INFLUENCE OF BRAIN-DERIVED NEUROTROPHIC FACTOR AND FAMILY HISTORY OF ALCOHOL DEPENDENCE ON ALCOHOL USE IN HEALTHY SOCIAL DRINKERS

by

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LIST OF ABBREVIATIONS

Abbreviation **Description**

AUD - Alcohol Use Disorder

BDNF - Brain Derived Neurotrophic Factor

DSM - Diagnostic and Statistical Manual of Mental Disorders

FH - Family History of Alcohol Dependence

NIAAA - National Institute on Alcohol Abuse and Alcoholism

PCI-DSS - Payment Card Industry Data Security Standard

QFI - Quantity Frequency Index of Alcohol Use

SAMHSA - Substance Abuse and Mental Health Services Administration

SNP - Single-Nucleotide Polymorphism

SSL - Secure Sockets Layer

$Val^{66}Met$ - BDNF $Val^{66}Met$ Polymorphism

$Val^{66}Val$ - BDNF $Val^{66}Val$ Genotype

ABSTRACT

Brain-derived neurotrophic factor (BDNF) is important for neuronal survival, differentiation and consolidation of synaptic strength. Studies have found increased alcohol use and genetic risk for alcohol dependence in individuals with the Val^{66}Met single-nucleotide polymorphism (SNP) of the BDNF gene, a genotype associated with decreased activity-dependent release of BDNF. However, the literature remains contentious with regard to this issue. The current study was designed to address this issue with two aims. Aim 1 examined the influence of the Val^{66}Met SNP and family history of alcohol dependence (FH) on alcohol use in healthy social drinkers. It was expected that the Val^{66}Met polymorphism would be associated with higher drinking levels compared to the Val^{66}Val genotype, and those participants with a combination of Val^{66}Met genotype and a positive FH would exhibit the most severe alcohol use profile. Results for Aim 1 indicated no significant effects of genotype on quantity/frequency of alcohol use; however, the Val^{66}Met group had an earlier age at first alcohol use. FH-positive participants had an earlier age at first drunken episode. There were no interactions of BDNF genotype x FH group. Correlational analyses revealed that quantity/frequency of alcohol use was positively related to perceived stress levels in the Val^{66}Met group, such that participants with higher stress levels tended to consume more alcohol. This relationship was not present in the Val^{66}Val group. Aim 2 examined stress-related changes in serum BDNF levels. It was expected that Val^{66}Met and Val^{66}Val groups

would have different stress-related changes in serum BDNF levels, and that the profile of the Val^{66}Met group would be associated with more severe alcohol use. Results of Aim 2 indicated that, across groups, serum BDNF levels decreased in response to stress, but there were no main effects or interactions of BDNF genotype or FH group. However, in the Val^{66}Met group, stress-related BDNF change (post- minus pre-stress) was related to age at first drink, such that earlier age of alcohol use was associated with a greater stress-related decrease in serum BDNF. This relationship was not present in the Val^{66}Val group. Taken together, the results of these two aims suggest that in healthy young social drinkers, the association between the Val^{66}Met SNP and alcohol use may be linked to stress vulnerability and behavioral risk factors (i.e., earlier initiation of alcohol use), which are known to be associated with the development of alcohol dependence.

I. SPECIFIC AIMS AND HYPOTHESES

Brain-derived neurotrophic factor (BDNF) is a secreted protein (Binder and Scharfman, 2004) that is important for neuronal survival, differentiation and consolidation of synaptic strength (Thoenin, 1995). Recent studies in animal models have suggested that decreased BDNF levels may also be associated with the genetic propensity to alcohol-drinking behaviors (Prakash *et al*., 2008). Studies in human participants have sought to confirm this relationship by examining the presence vs. absence of the Val^{66}Met single-nucleotide polymorphism (rs6265 SNP) of the BDNF gene, a genotype associated with decreased activity-dependent release of BDNF. A number of studies have found increased alcohol consumption and genetic risk for alcohol dependence among individuals with the Val^{66}Met polymorphism (see Ghitza *et al*., 2010 for review). However, the literature remains contentious with regard to this issue (see Nedic *et al*., 2013), and additional studies are required to elucidate the potential relationship between the Val^{66}Met SNP, serum BDNF levels, and alcohol use in human participants.

To this end, the current study had following specific aims.

Aim 1. Aim 1 extended previous research on alcohol use characteristics among individuals with the Val^{66}Met polymorphism compared to individuals with Val^{66}Val genotype by examining the impact of positive versus negative family history of alcohol dependence (FH+ versus FH-, a categorical variable) within each of these BDNF genotype groups : FH-/Val^{66}Met, FH+/Val^{66}Met, FH-/Val^{66}Val, FH+/Val^{66}Val.

Hypothesis 1a. Based on previous studies, it was expected that individuals with

the Val^{66}Met polymorphism would have higher levels of alcohol consumption, earlier initiation of alcohol use and drinking to excess, and greater endorsement of binge drinking habits compared to individuals in the Val^{66}Val group.

Hypothesis 1b. Examination of separate and combined effects of BDNF genotype and family history of alcoholism was expected to reveal that participants with the combination of a positive family history and the Val^{66}Met genotype would have a more severe profile of alcohol consumption characteristics.

Aim 2. Aim 2 examined potential differences in serum BDNF levels between genotype groups (Val^{66}Met vs. Val^{66}Val) before and after a mental and physical stress challenge in the laboratory setting. The potential relationship of the BDNF stress response to alcohol consumption characteristics, including quantitative family history of alcohol dependence (a continuous variable), was also examined.

Hypothesis 2a. Because the Val^{66}Met polymorphism has been associated with decreased activity-dependent release of BDNF, it was expected that participants within this group (relative Val^{66}Val genotype) would have a smaller increase (or greater decrease) in BDNF levels in response to stress**.**

Hypothesis 2b. It was predicted that the extent of BDNF increase in response to stress would be correlated with alcohol use characteristics in the Val^{66}Met group, such that a smaller stress-related increases (or larger decreases) in serum BDNF would be associated with a more severe profile of alcohol consumption characteristics. This relationship was not expected to be present (or if present, not as significant) in the Val^{66}Val group.

II. BACKGROUND AND SIGNIFICANCE

The human BDNF gene is located on short arm of chromosome 11. The most common BDNF polymorphism is a single-nucleotide substitution of A for G at nucleotide position 196, and this substitution produces a non-conservative substitution of a valine with a methionine at codon 66 of this gene (Egan *et al*., 2003; Chen *et al*., 2004) which is called $Val^{66}Met$ (rs6265) (Hong *et al*., 2011; Czira *et al*., 2012). The location of this SNP is in the prodomain of the BDNF gene and it results in impaired intracellular processing, trafficking, and extracellular secretion (Egan *et al*., 2003) and may also cause impairment in neural plasticity (Cheeran *et al*., 2008; Lamb *et al*., 2015). This SNP also affects memory and cognition (Hariri *et al*., 2003; Lamb *et al*., 2015), and is also associated with neuropsychiatric disorders including Alzheimer's disease, autism, depression, eating disorders and schizophrenia (Notaras *et al*., 2015).

Neurotrophins are synthesized as precursors (pro-neurotrophins), their protein products are structurally composed of a signal sequence, a pro-domain, and the mature domain. In the mature bioactive form, the pro-domain or the N-terminal fragment of approximately 120 amino acids is processed proteolytically by intracellular and/or extracellular enzymes (Seidah *et al*., 1996; Lee *et al*., 2001; Pang *et al*., 2004; Lu *et al*., 2005). The BDNF pro-region has a role as a molecular chaperone to assist in the folding of BDNF (Kolbeck *et al*., 1994), and post-translational mechanisms, including proteolytic cleavage of precursor proteins, generate diverse biological actions of proteins. The pro-BDNF (precursor of BDNF) has role in cell death, spine shrinkage and long term

depression, while BDNF promotes spine formation, spine survival and long term potentiation. (Woo *et al*., 2005; Teng *et al*., 2005; Deinhardt *et al*., 2011; Koshimizu *et al*., 2009). The secretion of BDNF is controlled by both constitutive and activity-regulated secretion pathways in neurons (Mowla *et al*., 2009), and the secretion of BDNF is controlled in a neuronal activity- and Ca2+-dependent manner (Lessmann *et al*., 2009) When the $Val^{66}Met$ SNP in the BDNF gene changes a valine to a methionine at codon 66 in the pro-region of human BDNF, this mutation impairs memory function, neuronal activity-dependent secretion of BDNF, and synapse sorting of BDNF protein (Egan *et al*., 2009).

BDNF and Addiction

It has long been known that BDNF plays an important role in neuronal growth, development, maintenance and function (Ghitza *et al*., 2010). However, more recently, researchers have focused on elucidating the role of BDNF in clinical conditions relevant to emotion, stress and addiction (Angelucci *et al*., 2005). Studies suggest that low levels of BDNF may be associated with negative mood and cognitive deficits (Sertoz *et al*., 2008). Other research indicates that BDNF levels may be linked to the reinforcing and rewarding effects of alcohol, as well as the genetic predisposition to alcohol dependence (Joe *et al*., 2007). Low serum levels of BDNF have been associated with increased levels of alcohol consumption, and polymorphisms in the genes coding for BDNF have been associated with emotional problems, substance misuse and appetitive disorders (Davis, 2008). These findings are supported by data indicating that BDNF provides trophic support to midbrain dopaminergic neurons, which play a critical role in drug reward and

relapse (Airaksinen and Saarma, 2002; see Gitza *et al*., 2010 for review). Further, a recent review of the literature on BDNF and addiction (Ghitza *et al*., 2010) concluded that BDNF acts as a negative modulator of alcohol reward, a function mediated primarily through the dorsal striatum (Jeanblanc *et al*., 2009; Logrip *et al*., 2008; see also McGough *et al*., 2004). It is worth noting that an opposite effect of BDNF has been observed for other addictive substances, such as psychostimulants and opiates, and in these cases, positive modulatory effects of BDNF on drug use appear to be mediated through the mesoaccumbens (Ghitza *et al*., 2010). A number of hypotheses have been advanced to explain these differential effects of BDNF on reward, including the notion that differences in BDNF modulation in psychostimulant and opiate reward versus alcohol reward reflect differences in the neurobiological substrates underlying the rewarding effects of these drugs (Ghitza *et al*., 2010).

Genotyping is one strategy that researchers have used to examine the potential relationship between BDNF and addiction. Previous studies have found that approximately 32% of the Caucasian population has the BDNF Val^{66}Met polymorphism, compared to 66% who are Val/Val homozygotes (see Colzato *et al*., 2011). Compared to Val/Val homozygotes (e.g., Val^{66}Val), studies of Val^{66}Met polymorphs have indicated reduced activity-dependent BDNF release, higher anxiety levels during a cold pressor task, greater alcohol consumption levels per week, and higher anticipatory cortisol responses (Colzato *et al*., 2011). Further, Wojnar *et al*., 2009 found a higher risk and earlier occurrence of relapse among Val^{66}Met individuals in treatment for alcohol dependence. However, the literature has not been consistent on this issue, with some

studies reporting no significant relationship between BDNF Val^{66}Met polymorphisms and specific alcohol-related phenotypes in medication-free participants with alcohol dependence (Grzywacz *et al*., 2010; Nedic *et al*., 2013; Muschler *et al*., 2011). Much of the research linking BDNF and addiction has been conducted in treatment-seeking, alcohol dependent populations. At present, the alcohol research literature is lacking a comprehensive study of the influence of BDNF polymorphisms, stress-induced BDNF levels and family history of alcohol dependence on the alcohol consumption characteristics of young, healthy, non-dependent social drinkers without psychiatric co-morbidity. The current study was designed to fill this void in the literature.

Alcohol, Family History and Stress

Acute stress activates the hypothalamic-pituitary-adrenal (HPA axis), initiating a cascade of events beginning with central nervous system (CNS) stimulation of hypothalamic corticotropin releasing hormone (CRH), which increases secretion of pituitary adrenocorticotropic hormone (ACTH) and glucocorticoid (cortisol) production from the adrenal glands. Heavy, acute alcohol administration is known to act as a stressor that stimulates HPA axis activity (Kovacs and Messingham, 2002; Ogilvie *et al*., 1998; Rasmussen *et al*., 2000). Studies on healthy volunteers have shown that alcohol effects on the hypothalamic-pituitary-adrenal (HPA) axis are dose dependent (Waltman *et al*., 1993). At least two studies of chronic, heavy drinkers in the general population have shown elevated cortisol levels (Gianoulakis *et al*., 2003; Thayer *et al*., 2006). However, persistent misuse of alcohol may dysregulate the HPA axis (Lovallo, 2006). In contrast to the general population, recently abstinent alcohol dependent participants typically exhibit

decreased cortisol levels and blunted HPA responses to stress challenges relative to control participants, even in the absence of group differences in self-reported mood state (Adinoff *et al*., 2005a; Lovallo, 2000). However, the literature remains contentious as to the persistence of HPA dysregulation with continued abstinence from addictive substance (Lovallo, 2006). Some studies report recovery of normal HPA function with continued abstinence (Iranmanesh *et al*., 1989). Adinoff *et al*. (2005a) note that the consistency of such work is confounded by uncertainties over the specific level at which the disruption of the HPA response may occur (e.g., at the level of the hypothalamus, pituitary, adrenal or feedback receptors). In a series of well-controlled studies, Adinoff and colleagues attempted to elucidate the mechanisms underlying HPA disruption in alcohol dependent populations (Adinoff *et al*., 2005b, c). Their results suggested that the defect in the HPA axes of alcohol dependent participants may be isolated at the level of the adrenal. It is important to note that not all alcohol dependent participants recover normal HPA function with continued abstinence, and Adinoff *et al*. (2005b,c) have also noted that HPA regulation may not be entirely normal even after recovery of normal diurnal cortisol patterns (Adinoff *et al*., 2005 b,c; see Lovallo, 2006 for review).

Other factors, such as one's family history of alcoholism (FH) may also contribute to variations in stress responsiveness. A positive family history of alcoholism (FH+) is commonly defined as having one or more alcoholic primary relatives, or two or more alcoholic secondary relatives (Mann *et al*., 1985). It has been established, largely through twin and adoption studies, that the heritability (the genetic component of the variance) of substance addiction lies between 40% and 70% (Goldman *et al*., 2005). The

heritability of alcoholism is approximately 50% (Goldman *et al*., 2005). Therefore, genetic and environmental risk factors for the development of addictive disorders are equally important. Because the current study focuses on healthy, social drinking college students, it is important to note that studies have shown that family history positive (FH+) students make up a substantial percentage (35%) of the college population (LaBrie *et al*., 2010). As in the general population, FH+ positive college students are at increased risk for problematic drinking and consequences as compared to their FH− peers (LaBrie *et al*., 2010).

The presence of a family history of alcoholism has been associated with decreased autonomic activity and a blunted endocrine system response to stress (Sorocco *et al*., 2006). For instance, a comparison of family history negative (FH-) boys aged 10-12 years with family history positive (FH+) boys whose fathers had been diagnosed with alcohol and psychoactive substance use disorders indicated lower salivary cortisol response to anticipatory stress in the FH+ group (Dawes *et al*., 1999; Moss *et al*., 1995). Further, a smaller plasma cortisol response to an alcohol challenge has been observed in the adult children of alcoholics in comparison to FH- controls (Croissant and Olbrich, 2004). Lovallo (2006) suggesting that pre-existing differences in top-down regulation of the HPA axis by signals from the limbic system and prefrontal cortex may explain why some participants continue to exhibit reduced cortisol and blunted HPA responses even in the absence of active alcohol dependence. This mechanism may also explain findings of reduced cortisol and blunted HPA responses in the offspring of alcoholics. Other researchers have suggested that blunted HPA responses in family history positive

participants may be related to the influence of the opioid neurotransmitter system at the level of the hypothalamus (see Wand *et al*., 1999).

BDNF and Stress

The BDNF gene has been identified as a stress vulnerability candidate gene (Hosang *et al*., 2014). As noted previously, studies suggest that compared to Val/Val homozygotes, Val^{66}Met polymorphs tend to have reduced activity-dependent BDNF release (Colzato *et al*., 2011). Val^{66}Met participants may also have higher anticipatory cortisol responses and greater anxiety during physical stressors such as the cold pressor task (Colzato *et al*., 2011). The results of Colzato *et al*. (2011) suggest that stress-related differences in Val^{66}Met polymorphs and Val/Val homozygotes may lie in their anticipatory stress responses rather than in their hormonal responses to acute stress challenge. Further, human participants with the Val^{66}Met allele may have differences, relative to their Val^{66}Val counterparts in brain areas linked to emotion. Studies suggest impairments in temporal-lobe cognitive performance, along with smaller hippocampal volumes and increased amygdala activity in response to unpleasant versus neutral pictorial stimuli (Stein *et al*., 2008).

Exposure to stress influences the expression and intracellular trafficking of the BDNF protein (Hosang *et al*., 2014). Studies in animal models have shown that exposure to a variety of stressors both acutely single stress exposure of 2–8 hours duration (Lee *et al*., 2008; Smith *et al*., 1995a; Ueyama *et al*., 1997) and chronically (10 days to 6 weeks) (Murakami *et al*., 2005; Nair *et al*., 2007; Rothman *et al*., 2012; Tsankova *et al*., 2006) can result in significant down-regulation both BDNF mRNA and protein levels

in the hippocampus. Figure 1 depicts the relationship between chronic stress and BDNF levels. Ultimately, stress-related modulation of BDNF signaling activity could increase risk for anxiety and alcoholism (see Maletic *et al*., 2007).

In contrast, animal studies have shown that the duration of stress differentially influences BDNF expression with short-duration stressors of less than 60 minutes evoking an induction in hippocampal BDNF expression and protein levels (Marmigère *et al*., 2003, Molteni *et al*., 2009, Neeley *et al*., 2011). However, the literature is sparse with regard to pre-/post-stress measures of BDNF in human participants. One study by Unternaehrer *et al*., (2012) examined quantitative DNA methylation of the BDNF gene in whole blood cells 10-minutes before, immediately after and 90 minutes after the Trier Social Stress test. In this study, the Trier Social Stress test was conducted in a room equipped with a standing microphone and a video camera in front of two desks. Participants had a 3-minute anticipation period prior to a 10-minute test period, during which they underwent a fictitious job interview and performed mental arithmetic in front of two research assistants wearing white doctor coats and using stop watches to check time. Participants were informed that they would be video- and speech-taped for later evaluation by the research staff. In this study, the researchers did not observe statistically significant changes in BDNF gene methylation over time. The researchers suggested that DNA methylation in BDNF may remain stable after a single short, non-recurring psychosocial stressor. They also noted previous studies showing changes in BDNF methylation after early life adversity (Roth *et al*., 2009), suggesting that BDNF methylation may have a long-term, rather than short-term role in stress adaptation.

However, it is important to note that a number of studies have acute changes in serum BDNF levels following bouts of high intensity aerobic exercise (Rojas Vega *et al*., 2006; Tang *et al*., 2008). This manipulation typically results in transient, moderate (20-40%) increases in serum BDNF levels, which return to baseline within 10-15 minutes following cessation of activity (Rojas Vega *et al*., 2006; Tang *et al*., 2008). In terms of duration, significantly elevated serum BDNF levels have been noted following 30 minute endurance bike rides (Ferris *et al*., 2007). To date, it is unclear whether changes in serum BDNF may be seen in response to more intense mental stressors (e.g., mathematical stressors such as the PASAT, Gronwall, 1977) of extended duration (e.g., $\geq$ 30 minutes) and/or in response to physical stressors such as the cold pressor task (von Baeyer *et al*., 2005).

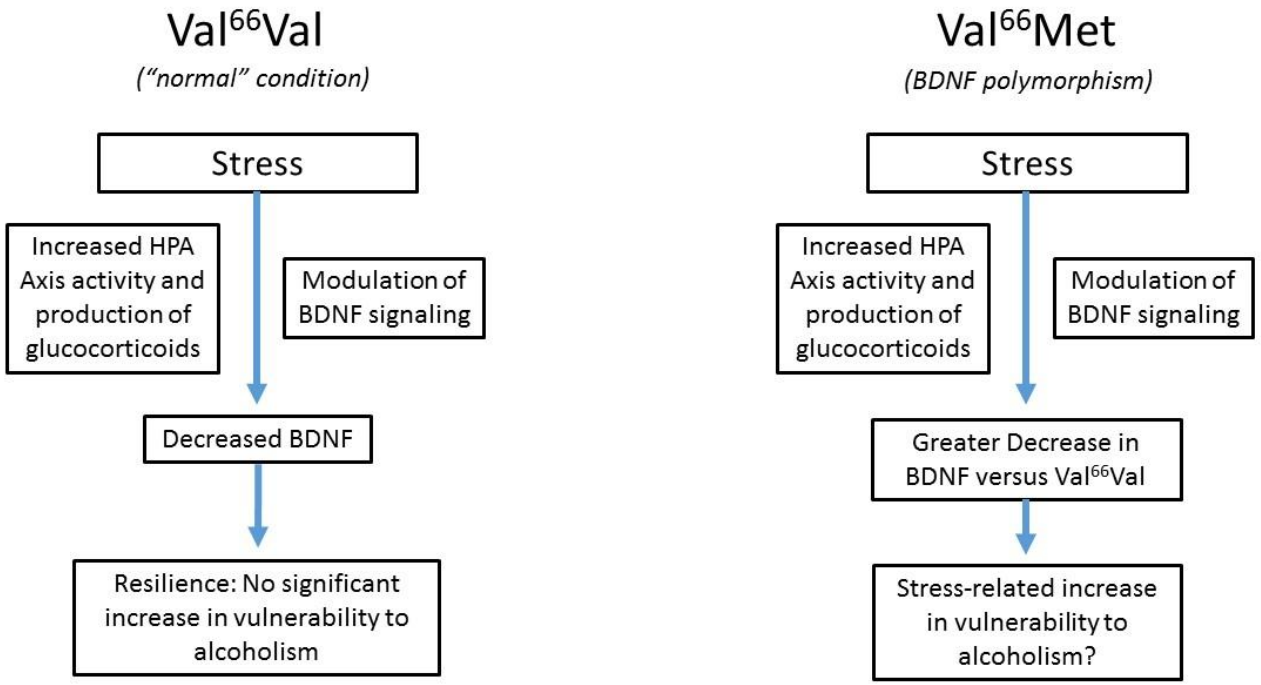


Figure 1. Proposed relationship between stress, BDNF and vulnerability to alcohol dependence (adapted from work by Maletic *et al*., 2007).

III. PRELIMINARY DATA

In 2013, a pilot study funded by a grant to Shobhit Sharma (PI) from the Texas Research Society on Alcoholism (TRSA) was conducted to examine potential differences in alcohol consumption between healthy social drinkers with the Val^{66}Met polymorphism versus Val/Val homozygotes. As mentioned previously, it has been established in the literature that approximately 32% of the Caucasian population has the BDNF Val^{66}Met polymorphism, compared to 66% who are Val/Val homozygotes and 2% who are Met/Met homozygotes (see Colzato *et al.*, 2011; Elzinga *et al.*, 2010; Gatt *et al.*, 2009; Lang *et al.*, 2005). Data from the TRSA pilot study (IRB protocol 2012D6270), which were collected in an ethnically/racially diverse sample, are in concordance with this distribution. In a random sample of 68 participants, 66.18% were Val^{66}Val, 29.41% were Val^{66}Met and 4.41% were Met^{66}Met (see Fig 2).

Participants who were Met^{66}Met homozygotes were removed from further analysis. Other participants who were removed included those who could supply only adoptive family history data, as well as those without significant social drinking histories (e.g., no lifetime history of alcohol consumption). The number of participants in the final sample, distributed across BDNF genotype and FH groups was as follows: FH-neg/Val^{66}Val (n=13), FH-pos/Val^{66}Val (n=26), FH-neg/Val^{66}Met (n=8), FH-pos/Val^{66}Met (n=10).

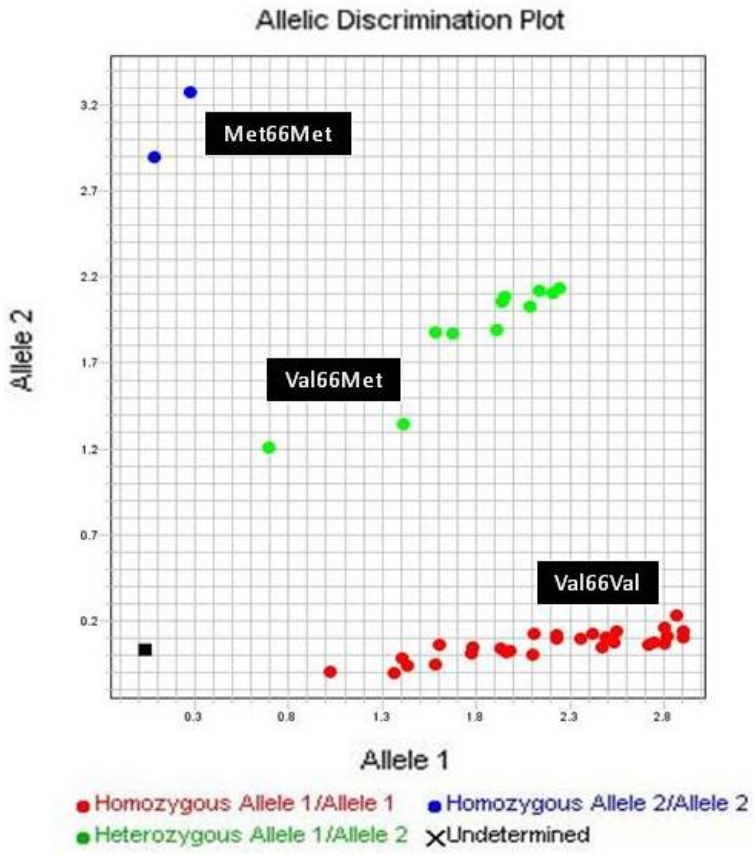


Figure 2. Allelic Discrimination Plot.

Statistical analyses indicated that the distribution of family history of alcohol dependence did not differ significantly between groups (p=0.41). BDNF Val^{66}Val and Val^{66}Met were demographically matched on age (mean = 22.3 years ± 3.68 years),

average parental education (mean = 14.2 years ± 3.03 years), gender distribution (approximately 36% male), and binge drinking in the previous 6 months (approximately 71% binge drinkers). Age at first drink (mean = 13.3 years ± 5.1 years) showed the expected trend toward earlier initiation within the family history positive group (p=0.11). Age at first drunken episode (mean = 16.9 years ± 2.6 years) was significantly higher within the family history positive group (p=0.01), which is consistent with previously published results (e.g., Kuperman *et al.*, 2013).

Quantity/frequency index of alcohol consumption (QFI; Cahalan *et al.*, 1969) was square root transformed to correct for moderate skewness. A 2 (BDNF group) x 2 (FH group) ANOVA was performed to examine potential effects of BDNF, FH and their interaction on total QFI, wine QFI, beer QFI and liquor QFI. A trend toward a main effect of BDNF group was noted for total QFI (p=0.09), which combines consumption of beer, wine and liquor. Contrary to the predictions of Hypothesis 1, in our preliminary group of participants, the Val^{66}Val group exhibited a higher quantity/frequency of alcohol consumption. When this effect was further examined by alcohol type, this pattern reached statistical significance for beer QFI (p=0.04). The Val^{66}Val group reported drinking an average of about 1 beer per day, whereas, the Val^{66}Met group consumed significantly less than 1 beer per day.

Wine consumption overall was quite low, and liquor consumption among the Val^{66}Met group was higher, but not significantly so (about 0.75 ounces of liquor per day for the Val^{66}Met group, where 1.5 ounces is considered the standard drink size for liquor; Cahalan *et al.*, 1969). No other significant effects were noted in the preliminary sample

of participants (*ps* > 0.44). Raw QFI values for the TRSA pilot sample are shown in Figure 3.

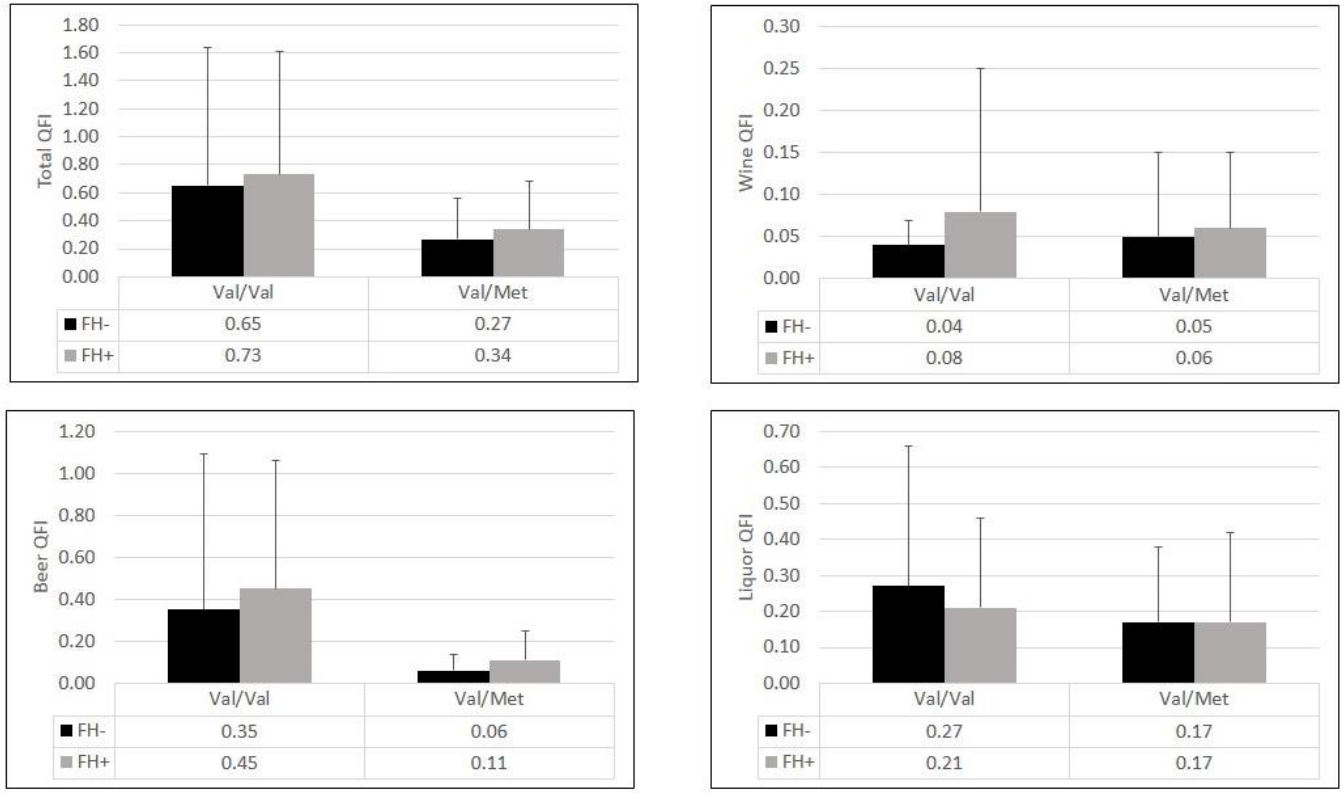


Figure 3. Raw QFI values for the TRSA pilot participants. Val/Val participants had a higher QFI for beer compared to the Val/Met group (p = .04). All other differences were non-significant.

IV. RESEARCH DESIGN AND METHODS

Aim 1: Genotyping and Survey

Aim 1 focused on the separate and combined effects of BDNF and FH on alcohol consumption. In addition to TRSA pilot data that had already been analyzed, additional recruitment and genotyping was needed to fulfill the goals of Aim 1. Power analyses using G*Power Version 3.0.10 with a medium effect size indicated that a minimum *N* of 82 would prove adequate statistical power (≥ 0.80) to detect between-subject effects of BDNF as well as their interaction. Based on genotype probability and TRSA pilot data, it was estimated that recruitment and genotyping of at least 40 additional participants would provide adequate numbers within each of the four groups: FH-neg/ $Val^{66}Val$, FH+/ $Val^{66}Val$, FH-/ $Val^{66}Met$, FH+/ $Val^{66}Met$. It is important to note that all recruitment materials specified that eligible participants would be recalled for Aim 2, which required participants to give a blood sample. Thus, individuals with an aversion to needles had the opportunity to opt out of participating in the study altogether.

The additional male and female social drinkers were recruited from the university community using advertisements and in-class announcements. Participants were also recruited through the Department of Psychology's Human Participants Pool/SONA System, which is used by students in Introductory Psychology and Statistics Laboratory courses to gain course credit. Regardless of the recruitment method, telephone/e-mail screening was used to determine study eligibility prior to the initial laboratory visit for genotyping. Exclusion criteria included any major medical illness that could affect brain function, current and/or past treatment for substance dependence, neurological

conditions, endocrine illness, history of head injury, history of psychiatric medical treatment, and/or clinically significant levels of stress, anxiety or depression. In general, participants were social drinkers meeting guidelines for moderate or low risk drinking as defined by the National Institute on Alcohol Abuse and Alcoholism (NIAAA, 2013); however, heavier drinking and binge drinking were acceptable as long as participants had not previously received a diagnosis of or treatment for alcohol dependence.

Survey. Eligible participants were paid $10 for participating in Aim 1. Participants were tested simultaneously in a dedicated computer research laboratory (accommodated 13 participants) in the Department of Psychology at Texas State University. Comprehensive informed consent and tracking information were obtained at this point. In addition to providing a cheek swab sample for genotyping purposes, participants completed in-depth questionnaires regarding anxiety, as well as familial and personal alcohol use characteristics. These questionnaires were administered via the internet using survey software provided by the Department of Psychology at Texas State. PCI-DSS compliant SSL technology was used to protect user information using both server authentication and data encryption.

In the survey, family history of alcohol dependence was measured using a standard pedigree questionnaire (Mann *et al*., 1985), which yields both quantitative (continuous) and dichotomous family history data (e.g., FH-positive and FH-negative groups). In this method, alcohol dependent first degree relatives (mother, father, sister, brother) are assigned 1 point and alcohol dependent second degree relatives (aunt, uncle, grandmother, grandfather) are assigned 0.5 point. The sum of each participants' family

history of alcohol dependence represented their final qFH score; scores greater than 1 indicated a positive family history of alcohol dependence. Alcohol consumption characteristics were measured using the Quantity/Frequency Index (Cahalan *et al*., 1969), which provides an estimate of the average volume (in ounces) of absolute ethanol consumed per day in the previous 6-month period. Information on age at first drink, age at first drunken episode, and time since alcohol was last consumed was also recorded. Binge drinking patterns over the past year were documented using the method of Cranford *et al*. (2006). BDNF has been implicated in disorders of stress, anxiety and depression, which are known precipitating factors for the development of substance abuse (Davis, 2008). In statistical analyses, levels of stress, anxiety and depression tend to be significantly inter-correlated. In this study, we chose to focus on self-reported levels of anxiety, which were measured using the Spielberger Trait Anxiety Questionnaire (STAI, Spielberger, 1983) and perceived stress (Perceived Stress Scale, Cohen *et al*., 1983). Where appropriate, the potential influence of stress or anxiety was addressed in statistical analyses.

Genotyping. Genotyping was accomplished through the collection of cheek swab samples. All samples were stored and processed through the Salivary Analysis Laboratory in the Department of Psychology at Texas State. Genomic DNA was extracted from buccal mucosa on a cotton swab using the BuccalAmp™ DNA Extraction Kit (Epicentre Biotechnologies, Madison, Wisconsin, USA). The rs6265 SNP ($Val^{66}Met$) of the *BDNF* gene was genotyped using Applied Biosystems (AB) TaqMan technology. The probe for genotyping the rs6265 was ordered through the TaqMan SNP Genotyping

assays (code C_11592758_10) AB assay-on-demand service. The final volume of the polymerase chain reaction was 20µl, which contained 10 µl of TaqMan Master Mix, 1 µl of 40x genotyping assay, 2 µl of DNA sample and 7 µl of water. The cycling parameters were as follows: 95°C for 10 minutes followed by 50 cycles of denaturation at 92°C for 15seconds and annealing/extension at 60°C for 1.5 minutes. Polymerase chain reaction plates were read on StepOnePlus™ instrument with version 2.1 software (Applied Biosystems).

Aim 2: Serum BDNF and Stress Challenge

Aim 2 focused on potential differences in serum BDNF levels between genotype groups ($Val^{66}Met$ vs. $Val^{66}Val$) before and after mental and physical stress challenge in the laboratory setting. Power analyses (G*Power 3.0.10) indicated that a minimum *N* of 25 participants, split between $Val^{66}Val$ and $Val^{66}Met$ genotypes would provide adequate power (≥ 0.80) to detect between- and within-subject differences and interactions. For this part of the project, family history of alcohol dependence was expressed as a continuous variable and was included in correlational analyses of serum BDNF change versus alcohol consumption characteristics in the two BDNF genotype groups.

Given that Aim 2 was a new addition to the proposal, two participant recruitment strategies were available to us. With IRB approval, we recalled participants from our database of individuals with known genotypes from the TRSA pilot study. We also tested new recruits from Aim 1 (see above). We used both strategies in an attempt to maximize the power of our limited budget. In both cases, all recruitment materials specifically indicated that blood draws were to be part of the project. Thus, individuals with an

aversion to needles had the opportunity to opt out of participating in the study altogether. Based on pilot data, we believed that we would need to allow for 20% loss of participants due to attrition and/or phlebotomy difficulties. Thus, we intended to recruit at least 30 genotyped individuals to participate in Aim 2. Individuals using hormonal birth control methods were excluded from the study.

Testing session. The timeline of the Aim 2 testing session is shown in Table 1. Upon arrival to the laboratory at 1pm, participants completed a separate consent document for Aim 2. They were informed that the testing session would involve completion of a mathematical task (PASAT, described below), as well as a physical stressor (cold pressor task, described below). Next, they were given a 15 minute rest break to encourage acclimation to the laboratory setting. During the rest break, participants did not engage in any stressful or study-related activities. Instead, they were offered neutral reading material and were allowed to use personal electronic devices, such as their smart phones. After the rest break, they then completed a baseline, self-report measure of anxiety state (Spielberger State Anxiety Inventory, SSAI; Spielberger, 1983). Next, they provided the first of two blood samples, followed by a second administration of the SSAI.

Stressors. The math task used in this study is the Paced Auditory Serial Additional Task (PASAT; Gronwall, 1977). The PASAT is a timed math task that challenges auditory information processing speed and flexibility, as well as calculation ability (Gronwall, 1977). In this task, participants attend to the auditory presentation of single-digit numbers and respond verbally to indicate the sum of the number just heard

and the number heard immediately prior. Numbers are presented with progressively faster rates of stimulus presentation, increasing the intensity of the task over time. This task has been shown to elicit autonomic arousal (Mathias *et al*., 2004), as well as self-reported increases in anxiety (Ceballos *et al*., 2012). Two approximately 13 minute blocks of the task were presented back-to-back. The PASAT was followed by a physical stressor, the cold pressor test (von Baeyer *et al*., 2005), in which participants were asked to immerse their hand in ice water (1 to 4 °C) for 1.5 minutes (Colzato *et al*., 2008).

Together, preparation and administration of the mental and physical stress sessions provided the 30 minutes of acute activity that is most likely to result in serum changes in BDNF (see Ferris *et al*., 2007). Immediately following administration of the stress tasks, participants completed a third SSAI and provided the second blood sample. After this, participants completed previously described (see Aim 1) questionnaires. A final SSAI was completed immediately prior to the end of the laboratory session.

Serum BDNF Assay. During the testing session, participants provided two blood samples: one before and one after completion of the stress tasks, described below. Blood from the antecubital vein was collected in sterile serum separator tubes (BD Vacutainer™ Venous Blood Collection Tubes). The blood samples were kept at room temperature for 15 min to allow for clotting, after which the samples were centrifuged at 1,100 rpm for 15 min. Serum was then harvested, aliquoted, and stored at −20 °C until analysis. Serum BDNF was quantified using an enzyme-linked immunosorbant assay (Human BDNF Quantikine Immunoassay, DBD00, R & D Systems) according to the manufacturer's instructions. The intra- and inter-assay coefficients of variation were 5% and 9%,

respectively. The serum samples were diluted 1:80 in the supplied sample diluent in the kit and assayed against a standard curve with a 500 pg/mL highest concentration. The supplied mouse anti-human BDNF–biotin primary antibody and strepavidin–HRP secondary antibody were used at a dilution factor of 1:1,000. After incubation with the provided substrate solution, the reaction was stopped with the addition of stop solution, and the plate was read at 450 nm using a spectrophotometric plate reader (Dynex MRXe plate reader with Revelation software).

Table1. Timeline of Aim 2 testing session

1 to 1:30 pm	1:30 to 2 pm	2 to 2:45 pm
Consent Form *Rest Break* Anxiety 1 Blood Sample 1 Anxiety 2	PASAT Cold Pressor Anxiety 3	Blood Sample 2 Anxiety 4 Questionnaires

Statistical Analysis Plan

Aim 1 focused on the separate and combined effects of BDNF and FH on alcohol consumption. Dependent variables included quantity/frequency indices (QFIs) of wine, beer and liquor, as well as total QFI, binge drinking indices, age at first drink, and age at first drunken episode. QFIs were square root transformed to correct for skewness. Separate 2 x 2 ANOCOVAs with between-subjects factors of BDNF genotype (Val/Val vs. Val/Met) and family history group (FH+ vs. FH-) and controlling for age and parental

years of education, were conducted for continuous dependent variables (age at first drink, age at first drunken episode, total QFI, beer QFI, wine QFI, and liquor QFI). Age was chosen as a covariate given the potential influence of legal drinking age (21 years) in a sample with age ranging from 18 to 30. Parental years of education was included as a covariate due to the influence of socioeconomic status on stress (Sapolsky, 2005). Categorical alcohol-related variables, such as presence versus absence of heavy episodic drinking in lifetime, prior 6 months and prior 1 month, and regular drinker status, were analyzed using Chi-Square against the aforementioned crossing of BDNF x FH groups. That is, distribution of variables was examined across four groups: FH-neg/ $Val^{66}Val$, FH+/ $Val^{66}Val$, FH-/ $Val^{66}Met$, and FH+/ $Val^{66}Met$. As mentioned previously, BDNF has also been implicated in disorders of stress and anxiety, which are known precipitating factors for the development of substance abuse (Davis, 2008). The relationship of perceived stress and square root transformed total QFI was examined separately for $Val^{66}Met$ and $Val^{66}Val$ groups using partial correlations, controlling for age and parental years of education.

Aim 2 focused on potential differences in serum BDNF levels between genotype groups ($Val^{66}Met$ vs. $Val^{66}Val$) before and after physical and mental stress challenge in the laboratory setting. To verify the aversive nature of the mental and physical stress challenge, repeated anxiety assessments (SSAI) were examined using repeated measures ANOVA. That is, a repeated measures ANOVA was conducted with time (four time points of SSAI assessment) as the within-subjects factor.

To address Aim 2, separate 2 x 2 repeated measures ANCOVAs were conducted

with serum BDNF as the DV and time (before and after stress) as the within-subjects factor and BDNF group (Val/Val versus Val/Met) as the between-subjects factor, controlling for average years of parental education. Parental years of education was included as a covariate due to the influence of socioeconomic status on stress (Sapolsky, 2005). Age was not included as a covariate in these analyses (versus Aim I) because the dependent variables were BDNF responses to stress rather than alcohol use characteristics.

Quantitative family history of alcohol dependence was expressed as a continuous variable and was examined along with other variables in separate correlational analyses of serum BDNF change (post- minus pre-stress) versus alcohol use characteristics. Further, an alternative statistical strategy involved the examination of anxiety as a potential covariate in these correlations. Anxiety (rather than perceived stress) was chosen as the most appropriate covariate for correlational analyses in Aim 2 because it was repeatedly measured throughout the Aim 2 testing session. As mentioned previously, anxiety is typically inter-correlated with perceived stress.

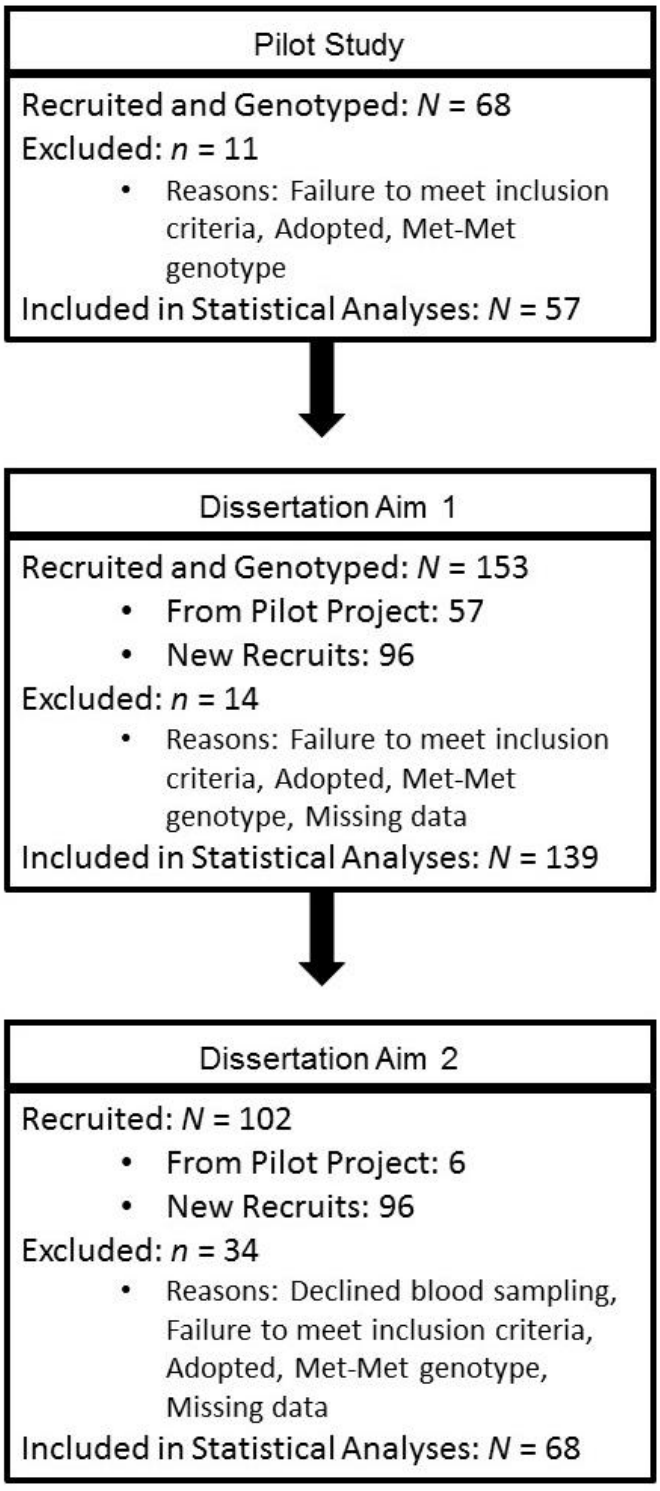


Figure 4. Flow diagram of participant recruitment and retention at each stage of the study.

V. RESULTS

Aim 1.

The first purpose of Aim 1, Hypothesis 1a, was to compare alcohol use characteristics among Val^{66}Met versus Val^{66}Val genotype groups. It was hypothesized that individuals with the Val^{66}Met polymorphism would have higher levels of alcohol consumption, earlier initiation of alcohol use and drinking to excess, and greater endorsement of binge drinking habits compared to individuals in the Val^{66}Val group. In addition, Aim 1, Hypothesis 1b involved examination of separate and combined effects of BDNF genotype (Val^{66}Met versus Val^{66}Val) and family history of alcohol dependence (FH+ versus FH-). An interaction of BDNF group x family history group was expected, in which participants with a positive family history of alcoholism and the Val^{66}Met genotype (FH+/Val^{66}Met) would have a more severe profile of alcohol consumption characteristics.

Background Characteristics. Statistical results are shown in Table 2. One hundred and thirty-nine participants (42 male) were tested in the Aim 1 component of the study. A flow chart for recruitment and testing is shown in Figure 4. No significant group differences or interactions were noted for age or years of parental education (*ps* > .29). The distributions of gender and race did not differ significantly across the BDNF x FH groups (*ps* > .17). However, ethnicity (Hispanic ethnicity within any racial group vs. Non-Hispanic ethnicity within any racial group) differed significantly across the BDNF x FH groups ($\chi^2(3) = 9.08$, $p = .03$). The inclusion of ethnicity as an additional independent variable in subsequent analyses associated with Aim 1 was not feasible due to small cell

sizes. Instead, this issue was addressed through the analysis of Non-Hispanics only. In addition, to enhance comparison to previous studies using an ethnically-homogenous Caucasian sample (e.g., Nedic *et al*., 2013), analyses were also repeated with White Non-Hispanic participants only.

Overall Model, Hypothesis I. Results of analyses with all participants are shown in Table 2. There was a main effect of BDNF group for age at first drink ($F(1,130) = 3.97$, $p = 0.048$), in which the Val^{66}Met group initiated alcohol use at an earlier age (M = 12.5 years, SD = 4.8 years) compared to the Val^{66}Val group (M = 13.5 years, SD = 3.7 years). No other significant effect or interactions were noted for age at first drink (ps > 0.22). Although all participants considered themselves to be social drinkers, seven percent of participants reported that they had never consumed enough alcohol at one sitting to become drunk. For those who reported having had at least one drunken episode in their lives, there was a main effect of FH group for the age at which their first drunken episode occurred ($F(1,121) = 4.52$, $p = 0.04$). The FH-positive participants reported an earlier age at first drunken episode (M = 16.1 years, SD = 2.1 years) compared to the FH-negative group (M = 17.1 years, SD = 2.3 years). No other significant effects were noted for age at first drunken episode (ps > 0.79). No significant effects were noted for BDNF, FH or their interaction on total QFI (ps > 0.39), or the QFIs for beer (ps > 0.56), wine (ps > 0.43) or liquor (ps > 0.24). The distribution of binge drinking for lifetime ($p = 0.59$) and prior 6-months ($p = 0.92$) did not differ significantly across BDNF x FH groups. Likewise, the distribution of self-identified regular drinkers did not differ significantly across the BDNF x FH groups ($p = 0.15$).

Non-Hispanics Only. Analyses were repeated with 68 Non-Hispanics (of any racial category): Val^{66}Val/FH-positive (n = 31), Val^{66}Val/FH-negative (n = 16), Val^{66}Met/FH-positive (n = 8), and Val^{66}Met/FH-negative (n = 13). The previously noted patterns of significance for alcohol use variables remained. For age at first drink, Val^{66}Met participants (M = 11.6 years, SD = 5.2 years) reported an earlier age at first drink compared to the Val^{66}Val group (M = 13.4 years, SD = 4.0 years), $F(1,61) = 5.12$, $p = 0.03$. For age at first drunken episode, FH-positive participants (M = 15.8 years, SD = 2.2) reported an earlier age compared to the FH-negative group (M = 17.2 years, SD = 2.4), $F(1,56) = 5.26$, $p = 0.03$). There were no significant BDNF or FH effects or interactions on total QFI ($p = 0.45$), or QFI for beer ($p = 0.18$), wine ($p = 0.65$), or liquor ($p = 0.65$). Groups did not differ on heavy episodic drinking over the lifetime ($p = .38$), in the prior 6-months ($p = 0.85$), or in the prior 1-month ($p = 0.65$). However, self-identified regular drinkers varied significantly in distribution across the BDNF x FH groups ($\chi^2(3) = 7.98$, $p = 0.046$). The greatest proportion of regular drinkers were found in the Val^{66}Val / FH-positive group.

White Non-Hispanics Only. Analyses were repeated with 55 White, Non-Hispanics: Val^{66}Val/FH-positive (n = 26), Val^{66}Val/FH-negative (n = 10), Val^{66}Met/FH-positive (n = 8), and Val^{66}Met/FH-negative (n = 11). Though this set of analyses was statistically underpowered, Val^{66}Met participants once again had an earlier age at first drink compared to Val/Val homozygotes (Val^{66}Met: M = 10.8 years, SD = 4.9 years; Val^{66}Val: M = 13.2 years, SD = 3.9 years; $F(1,49) = 3.83$, $p = 0.056$). FH and interaction effects for age at first drink were non-significant ($ps > 0.97$). FH-positive participants had

an earlier age at first drunken episode compared to FH-negative participants ($Val^{66}Met$: M = 15.5 years, SD =2.3; $Val^{66}Val$: M = 16.7 years, SD = 1.9; $F(1,46) = 4.10$, $p = 0.049$). BDNF genotype and interaction effects for age at first drunken episode were non-significant ($ps > 0.80$). There were no significant BDNF or FH effects or interactions on total QFI ($p = 0.65$), or QFI for beer ($p = 0.21$), wine ($p = 0.47$), or liquor ($p = 0.78$). Groups did not differ on heavy episodic drinking across the lifetime ($p = 0.60$), in the prior 6-months ($p = 0.78$), or in the prior 1-month ($p = 0.87$). Groups did not differ on distribution of regular drinkers ($p = 0.25$).

Table 2. Background and Alcohol Use Variables for AIM 1 Participants. Table shows means and standard deviations. Raw QFI values are shown in the table, whereas, square root transformed values were used for statistical analyses. *Significant difference in distribution across BDNF x FH groups, $p < .05$. †Significant main effect of BDNF group, $p < .05$. ††Significant main effect of family history of alcoholism, $p < .05$. No other significant effects were noted.

All Participants ($N = 139$)				
	Val^{66}Val		Val^{66}Met	
	FH- ($n = 30$)	FH+ ($n = 65$)	FH- ($n = 18$)	FH+ ($n = 26$)
% Male	40%	27%	44%	19%
% Hispanic*	43%	47%	24%	69%
% White	54%	61%	78%	77%
Age	21.5 (3.9)	20.5 (3.0)	20.9 (2.7)	20.7 (3.1)
Average Parental Education (years)	14.0 (2.6)	14.1 (2.3)	13.9 (2.1)	13.5 (3.0)
Perceived Stress (total)	24.57 (8.40)	26.40 (11.17)	25.28 (6.55)	25.96 (8.80)
Age at First Drink†	14.3 (3.5)	13.1 (3.7)	12.9 (5.0)	12.3 (4.8)
Age at First Drunken Episode††	17.0 (2.2)	16.1 (2.1)	17.4 (2.5)	16.2 (2.0)
Quantity Frequency Index				
Total	0.56 (1.01)	0.67 (0.86)	0.47 (0.51)	0.58 (0.81)
Beer	0.30 (0.69)	0.33 (0.59)	0.21 (0.33)	0.25 (0.46)
Wine	0.06 (0.17)	0.06 (0.13)	0.07 (0.16)	0.07 (0.10)
Liquor	0.20 (0.36)	0.28 (0.43)	0.19 (0.23)	0.26 (0.37)
% Heavy Episodic Drinking				
Lifetime	87%	85%	78%	92%
Last 6 Month	73%	75%	77%	81%
% Regular Drinkers	33%	17%	39%	27%

Correlations

In the analysis including all participants, and in the analysis including Non-Hispanics only (of any racial category), there were no significant relationships between perceived stress and alcohol use, regardless of BDNF genotype (All participants: *ps* > 0.29; Non-Hispanics: *ps* > 0.18). Likewise, there were no significant relationships

between the drinking expectancy of tension reduction and alcohol use, regardless of BDNF genotype (All participants: *ps* > 0.30; Non-Hispanics: *ps* > 0.26).

In the analysis including only White Non-Hispanics, perceived stress was not related to alcohol use in the Val^{66}Val group; however, there was a significant positive correlation between total QFI and perceived stress for the Val^{66}Met group ($r(17) = 0.50$, $p = 0.03$); see Figure 5. The Fisher r to z transformation was used to assess the significance of the difference between the correlation coefficients for the relationship between PSS and total QFI in the Val^{66}Val and Val^{66}Met groups. Results for the one-tailed test indicated a significant difference between the two correlations ($p = .02$).

Aim 2.

The purpose of Aim 2, Hypothesis 2a was to examine potential differences in serum BDNF levels between genotype groups (Val^{66}Met vs. Val^{66}Val) before and after a mental and physical stress challenge in the laboratory setting. It was expected that Val^{66}Met participants (relative to Val^{66}Val) would have a smaller increase (or perhaps a larger decrease) in BDNF levels in response to stress. Aim 2, Hypothesis 2b examined the potentially differential relationship of the BDNF stress response to alcohol consumption characteristics in the two BDNF genotype groups. For the Val^{66}Met group,

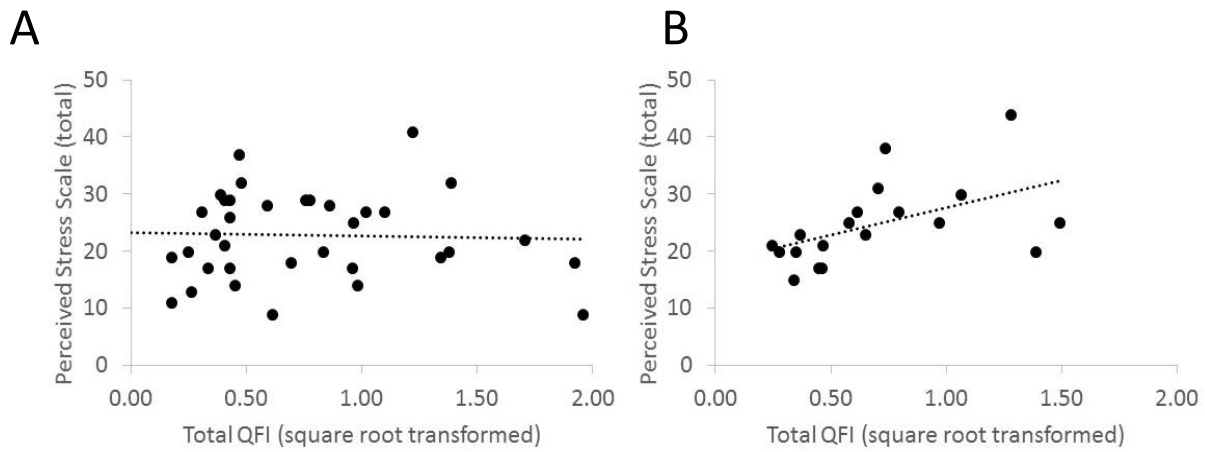


Figure 5. Differential Relationship between Quantity-Frequency of Alcohol Use and Perceived Stress in BDNF Genotype Groups. Panel A shows the relationship in the Val^{66}Met group, and Panel B shows the relationship in the Val^{66}Val group. The correlation reached significance in the Val^{66}Met group only ($r(17) = 0.50$, $p = 0.03$). Fisher's r to z transformation indicated a significant difference between correlation coefficients in Panel A versus Panel B ($p = .02$).

it was predicted that the extent of serum BDNF change in response to stress would be correlated with alcohol use characteristics, such that smaller stress-related increases (or greater stress-related decreases) in serum BDNF would be associated with a more severe profile of alcohol consumption characteristics. For the Val^{66}Val group, it was hypothesized that these relationships would be absent or present to a lesser extent versus the Val^{66}Met group. As part of our alternative analysis strategy for Aim 2, we also examined potential BDNF genotype differences in trait anxiety to determine whether or not this variable might serve as covariate in the analyses of interest (see partial correlations, below).

Background Characteristics. Sixty-eight participants (20 male) were included in the Aim 2 portion of the study. A flow diagram of recruitment and testing is shown in Figure 4. BDNF groups did not differ in age ($p = 0.62$) average years of parental education ($p = 0.30$), or perceived stress levels over the month prior to testing ($p = 0.97$). Distribution of gender ($p = 0.90$), ethnicity ($p = 0.73$), and race ($p = 0.07$) did not differ significantly across BDNF groups.

Manipulation Check. Prior to examining hypotheses 2a and 2b, a manipulation check was conducted to verify the aversive nature of the PASAT. Spielberger State Anxiety assessments from all four time points throughout the experimental session were compared. A significant within-subjects effect of time was noted ($F(3, 201) = 46.76$; $p < 0.001$), in which anxiety levels were highest immediately after the stressor compared to all other time points ($ps < 0.001$, Bonferroni corrected); see Figure 6. Further, the Val^{66}Met group had higher anxiety levels across the testing session compared to the

Val^{66}Val group (F(1,66) = 4.68, p = 0.03). The interaction of time x BDNF genotype group was non-significant (p= 0.85).

Hypothesis 2. For Aim 2, Hypothesis 2a, results indicated that across groups, BDNF levels decreased in response to the stressor (F(1,65) = 6.89, p = 0.01) and there were no significant BDNF genotype group differences, or time x BDNF genotype interactions, in average serum BDNF across the session (ps > 0.62). For Aim 2, Hypothesis 2b, correlational analyses were conducted separately in the Val^{66}Met and Val^{66}Val groups to examine the potential relationship between serum BDNF change in response to the stressor (continuous variable: BDNF pg/mL post-stress minus BDNF pg/mL pre-stress) and alcohol consumption characteristics (age at first drink, age at first drunken episode, age of initiation of regular drinking, total QFI, beer QFI, wine QFI and liquor QFI), as well as quantitative family history of alcohol dependence (continuous variable).

In the Val^{66}Met group, age at first drink was associated with a greater stress-related decrease in serum BDNF (post- minus pre-stress) (r(21) = 0.46, p = 0.03), as shown in Figure 7. This relationship was not present in the Val^{66}Val group (p = 0.38). The Fisher r to z transformation was used to assess the significance of the difference between the correlation coefficients for the relationship between BDNF change and age at first drink in the Val/Val and Val/Met groups. Results for the one-tailed test indicated a significant difference between the two correlations (p = .19). Correlations between serum BDNF change and other familial or personal alcohol use variables were statistically non-significant (ps > 0.08, most > 0.38). When the correlational analysis of age at first drink

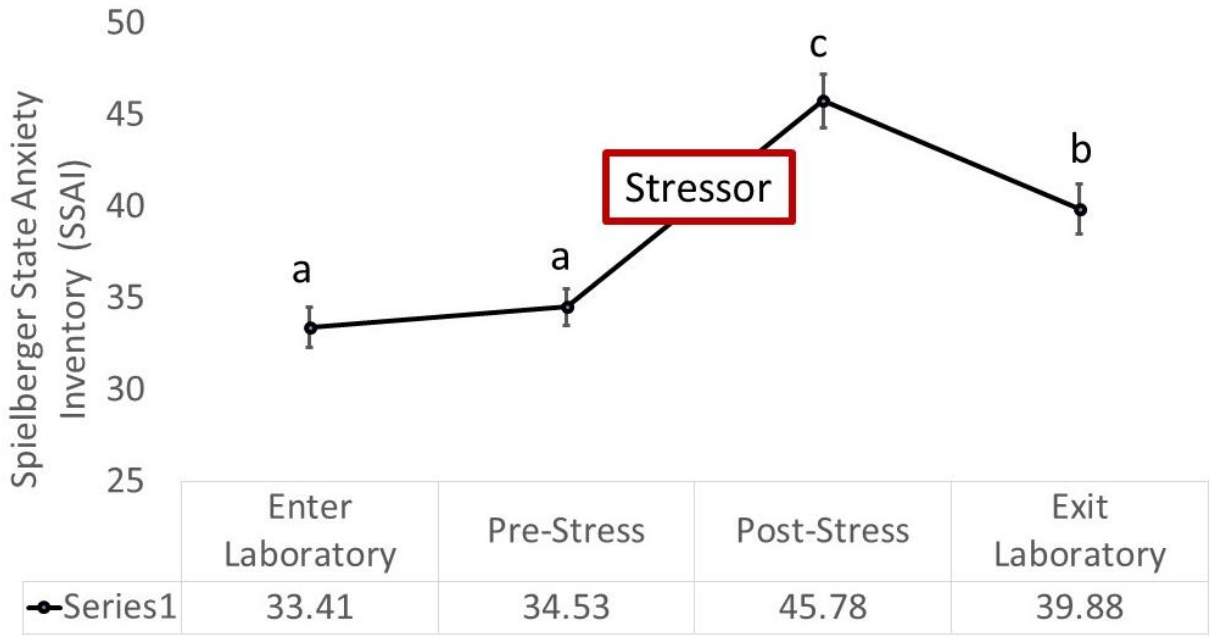


Figure 6. Stress manipulation increased mean anxiety level across all participants. Dissimilar superscripts denote significant difference, $p < .001$ (Bonferroni corrected). Error bars indicate standard error.

and stress-related BDNF change was repeated as a partial correlation controlling for parental education, the same pattern of results was obtained ($r(20) = 0.42$, $p = 0.05$) though significance was reduced.

Because the Val^{66}Met group reported higher average anxiety across the testing session (see Manipulation Check), partial correlations were repeated adding trait anxiety as a covariate. The same pattern of results was observed. There was no association between BDNF change and age at first drink for the Val^{66}Val group ($p = 0.33$); however, the association became even more significant for the Val^{66}Met group ($r(18) = .53$, $p = 0.02$).

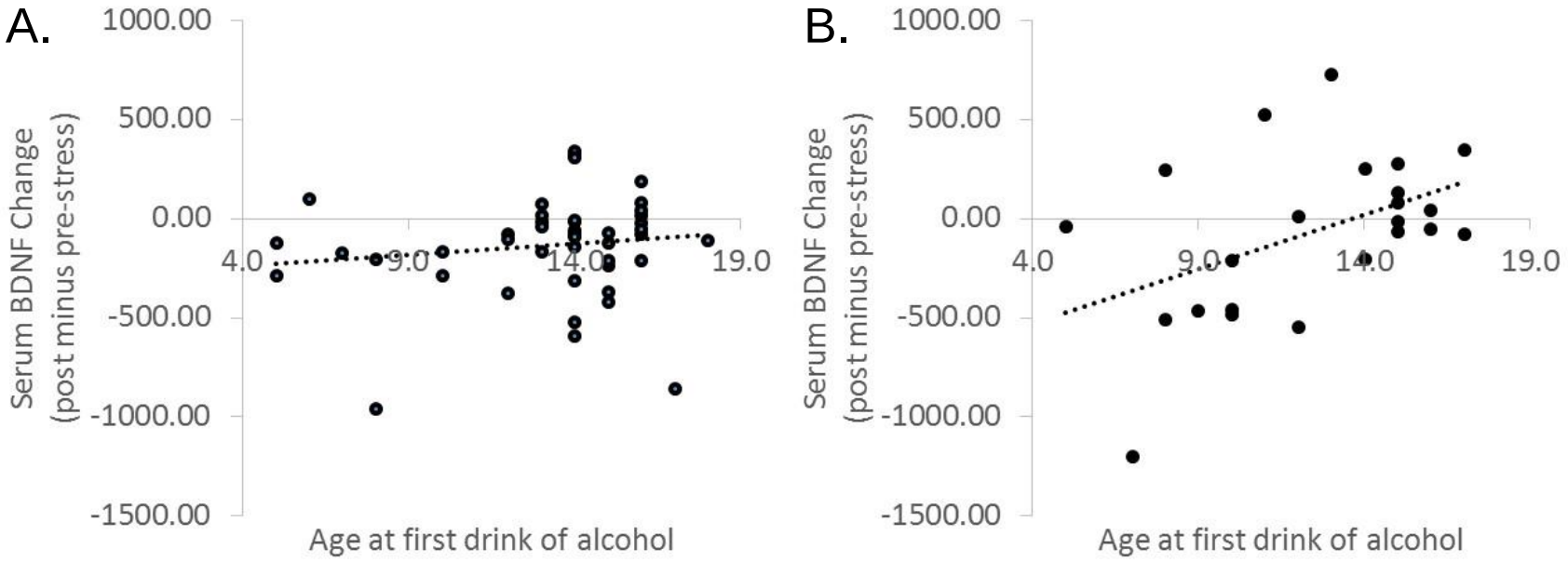


Figure 7. Differential relationships between serum BDNF change in response to stress and age at first drink. Graph A shows the non-significant correlation for the Val^{66}Val genotype group. Graph B shows the significant relationship for the Val^{66}Met genotype group.

VI. DISCUSSION AND FUTURE DIRECTIONS

Current literature suggests that individuals with the Val^{66}Met BDNF polymorphism may have increased alcohol consumption and genetic risk for alcohol dependence relative to those with the Val^{66}Val genotype (see Ghitza *et al*., 2010 for review). However, this finding is not universal, and the literature remains contentious with regard to this issue (see Nedic *et al*., 2013). Studies of this issue have historically disregarded the important influence of family history of alcohol dependence (FH) and potentially additive effects of positive FH and Val^{66}Met genotype. Aim 1 of the current study was designed to address this issue.

Building on a previous pilot study of healthy social drinkers, which failed to show a more severe pattern of alcohol use in the Val^{66}Met genotype group, additional participants were recruited to ensure adequate statistical power. In this larger and more diverse group of 139 participants, controlling for age and parental education, results indicated that the Val^{66}Met group began drinking at an earlier age and the FH-positive group had their first drunken episode at an earlier age. However, in contrast to the hypothesis for Aim 1, there were no interactions of BDNF group x FH group and no additive effects of these factors on alcohol consumption characteristics. Both main effects (BDNF group and FH group) support the existing literature. Earlier age of alcohol use is consistent with previous studies suggesting greater alcohol abuse or dependence risk in those with the Val^{66}Met genotype (see Ghitza *et al*., 2010 for review). The FH-positive group reported an earlier age for the first time they drank to excess, and this finding is consistent with work by Kuperman *et al*. (2013), who found that membership in a high

risk alcohol dependent family was a significant predictor of the initiation of alcohol use. We did not, however, find that family history positive participants had an earlier age at first drink (meaning consumption of any alcohol, even if given to them as a child) – only that they started drinking to excess at an earlier time point compared to their family history negative peers. One possible explanation of this might be that, while there was not a statistically significant difference in the distribution in gender across groups, the relatively larger number of female participants in the family history positive group may have diluted potential group differences on age at first drink.

Quantity/frequency measures of alcohol use did not differ significantly by BDNF group. However, this result might not be unexpected. It is also important to note that our participant sample was composed of young social drinkers, many of whom had not established a regular pattern of drinking. Instead, although the majority of participants reported binge drinking activity, most participants had a fairly low quantity/frequency of alcohol consumption equivalent to less than 1 to 2 drinks per day. It is possible that the relationship between the Val^{66}Met polymorphism and problem drinking (as evidence by high quantity/frequency index) may not become apparent until later in life, as the majority of studies on this topic have focused on older drinkers. It is possible that a longitudinal assessment of our participant sample might reveal more interesting associations between Val^{66}Met polymorphisms and alcohol use.

Further, relationships between stress and alcohol use were found exclusively in the Val^{66}Met group. Though the two genotype groups did not differ on the amount of perceived stress in the one month prior to study participation (a subjective index of

chronic stress), perceived stress was associated with quantity/frequency of alcohol use only in the Val^{66}Met group and not in the Val^{66}Val group. In general, the increased HPA-axis activity that accompanies stress tends to be associated with a decrease in BDNF (Maletic *et al*., 2007; Smith *et al*., 1995; Murakami *et al*., 2005), and a number of studies have shown hypersensitivity to stress among individuals with the Met allele (Elzinga *et al*., 2011; Murakami *et al*., 2005). In animal studies, activation of the BDNF signaling pathway inhibits alcohol consumption, suggesting that the opposite (inhibition of BDNF release) might predispose an organism for the development of alcohol dependence (McGough *et al*., 2004). In fact, BDNF heterozygous type mice have been shown to have a preference for alcohol consumption as compared to homozygotes (Hensler *et al*., 2003). Thus, in keeping with the model shown in Figure 1 (see Introduction section), the relationship between the Val^{66}Met polymorphism and alcohol consumption may proceed through genotype-specific differences in the relationship between chronic stress and BDNF release. If so, this pathway would also be consistent with previous studies showing interactive effects of recent life stress and the BDNF Val^{66}Met polymorphism on the development of other conditions such as anxiety and depression (Kim *et al*., 2007).

These issues were further examined through Aim 2, which focused on the influence of acute stress on BDNF release in the two genotype groups. It is important to note that the current study contributed to the literature as one of the few studies of serum BDNF levels during stress adaptation in human participants. Most of this work has been conducted in animal models. Of the existing studies, Unternaehrer *et al*. (2012) failed to find statistically significant changes in BDNF gene methylation as measured in whole

blood cells from human participants following the Trier Social Stress test. Other studies have shown transient, moderate increases in serum BDNF in response to high intensity aerobic exercise (Rojas Vega *et al*., 2006; Tang *et al*., 2008). The results of the current study indicated that across groups, BDNF levels decreased in response to the stressor, though there were no significant BDNF genotype group differences or time x BDNF genotype interactions in average serum BDNF across the session. Though the current study focused on peripheral serum levels of BDNF, the finding of stress-related decreases in BDNF is in keeping with animal studies in which unpredictable stress and chronic restraint stress led to decreases in hippocampal mRNA and protein levels of BDNF in mice and rats (Duman and Monteggia, 2006). In addition, corticosterone administration has been shown to produce decreases in hippocampal BDNF expression, further implicating the role of stress in BDNF dysregulation (Jacobsen and Mork, 2006). The potential relationship between serum BDNF levels and alcohol use characteristics was also examined in the two BDNF genotype groups. The only significant finding was that an earlier age at first drink was associated with a greater stress-related decrease in serum BDNF in the Val^{66}Met group, but not in the Val^{66}Val group. This finding remained significant when trait anxiety and parental education levels were controlled.

When the results of Aim 1 and Aim 2 are considered together, trends emerge. The Val^{66}Met group began drinking earlier (a risk factor for later problem drinking) and across studies, there were relationships between stress and alcohol use characteristics in the Val^{66}Met group that were not present for Val^{66}Val controls. Findings of both Aims support the notion that the increased risk for alcohol dependence, which has been

hypothesized in the research literature for the Val^{66}Met group, may proceed through stress-related mechanisms. There has been evidence in studies of animal models, which suggests that adolescent drinking may impact BDNF levels. In a study of rats that were exposed to ethanol during adolescence, BDNF concentration decreased in the hippocampus (Scheidt *et al*., 2105). Further, chronic alcohol treatment in rats decreases the expression of BDNF in the hippocampus and cortex (MacLennan *et al*., 1995). If this were true for human participants, it might create a cycle in which Val^{66}Met might be associated with earlier onset of alcohol use during adolescence (as it is in the current study), leading to decreased BDNF levels and greater stress vulnerability, which might predispose participants to higher levels of alcohol consumption later on. Our correlational results in the Val^{66}Met group in Aim 1 and Aim 2 certainly support this notion. However, it is not known if these participants will go on to develop alcohol-related problems. Though it is beyond the scope of the current project, additional longitudinal research is needed to fully address this issue.

Limitations and Future Directions

A number of limitations remain to be addressed in future research. For instance, we used a family history questionnaire that relied on self-report and participants' own perceptions of what constitutes problem drinking or drug use. Basically, participants were instructed to indicate that a relative had a problem with a substance if they had witnessed or heard about that person having negative consequences as a result of their use of that substance. A person may drink excessively (beyond NIAAA guidelines for moderate drinking, see Appendix for definitions of different drinking types) and yet not outwardly

demonstrate a significant number of negative consequences, as perceived by their relatives. The relatively liberal drinking norms for our geographical region (see Neal and Fromme, 2007) may have also influenced participants' perceptions of whether or not their relatives had a problem with alcohol use. Another limitation is that we used average parental education level as an indicator of socioeconomic status. A more precise measure would have incorporated both parental education level and parental income bracket. However, because our students are more likely to know their parents' education level versus their household income for the previous year, we chose to use parental education level as an indicator of socioeconomic status. Finally, we recognize that pre-existing chronic stress level might have influenced our participants' reactions to the acute laboratory stressor. However, it is important to note that participants in the Aim 2 portion of the study did not differ on their perceived stress levels over the previous month. This is a self-report of subjective stress. A more precise measure of chronic stress would include objective measures (such as diurnal cortisol levels) over the previous month. Along these same lines, inclusion of cortisol samples throughout the Aim 2 testing session would have given a more objective measure of participants' stress levels in response to the PASAT. These types of measures would have helped to disentangle chronic and acute stress influences on BDNF levels in Aim 2. Unfortunately, these measures were beyond the scope of the current study but may be a direction for future research.

From an experimental perspective, our claim that serum BDNF changes were due to the stress manipulation would be enhanced by the inclusion of a control task (e.g., a non-stressful video game), as well as direct physiological measures of the stress response

(e.g., heart rate, blood pressure, cortisol levels). This would have useful for detecting the presence of any non-experiment-related changes in BDNF during the testing session and would allow us to definitively state that the stress-related BDNF changes were caused by the stress manipulation alone. However, there was a significant stress-related change in self-reported anxiety levels over the course of the testing session, suggesting that the stressor was sufficiently aversive. As mentioned previously, it would be interesting to follow these research participants longitudinally to track their alcohol use and to determine whether or not their earlier age for the initiation of alcohol use might ultimately be associated with problem drinking later on in the $Val^{66}Met$ group (versus $Val^{66}Val$).

APPENDIX SECTION

Screening Questions for Study Enrollment

Participant Number: _____
Date: _____
The following questions ask about eating, drinking and sleeping patterns, medications you take, and related topics. These questions will help us assess whether any of these factors might influence the saliva tests that will be used in this study. Please think about your current situation, as well as what you expect to be doing for the next 6 months or so, as we many contact you for a follow-up study.

Please respond by writing YES or NO for each question unless otherwise indicated.

1. Do you have a regular sleep cycle most days of the week; that is, do you sleep at night and stay awake during the day? Will this schedule likely be the same for the next 6 months or so?

2. Are you taking any prescription medications? This includes birth control pills/patch/etc. If so, please list the name of the medication, the dose and how often you take it.

3. Are you taking any over-the-counter medications or supplements that you would not be willing to stop taking during the second part of the study when saliva tests will be conducted?

4. Do you have any chronic medical or psychiatric conditions or diagnoses that do not require medication? This could include previous treatment for alcohol abuse or drug dependence.

5. WOMEN ONLY: Are you currently pregnant and/or do you plan to become pregnant within the study period (including the next 6 months or so)?

AIM 1 SURVEY QUESTIONS

1. First, type your Participant Number in the blank below. This information will be provided by the researcher. You cannot move forward with the survey until you type in your Participant Number. Please DO NOT type your NAME anywhere on this survey.
2. What is your current age?
3. What is your gender?
4. Which ethnic group do you identify yourself as? You may choose more than one. (Please note that these are the ethnic categories used by the U.S. National Institutes of Health)

    - Hispanic or Latino or Spanish origin: a person of Cuban, Mexican, Puerto Rican, South or Central American, or other Spanish Culture or origin, regardless of race.
    - Not Hispanic or Latino or Spanish Origin

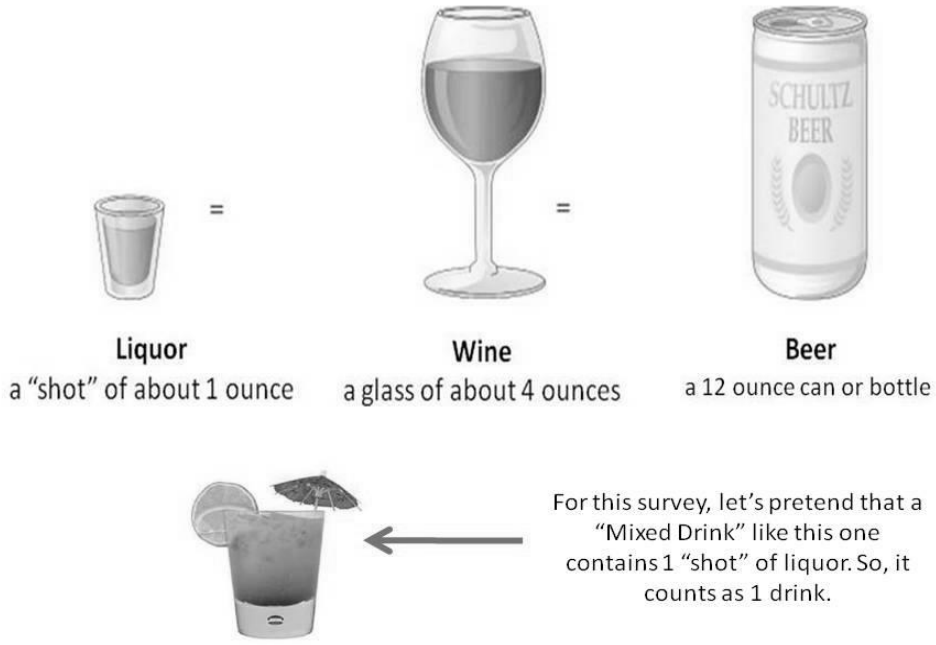
5. Which racial group do you identify yourself as? You may choose more than one. (Please note that these are the racial categories used by the U.S. National Institutes of Health)

    - American Indian or Alaska Native: A person having origins in any of the original peoples of North, Central, or South America, and who maintains tribal affiliations or community attachment.
    - Asian: A person having origins in any of the original peoples of the Far East, Southeast Asia, or the Indian subcontinent including, for example, Cambodia, China, India, Japan, Korea, Malaysia, Pakistan, the Philippine Islands, Thailand, and Vietnam.
    - Black or African American or Haitian: A person having origins in any of the black racial groups of Africa.
    - Native Hawaiian or Other Pacific Islander: A person having origins in any of the original peoples of Hawaii, Guam, Samoa, or other Pacific Islands.

- White: A person having origins in any of the original peoples of Europe, the Middle East, or North Africa. Other (please specify)

6. How many years of school have you COMPLETED? (For instance, if you are in your sophomore year of college, then you would say that you have completed 13 years of education thus far)
7. Do you have a job at which you work for wages or salary?
8. If you have a job at which you work for wages or salary, what kind of work do you normally do?
9. What is the highest level of education completed by your father?
10. What is your father's usual job?
11. What is your mother's highest level of education?
12. What is your mother's usual job?

Please take a moment to familiarize yourself with the size of a "standard drink" of alcohol.



13. When you do consume alcohol, how many drinks do you typically have during one drinking episode (like an evening out or a day at the river, for example)? Please be as descriptive as possible. For example, a good answer would include quantity and type such as "Three Mixed Drinks" or "Three bottles of Coors Light beer".

14. What is the highest number of drinks that you've ever consumed during one drinking episode? Please be as descriptive as possible. For example, a good answer would include quantity and type such as "Six Mixed Drinks" or "Six bottles of Coors Light beer" or "One Half-Gallon of Bacardi Rum".

15. When you consumed your highest number of drinks ever, over how many hours did you drink them?

16. SINCE YOU FIRST STARTED DRINKING ALCOHOL, have you ever engaged in "binge drinking", that is, have you ever consumed 4 or more drinks (for women) or 5 or more drinks (for men) over the course of a drinking episode?

17. TYPICALLY, when/if you "binge drink", how many drinks do you usually consume during one drinking episode? Please be as descriptive as possible. For example, a good answer would include quantity and type such as "Four Mixed Drinks" or "Four bottles of Coors Light beer".

18. When/if you "binge drink", over how many hours do you usually drink?

19. When/if you "binge drink", what types of alcohol do you consume most often? (check all that apply).

- ☐ Wine
- ☐ Beer
- ☐ Liquor (such as shots or mixed drinks)
- ☐ Other, please specify.

20. In the <u>past 6 months</u>, have you had any periods of "binge drinking"?

If so, how many times, or how often?

21. In the past 3 months, have you had any periods of "binge drinking"? If so, how many times or how often?

22. In the past month, have you had any periods of "binge drinking"? If so, how many times or how often.

23. In the past two weeks, have you had any periods of "binge drinking"? If so, how many times or how often.

24. Would you say that "binge drinking" is your typical drinking pattern?

These next questions concern your family history of alcohol and drug use. Please answer to the best of your ability. Note that a "problem with alcohol" simply means that YOU consider this person to have a problem.

The first set of questions will refer to your "primary" relatives, that is, your mother, father, sisters and brothers.

25. Think about the alcohol and drug use patterns of your mother. (check one)

- ☐ I have no knowledge of this relative's alcohol and drug use history.
- ☐ This person NEVER used alcohol or drugs in their life.
- ☐ This person SOCIALLY used alcohol or drugs – like at parties or events with others.
- ☐ This person had a PROBLEM with ALCOHOL only.
- ☐ This person had a PROBLEM with DRUGS only.
- ☐ This person had a PROBLEM with both ALCOHOL AND DRUGS.

26. When you answered these questions about your mother, you were referring to… (check one)

- ☐ Your natural mother (birth mother).
- ☐ Your adopted mother or your step-mother.
- ☐ Other (please specify)

27. Now, think about the alcohol and drug use patterns of your father. (check one)

- ☐ I have no knowledge of this relative's alcohol and drug use history.
- ☐ This person NEVER used alcohol or drugs in their life.
- ☐ This person SOCIALLY used alcohol or drugs – like at parties or events with others.
- ☐ This person had a PROBLEM with ALCOHOL only.
- ☐ This person had a PROBLEM with DRUGS only.
- ☐ This person had a PROBLEM with both ALCOHOL AND DRUGS.

28. When you answered these questions about your father, you were referring to… (check one)

- Your natural father (birth father).
- Your adopted father or your step-father.
- Other (please specify)

29. Now, think about yourself. (check one)

- I have NEVER used alcohol or drugs in my life.
- I have SOCIALLY used alcohol or drugs – like at parties or events with others.
- I have had a PROBLEM with ALCOHOL only.
- I have had a PROBLEM with DRUGS only.
- I have had a PROBLEM with both ALCOHOL AND DRUGS.
- Other (please specify)

30. Now, think of your own brothers. How many of your own brothers have had a problem with BOTH ALCOHOL AND DRUGS? If none of them have had a problem, write "0". If you don't have any brothers, write "N/A", and if you don't know, write "I don't know."

31. How many of your own brothers have had a problem with ALCOHOL ONLY? That is, a problem with alcohol, but no problem with drugs?

32. How many of your own brothers have had a problem with DRUGS ONLY? That is, a problem with drugs, but no problem with alcohol.

33. Now, think of your own sisters. How many of your own sisters have had a problem with BOTH ALCOHOL AND DRUGS? If none of them have had a problem, write "0". If you don't have any sisters, write "N/A", and if you don't know, write "I don't know."

34. How many of your own sisters have had a problem with ALCOHOL ONLY? That is, a problem with alcohol, but no problem with drugs.

35. How many of your own sisters have had a problem with DRUGS ONLY? That is, a problem with drugs, but no problem with alcohol.

The next set of questions will cover your "secondary" relatives; that is, your grandparents and your aunts and uncles on both sides of your family tree. Maternal relatives are from your mother's side of the family, and paternal relatives are from your father's side of the family.

36. Please choose the statement that best describes your MATERNAL GRANDMOTHER, that is, your mother's mother. (check one)

- ☐ I have no knowledge of this relative's alcohol and drug use history.
- ☐ This person NEVER used alcohol or drugs in their life.
- ☐ This person SOCIALLY used alcohol or drugs – like at parties or events with others.
- ☐ This person had a PROBLEM with ALCOHOL only.
- ☐ This person had a PROBLEM with DRUGS only.
- ☐ This person had a PROBLEM with both ALCOHOL AND DRUGS.

37. Please choose the statement that best describes your MATERNAL GRANDFATHER, that is, your mother's father. (check one)

- ☐ I have no knowledge of this relative's alcohol and drug use history.
- ☐ This person NEVER used alcohol or drugs in their life.
- ☐ This person SOCIALLY used alcohol or drugs – like at parties or events with others.
- ☐ This person had a PROBLEM with ALCOHOL only.
- ☐ This person had a PROBLEM with DRUGS only.
- ☐ This person had a PROBLEM with both ALCOHOL AND DRUGS.

38. Please choose the statement that best describes your PATERNAL GRANDMOTHER, that is, your father's mother. (check one)

- ☐ I have no knowledge of this relative's alcohol and drug use history.
- ☐ This person NEVER used alcohol or drugs in their life.
- ☐ This person SOCIALLY used alcohol or drugs – like at parties or events with others.
- ☐ This person had a PROBLEM with ALCOHOL only.
- ☐ This person had a PROBLEM with DRUGS only.
- ☐ This person had a PROBLEM with both ALCOHOL AND DRUGS.

39. Please choose the statement that best describes your PATERNAL GRANDFATHER, that is, your father's father. (check one)

    - ☐ I have no knowledge of this relative's alcohol and drug use history.
    - ☐ This person NEVER used alcohol or drugs in their life.
    - ☐ This person SOCIALLY used alcohol or drugs – like at parties or events with others.
    - ☐ This person had a PROBLEM with ALCOHOL only.
    - ☐ This person had a PROBLEM with DRUGS only.
    - ☐ This person had a PROBLEM with both ALCOHOL AND DRUGS

40. Think of your MATERNAL UNCLES, that is, your mother's brothers. How many of your maternal uncles had a problem with BOTH ALCOHOL AND DRUGS? If none of them have had a problem, write "0". If you don't have any maternal uncles, write "N/A", and if you don't know, write "I don't know."

41. How many of your MATERNAL UNCLES have had a problem with ALCOHOL ONLY? That is, a problem with alcohol, but no problem with drugs?

42. How many of your MATERNAL UNCLES have had a problem with DRUGS ONLY? That is, a problem with drugs, but no problem with alcohol.

43. Think of your MATERNAL AUNTS, that is, your mother's sisters. How many of your maternal aunts had a problem with BOTH ALCOHOL AND DRUGS? If none of them have had a problem, write "0". If you don't have any maternal uncles, write "N/A", and if you don't know, write "I don't know."

44. How many of your MATERNAL AUNTS have had a problem with ALCOHOL ONLY? That is, a problem with alcohol, but no problem with drugs?

45. How many of your MATERNAL AUNTS have had a problem with DRUGS ONLY? That is, a problem with drugs, but no problem with alcohol.

46. Think of your PATERNAL UNCLES, that is, your father's brothers. How many of your paternal uncles had a problem with BOTH ALCOHOL AND DRUGS? If none of them have had a problem, write "0". If you don't have any maternal uncles, write "N/A", and if you don't know, write "I don't know."

47. How many of your PATERNAL UNCLES have had a problem with ALCOHOL ONLY? That is, a problem with alcohol, but no problem with drugs?

48. How many of your PATERNAL UNCLES have had a problem with DRUGS ONLY? That is, a problem with drugs, but no problem with alcohol.

49. Think of your PATERNAL AUNTS, that is, your father's sisters. How many of your paternal aunts had a problem with BOTH ALCOHOL AND DRUGS? If none of them have had a problem, write "0". If you don't have any maternal uncles, write "N/A", and if you don't know, write "I don't know."

50. How many of your PATERNAL AUNTS have had a problem with ALCOHOL ONLY? That is, a problem with alcohol, but no problem with drugs?

51. How many of your PATERNAL AUNTS have had a problem with DRUGS ONLY? That is, a problem with drugs, but no problem with alcohol.

The next set of questions are VERY IMPORTANT. Here, you will be asked to describe your own patterns of alcohol use.

52. How old were you when you took your first drink of alcohol, even if it was given to you as a child?

53. How old were you the first time you "got drunk"? For example – the first time you drank to excess and felt dizzy. If you have never been "drunk", type "I have never been drunk."

54. How old were you the first time you started drinking on a regular basis? For example – established a regular pattern of drinking every weekend. If you don't drink on a regular basis and never have, simply type "NEVER" in the blank.

55. How long ago did you have your last alcoholic beverage? If possible, type how many days since your last drink. For example – "1 day ago". If it's a shorter period of time, describe this in words. For example – "I am drinking right now."

56. In the previous 6 months, how often did you have any kind of beverage containing alcohol, whether it was wine, beer, whisky, or any other alcoholic drink?

- ☐ Three or more times per day
- ☐ Twice per day
- ☐ Every day or nearly every day
- ☐ Three or four days per week
- ☐ One or two days per week
- ☐ Two or three times per month
- ☐ About once a month
- ☐ At least one time in the last 6 months
- ☐ No alcohol at all in the last 6 months

57. In the previous 6 months, how often did you typically drink WINE?
    - Every day
    - 5 to 6 days per week
    - 3 to 4 days per week
    - 1 to 2 days per week
    - 3 times a month or less
    - I did not drink WINE at all

58. On a day when you drank WINE, how much did you typically drink? For example, if you consumed one 4 ounce glass, you would type "One glass".

59. When you drink wine, what type do you usually drink?

    - Table wine (examples include red or white wine, champagne or sparkling wine)
    - Fortified wine (examples include high end types like Madeira, Port, Sherry, Marsala and Vermouth, AND/OR low-end types like MadDog 20/20, Thunderbird, Wild Irish Rose and Night Train)

60. In the previous 6 months, how often did you typically drink BEER?

    - Every day
    - 5 to 6 days per week
    - 3 to 4 days per week
    - 1 to 2 days per week
    - 3 times a month or less
    - I did not drink BEER at all

61. On a day when you drank BEER, how much did you typically drink? For example, if you consumed one six-pack of bottles, you would type "one six-pack of beer".

62. When you drink beer, what type do you usually drink?

    - Light beer (example: Bud Light)
    - Regular Beer (example: Budweiser)
    - Domestic Beer (examples include beers like Coors, Budweiser, Michelob, Miller, Milwaukee's Best, etc.)
    - Imported Beer (examples include beers like Guinness, Fosters, Heineken, Corona, Dos Equis, Modelo, Tecate, Red Stripe)
    - Beer purchased at a liquor store
    - Beer purchased at a convenience store
    - Other (please specify)

63. In the previous 6 months, how often did you typically drink LIQUOR? Remember, LIQUOR can mean a “shot” or a mixed drink. Some liquor examples include tequila, vodka, whisky, rum, gin, brandy, etc.

- Every day
- 5 to 6 days per week
- 3 to 4 days per week
- 1 to 2 days per week
- 3 times a month or less
- I did not drink LIQUOR at all

64. On a day when you drank LIQUOR, how much did you typically drink? For example, if you usually have a couple of mixed drinks that contain liquor, you should type “two mixed drinks”.

65. When you drink LIQUOR, what type do you usually drink?

- Tequila (example: Jose Cuervo, margaritas)
- Vodka (example: Stolichnaya “Stoli” or SKYY)
- Whisky (includes Jack Daniels, Scotch, Bourbon, etc.)
- Rum (example: Bacardi)
- Gin (example: Bombay Sapphire or Tanqueray)
- Brandy (example: Cognac)
- Other (please specify)

The next set of questions will ask about your stress and anxiety levels.

66. Read each statement below and indicate HOW YOU GENERALLY FEEL using this scale: not at all, somewhat, moderately so, very much so.

I feel calm.
I feel secure.
I am tense.
I feel strained.
I feel at ease.
I feel upset.
I am presently worrying over possible misfortunes.
I feel satisfied.
I feel frightened.
I feel comfortable.
I feel self-confident.
I feel nervous.
I am jittery.
I feel indecisive.

I am relaxed.
I feel content.
I am worried.
I feel confused.
I feel steady.
I feel pleasant.

67. Now, read each statement below and indicate how you are feeling RIGHT NOW, AT THIS MOMENT using this scale: not at all, somewhat, moderately so, very much so.

I feel calm.
I feel secure.
I am tense.
I feel strained.
I feel at ease.
I feel upset.
I am presently worrying over possible misfortunes.
I feel satisfied.
I feel frightened.
I feel comfortable.
I feel self-confident.
I feel nervous.
I am jittery.
I feel indecisive.
I am relaxed.
I feel content.
I am worried.
I feel confused.
I feel steady.
I feel pleasant.

68. The next section asks about your feelings DURING THE LAST MONTH. In each case, you will be asked to indicate HOW OFTEN you felt or thought in a certain way, using this scale: never, almost never, sometimes, fairly often, very often.

In the last month, how often have you been upset because of something that happened unexpectedly?

In the last month, how often have you felt that you were unable to control the important things in your life?

In the last month, how often have you felt nervous and "stressed"?

In the last month, how often have you dealt successfully with irritating life hassles?

In the last month, how often have you felt that you were effectively coping with important changes that were occurring in your life?

In the last month, how often have you felt confident about your ability to handle your personal problems?

In the last month, how often have you felt that things were going your way?

In the last month, how often have you found that you could not cope with all the things that you had to do?

In the last month, how often have you been able to control irritations in your life?

In the last month, how often have you felt that you were on top of things?

In the last month, how often have you been angered because of things that happened that were outside of your control?

In the last month, how often have you found yourself thinking about things that you have to accomplish?

In the last month, how often have you been able to control the way you spend your time?

In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?

The next set of questions will ask about your general health.

The following question related to your usual sleep habits during the PAST MONTH only. Your answers should indicate the most accurate reply for the majority of days and nights in the past month. Please answer all of the questions.

69. During the past month, what time have you usually gone to bed at night?

70. During the past month, how long (in minutes) has it usually taken you to fall asleep each night?

71. During the past month, what time have you usually gotten up in the morning?

72. During the past month, how many hours of actual sleep did you get at night? (This may be different than the number of hours you spent in bed.)

73. During the past month, how would you rate your sleep quality overall?

- ☐ Very good
- ☐ Fairly good
- ☐ Fairly bad
- ☐ Very bad

Want to learn more about alcohol use and/or misuse?

There are many sources of help available ranging from local counselors to national hotlines. For more information about alcohol use and misuse, please see the following resources (you may wish to print this page for your records).

Texas State University Alcohol Resource Center:
601 University Drive, San Marcos, Texas
78666 -- 5-4.1 LBJ Student Center Phone:
(512) 245-3601; Fax: (512) 245-7601; E-
mail: adrcoffice@txstate.edu

U.S. Department of Health and Human Services:
Substance Abuse and Mental Health Services Administration's National Clearinghouse for Alcohol and Drug Information. (Contains links to a variety of addiction information and treatment resources) http://ncadi.samhsa.gov/
Phone: (800) 729-6686

You are finished!

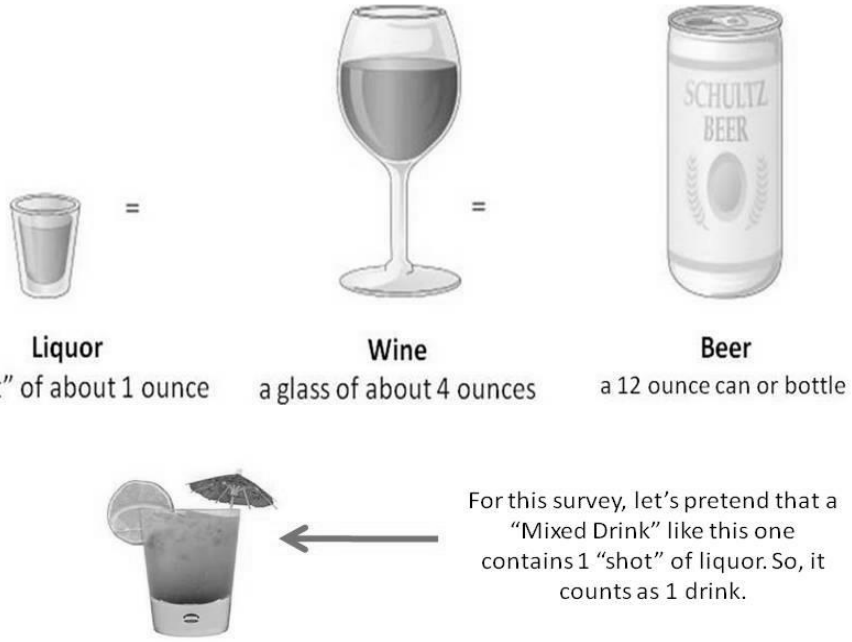
You are now finished with the survey. We may contact you again to invite you to participate in another part of the study. If any of your contact information changes, please let Dr. Ceballos know by sending her an e-mail (nc18@txstate.edu)

AIM 2 SURVEY QUESTIONS

1. First, type your Participant Number in the blank below. This information will be provided by the researcher. You cannot move forward with the survey until you type in your Participant Number. Please DO NOT type your NAME anywhere on this survey.

2. How long ago did you have your last alcohol beverage? If possible, type how many days since your last drink. For example -- "1 day ago". If it's a shorter period of time, describe this in words. For example -- "I am drinking right now".

Take a moment to familiarize yourself with the size of a standard drink of alcohol.



3. In the previous 6 months, how often did you have any kind of beverage containing alcohol, whether it was wine, beer, whisky, or any other alcoholic drink?

- ☐ Three or more times per day
- ☐ Twice per day
- ☐ Every day or nearly every day
- ☐ Three or four days per week
- ☐ One or two days per week

- Two or three times per month
- About once a month
- At least one time in the last 6 months
- No alcohol at all in the last 6 months

4. In the previous 6 months, how often did you typically drink WINE?

- Every day
- 5 to 6 days per week
- 3 to 4 days per week
- 1 to 2 days per week
- 3 times a month or less
- I did not drink WINE at all

5. On a day when you drank WINE, how much did you typically drink? For example, if you consumed one 4 ounce glass, you would type "One glass".

6. When you drink wine, what type do you usually drink?

- Table wine (examples include red or white wine, champagne or sparkling wine)
- Fortified wine (examples include high end types like Madeira, Port, Sherry, Marsala and Vermouth, AND/OR low-end types like MadDog 20/20, Thunderbird, Wild Irish Rose and Night Train)

7. In the previous 6 months, how often did you typically drink BEER?

- Every day
- 5 to 6 days per week
- 3 to 4 days per week
- 1 to 2 days per week
- 3 times a month or less
- I did not drink BEER at all

8. On a day when you drank BEER, how much did you typically drink? For example, if you consumed one six-pack of bottles, you would type "one six-pack of beer".

9. When you drink beer, what type do you usually drink?

- Light beer (example: Bud Light)
- Regular Beer (example: Budweiser)
- Domestic Beer (examples include beers like Coors, Budweiser, Michelob, Miller, Milwaukee's Best, etc.)
- Imported Beer (examples include beers like Guinness, Fosters, Heineken, Corona, Dos Equis, Modelo, Tecate, Red Stripe)
- Beer purchased at a liquor store
- Beer purchased at a convenience store
- Other (please specify)

10. In the previous 6 months, how often did you typically drink LIQUOR? Remember, LIQUOR can mean a "shot" or a mixed drink. Some liquor examples include tequila, vodka, whisky, rum, gin, brandy, etc.

- Every day
- 5 to 6 days per week
- 3 to 4 days per week
- 1 to 2 days per week
- 3 times a month or less
- I did not drink LIQUOR at all

11. On a day when you drank LIQUOR, how much did you typically drink? For example, if you usually have a couple of mixed drinks that contain liquor, you should type "two mixed drinks".

12. When you drink LIQUOR, what type do you usually drink?

- Tequila (example: Jose Cuervo, margaritas)
- Vodka (example: Stolichnaya "Stoli" or SKYY)
- Whisky (includes Jack Daniels, Scotch, Bourbon, etc.)
- Rum (example: Bacardi)
- Gin (example: Bombay Sapphire or Tanqueray)
- Brandy (example: Cognac)
- Other (please specify)

13. Since your last participation in this study, have you engaged in "binge drinking", that is, have you consumed 4 drinks (for women) or 5 drinks (for men) over the course of a drinking episode?
14. How many binge drinking episodes have you had since your last participation in our study?
15. How many drinks did you usually consume during one drinking episode?
16. And, over how many hours did you usually drink these?

The following set of questions will ask about your current and typical levels of stress, anxiety and depression.

The next set of questions will ask about your stress and anxiety levels.

17. Read each statement below and indicate HOW YOU GENERALLY FEEL using this scale: not at all, somewhat, moderately so, very much so.

    I feel calm.
    I feel secure.
    I am tense.
    I feel strained.
    I feel at ease.
    I feel upset.
    I am presently worrying over possible misfortunes.
    I feel satisfied.
    I feel frightened.
    I feel comfortable.
    I feel self-confident.
    I feel nervous.
    I am jittery.
    I feel indecisive.
    I am relaxed.
    I feel content.
    I am worried.
    I feel confused.
    I feel steady.
    I feel pleasant.

18. Now, read each statement below and indicate how you are feeling RIGHT NOW, AT THIS MOMENT using this scale: not at all, somewhat, moderately so, very much so.

    I feel calm.
    I feel secure.
    I am tense.
    I feel strained.
    I feel at ease.
    I feel upset.
    I am presently worrying over possible misfortunes.
    I feel satisfied.
    I feel frightened.
    I feel comfortable.
    I feel self-confident.
    I feel nervous.
    I am jittery.
    I feel indecisive.
    I am relaxed.
    I feel content.
    I am worried.
    I feel confused.
    I feel steady.
    I feel pleasant.

19. The next section asks about your feelings DURING THE LAST MONTH. In each case, you will be asked to indicate HOW OFTEN you felt or thought in a certain way, using this scale: never, almost never, sometimes, fairly often, very often.

    In the last month, how often have you been upset because of something that happened unexpectedly?

    In the last month, how often have you felt that you were unable to control the important things in your life?

    In the last month, how often have you felt nervous and "stressed"?

    In the last month, how often have you dealt successfully with irritating life hassles?

    In the last month, how often have you felt that you were effectively coping with important changes that were occurring in your life?

In the last month, how often have you felt confident about your ability to handle your personal problems?

In the last month, how often have you felt that things were going your way?

In the last month, how often have you found that you could not cope with all the things that you had to do?

In the last month, how often have you been able to control irritations in your life?

In the last month, how often have you felt that you were on top of things?

In the last month, how often have you been angered because of things that happened that were outside of your control?

In the last month, how often have you found yourself thinking about things that you have to accomplish?

In the last month, how often have you been able to control the way you spend your time?

In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?

The next set of questions will ask about your general health.

The following question related to your usual sleep habits during the PAST MONTH only. Your answers should indicate the most accurate reply for the majority of days and nights in the past month. Please answer all of the questions.

20. During the past month, what time have you usually gone to bed at night?

21. During the past month, how long (in minutes) has it usually taken you to fall asleep each night?

22. During the past month, what time have you usually gotten up in the morning?

23. During the past month, how many hours of actual sleep did you get at night? (This may be different than the number of hours you spent in bed.)

24. During the past month, how would you rate your sleep quality overall?
    - Very good
    - Fairly good
    - Fairly bad
    - Very bad

The next few questions are for women only.

25. Please write today's date here _____. Now, please write the date of your last menstrual period here _____.

26. Are you currently taking any hormonal forms of prescription birth control, such as the pill, patch, shot or Nuvaring?

    If so, please list the name of the prescription birth control and how long you've been taking it.

These questions are for everyone (males and females).

27. Have you had any recent physical illnesses?
    If so, are you currently taking any medications? List them and indicate how long you've been taking them.
28. Have you started taking any new prescription medications since your last participation in our study? If so, please list them and tell us how long you've been taking them.

Want to learn more about alcohol use and/or misuse?

There are many sources of help available ranging from local counselors to national hotlines. For more information about alcohol use and misuse, please see the following resources (you may wish to print this page for your records).

Texas State University Alcohol Resource Center:
601 University Drive, San Marcos, Texas
78666 -- 5-4.1 LBJ Student Center Phone:
(512) 245-3601; Fax: (512) 245-7601; E-
mail: adrcoffice@txstate.edu

U.S. Department of Health and Human Services:
Substance Abuse and Mental Health Services Administration's National Clearinghouse for Alcohol and Drug Information. (Contains links to a variety of addiction information and treatment resources) http://ncadi.samhsa.gov/ Phone:

(800) 729-6686

You are now finished with the survey!

List of Definitions

Moderate alcohol consumption: "up to 1 drink per day for women and up to 2 drinks per day for men" (National Institute on Alcohol Abuse and Alcoholism, 2016).

Binge Drinking: "a pattern of drinking that brings blood alcohol concentration levels to 0.08 g/dL. This typically occurs after 4 drinks for women and 5 drinks for men—in about 2 hours. The Substance Abuse and Mental Health Services Administration defines binge drinking as drinking 5 or more alcohol drinks on the same occasion on at least 1 day in the past 30 days" (National Institute on Alcohol Abuse and Alcoholism, 2016).

Heavy Drinking: "The Substance Abuse and Mental Health Services Administration defines heavy drinking as drinking 5 or more drinks on the same occasion on each of 5 or more days in the past 30 days" (National Institute on Alcohol Abuse and Alcoholism, 2016).

Alcohol Use Disorder: "Under DSM–5, anyone meeting any two of the 11 criteria during the same 12-month period receives a diagnosis of AUD. The severity of an AUD—mild, moderate, or severe—is based on the number of criteria met" (National Institute on Alcohol Abuse and Alcoholism, 2016).

Criteria include 1) drinking more or longer than intended, 2) wanting to cut down or stop drinking or trying to and failing, 3) spending a lot of time drinking, being sick, or getting over the aftereffects, 4) experiencing craving (a strong need, or urge, to drink), 5) finding that drinking (or being sick from drinking) often interferes with taking care of home or family, or causes job troubles or school problems, 6) continuing to drink even though it causes trouble with family or friends, 7) giving up or cutting back on important

pleasurable activities in order to drink, 8) getting into situations while or after drinking that increase the chance of injury, 9) continuing to drink even though alcohol becomes associated with depression, anxiety or other health problems, or after a memory blackout, 10) having to drink much more than before in order to achieve the same effect or finding that the usual number of drinks has less of an effect, 11) experiencing withdrawal symptoms when the effects of alcohol wear off (examples include trouble sleeping, shakiness, irritability, anxiety, depression, restlessness, nausea, or sweating, or sensing things that are not there (National Institute on Alcohol Abuse and Alcoholism, 2016).

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