations in the TACR1 gene that predicted not only the clinical phenotype of alcohol dependence, but also a neurobiological intermediate phenotype, BOLD activation during alcohol craving. 16 TACR1 SNPs predicted activation in response to alcohol cues in brain areas related to emotion and learning. Specifically, the SNPs were significantly related to activity in the putamen and caudate, areas involved in learning and memory. They were also significant associations with areas involving emotional perception and processing, including the amygdala and cingulate, as well as areas involved in executive function and decision making, including the insula and orbitofrontal cortex. This supports the idea that TACR1 has a role in the formation or maintenance of the association between stress, negative affect, and depression and alcohol use.

One SNP, rs3771863, was predictive of AD symptom count and BOLD activation in response to alcohol cues, regardless of major depression status (Figure 2). It also was predictive of AD symptom count in the SAGE Genome Wide Association Study. However, it is unlikely that this SNP is a functional determinant of TACR1 receptor structure or activity because it is within an intron in the gene. The functions of introns remain largely unknown, although some have been implicated in RNA slicing (Mattick, 2004). Thus, it is not a good candidate as a point of potential pharmacological intervention. It might be in high LD with a functional SNP in the 5' untranslated or promoter region, which is more likely to alter TACR1 receptor number, structure, and function (Figure 6).

However, one SNP with potential functional significance, rs1106855, was associated with BOLD activation in response to alcohol cues (Figure 4). This SNP is in an intron, but is located within a stop codon. Therefore, it could affect the amount of mRNA produced by a cell and therefore alter the number of receptors or the binding properties of the TACR1 receptors.

For two other SNPs that predicted BOLD activation in response to alcohol cues (rs3771810 and rs12477553) (Figures 1 and 3), major depression status moderated the relationships with AD symptom counts. Neither SNP was associated with AD symptom count in the SAGE sample. This finding parallels previous research regarding TACR1 antagonists as a treatment for AD for depression. That is, TACR1 antagonism has proven most effective as a treatment for depression in those with higher Substance P levels and correspondingly, more severe depression (Bondy et al., 2003; Murtra et al., 2000). For these people, AD could be secondary to a primary depression diagnosis, one of many potential etiologies for AD.

Each of the SNPs associated with AD symptom count in the imaging sample or the SAGE sample (rs3771863, rs3771810, rs1106855, rs124477553) are in regions of the TACR1 gene with high potential for functional significance (Figure 6). However, the association with BOLD activation in response to alcohol cues identified many more potential SNPs of interest. As an intermediate phenotype, it is closer to the gene's neurobiological activity, and thus was able to detect smaller, but potentially important effects of SNPs on this intermediate phenotype and the corresponding clinical phenotype (Figure 5).

These findings must be interpreted within the limitations of this study. First, because only common SNP variants were used in these analyses, the potential effects of rare SNP variants could have been missed. Deep sequencing is a new technique which might reveal rare, but functional SNPs and should be applied to the TACR1 gene in future studies.

Moreover, although the sample was not underpowered to find BOLD effects during the craving task or to detect associations between SNPs intermediate phenotype and the clinical phenotype, the number of statistical tests performed was extremely high. Despite the fact that linkage disequilibrium among SNPs within a gene decreases the effective number of statistical tests, the ability to detect the effect of a single SNP on BOLD activation remains limited. All SNPs and corresponding genes interact with thousands of other SNPs and hundreds of other genes to influence cellular activity. Activity at the cellular level then influences tissue specific functions, especially neurobiological function in different brain areas. The interactions among genes are not always additive either; some genes work to decrease the activity of other genes and their products. Therefore any statement linking a complex phenotype solely to the function of one gene is an over-simplification. Personalized medicine based on single gene function is therefore limited and a cellular systems approach, combining the study many genes or many thousands of SNPs that underlie neural activity at once, might be more fruitful alternative.

Another limitation of this study was the use of DSM-IV-TR AD symptom count as the clinical phenotype because two people can be diagnosed with AD without sharing even one symptom. Thus, as a phenotype, it is not uniform or very informative. This

underscores the importance of intermediate phenotypes, such as BOLD activation, in the parsing of AD variation to aid in the development of novel medications. Better assessments of AD do exist, such as the Alcohol Use Disorders Identification Test (AUDIT, Barbor et al., 1992) and the Alcohol Dependence Scale (ADS, Skinner & Allen, 1982). Unlike AD symptom count, BOLD response does predict scores on these measures (Claus et al., in submission). However, these variables were unavailable for the SAGE GWAS sample, so AD symptom count was used in both analyses so that a direct comparison could be made between the two samples.

An additional limitation of this study was the lack of a stress measurement. Because TACR1 is involved in the stress response and stress is highly associated with both alcohol dependence and depression, measures of stress should be included in future studies. The design could be further improved by manipulating stress prior to or during exposure to alcohol cues. Furthermore, the disentanglement of stress, negative affect, and depression as separate constructs, in addition to distinct operationalization would further work in this area immensely. Perhaps they are different manifestations of the sample physiological response or negative affect and depression are results of external stress.

A final, but important, limitation of this study was previously described as a strength of this study: the use of BOLD signal as an intermediate phenotype. This approach has high statistical power to detect small effects, but it remains controversial. The idea that brain function is intermediate between genetic and phenotypic profiles is widely accepted and BOLD response is a representation of brain activity. However, the BOLD response itself is based upon a series of assumptions and indirect evidence that

it reflect neural activity. Solid evidence linking BOLD to changes in neural activity would strengthen all results based on this intermediate phenotype. Additionally, the mean percent BOLD signal change for each region of interest (whole brain, incentive mask, and control mask) was used as a variable in these analyses. How this maps onto a meaningful effect size measure is unclear. Furthermore, the use of BOLD response in response to this particular task may not be the most appropriate endophenotype. There is evidence that the BOLD response to the task is dependent on current levels of drinking and age (Claus et al., in submission). Endophenotypes are, by definition, unchanging based on the organism's state as they are supposed to reflect enduring traits (Cannon & Keller 2006; Gottesman & Gould 2003).

In sum, despite these caveats, this study adds to the literature regarding the role of TACR1 receptors in alcohol dependence and depression. SNPs with potential functional significance were identified and areas of that TACR1 gene that are most likely to be functional were identified. These SNPs should also be considered as potential biomarkers, if not points of pharmacological interventions. TACR1 receptor antagonism as a form of treatment should be examined in less heterogeneous groups, as it might be very effective for those with primary or secondary alcohol dependence in addition to a diagnosis of major depression.

Participant Characteristics	Mean	SD	Range
Age	31.86	9.72	21 - 56
Estimated verbal IQ (WASI)	54.80	10.11	28 – 76
Alcohol dependence (ADS)	13.47	8.17	1 - 43
Average drinks per drinking day (past month)	7.35	4.16	.7 – 39
Proportion of drinking days	0.59	0.25	.07 - 1
Proportion of binge drinking days	0.43	0.28	0 - 1
Proportion of Current Cigarette Smokers	0.49	n/a	0-1
Depressive symptoms (BDI)	11.98	9.64	0 – 47
Anxiety symptoms (BAI)	9.59	8.92	1 - 40

Table 1. This ethnically diverse adult sample included heavy binge drinkers, with mild to severe depression and anxiety symptoms. One half of the participants were current cigarette smokers.

SNP	Incentive Largest Cluster Size, contiguous voxels	Control Largest Cluster Size, contiguous voxels	Whole Brain Largest Cluster Size, contiguous voxels
<b>rs3755459</b>	468 (0.002)	<b>1145 (0.000000119)</b>	<b>4189 (0.0000000000227)</b>
rs3821320	99 (0.186)	396 (0.0011)	664 (0.0034)
<b>rs3771863</b>	119 (0.0137)	383 (0.00132)	<b>3348 (0.000000000143)</b>
rs2024512	26 (0.601)	273 (0.00738)	506 (0.0143)
rs3771869	26 (0.601)	269 (0.00789)	392 (0.00133)
rs6546952	159 (0.0767)	253 (0.0102)	390 (0.0446)
rs3771836	90 (0.213)	229 (0.0158)	812 (0.000954)
rs3771811	44 (0.447)	204 (0.0245)	341 (0.0739)
rs3771846	39 (0.485)	203 (0.0252)	309 (0.109)
rs4439987	59 (0.35)	172 (0.0442)	196 (0.352)
rs12477554	33 (0.536)	171 (0.0455)	488 (0.0171)
rs10168354	79 (0.254)	170 (0.0461)	177 (0.428)
rs11680998	184 (0.0548)	170 (0.0474)	591 (0.00676)
rs3755468	84 (0.234)	166 (0.0498)	233 (0.237)
rs3771810	254 (0.221)	133 (0.0498)	774 (0.00133)
rs6546951	562 (0.000795)	82 (0.275)	390 (0.0446)
<b>rs1106855</b>	244 (0.222)	40 (0.51)	<b>2138 (0.000000119)</b>

Table 2. SNPs associated with BOLD activation, largest cluster sizes per mask and in the whole brain, bold & italic values significant at  $p < 0.05$ , corrected for multiple tests.

SNP	Position	Top Strand	Corr/Trend P	Corr/Trend -log10 P
rs10490308	75335883	[A/C]	0.005371097	2.269936968
rs11688000	75146665	[A/G]	0.027260033	1.56447363
rs3771863	75273222	[A/G]	0.033508291	1.474847722

Table 3. TACR1 SNPs associated with AD symptom count in the Study of Alcohol Genetics and Environment Genome Wide Association Study,  $p < 0.05$ , uncorrected for multiple comparisons.

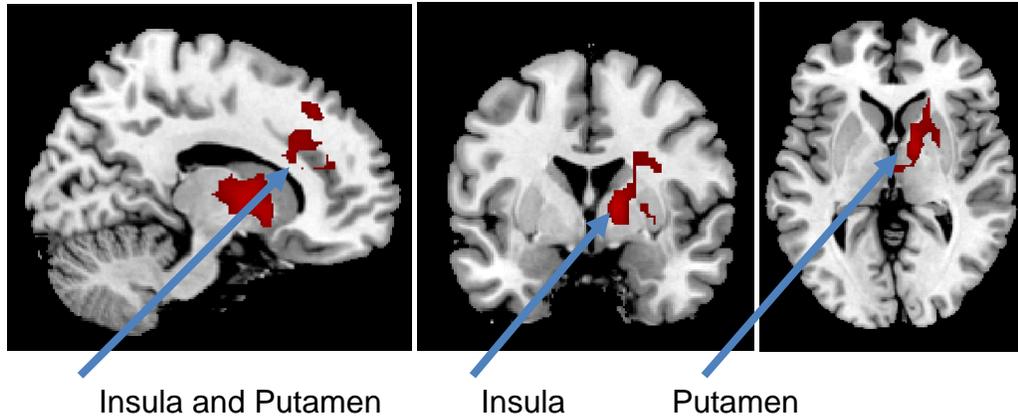


Figure 1. BOLD activation significantly associated with rs3771810 genotypes, corrected map,  $z > 2.32$ .

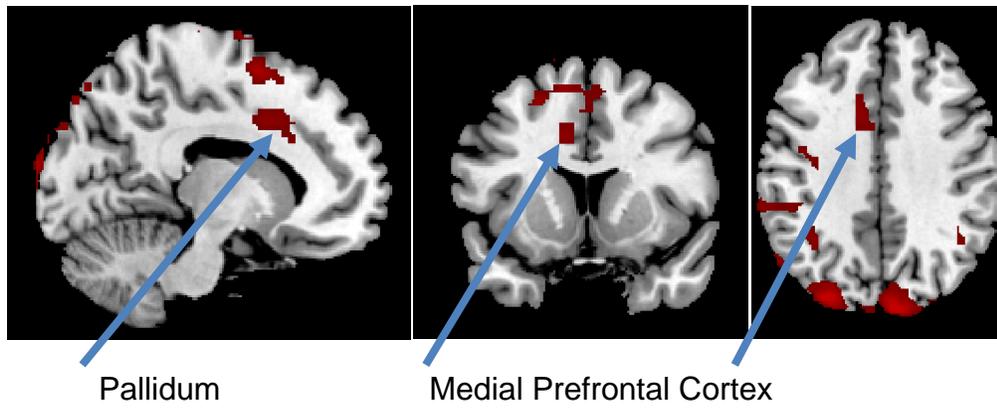


Figure 2. BOLD activation significantly associated with rs3771863 genotypes, corrected map,  $z > 2.32$ .

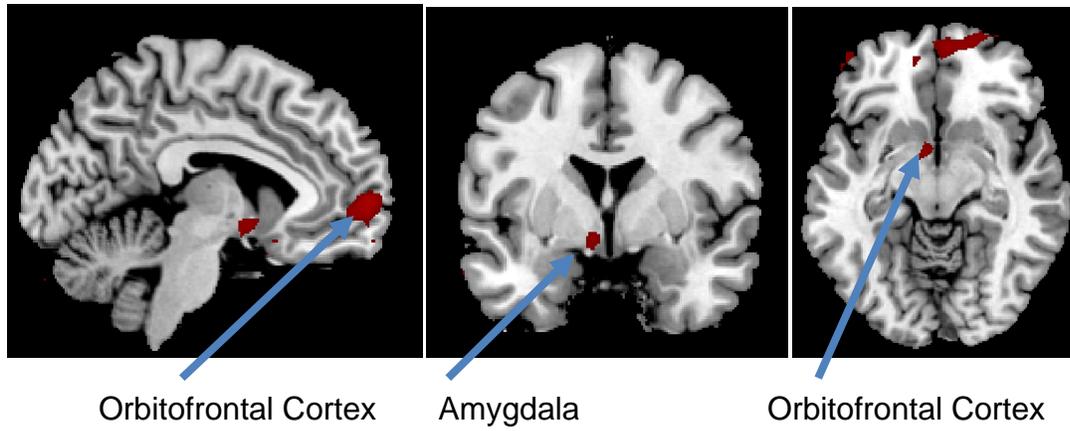


Figure 3. BOLD activation significantly associated with rs12477554 genotypes, uncorrected map,  $z > 2.32$

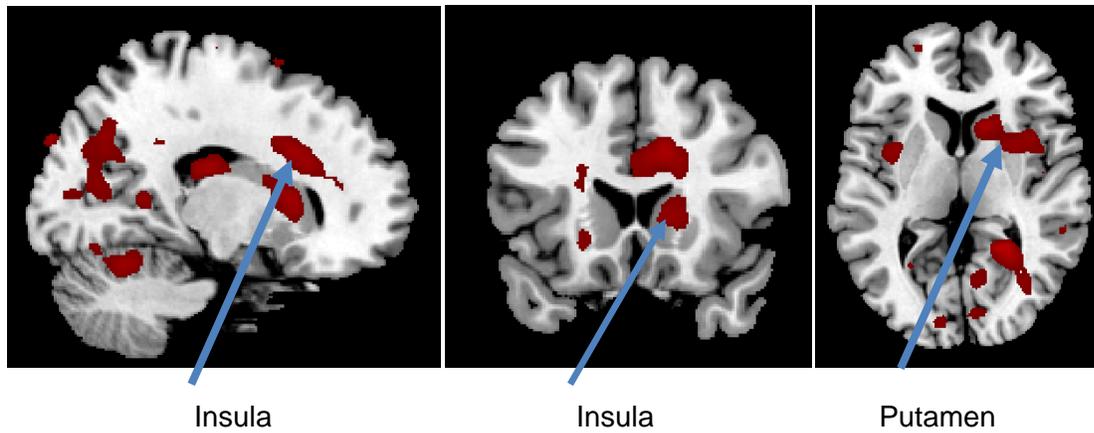


Figure 4. BOLD activation associated with rs1106855 genotypes, uncorrected map,  $z > 2.32$ .

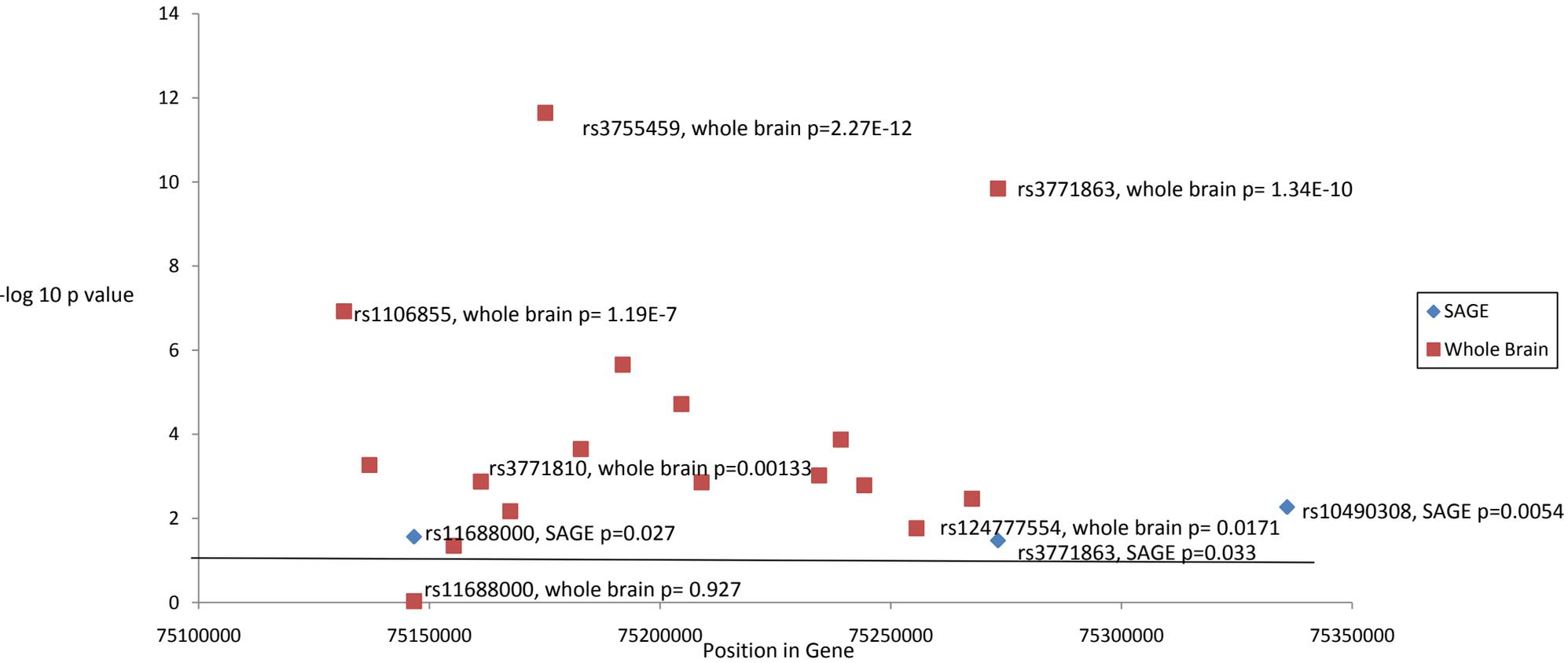


Figure 5. Significant Associations between SNPs and BOLD activation and significant associations between SNPs and AD symptom count in the SAGE GWAS, a value above 1.5 indicates a statistical significant association.

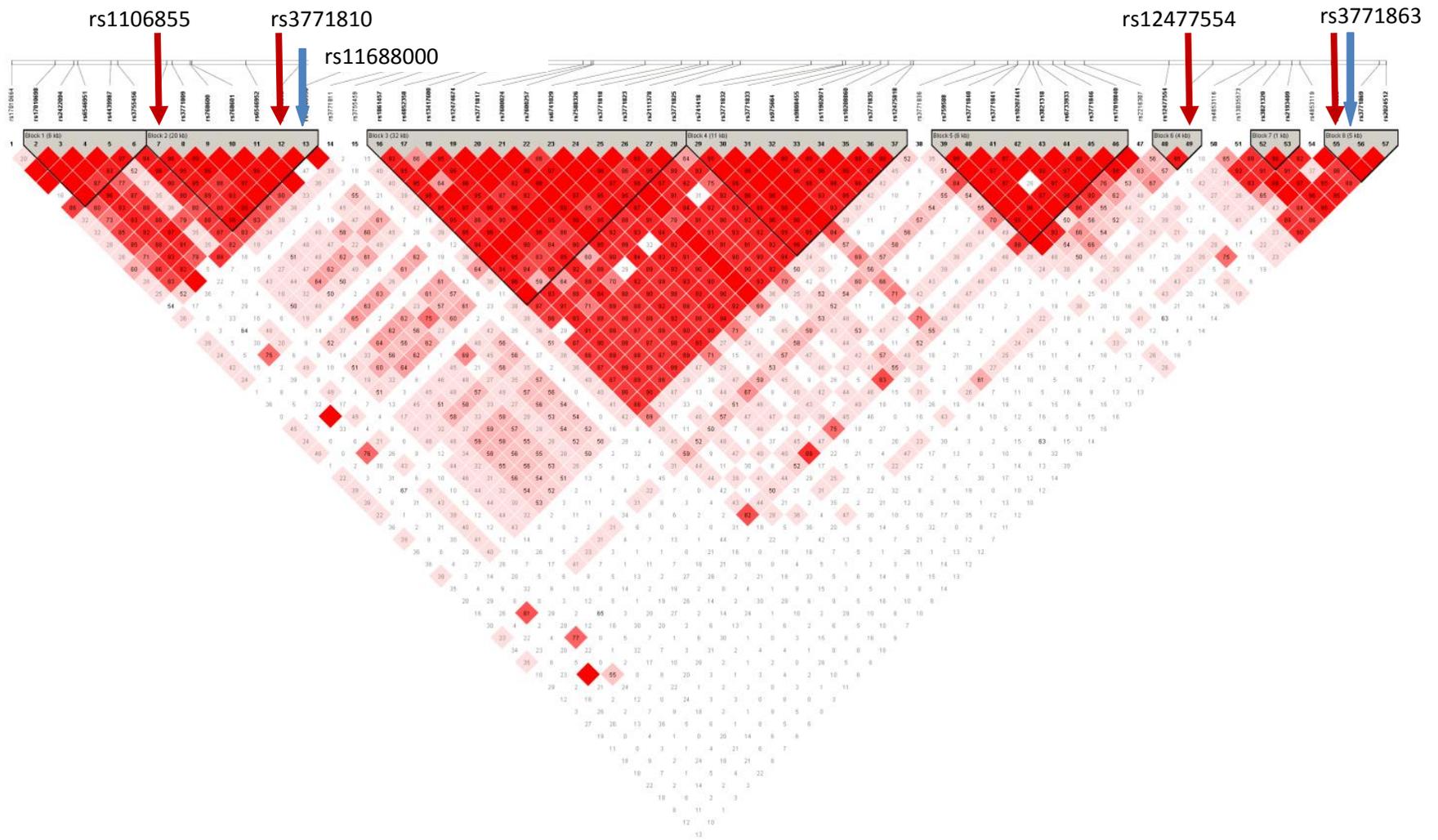


Figure 5. Linkage Disequilibrium plot of TACR1 gene. SNPs previously identified in the literature that were also significantly associated with BOLD response to alcohol cues are indicated with red arrows. SNPs that were significantly associated with AD symptom count in SAGE are indicated with blue arrows.

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