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Utilizing Human Induced Pluripotent Stem Cells in the Study of Alcohol Use Disorder

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Utilizing Human Induced Pluripotent Stem Cells in the Study of Alcohol Use Disorder

Richard Lieberman, Ph.D.

University of Connecticut

2016

Abstract: Alcohol use disorder is a complex, heterogeneous disorder that affects \( \approx 14\% \) of the U.S. population. Much of what is known about the molecular effects of alcohol on the brain has been derived from studies utilizing rodent or human post-mortem tissue model systems. The advent of induced pluripotent stem cell (iPSC) technologies allows for the examination of phenotypically- and genetically-characterized human neural cells \textit{in vitro}. Much of the work presented in this dissertation explores the utility of iPSCs in the study of alcohol use disorder. We start by exploring whether human iPSC-derived neural cells can recapitulate the findings in animal models examining the molecular effects of acute and chronic alcohol exposure on ligand gated ion channel function and gene expression (chapter 2). Next, we utilize iPSCs in the study of a common single nucleotide polymorphism in the \textit{GABRA2} gene that has been linked to the development of AUD (chapter 3). Finally, we use in a large human sample to identify a novel gene \times environment interaction for a polymorphism in the \textit{FKBP5} gene that moderates heavy alcohol consumption in the setting of early life trauma, and explore the molecular implications of the polymorphism in human iPSC-derived neural cultures (chapters 4 and 5). This work suggests that iPSC neural differentiation is a promising tool in the study of complex psychiatric disorders such as drug addiction.
Utilizing Human Induced Pluripotent Stem Cells in the Study of Alcohol Use Disorder

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B.A., Assumption College, 2008
M.S., University of Hartford, 2010

A Dissertation
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy
at the
University of Connecticut
2016
Approval Page

Doctor of Philosophy Dissertation

Utilizing Human Induced Pluripotent Stem Cells in the Study of Alcohol Use Disorder

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University of Connecticut

2016
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Introduction

Alcohol Use Disorder

It is estimated that over 60% of the U.S. population consumes alcohol, most of which drink at moderate, safe amounts, defined by the National Institute of Alcohol Abuse and Alcoholism (NIAAA) as 7 drinks per week for women with no more than three consumed in one day, and 14 drinks per week for men with no more than four consumed in one day. Moderate alcohol consumption has been shown to have health benefits including reducing risk of stroke, heart disease (O'Keefe et al., 2007), and dementia (Deng et al., 2006). But the same moderate alcohol consumption also has adverse affects on health including liver cirrhosis and increased risks of cancers, particularly of the mouth and throat (Corrao et al., 2004), highlighting the drug's complexity and heterogeneity. Furthermore, the benefits of alcohol consumption create a sharp J-shaped curve, with heavy daily alcohol consumption associated with greater overall mortality and increased risks of stroke (O'Keefe et al., 2007) and dementia (Deng et al., 2006). Heavy drinking is defined by the NIAAA as consumption of five or more drinks in a single occasion five or more times within the past 30 days, and in addition to the detrimental physical effects, continued heavy drinking puts an individual at greater risk of developing alcohol use disorder.

Alcohol use disorders (AUDs) are among the most prevalent psychiatric disorders worldwide (Grant et al., 2004b, Wittchen et al., 2011), leading to a reduced quality of life in affected individuals (Donovan et al., 2005) and significantly contributing to global disability (Whiteford et al., 2013). AUDs are diagnosed by 11 criteria set forth in the fifth version of
the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (Table 1). To be diagnosed with AUD an individual must meet at least two of the 11 criteria within a 12-month period. New to this edition of the DSM-V, the severity of diagnosis differs based on the number of criteria met; 2-3 for mild, 4-5 for moderate, and greater than 6 for a diagnosis of severe AUD (Hasin et al., 2013). A recent epidemiological study of more than 36,000 participants between 2012-2013 found the 12-month prevalence of AUD in the United States was 13.9%, with 29.1% of participants meeting DSM-V diagnostic criteria for AUD within their lifetime. 12-month and lifetime AUD was higher in men (17.6% and 36%) than women (10.4% and 22.7%), and was highest in individuals between the ages of 18-29 (26.7% and 37%) (Grant et al., 2015). The staggering prevalence of excessive alcohol use has a high economic cost to society, which in 2006 was greater than $223 billion, and included costs associated with lost productivity, healthcare, and legal fees (Bouchery et al., 2011).

**Risk Factors for Alcohol Use Disorder**

Despite the large personal and economic costs associated with AUD, less than 20% of affected individuals seek treatment (Grant et al., 2015). While there are currently three FDA approved medications for the treatment of AUD, the small fraction of affected individuals that seek treatment is in part due to the poor efficacy and adverse effects of currently approved medications. This has led researchers to identify risk factors that influence development of AUD, which, in addition to contributing to the understanding of
### Table 1.1. DSM-5 Criteria for Diagnosis of Substance Use Disorder

Diagnosis of Alcohol Use Disorder Requires Meeting Two or More Criteria Within a 12-Month Period:

<table>
<thead>
<tr>
<th>Criteria</th>
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<tbody>
<tr>
<td>1. Hazardous Use</td>
</tr>
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<td>2. Social/Interpersonal Problems Related to Use</td>
</tr>
<tr>
<td>3. Neglected Major Roles to Use</td>
</tr>
<tr>
<td>4. Use Larger Amounts for Longer Periods of Time</td>
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<tr>
<td>5. Repeated Attempts to Quit or Control Use</td>
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<tr>
<td>6. Much Time Spent Using</td>
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<td>7. Physical or Psychological Problems Related to Use</td>
</tr>
<tr>
<td>8. Activities Given Up to Use</td>
</tr>
<tr>
<td>9. Withdrawal Symptoms</td>
</tr>
<tr>
<td>10. Tolerance to Acute Effects</td>
</tr>
<tr>
<td>11. Craving</td>
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</tbody>
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<table>
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<tr>
<th>Number of Criteria</th>
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<tr>
<td>2-3</td>
<td>Mild</td>
</tr>
<tr>
<td>4-5</td>
<td>Moderate</td>
</tr>
<tr>
<td>&gt;6</td>
<td>Severe</td>
</tr>
</tbody>
</table>
the disease, may help to identify at-risk individuals to curb or prevent future development of AUD.

The risks associated with AUD come from both environmental and genetic influences, and as discussed later, there is a complex interaction between the two. Environmental factors such as exposure to alcohol (as one cannot become dependent on alcohol without exposure (Gelernter and Kranzler, 2009)) and stressful or traumatic events are predictors for increased drinking and development of AUD. Indeed, acute and chronic stress experienced in adulthood leads to increased alcohol consumption and vulnerability to AUD (Sinha, 2008), but additionally stress or trauma experienced early in development can have long-lasting effects on hypothalamic-pituitary-adrenal (HPA) axis functioning, leading to increased risk of AUD in adulthood (Sinha, 2008, Enoch, 2011). However, the mechanisms by which early life exposure to stress or trauma increases the risk of AUD in adulthood are complex, as not every maltreated child develops psychopathology. As discussed later, this suggests that genetics interact with environmental influences to shape risk.

In addition to environmental factors, there is a significant genetic component to AUD that was originally indicating by the groundbreaking findings of family, twin, and adoption studies, and then further elucidated by the use of modern-day genetic approaches. Astute observers over the course of early history noticed that excessive alcohol use “runs in families” (Devor and Cloninger, 1989). Observations in the clinical field support this notion, including those made by Crothers (1909) over the course of a 35-year career, observing that of the over 4400 “inebriates” he treated, over 70% had a family member whom also suffered from the disorder (Crothers, 1909). Meta analysis on the
following decades worth of research examining family histories in affected individuals provided further support for the familial transmission of AUD, proposing that the offspring of individuals with AUD were approximately three to five times more likely to develop AUD during their lifetime than offspring of non-affected individuals (Cotton, 1979).

Indeed, one of the most reliable predictors of AUD is a positive family history, but due to shared environments between families it is difficult to specifically quantify the genetic contribution of AUD using these studies (McGue, 1999). Therefore, some of the best studies to tease apart the genetic component of AUD from environmental influences are twin and adoption studies. Twin studies set out to examine traits in monozygotic and dizygotic twins. Since monozygotic twins are genetically identical and dizygotic twins are about 50% genetically identical, a trait that has a large genetic component will have a greater concordance among monozygotic than dizygotic twins (Devor and Cloninger, 1989). When examining AUD among twins, higher concordance rates have been reported in monozygotic vs. dizygotic twins in a large sample of over 8900 Swedish twins across multiple birth cohorts ranging from 1902-1949 (Kendler et al., 1997) and in a U.S.-based study containing over 3500 twins (Prescott and Kendler, 1999). These studies, coupled with numerous other studies examining AUD concordance among twins, indicate that the genetic component of AUD is large (Devor and Cloninger, 1989). In fact, a significant strength of examining monozygotic and dizygotic twins is the ability to calculate heritability, the proportion of the trait attributed to genetic factors, which for AUD is estimated between 50-60% (McGue, 1999).

Another design that aims to separate the genetic and environmental components of a trait are adoption studies, which examine offspring of affected individuals reared in
environments away from the biological parent. Goodwin et al. (1973) examined a sample of 133 male adoptees, 55 of which had a biological parent with AUD, and found that significantly more adoptees with a parent with AUD had a history of drinking problems (Goodwin et al., 1973). Further adoption studies have confirmed the finding that adopted children of alcoholics have higher rates of AUD than adopted children of non-alcoholics, (McGue, 1999), although gender differences have been observed, such that male adoptees with a biological parent with AUD were at greater risk (Devor and Cloninger, 1989, Goodwin et al., 1977).

The genetic component of AUD identified through family, twin, and adoption studies led to the search for “risk genes;” genes harboring genetic variants such as single nucleotide polymorphisms (SNPs) that associate with development of AUD in a population sample. Some of the most replicable AUD-associated SNPs are found within genes encoding enzymes that metabolize alcohol and its toxic downstream byproduct acetaldehyde. Accumulation of acetaldehyde leads to flushing, dizziness, and nausea. Functional genetic variation within two genes, *ADH1B* (alcohol dehydrogenase 1B) and *ALDH2* (aldehyde dehydrogenase 2), results in overactive and underactive enzymatic function, respectively. In turn, the altered enzymatic function leads to an over-accumulation of acetaldehyde, which is responsible for the adverse “flushing reaction” that occurs when people carrying the polymorphisms in either of these genes consume alcohol (Crabb et al., 1989). The genetic variants within *ADH1B* and *ALDH2* are protective, as subjects carrying these mutations have lower rates of AUD due to the reduced ability to consume alcohol without becoming ill (Thomasson et al., 1991). Additional functional polymorphisms located within
genes encoding alcohol metabolizing enzymes have also been linked to AUD, particularly in a cluster of genes located on chromosome 4 (Gelernter and Kranzler, 2009).

As discussed in subsequent sections, alcohol is a non-specific drug that exerts its behavioral effects by targeting many neurotransmitter receptors and systems. SNPs within genes encoding the targets for the molecular actions of alcohol would therefore be obvious candidates to link to development of AUD. Indeed, greater risk for AUD has been associated with SNPs located in genes encoding targets of alcohol including the μ-opiod receptor (OPRM1), muscarinic acetylcholine receptor M2 (CHRM2), dopamine D2 receptor (DRD2), serotonin transporter (SLC6A4), NR2A subunit of the NMDA receptor (GRIN2A), neurosteroid-synthesizing enzymes 5α-reductase type I (SRD5A1) and aldo-keto reductase family 1, member 3 (AKR1C3), among many others (Gelernter and Kranzler, 2009, Dick and Bierut, 2006, Milivojevic et al., 2014, Domart et al., 2012, Rietschel and Treutlein, 2013). However, recent meta-analysis examining SNPs in over 50 candidate genes found that several common variants associated with AUD could not be replicated in the larger compiled sample. The only significant AUD-associated SNP that withstood the meta-analysis was rs279858 in GABRA2, which encodes the α2 subunit of the GABA_A receptor (Olfson and Bierut, 2012). This synonymous SNP resides in exon 5 and is a T-to-C substitution where the C-allele is overrepresented in individuals with AUD (Covault et al., 2004, Edenberg et al., 2004). The GABRA2 gene resides within a cluster of four GABA_A receptor-encoding genes on chromosome 4p12, with the AUD-associated SNP located in a haplotype block of SNPs in strong linkage disequilibrium spanning the 3’ end of GABRA2 and the 5’ end of the adjacent GABRG1 gene (Covault et al., 2008), and associations between haplotype-tagging SNPs in this region with AUD have been some of the most well-
replicated. However, the molecular functions of genetic variation within this region that lead to increased risk of AUD have remained elusive, potentially due to the limitation of applicable model systems since this synonymous SNP may not have functional effects itself, but rather may be in linkage with a functional variant. Intriguingly, *GABRA2* genetic variation is associated with differences in fast beta-frequency brain activity measured via electroencephalogram (Edenberg et al., 2004), and activation of brain areas associated with addiction to reward or alcohol cues (Villafuerte et al., 2012, Kareken et al., 2010, Heitzeg et al., 2014), suggesting that genetic variation within this chromosomal region that is tagged by rs279858 in *GABRA2* may in part exert effects at the level of brain development and connectivity.

Complex interactions between genes and environment also contribute to risk of AUD, although currently identified interactions between genetic variants and environmental influences are limited. One reported gene x environment interaction is with a functional repeat polymorphism located in *SLC6A4*, which encodes the serotonin transporter. This variant gives rise to “short” and “long” alleles where the short allele is associated with reduced transcriptional activity and serotonin transporter function. Originally identified to moderate the influence of stressful life events on depressive symptoms (Caspi et al., 2003), the short allele has since been found to moderate the effect of past-year negative life events on alcohol consumption, such that carriers of the short allele that experienced negative life events reported greater frequencies of drinking and heavy drinking (Covault et al., 2007). Another gene x environment interaction identified to moderate risk of AUD is within gene encoding the corticotropin-releasing hormone receptor 1 (*CRHR1*), which is a component of the HPA-axis. Individuals homozygous for the
C-allele of the haplotype-tagging SNP rs1876831 in CRHR1 that reported higher prevalence of stressful life events had a higher percentage of lifetime heavy drinking, drank greater amounts of alcohol per occasion, and reported earlier ages of drinking onset (Blomeyer et al., 2008, Schmid et al., 2010). The HPA-axis is an intriguing system to explore other candidate gene x environment interactions, since long-lasting dysregulation of HPA-axis activity in some, but not all, individuals that are exposed to early life stress/trauma suggests an underlying genetic component. One gene that has recently been a primary center of focus due to its association with the development of psychiatric disorders including depression, anxiety, and post-traumatic stress disorder in trauma-exposed individuals is FKB5, which encodes the FK506 binding protein 51, which is a regulator of glucocorticoid receptor activity (Zannas and Binder, 2014b, Binder, 2009). Research identifying a gene x environment interaction with FKB5 on alcohol consumption and examination of molecular effects of genotype are discussed extensively in chapters four and five.

Pharmacologic Actions of Acute Alcohol on Neuronal GABAA and NMDA Receptors

Alcohol was originally hypothesized to exert its acute behavioral effects through interactions with and perturbations of lipids in the neuronal membrane, in what is known as the “lipid theory.” However, these findings were refuted due to the high concentrations of alcohol needed to observe biologic effects on membrane lipids (Peoples et al., 1996). Alcohol has since been hypothesized to exert its acute behavioral effects through interactions with membrane-bound proteins, mainly neurotransmitter receptors. Although classified as a depressant, acute alcohol exposure produces both sedative and stimulatory
behavioral effects, likely due to its pharmacological actions on a multitude of neurotransmitter systems, including modulation of dopamine, serotonin, acetylcholine, and glycine signaling (Spanagel, 2009). Due to the behavioral effects of acute alcohol and the symptoms of alcohol withdrawal syndrome, its modulation of γ-aminobutyric acid type A (GABA_A) and N-methyl-D-asparate (NMDA) ligand-gated ionotropic receptor function has been studied in depth.

GABA is the primary inhibitory neurotransmitter in the human brain. The majority of fast inhibitory neurotransmission is mediated via GABA's actions on ionotropic GABA type-A receptors (GABA_A). GABA_A receptors are heteropentameric, composed of a diverse set of 19 subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3), each with four transmembrane domains, that surround a pore permeable to chloride (Olsen and Sieghart, 2009). The most common GABA_A receptor subunit composition is two α, two β, and 1 γ subunit, with the α1β2γ2 and α2β3γ2 combinations being the most abundant in adult cortex (Rudolph et al., 2001). Expression of subunits varies by brain region and over the course of development (Fillman et al., 2010), leading to heterogeneity of GABA_A receptors with estimates of over 500 different receptor subtypes (Sieghart and Sperk, 2002). Interestingly, the subunit stoichiometry is a major determinant of the electrophysiological and pharmacological properties of the receptor (Barnard et al., 1998). Electrophysiological, expression of α1-6 subunits together with the β3 and γ2 subunits in recombinant GABA_A receptors in mouse fibroblasts or HEK-293 cells changed the receptor’s sensitivity to GABA and channel kinetics, including activation, deactivation, and desensitization rates (Picton and Fisher, 2007). The α subunit is not solely responsible for determining receptor properties, as differences in GABA sensitivity and channel kinetics have also been observed when
expressing either the γ2 or the δ subunit together with the α1 and β3 subunits (Haas and Macdonald, 1999). Pharmacologically, a number of compounds exert effects on GABA_A receptors, including benzodiazepines, barbiturates, and steroids, among others, and the efficacy of these modulatory compounds varies for receptors comprised of different subunits (Sieghart and Sperk, 2002). As an example, the binding site for classical benzodiazepines such as diazepam resides between the α and γ subunits of the GABA_A receptor, and depending on which subunits are expressed, can give rise to benzodiazepine-sensitive and -insensitive receptor subtypes (Sieghart, 1995). Together, these findings suggest that the diversity of GABA_A receptor subunits can give rise to a variety of functionally distinct receptors.

Alcohol was originally hypothesized to interact with GABA_A receptors due to similarities in the effects of alcohol, benzodiazepines, and barbiturates, including anxiolysis, sedation, hypnosis, impaired motor control, and impaired cognitive function (Hevers and Luddens, 1998, Kumar et al., 2009). Furthermore, patients being treated for alcohol-withdrawal symptoms are given benzodiazepines but need greater doses than those usually effective, indicating development of cross-tolerance between drugs and suggesting shared molecular mechanisms of action (Harris, 1990). Rodent neurons have been used extensively to investigate the electrophysiological effects of acute alcohol on GABA_A receptors, with varying results that highlight the complexity of both the molecular actions alcohol and the brain. Current opinion is that alcohol potentiates GABA_A receptor-mediated chloride influx at concentrations that are physiologically relevant to human consumption (below 100mM), although results vary by the brain region examined, experimental method, and the concentration of alcohol. Studies using hippocampal,
cortical, cerebellar, and amygdala slice preparations have shown that alcohol’s effect on GABA-gated chloride current is heterogeneous, with some studies reporting a potentiating effect of acute alcohol and others showing no effect (Weiner and Valenzuela, 2006, Kumar et al., 2009). A possibility for the reported inconsistencies of alcohol’s effects at GABA_A receptors could be that only a subset of neurons are susceptible to alcohol, suggested by the results of Signore and Yeh (2000), who reported that in cortical pyramidal neurons a majority of cells were insensitive to alcohol, with only 20% of cells showing potentiation in response to 25mM acute alcohol exposure (Signore and Yeh, 2000). Similar results suggesting cell-specific sensitivity to alcohol were obtained using cultured mouse hippocampal and cortical cells, which showed a potentiating effect of alcohol in roughly 70% of cells examined (Aguayo, 1990). Inconsistencies may also arise from the diversity of GABA_A receptor subtype, where only subpopulations of GABA_A receptors are sensitive to alcohol. Using oocytes, Wallner et al. (2003) reported that GABA_A receptors that contain the δ-subunit, which are primarily located extrasynaptically in neurons, are potentiated by very low concentrations of alcohol that are achieved with social drinking (Wallner et al., 2006). Taken together, these two results suggest that finding neurons that respond to alcohol in the brain may be challenging, and could explain some of the discrepancies in reports examining the effects of acute alcohol. Furthermore, the potentiating effects of alcohol at the GABA_A receptor appear to be biphasic. In rat hippocampal tissue, Sanna et al. (2004) reported that alcohol has a direct effect on both spontaneous and evoked GABA responses within the first 10 minutes of exposure, but the effect is not observed after this early stage in the presence of finasteride, which blocks synthesis of neurosteroids, suggesting that the potentiating effect of alcohol on GABA_A receptors is direct early on but
at later time points indirectly requires brain steroidogenesis (Sanna et al., 2004). These findings highlight the complexity of alcohol’s actions at the GABA<sub>A</sub> receptor, and suggest a benefit to using human neurons to further elucidate mechanisms of alcohol’s inhibitory effects on the brain.

Glutamate is the primary excitatory neurotransmitter in the human brain. Fast excitatory neurotransmission is mediated via glutamate’s actions at ionotropic glutamate receptors, of which there are currently three known classes identified by their synthetic ligands; α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA) (Traynelis et al., 2010). While alcohol has been shown to modulate all three types of ionotropic glutamate receptors, NMDA receptors in particular have been a primary focus for alcohol research due to their unique contribution to cellular physiology, including synaptic transmission, synaptic plasticity, and excitotoxicity (Nagy, 2004). NMDA receptors are heterotetramers, comprised of two obligatory NR1 subunits and two additional subunits, conventionally consisting of NR2 (A-D), although recent studies have elucidated functional roles for modulatory NR3 subunits (Cull-Candy and Leszkiewicz, 2004, Cavara and Hollmann, 2008). The subunits of the NMDA receptor form an ion channel permeable to cations with a high permeability to calcium, which has important intracellular functions. NMDA receptors are activated under specific conditions that distinguish them from AMPA and kainate receptors. First, NMDA receptors require the binding of both the agonist glutamate, which binds to the NR2 subunit, and the co-agonist glycine (or serine), which binds to the NR1 subunit. Furthermore, at normal resting membrane potential conventional NMDA receptors are blocked by a magnesium ion that resides in the channel pore, which is only removed upon cellular depolarization, making
the NMDA receptor a “coincidence detector” since it is only activated under specific conditions (Nagy, 2008b). Similar to GABA$_A$ receptor previously discussed, the subunits of the NMDA receptor dictate the channel properties, including channel opening and closing kinetics and its pharmacological targets. For example, NMDA receptors containing NR1/NR2A subunits have faster deactivation kinetics than receptors containing NR1/NR2B, while inclusion of the NR2B subunit makes the receptor susceptible to the antagonizing actions of ifenprodil (Cull-Candy and Leszkiewicz, 2004).

Early studies using implanted electrodes in mammals found that acute alcohol exposure at concentrations relevant to human consumption altered the firing rate of neurons in multiple brain areas (Klemm et al., 1976, Klemm and Stevens, 1974). Investigations into the specific mechanisms for the altered firing rate led researchers to discover that the NMDA receptor-mediated currents are reproducibly and potently attenuated by low doses of alcohol. Some of the earliest work implicating the NMDA receptor as a target for acute alcohol was done in rat hippocampal cells, where pharmacologically isolated NMDA receptor-mediated currents were found to be rapidly and reversibly inhibited by low doses of alcohol to a greater extent than other ionotropic glutamate receptors (Lovinger et al., 1990, Lovinger et al., 1989). Continued investigation into alcohol’s actions at NMDA receptors revealed that subunit stoichiometry is a determinant of alcohol’s inhibitory effects on NMDA currents. For example, NMDA receptors expressed in oocytes assembled with NR1/NR2A and NR1/NR2B subunits were inhibited more than receptors assembled with NR1/NR2C and NR1/NR2D subunits (Mirshahi and Woodward, 1995). Additionally, investigation of native NMDA receptors in cultured rodent neurons found that the attenuating effect of alcohol at NMDA receptors
corresponded to the receptor’s ability to be antagonized by ifenprodil, which acts on NR2B-containing receptors, suggesting that NR2B-containing receptors are particularly susceptible to the actions of alcohol (Lovinger, 1995). The mechanisms by which alcohol inhibits the NMDA receptor are poorly understood, as no direct binding site has been clearly identified, although hypothetical binding sites have been suggested to reside between transmembrane domains 3 and 4 (Moykkynen and Korpi, 2012, Smothers and Woodward, 2006). A promising idea on how alcohol regulates NMDA receptors is through intracellular mechanisms. NMDA receptor subunits, in particular NR2B, have long intracellular C-terminal domains that dictate the receptors trafficking, localization, and function via numerous post-translational modulatory sites (Chen and Roche, 2007). Indeed, acute alcohol was found to regulate Fyn kinase-mediated phosphorylation of the intracellular tail of the NR2B subunit in rodent brain slices following 10 minutes of exposure, which lead to an increase in channel function and a tolerance to the acute effects of alcohol (Yaka et al., 2003a). Interestingly, alcohol’s affect on Fyn kinase-mediated phosphorylation of the NR2B subunit was found in the hippocampus but not the prefrontal cortex, suggesting brain-region specific post-translational modifications of the NMDA receptor by acute alcohol.

Molecular Adaptations of GABA\textsubscript{A} and NMDA Receptors to Chronic Alcohol

The development of tolerance to the acute effects of alcohol is one of the criteria listed in the DSM-V for a diagnosis of alcohol use disorder. Tolerance is defined as a diminished behavioral response to a drug over repeated exposures. At the molecular level tolerance is, in part, mediated via adaptations that occur within the targets of alcohol,
including membrane-bound ion channels such as the GABA$_A$ and NMDA receptor. The adaptations attempt to return normal functioning to these receptors in the chronic presence of alcohol, characterized as 24 hours of more of exposure to the drug (Pietrzykowski and Treistman, 2008). The molecular changes that result in altered GABA$_A$ and NMDA receptor function are a type of homeostatic plasticity, allowing the brain to retain a balance between excitatory and inhibitory signaling that has been dysregulated (Clapp et al., 2008).

GABA$_A$ receptors down-regulate their function following chronic alcohol exposure to counteract the persistent potentiating effects of the drug at these receptors. Early work demonstrated that chronic exposure to alcohol in vivo and in vitro greatly reduced agonist-evoked GABA$_A$-mediated chloride flux (Morrow et al., 1988, Buck and Harris, 1991). Consistent with these results, rats chronically treated with alcohol showed a 30% attenuation of GABA$_A$-mediated chloride currents in hippocampal cells compared to sham treated animals measured using whole cell patch clamp electrophysiology. Interestingly, the reduced GABA$_A$ receptor chloride flux was not observed in other brain regions including the frontal cortex, parietal cortex, and inferior colliculus, suggesting brain region-specific modification of GABA$_A$ receptors following chronic alcohol (Kang et al., 1996). The mechanisms by which GABA$_A$ receptors alter their function to adapt to the alcohol-containing environment may include subunit switching, since the subunit composition dictates the receptor’s functional properties. Expression of GABA$_A$ subunits following chronic alcohol has been examined in animal models and human post-mortem brain samples. The most consistent findings of these studies are an upregulation of the $\alpha4$ subunit and a downregulation of the $\alpha1$ subunit (Clapp et al., 2008, Devaud et al., 1995).
Interestingly, expression of these subunits in oocytes revealed that GABA_A receptors containing the α4 subunit have faster channel deactivation rates, while α1 subunit containing receptors have longer open duration (Picton and Fisher, 2007), supporting the idea that subunits are switched to compensate for the potentiating effects of alcohol. Another method by which GABA_A receptors may adapt to chronic alcohol is via posttranslational modifications. Tyrosine kinase phosphorylation of the γ2 and β2, but not the α1 subunit, was observed following chronic alcohol exposure in cultured cortical mouse neurons (Marutha Ravindran and Ticku, 2006). Furthermore, mutant mice lacking a specific isoform of protein kinase C (PCK) showed reduced responses to alcohol exposure (Harris et al., 1995), suggesting that PKC-mediated modifications may be a method by which GABA_A receptors adapt to an environment with alcohol present.

NMDA receptors also undergo modifications in response to chronic alcohol exposure. Electrophysiological evidence suggests that NMDA receptor function is enhanced in the presence of chronic alcohol in order to counteract the inhibitory effects of the drug at these receptors. Early in vitro evidence of increased NMDA receptor function was obtained using cultured mammalian neurons chronically treated with alcohol (4-7 days), reporting that NMDA receptor-mediated calcium currents in cortical and hippocampal neurons were greater in cultures treated with alcohol (Smothers et al., 1997, Ahern et al., 1994). Electrophysiological changes to chronic alcohol exposure are accompanied by an upregulation of genes encoding NMDA receptor subunits in cultured rat cortical and hippocampal neurons (Nagy et al., 2003, Hu et al., 1996), while an upregulation of the NR2A and NR2B subunits were seen in the frontal cortex and hippocampus of chronically alcohol treated rats (Follesa and Ticku, 1995). Interestingly, there is increased
binding of radiolabeled NMDA receptor antagonists in human post-mortem brain samples derived from alcoholics (Freund and Anderson, 1996), which suggests that the number of NMDA receptors is increased in response to chronic alcohol. Indeed, increased number of NMDA receptors and increased synapse size were observed in rodent hippocampal cultures exposed to alcohol for 4 days (Carpenter-Hyland et al., 2004, Clapp et al., 2008).

The precise mechanisms by which NMDA receptor subunit expression is upregulated in response to chronic alcohol remain poorly understood, although one study found demethylation of the NR2B subunit gene (grin2b) in primary mouse hippocampal cultures following 5 days of alcohol exposure (Marutha Ravindran and Ticku, 2005), suggesting that alcohol-related changes in methylation status may play a role. It is important to note that not all studies reported changes in NMDA subunit expression to alcohol (Nagy, 2008b). In fact, using a primate model of chronic alcohol exposure, which is more applicable than rodent models to the human condition, a decrease in NMDA receptor subunit expression was observed in prefrontal cortex following months of alcohol self-administration (Acosta et al., 2010). In agreement with this result, decreased mRNA expression of the genes encoding the NR1, NR2A, and NR2B subunits was observed in post-mortem human brain samples derived from alcoholics with cirrhosis of the liver (Ridge et al., 2008), and decreased NR1 protein expression was found in the locus coeruleus of alcoholics (Karolewicz et al., 2008).

The homeostatic adaptations that occur in GABA<sub>A</sub> and NMDA receptors in response to chronic alcohol can be deleterious to the individual upon cessation of alcohol consumption, leading to adverse symptoms experienced during alcohol withdrawal, including seizures. Due to the attenuation of GABA<sub>A</sub> and the enhancement of NMDA
receptors, the brain enters a state of hyperexcitability when alcohol is suddenly withdrawn (Clapp et al., 2008). For example hippocampal explant cultures were found to be hyperexcitable following withdrawal from chronic alcohol exposure, and this hyperexcitability was NMDA-receptor dependent (Hendricson et al., 2007). In agreement with this, NMDA receptor antagonists have been shown to block alcohol withdrawal associated seizures and prevent alcohol withdrawal-associated neurotoxicity in chronically treated cerebellar granule cells (Hoffman et al., 1995). In humans, alcohol withdrawal symptoms are treated with GABA\textsubscript{A} receptor-potentiating pharmacological agents including the benzodiazepine diazepam (Finn and Crabbe, 1997). Taken together, these findings clearly indicate the involvement of molecular adaptations within GABA\textsubscript{A} and NMDA receptors in alcohol withdrawal symptoms.

**Pharmacologic Treatments for Alcohol Use Disorder**

Despite the prevalence of alcohol use disorder in modern society there are currently only three FDA-approved treatments for the disorder, and these medications do not work for everyone, likely due to the heterogeneous, complex nature of AUDs that leads to diversity among affected individuals (Litten et al., 2012). The poor outcomes and side effects related to current approved treatments are some factors that contribute to the small number of treatment-seeking alcoholics, with less than 20% of affected individuals seeking treatment (Grant et al., 2015). The oldest FDA-approved treatment, disulfiram, which was approved for use treating AUD in 1950, acts by inhibiting aldehyde dehydrogenase, leading to an accumulation of acetaldehyde if the individual consumes alcohol, causing unpleasant feelings such as flushing, headache, and nausea (Heilig and Egli, 2006). Although the
unpleasant feelings are strong deterrents to drink, many doctors are not in favor of prescribing disulfiram due to the poor success rate, given that individuals can simply stop taking the medication before drinking (Miller, 2008). The second FDA approved treatment approved in 1994, naltrexone, acts as an antagonist to the μ isoform of opioid receptors thereby blocking the pleasurable effects of alcohol consumption (Miller, 2008). In numerous studies treatment with naltrexone was found to be efficacious in reducing the risk of relapse to heavy drinking (Heilig and Egli, 2006), and interestingly its efficacy in preventing relapse is moderated by a polymorphism in the gene encoding the μ opioid receptor \((OPRM1)\) (Oslin et al., 2003), suggesting that naltrexone treatment may be more beneficial in some individuals. The third treatment is acamprosate, which was approved by the FDA in 2004 (Miller, 2008) and acts as a regulator of excitatory and inhibitory neurotransmission by targeting GABA, NMDA, and metabotropic glutamate receptors (Heilig and Egli, 2006). Acamprosate is currently the most widely prescribed medication for AUD due its safety and efficacy in maintaining abstinence from alcohol use (Mason and Heyser, 2010). Similar to naltrexone, efficacy of acampraste in treating AUD was moderated by polymorphisms within two genes encoding subunits of the GABA\(_A\) receptors \((GABRA6\) and \(GABRB2)\) (Ooteman et al., 2009), implicating that genetic background may be a source of variability between responders and non-responders to medications, and further suggesting that genetic screens may be advantageous in prescribing treatments for AUD.

As previously described, chronic alcohol use results in compensatory changes in excitatory/inhibitory neurotransmission via changes in many receptors including GABA\(_A\) and NMDA receptors. Acamprosate is currently the only FDA approved medication that targets the known receptors that are significantly modulated by chronic alcohol. Current
knowledge of what systems alcohol targets and modulates has led studies to investigation of compounds that modulate neurotransmission as future treatments for AUD. Such potential medications include baclofen, which modulates presynaptic metabotropic GABA_B receptors, ondansetron, which targets a class of serotonin receptors (5-HT3), varenicline, which targets nicotinic acetylcholine receptors, and topiramate, which is currently FDA-approved for use as an anticonvulsant and acts by modulating a number of receptors including glutamatergeric kainate and NMDA receptors, GABA_A receptors, as well as voltage gated sodium and potassium channels (Heilig and Egli, 2006, Miller, 2008). Additional research examining the molecular effects of alcohol on the human brain may identify novel targets of alcohol that lead to better treatment options. Furthermore, as indicated by studies examining genetic moderators of naltrexone and acamprosate treatments (Oslin et al., 2003, Ooteman et al., 2009), pharmacogenomics research promises better patient-medication matching and should play a large role in the future treatment of AUD. This has recently been demonstrated for topiramate, where efficacy of the drug for reducing heavy drinking in alcoholics was moderated by a polymorphism in the GRIK1 gene, which encodes the GluK1 subunit of the kainate receptor, a target for topiramate’s pharmacologic action. In this study, Kranzler et al. (2014) reported that homozygotes for the rs2832407 C-allele treated with topiramate reduced their heavy drinking days significantly more than placebo, an effect that was not observed in carriers of the A-allele (Kranzler et al., 2014a), and importantly the effect of topiramatae on reduction of heavy drinking days was maintained 3- and 6-months after treatment in rs2832407 C/C subjects (Kranzler et al., 2014b).
Induced Pluripotent Stem Cells in the Study of Psychiatric Disorders

Much of what we know about the molecular actions of alcohol on the brain has been derived from the study of rodent models and post-mortem human brain samples, which may not accurately reflect responses in human cells or the human condition of AUD. Therefore, an in vitro model system of phenotypically- and genetically-characterized human neural cells would be beneficial for the examination of the molecular effects of alcohol and AUD-associated genetic variants in human tissue. The advent of induced pluripotent stem cell technologies has revolutionized the field of stem cell research, with promising use for the study of genetically heterogeneous and difficult-to-model psychiatric disorders. Induced pluripotent stem cells (iPSCs) were first generated in 2006 by reprogramming mouse fibroblasts to a state of pluripotency through the expression of four transcription factors (OCT3/4, KLF4, SOX2, and c-Myc) via viral transduction (Takahashi and Yamanaka, 2006). Human iPSCs were generated the following year using the same methodology (Takahashi et al., 2007). Cellular reprogramming resets the epigenetic landscape to state similar to that observed in human embryonic stem cells, although iPSC methylation patterns differ between cell lines and harbor signatures from their tissue of origin (Kim et al., 2010, Lister et al., 2011). Despite differences in epigenetic profiles between embryonic and induced stem cells, a defining characteristic of iPSCs is that they have the capacity to differentiate into all cell types from the three germ layers, including neural cells, making them a powerful tool for in vitro examination of difficult to obtain human neural tissue (FIGURE 1.1).

To date, numerous protocols have been used to differentiate iPSCs into various neuronal cell types, including cultures that are enriched for glutamate (Zeng et al., 2010),
Patient-derived somatic cells can be reprogrammed to a state of pluripotency through expression of transcription factors; generating patient-specific induced pluripotent stem cells (iPSC). Through various protocols, iPSCs can be driven to differentiate into any cell type derived from the three embryonic germ layers. These differentiated cells can then be used as in vitro models of disease or for toxicology and drug screening studies, as well as used for regenerative medicine in cell replacement therapies. Picture taken from Yamanaka (2009) “A Fresh Look at iPS Cells.”
dopamine (Swistowski et al., 2010), interneuron (Liu et al., 2013), and serotonin neurons (Shimada et al., 2012), providing researchers a minimally invasive method to explore molecular phenotypes of psychiatric disorders in human neural cultures. Although the field is in its infancy, iPSC technology has been used to study numerous psychiatric disorders where in vitro models were previously limited or even unobtainable, including complex neurogenetic disorders such as Angelman and Prader-Willi syndrome (Chamberlain et al., 2010, Germain et al., 2014), schizophrenia (Brennand et al., 2011), non-syndromic autism spectrum disorder (Mariani et al., 2015), DISC1-related mental disorders (Wen et al., 2014), and bipolar disorder (Madison et al., 2015), among others. However, research utilizing iPSCs to explore drug addictions such as AUD, including the molecular effects of drug exposure or addiction-associated genetic variants, is lacking, potentially due to the challenges posed by the large environmental influence and genetically heterogeneous nature of AUD.

**Thesis Objectives**

iPSC technologies are promising new tools in the study of psychiatric disorders including AUD. However, work examining their utility in exploration of drug addiction is limited, and is nonexistent in the study of AUD. Nonetheless, if iPSC-derived neural cultures express functional targets of alcohol and are able recapitulate molecular effects of alcohol exposure identified in rodent or post-mortem human samples, iPSC technologies promise new insights into mechanisms of AUD. Exploring the utility of iPSCs for AUD research is the central objective of this thesis work. To achieve this objective, the following questions are addressed: 1) Do human iPSC-derived neural cells express functional targets
of alcohol, and does the effect of acute and chronic alcohol exposure recapitulate findings observed in rodent and human post-mortem model systems? 2) Can iPSC neural differentiation provide new insights into the molecular effects associated with genetic variation within the AUD-risk gene GABRA2? 3) Is genetic variation within the psychiatric disorder-associated gene FKBP5 predictive of alcohol consumption in humans and 4) can iPSC-derived neural cells recapitulate reported effects of FKBP5 genetic variation on mRNA expression previously observed in peripheral blood cells? The results presented in this dissertation support the utilization of iPSCs for researching the molecular effects of alcohol and AUD-associated polymorphisms on human neural cells in vitro by arguing that iPSC-derived neural cells express functional targets of alcohol and display adaptations to acute and chronic alcohol exposure previously reported in other model systems. Furthermore, polymorphisms associated with development of AUD and increased alcohol consumption in a large human sample can be examined in vitro using human iPSC neural differentiation.
Chapter 2.

Pilot Study of iPS-Derived Neural Cells to Examine Biologic Effects of Alcohol on Human Neurons In Vitro


Abstract

**Background:** Studies of the effects of alcohol on NMDA receptor function and gene expression have depended on rodent or post-mortem human brain models. Ideally, the effects of alcohol might better be examined in living neural tissue derived from human subjects. In this study, we used new technologies to reprogram human subject-specific tissue into pluripotent cell colonies and generate human neural cultures as a model system to examine the molecular actions of alcohol. **Methods:** Induced pluripotent stem (iPS) cells were generated from skin biopsies taken from 7 individuals, 4 alcohol dependent subjects and 3 social drinkers. We differentiated the iPS cells into neural cultures and characterized them by immunocytochemistry using antibodies for the neuronal marker beta III-tubulin, glial marker s100β, and synaptic marker synpasin1. Electrophysiology was performed to characterize the iPS-derived neurons and measure the effects of acute alcohol exposure on the NMDA receptor response in chronically alcohol exposed and non-exposed neural cultures from one non-alcoholic. Finally, we examined changes in mRNA expression of the NMDA receptor subunit genes GRIN1, GRIN2A, GRIN2B, and GRIN2D after 7 days of alcohol exposure and after 24-hour withdrawal from chronic alcohol exposure. **Results:** Immunocytochemistry revealed positive staining for neuronal, glial, and synaptic
markers. iPS-derived neurons displayed spontaneous electrical properties and functional ionotropic receptors. Acute alcohol exposure significantly attenuated the NMDA response, an effect that was not observed after 7 days of chronic alcohol exposure. After 7 days of chronic alcohol exposure, there were significant increases in mRNA expression of \textit{GRIN1}, \textit{GRIN2A}, and \textit{GRIN2D} in cultures derived from alcoholic subjects but not in cultures derived from non-alcoholics. \textbf{Conclusions:} These findings support the potential utility of human iPS-derived neural cultures as \textit{in vitro} models to examine the molecular actions of alcohol on human neural cells.

\textit{Introduction}

Alcohol use disorders, including alcohol abuse and dependence, are among the most prevalent mental disorders in the United States, with approximately 8.5\% of the US population being affected by them during a one-year period (Grant et al., 2004a). Despite the high prevalence of these disorders, the molecular mechanisms underlying many of the behavioral effects of alcohol and those that lead to the development of tolerance and dependence are not well understood (Lewohl et al., 2000, Hanchar et al., 2006).

Ligand-gated ion channels, including the N-methyl-D-aspartate (NMDA) and GABA\textsubscript{A} receptors are targets for the actions of alcohol (Vengeliene et al., 2008) and these may play a role in the development of alcohol use disorders. In the central nervous system (CNS), NMDA receptors play important roles in synaptic transmission, synaptic plasticity (such as long-term potentiation), and excitotoxicity (Nagy, 2004). The conventional NMDA receptor is a tetrameric structure consisting of two NR1 and two NR2 (NR2A-D) subunits (Bigge, 1999) that surround a cation channel with a high permeability to calcium (Cavara and
Hollmann, 2008). Under normal resting conditions the NMDA receptor is blocked by magnesium (Mg$^{2+}$) ions, which reside inside the channel pore. This blockade is removed upon membrane depolarization in conjunction with the combined binding of the agonist glutamate and the co-agonist glycine. Because these channels are activated only when electrical and chemical signals are present simultaneously, NMDA receptors are thought to act as coincidence detectors (Nagy, 2008b).

Research using both in vivo and in vitro models has established that both acute and chronic exposure to alcohol affects the NMDA receptor. Acutely, alcohol acts as an NMDA antagonist (Tomberg, 2010), leading to significant decreases in the size of NMDA excitatory post-synaptic potentials (EPSPs) (Nie et al., 1994) and inhibition of NMDA-dependent long-term potentiation (LTP) (Puglia and Valenzuela, 2010). Chronic exposure to alcohol leads to an increased density of receptors and facilitation of NMDA receptor functioning, due to receptor subunit phosphorylation and increased expression of various NMDA receptor subunit mRNAs (Nagy, 2008b). Significantly increased expression after chronic alcohol exposure has been observed in NR1 subunit mRNA in the amygdala (Floyd et al., 2003) and protein in the hippocampus (Maler et al., 2005), in NR2A and NR2B subunit mRNA in the cortex and hippocampus (Follesa and Ticku, 1995) and protein in the hippocampus (Maler et al., 2005). After a 48-hour withdrawal period, NR1, NR2A, and NR2B mRNA levels returned to baseline in the hippocampus and cortex (Follesa and Ticku, 1995), and protein levels were returned to baseline in the amygdala (Roberto et al., 2006) and the cortex (Kalluri et al., 1998). The changes observed in NMDA receptor expression and function are believed to contribute to the development of alcohol tolerance and dependence, and the
CNS hyperexcitability and excitotoxicity observed during alcohol withdrawal (Nelson et al., 2005, Nagy, 2008a, Nagy, 2008b)

Other studies have compared post-mortem human brain tissue derived from alcoholics and non-alcoholics (Lewohl et al., 1997, Lewohl et al., 2000). NMDA receptor NR1, NR2A, and NR2B subunit mRNA expression in both the superior frontal and primary motor cortices was significantly lower in alcoholic individuals with cirrhosis of the liver than either non-alcoholic controls or alcoholics without co-morbid disease (Ridge et al., 2008). Studies using post-mortem human brain tissue bypass the questions of relating animal models to humans. However, using post-mortem brain tissue does not allow for examination of electrophysiological effects of alcohol or controlled exposure to alcohol on gene expression. In addition, factors including gender, brain pH, age of death, ethnicity, history of medication, and post-mortem interval also contribute to variability among post-mortem samples (Hynd et al., 2003, Preece and Cairns, 2003).

One way potentially to bypass the difficulties that arise with the use of animal and post-mortem human brain models is to derive pluripotent cells from samples taken from living participants, and then differentiate these pluripotent cells into the tissue of interest. Takakashi and Yamanaka reported that it was possible to reprogram mouse or human fibroblasts into a pluripotent state by virally introducing 4 factors into the cell culture, generating induced pluripotent stem (iPS) cells (Takahashi et al., 2007, Takahashi and Yamanaka, 2006). Subsequently, other groups have used protocols developed for human embryonic stem (hES) cells to differentiate iPS cells into neural cultures containing neurons and glia (Hu et al., 2010, Johnson et al., 2007). iPS cell derivation and neural
differentiation provides a minimally invasive method to obtain and examine subject-specific neural tissue.

The ability to generate neurons from participant fibroblasts has made it possible to examine and study living neurons from individual subjects with different diseases (Chamberlain et al., 2008). Current research has focused on the use of iPS cell technologies to examine mechanisms of neurodegenerative disease such as amyotrophic lateral sclerosis, spinal muscular atrophy, and Parkinson’s disease (Yamanaka, 2009), and genetic, neurodevelopmental diseases including Angelman syndrome and Prader-Willi syndrome (Chamberlain et al., 2010). To our knowledge, there are no reports of research using human iPS-derived neural cells as a model for the molecular actions of alcohol and the development of alcohol use disorders.

Here, we present results focusing on the NMDA receptor to explore the potential use of human iPS-derived neural cells to examine the biological and molecular effects of alcohol. Electrophysiological recordings revealed that iPS-derived neurons in our cultures were able to generate action potentials, exhibited spontaneous synaptic activity, and expressed functional GABA<sub>A</sub>, AMPA, and NMDA receptors. Chronic effects of alcohol exposure were also detectable using electrophysiological and gene expression analysis.

**Materials and Methods**

*iPS Cell Derivation and Culture*

Fibroblasts cell lines were generated from skin punch biopsies taken from participants enrolled in clinical studies of the effects of alcohol in social drinkers and a treatment trial for problem drinkers at the University of Connecticut Health Center (UCHC).
Participants signed a consent form that was approved by the UCHC institutional review board and a punch biopsy sample was taken from the inner, upper arm. Biopsy samples were finely minced and cultured in Dulbecco’s Modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (FBS, Invitrogen) to establish primary fibroblast cultures. Fibroblast lines used in the current report were generated from 2 women and 5 men. Four of the participants met criteria for current DSM-IV alcohol dependence, including tolerance to alcohol, while three participants were classified as non-alcohol dependent social drinkers. Cell lines derived from non-alcohol dependent social drinkers are labeled 1, 2, and 3, while alcohol-dependent participants are labeled 4, 5, 6, and 7. The two cell lines derived from women are numbers 4 and 6.

Human fibroblast cells were reprogrammed by the UCHC Stem Cell Core using retrovirus constructs expressing 5 reprogramming factors (OCT4, SOX2, KLF4 c-MYC, and LIN28). iPS cell colonies were identified based on characteristic morphology approximately 2-4 weeks after viral transduction. Selected colonies were expanded as separate clones and the expression of pluripotency markers SSEA-3/4 and NANOG was verified by the UCHC Stem Cell Core using immunocytochemistry.

iPS cells were cultured and expanded by growth on a layer of irradiated mouse embryonic fibroblasts (MEFs) using human embryonic stem (hES) cell media containing Dulbecco’s Modified Eagle Medium with F12 (DMEM/F12, 1:1 ratio) (Invitrogen) supplemented with 20% Knockout Serum Replacer (Invitrogen), 1x non-essential amino acids (Invitrogen), 1mM L-glutamine solution (Invitrogen), 0.1mM β-mercaptoethanol (MP Biomedicals, Solon, OH, USA), and 4ng/mL basic fibroblast growth factor (bFGF) (Millipore, Billerica, CA, USA). Colonies were observed on a daily basis, and any colonies exhibiting
spontaneous differentiation were removed. iPS media was replaced on a daily basis. iPS cells were passed every 7 days onto irradiated MEFs using a 1mg/mL dispase (Invitrogen) in DMEM/F12.

**iPS Cell Neural Differentiation**

iPS cells were differentiated into neural cells using an established protocol (#SOP-CH-207 Rev A) developed by the WiCell Institute (www.wicell.org, Madison, WI, USA) for the neural differentiation of hES cells. Confluent iPS colonies were harvested from 10 cm plates using 1mg/mL dispase in DMEM/F12. iPS cells were cultured in suspension for 4 days in 75mL low-adhesion flasks using hES cell media without the addition of bFGF to allow for the formation of embryoid bodies (EBs). Half of the media was changed daily. After 4 days, EBs were collected, centrifuged, and washed in phosphate buffered saline (PBS) and placed into new 75mL low adhesion flasks in a neural induction media [DMEM/F12 supplemented with 1x N2 supplement (Invitrogen), 1x non-essential amino acids and 2µg/mL heparin (Sigma-Aldrich, St. Louis, MO, USA)] to allow for the formation of neural epithelial (NE) cells. Half of the media was changed every other day. After 2-4 days in suspension, NE cells were collected and plated onto laminin (20µg/mL in DMEM/F12) coated 6-well cell culture plates to allow the NE cells to adhere to the surface. Cells were cultured in neural induction media, and half of the media was changed every other day. NE cells plated on laminin were cultured for 10-14 days. During this time, formation of neural tube-like rosettes was observed in the culture. NE cells were collected by mechanical detachment, centrifuged, and re-suspended in neural induction media and incubated in 75mL low adhesion flasks for 7-14 days with half of the media changed every
other day. NE cells were then collected, pelleted, and re-suspended in neural differentiation media [Neurobasal media (Invitrogen) supplemented with 1x non-essential amino acids, 4x B27 Supplement (Invitrogen), 1µM dibutyryl-cAMP (Enzo Life Sciences, Farmingdale, NY, USA), and 200µM ascorbic acid (Sigma-Aldrich)]. NE cells were then mechanically dissociated by pipetting and plated onto 13mm glass coverslips (Fisher Scientific, Pittsburg, PA, USA) pre-coated with polyornithine (0.1mg/mL in water) and Matrigel (0.67mg/mL in DMEM/F12) (BD Biosciences, Bedford, MA, USA) coated at a density of 100,000 cells per coverslip. Cells were allowed to adhere to coverslips for 24 hours, and then cultured in neural differentiation media supplemented with 10ng/mL brain derived neurotrophic factor (BDNF) (Peprotech, Rocky Hill, NJ, USA), 10ng/mL glial derived neurotrophic factor (GDNF) (Peprotech), 10ng/mL insulin-like growth factor (IGF) (Peprotech), and 1µg/mL laminin (Sigma-Aldrich) to allow for the differentiation of neural cells. Half of the media was changed 3 times per week, and neural cells were cultured for 12 weeks or more.

**Chronic alcohol treatment:** Cultures were fed daily for 7 days with neural differentiation media containing 50 mM ethanol (0.23 mg/dl). Evaporation of alcohol following daily media changes in unsealed culture plates is expected to produce a gradual reduction of alcohol each day. Evaporation of alcohol was examined in sister culture dishes not containing neural cells by measurement of media alcohol concentrations at 2, 4, 6, 8, 10 and 24 hours post-feeding using an AM1 Alcohol Analyzer (Analox Instruments Ltd, The Vale, London, UK). Alcohol concentration was gradually reduced with a ~19-hour half-life, such that by the end of 24 hours the average alcohol concentration was 18.1±2.0 mM (0.07
mg/dl). This daily replenishment of alcohol followed by gradual loss to evaporation in unsealed culture dishes provides a pattern of exposure more similar to that in human daily heavy drinkers than would continuous exposure to 50 mM ethanol in a sealed culture system.

**Immunocytochemistry**

 Neural cultures were fixed with 4% paraformaldehyde in PBS (Invitrogen) for 45 min and stained with mouse monoclonal antibody to beta III-tubulin (Millipore, 1:500), and either rabbit polyclonal antibody to s100β (Abcam, Cambridge, MA, USA 1:250) or synapsin1 (Millipore, 1:500). All primary antibodies were diluted in 0.1% bovine serum albumin in PBS. Appropriate donkey anti-mouse and donkey anti-rabbit alexa fluor 488 and 594 (Invitrogen, 1:600) secondary antibodies were used for visualization.

**Electrophysiology**

 Electrophysiological recordings were obtained from neurons from subject 1 (a non-alcoholic) using previously described techniques (Lemtiri-Chlieh and Levine, 2010). Neurons were selected for recording based on neural morphology (neurite projections and pyramidal-shaped soma). Artificial cerebrospinal fluid (aCSF) containing 125mM NaCl, 2.5mM KCl, 1.25mM NaH2PO4, 1mM MgCl2-6H2O, 25mM NaHCO3, 2mM CaCl2, and 25mM dextrose was perfused into the recording chamber at 1mL/min at room temperature. To examine NMDA responses to locally applied glutamate, the bath solution contained the AMPA receptor antagonist DNQX (10µM) under Mg2+ free conditions to relieve the voltage-dependent Mg2+ block of NMDA receptors.
To examine the generation of action potentials, spontaneous synaptic activity, and AMPA and NMDA responses, an internal recording solution was used containing 4mM KCl, 125mM K-Gluconate, 10mM HEPES, 10mM phosphocreatine, 1mM EGTA, 0.2mM CaCl2, 4mM Na2-ATP, and 0.3mM Na-GTP (pH 7.3). To examine GABA responses, the pipette solution contained 130mM KCl, 10mM HEPES, 10mM phosphocreatine, 1mM EGTA, 0.1mM CaCl2, 1.5mM MgCl2, 4mM Na2-ATP, and 0.3mM Na-GTP (pH 7.3), which allowed GABA-induced synaptic currents to be recorded at normal resting membrane potential. Whole cell current clamp recordings were used to examine the ability of cells to generate action potentials and to observe AMPA responses to 50μM glutamate (in the presence of Mg) and NMDA responses to a combination of 50μM glutamate and 10μM glycine (in the presence of DNQX and the absence of Mg2+). Whole cell voltage clamp recordings were used to examine spontaneous synaptic activity in the neural culture as well as to observe responses to 50μM GABA. To elicit AMPA, NMDA, and GABA responses, a micropipette containing the appropriate solution was placed adjacent to the recorded cell. The solution was puffed onto the cell using 5psi of positive pressure.

To examine the effects of acute alcohol exposure on the NMDA response in both control and chronically ethanol-treated neural cultures, a micropipette filled with 50μM glutamate and 10μM glycine was placed next to a patched neuron. Whole cell recordings were done in current clamp mode. aCSF used contained 10μM DNQX and did not contain Mg2+. The glutamate/glycine solution was puffed onto the cell every 30 sec and the responses were recorded. A baseline recording was taken for 5 min, and then a Mg2+ free aCSF supplemented with 50mM alcohol was perfused into the recording chamber. aCSF containing alcohol was perfused onto the cell for 5 min, with the glutamate/glycine puff
occurring every 30 sec. Peak amplitude of the responses in the final 2 min of the baseline period and the final 2 min of the acute alcohol exposure period were calculated using the Axon program. Alcohol effects were analyzed via paired t-test using Graphpad Prism software.

To observe the effects of acute alcohol exposure on GABA$_\alpha$ and AMPA responses, the same paradigm was used as described above, except the solution being puffed onto the cell was either 50 µM GABA or 50 µM glutamate to elicit GABA$_\alpha$ or AMPA responses, respectively. AMPA responses were observed using an external solution containing 1mM Mg$^{2+}$ without the addition of DNQX.

**Effects of Alcohol on NMDA Subunit mRNA Expression**

To examine the effects of chronic alcohol exposure on NMDA subunit mRNA expression, iPS-derived neural cells from 7 subjects (4 alcoholics and 3 non-alcoholics) were cultured for 15-18 weeks and subjected to three treatment conditions; a 7-day sham treatment, a 7-day alcohol treatment, and a 7-day alcohol treatment with a 24-hr withdrawal period (n=4-7 coverslips per subject per condition). Neural differentiation media with or without 50 mM alcohol was replaced daily. In the condition examining withdrawal, after 7 days of alcohol treatment, alcohol-containing media was replaced with normal neural differentiation media for 24 hours.

RNA was extracted from neural cultures using TRIzol Reagent (Invitrogen) following the manufacturer's directions and cDNA was synthesized from 2µg RNA from all conditions using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).
Neural cell culture cDNA was analyzed by real time polymerase chain reaction (RT-PCR) using an Applied Biosystems 7500 instrument and TaqMAN Assay on Demand (Applied Biosystems) probes and primers for: *GRIN1* (Hs00609557_m1), *GRIN2A* (Hs00168219_m1), *GRIN2B* (Hs00168230_m1), and *GRIN2D* (Hs00181352_m1). Expression of these genes was normalized relative to the expression of the housekeeping gene *GUSB* (Applied Biosystems, 4326320E) co-amplified with target genes. PCR cycles were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. A standard curve consisting of a 4-level 2-fold serial dilution series of pooled cDNA taken from all samples in the experiment was used to determine relative mRNA expression levels. Expression after 7 days of alcohol exposure and 7 days of alcohol exposure plus a 24-hour withdrawal period was normalized to each subject's sham condition. Data were analyzed for statistical significance with mixed model repeated measures test using SPSS software.

**Results**

*Human iPS Cell Neural Differentiation*

To generate subject-specific tissue, we differentiated iPS cells derived from 7 human subjects, 4 of whom met criteria for current alcohol dependence, including alcohol tolerance, into neural cell cultures using an established protocol for the neural differentiation of hES cells (Figure 2.1A-F). After 10 weeks in neural differentiation medium, iPS-derived cell cultures contained beta-III tubulin and s100β positive cells
Figure 2.1. Neural Differentiation Timeline

(A) Timeline of neural differentiation includes culturing iPS cell colonies (B) to confluence for 7 days, followed by culture in suspension for 4 days to allow for the formation of embryoid bodies, followed by incubation in neural induction media and growth on a laminin substrate to trigger the formation of neuroepithelial cells displaying neural tube-like rosette structures, which are expanded in suspension (C) culture for several days prior to plating dissociated cells on coverslips. Dissociated cells are cultured in a neural differentiation media containing the growth factors BDNF, GDNF, and IGF to promote the differentiation of neural cells (D, arrows indicate neural cell bodies, red arrow indicates recording electrode for performing electrophysiology). Dissociated neural cells were then cultured for 12-18 weeks in neural differentiation media. At 10-weeks post-plating neural cultures displayed neuronal lineage marker beta III-tubulin and glial cell lineage s100β positive cells (E), indicating that iPS cells differentiated into cultures containing a mixed culture of neurons and glia. Focal synapsin-1 immunostaining along beta III tubulin (F) positive processes were observed indicating the formation of synaptic specializations within the culture.
(Figure 2.1E), indicating that human iPS cells were successfully differentiated into cultures containing neurons and glia. Further immunostaining revealed positive synapsin-1 staining (Figure 2.1F), suggesting that synaptic specializations were forming within iPS-derived neural cultures.

*Electrophysiology*

We examined electrophysiological properties of neurons, selected for recording based on morphology (neurite projections and pyramidal-shaped soma), in iPS-derived neural cultures from one non-alcoholic subject, including the ability to generate action potentials and spontaneous synaptic activity. Cells that did not generate action potentials (7 of 58 cells examined) were excluded from further analysis. Consistent with reports on the developmental time course of hES cell-derived neural cells (Johnson et al., 2007), we observed that, after several weeks in culture, neurons developed resting membrane potentials of -50 to -60mV, and beginning at 10-12 weeks post-plating, iPS-derived neurons generated trains of action potentials in response to current injection (Figure 2.2A). Spontaneous synaptic activity with both fast and slow components typical of glutamatergic synaptic transmission was observed (Figure 2.2B).

To further characterize iPS-derived neurons, we examined whether functional GABA<sub>A</sub>, AMPA, and NMDA ionotropic receptors were expressed. Functional GABA<sub>A</sub> receptors were observed using voltage-clamp mode where a 50μM GABA puff produced an inward current response (Figure 2.2C). In total, 20 of 20 cells tested responded to GABA. Functional AMPA receptors were observed in current-clamp mode where 50μM glutamate
Whole-cell patch recordings from 12-week iPS-derived neurons from a non-alcohol dependent subject demonstrate functional neuronal properties, including generation of action potentials, spontaneous synaptic activity, and ionotropic receptor currents. (A) Neurons generate trains of action potentials in response to current injection. (B) Spontaneous synaptic activity (Inset) close-up of a spontaneous event demonstrates fast and slow kinetic components of the event. (C) GABA receptor current response to a 200 ms puff of 50μM GABA indicates that functional GABA receptors are present (arrow indicates when GABA was applied). (D) Depolarization response to a 200 ms puff of 50μM glutamate in the presence of Mg²⁺ indicates that functional AMPA receptors are expressed. Response was completely abolished when the AMPA antagonist DNQX (10μM) was perfused into the recording chamber (arrows indicate when glutamate was applied). (E) NMDA channel mediated depolarization in response to a 200 ms puff of 50μM glutamate and 10 μM glycine in a magnesium-free recording solution that contained the AMPA receptor antagonist DNQX (10μM). The response was completely abolished when NMDA receptor antagonist CPP (6μM) was perfused into the recording chamber (Dashed arrows indicate when glutamate plus glycine solution was applied).
puffs produced a depolarization of the membrane in the presence of 1mM Mg^{2+}. In total, 9 of 9 cells tested responded to glutamate administration under these conditions. This response to glutamate was blocked by the administration of the AMPA antagonist DNQX (Figure 2.2D). To identify NMDA glutamate receptor responses, a Mg^{2+} free recording solution containing DNQX to block AMPA receptor activity was used. The puffing pipette contained 50\mu M glutamate plus 10\mu M glycine and produced a depolarization of the membrane. This response was blocked when the NMDA antagonist CPP (6\mu M) was administered in the bath solution (Figure 2.2E). In total, 27 of 29 cells examined demonstrated functional NMDA responses.

We next explored the effects of acute alcohol exposure on the NMDA response, which in other model systems was attenuated by acute exposure to alcohol (Nie et al., 1994, Puglia and Valenzuela, 2010). In iPS-generated neurons, an acute exposure to 50mM alcohol significantly attenuated the peak amplitude of NMDA responses (p=0.036, Figure 2.3C). To further explore the use of human iPS-derived neural cells as a model to examine the effects of alcohol exposure, we explored whether tolerance to acute alcohol exposure could be observed in the culture after chronic alcohol treatment. Neural cultures were treated daily with 50mM alcohol for 7 days and alcohol withdrawn for 1 hr following transfer of coverslips to the recording chamber, and the acute effects of alcohol were measured. In neurons exposed to alcohol for 7 days, re-exposure to 50mM alcohol did not attenuate the NMDA response (p=0.77, Figure 2.3D). In contrast to results for NMDA receptors, no significant changes were observed following acute 50 mM alcohol exposure for GABA_A (n=4, p=0.27, data not shown) or AMPA receptors (n=5, p=0.19, data not shown). As we did not observe significant changes in responses of these receptors to acute
Figure 2.3. Acute Alcohol Attenuation of NMDA Response is Not Observed After 7-Days of Alcohol Exposure.

Whole-cell patch clamp recordings from neurons derived from a non-alcohol dependent subject. (A) Example of the time course illustrating the inhibition of the NMDA response by acute alcohol exposure (50 mM) in a naïve neuron. (B) Example of the lack of effect of alcohol on NMDA receptors following 7-day exposure to 50 mM alcohol. (C) Group data for the effect of acute alcohol which significantly reduced the amplitude of the NMDA response (n=8, paired t-test, p=.036). (D) Group data for 7-day alcohol pre-treated cells showing no significant effect of bath applied alcohol on NMDA response (n=10, paired t-test, p=.77). Differences in the mean amplitude of the baseline depolarization between naïve and chronically treated cells (panels C and D) relate in large measure to variation in the distance of the puffing pipette from the patched cells examined.
alcohol exposure, we did not examine the effect of a 7-day chronic alcohol treatment on GABA_A or AMPA responses to acute alcohol.

**Effect of Repeated Alcohol Exposure on NMDA Subunit mRNA Expression**

To examine whether chronic alcohol treatment produced changes in NMDA subunit mRNA expression, 15-18 week old human iPS-derived neural cultures derived from 4 alcoholic and 3 non-alcoholic donor subjects were fed daily with media containing 0 or 50mM alcohol and the levels of expression relative to sham treated cells were examined for the NMDA receptor subunit genes *GRIN1* (NR1 subunit), *GRIN2A* (NR2A subunit), *GRIN2B* (NR2B subunit) and *GRIN2D* (NR2D subunit) (Figure 2.4). For neural cultures derived from alcoholic subjects, significant treatment effects were seen after 7 days of alcohol exposure for *GRIN1* (F(1,37)=10.8, p=0.002), *GRIN2A* (F(1,39)=13.5, p=0.001), and *GRIN2D* (F(1,37)=16.1, p<0.001) mRNA levels. A trend towards significance was seen for *GRIN2B* (F(1,37)=3.7, p=0.06). Following a 24-hour withdrawal period, the expression of *GRIN1* in alcoholic-derived cultures remained significantly elevated (F(1,40)=5.0, p=0.03), the expression of *GRIN2A* continued to increase (F(1,40)=33.6, p<0.001), and the expression of *GRIN2B* and *GRIN2D* returned to baseline levels (F(1,43)=0.2, p=0.64, and F(1,40)=0.6, p=0.44, respectively). In contrast, following 7-day alcohol exposure of neural cultures derived from non-alcohol dependent subjects there was no change in the expression of the four NMDA subunit genes (*GRIN1* F(1,29)=1.0, p=0.32, *GRIN2A* F(1,25)=0.06, p=0.82, *GRIN2B* F(1,29)=0.75, p=0.39, *GRIN2D* F(1,27)=0.26, p=0.62). Withdrawal effects on mRNA concentration were available for only 2 of 3 cultures derived from non-alcoholics, precluding a meaningful analysis of that condition.
Subject-to-subject differences in responses were noted (Fig. 2.5 A-D), with a greater proportion of alcoholic subjects (4 out of 4) showing at least a 50% increase in expression for one or more NMDA receptor subunits compared to non-alcoholics (1 of 3) after a 7-day exposure.

**Discussion**

The objective of this study was to explore the potential utility of human iPS cells as a model to examine the biological actions of alcohol on human neural cells. We found that NMDA receptor function and mRNA expression in human iPS-derived neural cells responded to acute and chronic alcohol exposure similarly in several respects to that seen in rodent models. Specifically, acute exposure attenuated the NMDA but not AMPA receptor response, consistent with previous findings (Puglia and Valenzuela, 2010, Nie et al., 1994, Nagy, 2004, Dodd et al., 2000). In contrast we did not observe acute alcohol enhancement of GABA_A responses, which has been observed in some, but not all, reports of alcohol effects in dissociated cell rodent cultures (Kumar et al., 2009, Yamashita et al., 2006). GABA_A responses to acute alcohol may in part be dependent on indirect effects of alcohol on GABA_A receptors mediated by neuroactive steroids (Sanna et al., 2004). We also observed that 7-day alcohol exposure significantly increased NMDA receptor subunit mRNA expression, which parallels previous findings in rodent models (Nagy, 2008b, Hu et al., 1996, Follesa and Ticku, 1995). Our results suggest that human iPS-derived neural cells may provide a useful experimental model to examine mechanisms underlying changes in NMDA function and expression in response to acute and chronic alcohol, which remain incompletely understood (Hu et al., 1996, Hanchar et al., 2006).
Neural cells (age 12-15 weeks) derived from 7 different subjects, 3 non-alcoholics and 4 alcoholics, were treated daily with 50 mM alcohol for 7 days and levels of GRIN1, GRIN2A, GRIN2B, and GRIN2D mRNA expression was analyzed using RT-PCR. No significant changes were observed in the expression of these NMDA receptor subunit genes after 7-day alcohol exposure (relative to the sham condition) in neural cells derived from the group of non-alcoholic donor subjects (left group of data panels A-D). In contrast significant increases in mRNA levels compared to the sham condition were seen for neural cells derived from the alcoholic subject group for NMDA subunit genes (A) GRIN1, (B) GRIN2A, and (D) GRIN2D, while a trend towards significance was observed for (C) GRIN2B (p=0.06). Scatter plot symbols represent results from individual culture wells together with mean and SEM (significance level compared with sham condition * < 0.05, ** <0.01, *** <0.001).
Figure 2.5. Variability Between Subjects in NMDA Subunit mRNA changes following Alcohol Exposure and Withdrawal.

Individual subject data normalized to each subject’s average sham NMDA receptor gene expression normalized to GUSB. iPS-derived neural cells from non-alcohol dependent (Non-AD) subjects are indicated on the left side of the x-axis, while neural cell generated from alcohol-dependent (AD) subjects are indicated on the right. Only one non-AD subject demonstrated an increase in NMDA mRNA expression following 7 days of alcohol exposure. In contrast, under these conditions, all four AD subjects demonstrated increased expression of GRIN1, which returned to baseline levels in three of the four subjects. Three AD subjects demonstrated increased GRIN2A expression, which continued to increase after 24 hours of withdrawal in all three subjects. Two AD subjects showed increases in GRIN2B and GRIN2D expression; with the exception of GRIN2B in one AD subject, these changes returned to baseline after withdrawal. (Symbols: open bars – sham condition; solid bars – 7-day of alcohol exposure; gray bars – 7-day alcohol + 24-hour withdrawal; error bars represent SEM).
Perhaps more significantly, iPS-derived neural cells may provide a model to examine between-subject differences in molecular and physiologic responses to acute or chronic alcohol exposure, and in gene function with respect to between-subject genetic variation. Although preliminary, our results suggest that patterns of NMDA mRNA expression change following repeated alcohol treatments vary from subject to subject. Further, increases in expression may be more common in neural cells derived from alcoholic subjects than from non-alcoholics. This finding suggests that heritable traits may render gene expression in the NMDA system more reactive to effects of alcohol in persons predisposed to AD and may thereby contribute to the development of tolerance to the inhibitory effects of alcohol on the NMDA receptor activity. Although the list of genetic markers associated with alcohol dependence is expanding (Edenberg and Foroud, 2006, Enoch et al., 2009b, Kimura and Higuchi, 2011), the biological correlates of polymorphisms reported to be associated with alcohol dependence are in large part unknown. iPS cell technologies may provide a powerful tool with which to study the functional effects on neural tissue of alleles identified with alcohol dependence risk.

Future research might also use iPS cells as models to examine drug therapies for the treatment of alcohol use disorders or symptoms associated with alcohol withdrawal. A promising potential use for iPS cell technologies is to identify new drugs and drug targets, as well as to screen drugs for off-target toxicities (Cundiff and Anderson, 2011). Three of the most widely prescribed medications to treat alcohol use disorders include disulfiram, which disrupts alcohol metabolism, naltrexone, which antagonizes opioid receptors, and acamprosate, which disrupts glutamate signaling. However, each of these drugs has adverse effects associated with their use, and treatment efficacy varies among individuals,
and this variation may be associated with different genotypes (Miller, 2008, Johnson et al., 2011, Ray et al., 2009). Additional research is currently focusing on additional drugs for the treatment of alcohol use disorders, such as topiramate, which targets glutamate and GABA\textsubscript{A} receptors (Miller, 2008, Johnson, 2010). Our results show that functional glutamate and GABA\textsubscript{A} receptors are expressed in iPS-derived neural cultures. Because the use of iPS cell technologies allows us to obtain neural cultures from individuals with different genotypes, we propose that these cells could be used as models to evaluate drug therapies and their adverse effects in the treatment of alcohol use disorders. While the use of iPS cells to model pathophysiology and to screen potential drug therapies is still in its early stages, further research can be expected to clarify the potential benefits and limitations of this approach.

The use of human iPS cells to study the action of alcohol is a novel and promising means to evaluate group differences in the response to alcohol exposure. However, much remains to be learned about this approach and additional research is needed to address our study's limitations. Our electrophysiologic studies were limited to the examination of cells from a single non-alcoholic subject. Examination of receptor responses from cultures derived from multiple alcoholic and non-alcoholic subjects are needed to provide electrophysiologic validation of the expression results. Secondly, we isolated NMDA responses using a low magnesium solution containing DNQX, which allowed the natural agonists (glutamate and glycine) to activate the receptor. Others have reported that inhibition of NMDA receptor currents in response to alcohol is greater in the presence of magnesium when the synthetic agonist NMDA is used (Martin et al., 1991). Future studies may benefit from investigating the effect of alcohol under such conditions. Third, our puff
application of neurotransmitters produced a local dilution of alcohol during recordings to examine alteration of receptor responses by bath-applied alcohol. Use of a multi-barrel pipette for puff application could limit dilution of local alcohol concentration during receptor activation in such experiments. Fourth, as with any in vitro study, the neural cells we examined were not in their native environment and variation in culture conditions or well-to-well variability may impact patterns of gene expression (Newman and Cooper, 2010). Fifth, although the genetic sequence differences contributing to risk of alcohol dependence in neural cells derived from AD individuals are likely to be maintained in iPS cells, the environmental effect of chronic alcohol exposure in AD donor subjects (e.g. epigenetic, transcriptional or post-translational effects of alcohol on neural cells) are not likely to be preserved during reprogramming. Sixth, the results generated from our pilot data do not identify whether changes in mRNA expression directly lead to changes in NMDA receptor activity. Post-translational effects of alcohol on NMDA receptors have also been reported. Fyn kinase-mediated phosphorylation of tyrosine residues on the long intracellular tail of the NR2B subunit is stimulated by acute alcohol exposure and is thought to contribute to the development of acute tolerance to the inhibitory effects of alcohol on the NMDA receptor (Yaka et al., 2003b, Miyakawa et al., 1997). Finally, although our pilot study has focused on an examination of the effects of alcohol on the expression of NMDA receptor genes, study of other neurotransmitter systems, including GABA_A, in iPS derived neurons will also be important.

In summary, this study demonstrates that human iPS-derived neural cells provide a promising new model system to examine the molecular and biological effects of alcohol on human nervous tissue. To our knowledge, this is the first study to examine the effects of
acute and chronic alcohol exposure on human neurons derived from iPS cells. Using this subject-specific tissue, future research should aim to identify and examine molecular differences in neural cultures derived from individuals differentiated by the presence of alcohol tolerance and genetic markers of risk for alcohol dependence.
Chapter 3.

_GABRA2_ Alcohol Dependence Risk Allele is Associated with Reduced Expression of Chromosome 4p12 GABA\(_A\) Subunit Genes in Human Neural Cultures


Abstract

Background: Genetic variation in a region of chromosome 4p12 that includes the GABA\(_A\)-subunit gene _GABRA2_ has been reproducibly associated with alcohol dependence (AD). However, the molecular mechanisms underlying the association are unknown. This study examined correlates of _in vitro_ gene expression of the AD-associated _GABRA2_ rs279858*C-allele in human neural cells using an induced pluripotent stem cell (iPSC) model system.

Methods: We examined mRNA expression of chromosome 4p12 GABA\(_A\) subunit genes (_GABRG1, GABRA2, GABRA4, _and_ GABRB1_ in 36 human neural cell lines differentiated from iPSCs using quantitative PCR and Next Generation RNA Sequencing. mRNA expression in adult human brain was examined using the BrainCloud and Braineac datasets.

Results: We found significantly lower levels of _GABRA2_ mRNA in neural cell cultures derived from rs279858*C-allele carriers. Levels of _GABRA2_ RNA were correlated with those of the other three chromosome 4p12 GABA\(_A\) genes, but not other neural genes. Cluster analysis based on the relative RNA levels of the four chromosome 4p12 GABA\(_A\) genes identified two distinct clusters of cell lines, a low-expression cluster associated with rs279858*C-allele carriers and a high-expression cluster enriched for the rs279858*T/T genotype. In contrast, there was no association of genotype with chromosome 4p12 GABA\(_A\) gene expression in post-mortem adult cortex in either the BrainCloud or Braineac datasets.
Conclusions: AD-associated variation in GABRA2 is associated with differential expression of the entire cluster of GABA subunit genes on chromosome 4p12 in human iPSC-derived neural cell cultures. The absence of a parallel effect in post-mortem human adult brain samples suggests that AD-associated genotype effects on GABA expression, although not present in mature cortex, could have effects on regulation of the chromosome 4p12 GABA cluster during neural development.

Introduction

GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the human brain (Olsen and Sieghart, 2009). The fast inhibitory effect of GABA in the mature nervous system is mediated through GABA type-A (GABA_{A}) receptors, which are heteropentameric ligand-gated ion channels permeable to chloride (Hevers and Luddens, 1998). GABA_{A} receptors are distributed throughout the brain and composed of a diverse set of 19 subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3) that vary in expression across brain regions (for a review, see (Olsen and Sieghart, 2009). The various GABA_{A} receptor subtypes differ in subunit stoichiometry, a major determinant of their diverse pharmacological and electrophysiological properties (Barnard et al., 1998, Olsen and Sieghart, 2009). The majority of GABA_{A} receptors contain two α, two β, and 1 γ subunit, with the α1β2γ2 and α2β3γ2 combinations comprising 75-85% of GABA_{A} receptors in adult cortex (Rudolph et al., 2001).

In the human genome, the majority of GABA_{A} subunit genes are clustered on four chromosomes: 4p12 (β1, α4, α2, γ1), 5q34 (β2, α6, α1, γ2), 15q11 (β3, α5, γ3) and Xq28 (θ, α3, ε) (Steiger and Russek, 2004). Homologous clusters of genes are found on mouse
chromosomes 5, 11, 7 and X and are thought to have derived from a single ancestral $\alpha\beta\gamma$ cluster by gene duplication (Russek, 1999). Positioning of GABA$_A$ genes in tandem is believed to facilitate coordinated and tissue-specific co-regulation of gene expression by allowing clustered genes to share regulatory elements (Steiger and Russek, 2004, Barnard et al., 1998). Co-regulation of genes encoding the GABA$_A$ receptor subunits is evidenced by variation in RNA levels in post-mortem human brain samples over a range of developmental ages. At earlier stages of development, chr4p12 GABA$_A$ genes are expressed at higher levels than chromosome 5q34 genes. However, during development, chr4p12 genes are down regulated while chromosome 5q34 genes are up regulated (Fillman et al., 2010). Similarly, in rhesus monkeys, there is a developmental up regulation of the $\alpha1$ subunit and a down regulation of the $\alpha2$ subunit in the dorsolateral prefrontal cortex, which coincide with decreases in the decay time of miniature inhibitory post-synaptic potentials (Hashimoto et al., 2009).

Alcohol use disorders are prevalent, affecting more than 8% of the U.S. population (Grant et al., 2004a). They have multiple and complex etiologies including a strong genetic component, with heritability estimates ranging from 50-60% (Gelernter and Kranzler, 2009). Among the most widely studied genetic associations with alcohol dependence (AD) are those involving single nucleotide polymorphisms (SNPs) contained in a ~140 kb haplotype block spanning the 3’-region of GABRA2 and the adjacent intergenic GABRA2-GABRG1 region on chr4p12 (Covault et al., 2008, Edenberg et al., 2004, Covault et al., 2004). The association of SNPs in this region with AD has been replicated in multiple studies and across different populations (Ittiwut et al., 2012, Enoch et al., 2009a, Lappalainen et al., 2005, Bauer et al., 2007, Fehr et al., 2006, Soyka et al., 2008, Li et al., 2014), but lack of
replication has also been reported (Matthews et al., 2007, Lydall et al., 2011). SNPs in this haplotype block have also been associated with drug dependence (Agrawal et al., 2006), suggesting that variability in replication may relate in part to differing co-morbidities among samples. The most commonly examined tag-SNP in this region, rs279858, a synonymous SNP in exon 5 of GABRA2, was the only candidate marker associated with AD in a genome-wide analysis of candidate genes (Olfson and Bierut, 2012). The molecular mechanism by which GABRA2 polymorphisms influence risk for AD is unknown. The absence of a linked coding variant suggests that AD-associated variation in this region is in linkage disequilibrium with an as-yet-unidentified functional variant that influences the developmentally regulated expression of GABRA2 or adjacent chr4p12 GABA_A subunit genes. This could result in subtle differences in neural connectivity and subsequent behavioral effects. Support for this hypothesis comes from reports that SNPs in GABRA2 are associated with intermediate neural phenotypes, including fast beta frequency electroencephalographic (EEG) activity (Edenberg et al., 2004, Lydall et al., 2011), increased activation of the insular cortex during reward anticipation (Villafuerte et al., 2012), differences in activation of the medial frontal cortex and ventral tegmental area by alcohol cues (Kareken et al., 2010) and increased activation of the nucleus accumbens in reward anticipation paradigms (Heitzeg et al., 2014).

To explore molecular effects of AD-associated variants in the GABRA2 haplotype block, we used an induced pluripotent stem cell (iPSC) model system (Takahashi and Yamanaka, 2006, Takahashi et al., 2007). Generation of iPSCs from individuals with known genotypes and subsequent differentiation into neural cells provides an opportunity to explore molecular phenotypes of AD-associated genetic variants in vitro. We examined
neural cell cultures differentiated from 36 iPSC lines from 21 donor subjects with different GABRA2 rs279858 genotypes and RNA expression of genes encoding GABA<sub>A</sub> receptor subunits was examined. Our results indicate that chr4p12 AD risk alleles are associated with differences in RNA expression of all four GABA<sub>A</sub> genes located in this region in this in vitro model system.

**Materials and Methods**

*iPSC Generation*

Fibroblast cell lines were generated from skin punch biopsies donated by participants enrolled in a study of the effects of alcohol in social drinkers (9 males) (Milivojevic et al., 2014) or in a clinical trial for heavy drinkers (10 males and 2 females) (Kranzler et al., 2014a) at the University of Connecticut Health Center (UCHC). All fibroblast samples were from Caucasian donors. Fibroblasts were reprogrammed to pluripotency by the UCHC Stem Cell Core using retrovirus constructs expressing five factors (OCT4, SOX2, KLF4, c-MYC, and LIN28) for 34 lines or sendai virus constructs expressing four factors (OCT4, SOX2, KLF4, c-MYC) for 2 lines. 2 to 4 weeks after viral transduction, individual iPSC colonies were identified by morphological characteristics and selected clones were expanded and cultured as separate cell lines in human embryonic stem cell media on a feeder layer of irradiated mouse embryonic fibroblasts.

*Neural Differentiation*

Neural cells generated from 36 iPSC lines were examined, including two independent lines from each of 15 donors and one line each from an additional 6 donors.
iPSCs were differentiated into forebrain lineage neural cultures using an embryoid body-based protocol developed by the WiCell Institute (#SOP-CH-207 Rev A, www.wicell.org, Madison, WI). For details on the neural differentiation methods used see (Lieberman et al., 2012). Mature cultures (12 weeks post-neural plating) generated using this protocol contain a mixture of glial cells and neurons with spontaneous electrical activity, functional ionotropic GABA_A and glutamate receptors, and the ability to generate action potentials (Lieberman et al., 2012). A comparison of transcriptome profiles for human iPSC-derived forebrain lineage neural cultures and human post-mortem brain tissue suggests that 6-12 week iPSC neural cultures have a gene expression profile most similar to first trimester forebrain tissue (Brennand et al., 2014, Mariani et al., 2012).

**Reverse Transcription- Quantitative Polymerase Chain Reaction (RT-qPCR)**

Neural cultures 12-17 weeks post-plating were mechanically harvested and RNA extracted using TRlzol (Life Technologies, Carlsbad, CA). RNA concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Pittsburgh, PA) and cDNA was synthesized using a High Capacity cDNA Reverse Transcription kit (Life Technologies) using 2 µg of total RNA. Relative transcript levels represented in the resulting cDNA were measured by real-time qPCR using an Applied Biosystems 7500 instrument and FAM-labeled TaqMAN Assays (Life Technologies) for: GABRA1 (Hs00971228_m1), GABRA2 (Hs00168069_m1), GABRA4 (Hs00608034_m1), GABRB1 (Hs00181306_m1), GABRG1 (Hs00381554_m1), GABRG2 (Hs00381554_m1), GABRD (Hs00181309_m1), RBFOX3 (Hs01370653_m1), GAD2 (Hs00609534_m1), and SLC17A7 (Hs00220404_m1). VIC-labeled GUSB (β-glucuronidase, 4326320E) was used as
a within-well reference gene for normalization. Each sample was assayed in triplicate and a standard curve using cDNA generated from mature iPSC-derived neural cells from one subject was included on each qPCR assay plate to allow pooling of relative RNA expression levels among all samples. Ct values for target genes and the internal reference gene were converted to relative expression levels using the standard curve method, which compares each experimental sample with a calibrator reference RNA sample whose RNA level for each probe is defined as 1 (Wong and Medrano, 2005). PCR cycles were: 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds.

**Allelic Expression**

TaqMan (Life Technologies) FAM- and VIC-labeled genotyping probe and primer sets targeting exonic SNPs in GABRG1 (rs976156, C_8723970_1_), GABRA2 (rs279858, C_2073557_1_), GABRA4 (rs7660336, C_11278069_10), GABRB1 (rs10028945, C_2119811_30), and AKR1C3 (rs12529, C_8723970_1_) were used to examine allelic expression bias of the chr4p12 GABA_A cluster. 20μL TaqMan reactions containing 2μL of cDNA from iPSC-derived neural cells or genomic DNA from human fibroblasts were carried out in triplicate using Universal Master Mix II (Life Technologies) and an Applied Biosystems 7500 instrument. ΔC_T values were generated as the difference between FAM- and VIC-probes for the iPSC-derived neural cell cDNA samples or heterozygote fibroblast gDNA for each SNP. Consistent with other studies (Lin et al., 2012, Serre et al., 2008), we used a threshold for allelic imbalance as a 50% increase in RNA from one of the two alleles (i.e. ±0.5ΔC_T).
Post-mortem human brain datasets.

To compare our results from *in vitro* neural cultures with adult human brain GABA_A gene expression *in vivo*, we examined data from two publicly available post-mortem datasets: Brain Cloud and Braineac. For the Brain Cloud sample the microarray gene expression dataset (http://braincloud.jhmi.edu) (Colantuoni et al., 2011) was merged with SNP genotype data for each sample (dbGap dataset phs000417.v2.c1). We selected all adult Caucasian cases age 18-80 yo (mean=43.1) with an RNA integrity number ≥7.0 (mean RIN=8.25), resulting in a sample of n=60. The SNP dataset did not include rs279858, but rather a nearby marker, rs279856, tightly linked to rs279858 in Caucasians (r²=0.91; http://www.broadinstitute.org/mammals/haploreg/haploreg_v3.php). The Braineac dataset [http://braineac.org/; UK Brain Expression Consortium, (Trabzuni et al., 2011)] consists of microarray gene expression datasets for each of 134 Caucasian subjects together with whole genome SNP data. Donors ranged in age from 16-102 yo (mean=59.0) and RNA samples had an average RIN of 3.85 (range=1-8.5). The Braineac public dataset does not allow the selection of a subset of subjects based on age or RIN. Both rs279858 and rs279856 were reported in this dataset and were in complete LD (r²=1.0).

**RNA Sequencing**

RNA from 4 wells per neural culture was pooled as input for Next Generation Sequencing (NGS). RINs ranged from 7-9.7, with an average of 8.63. Poly-A selected (n=5) or ribosomal-RNA depleted (n=11) RNA was used to generate randomly primed libraries (200-500 bp inserts). NGS was conducted at the Genomics Core of the Yale Stem Cell Center using Illumina TruSeq Chemistry for library preparation and the Illumina HiSeq
2000 platform to generate 100-bp reads (average 44 million reads per sample). Sequence reads were aligned using Bowtie and TopHat (Trapnell et al., 2009) and gene expression levels quantified using Cufflinks (Trapnell et al., 2010) using a computational pipeline in the Department of Computer Science and Engineering, UConn-Storrs. Sequence tag reads were aligned to reference human genome hg19 and results normalized for exon length and the total sequence read number for each sample to generate Reads Per Kilobase of exon per Million mapped reads (RPKM).

**Statistical Analysis**

Statistical analysis was conducted using SPSS software v22 (IBM, Armonk, NY). Linear mixed-effects models were used to compare gene expression as a function of genotype or cluster membership to account for biological replicates for a subset of lines (biological replicates were available for 15 lines for qPCR data and 6 lines for RNA sequence data). Analysis was repeated using a single value per donor subject by averaging biological replicates and using t-tests for comparison of group means. Statistically significant contrasts identified using mixed-effects models were also seen using t-tests. Pearson correlations and two-step cluster analysis were used to examine the correlation in expression of GABA<sub>A</sub> genes and to group samples based on patterns of chromosome 4 GABA<sub>A</sub> gene expression. One-way ANOVA was used to compare adult brain RNA expression as a function of genotype. P values of ≤0.05 were considered significant.

**Results**

*GABRA2 RNA Expression Differs by rs279858 Genotype*
Mixed-effects models examining RT-qPCR GABRA2 RNA levels revealed a significant effect of GABRA2 rs279858 genotype (F=3.7, df=2,15.8, p=0.046). Neural cell cultures derived from rs279858*C-allele donor subjects had lower levels of GABRA2 RNA than T-allele homozygotes, Figure 3.1A (T/T vs. C/T F=4.9, df=1,20, p=0.039, T/T vs. C/C F=6.1, df=1,12.1, p=0.03, C/C vs. C/T F=0.10, df=1,10.8, p=0.76). In contrast, there was no significant effect of rs279858 genotype on RNA expression of RBFOX3 (Figure 3.1B, F=0.562, df=2,19.3, p=0.58), which encodes the neuron-specific transcription factor NeuN, suggesting that the between-sample differences in GABRA2 expression were not due to variability in the capacity of iPSC lines to differentiate into neurons.

**RNA levels for Chromosome 4p12 GABA<sub>A</sub> Genes are Correlated and Identify Two Clusters of iPSC Lines.**

RT-qPCR analysis revealed a significant correlation of RNA expression among the four consecutive chr4p12 GABA<sub>A</sub> subunit genes (GABRG1, GABRA2, GABRA4, and GABRB1) in iPSC-derived neural cultures (Table 3.1). This suggests that AD-associated genetic variation in GABRA2 may have regional effects on the expression of all four GABA<sub>A</sub> subunit genes. There were no correlations between RNA levels for any of the chr4p12 GABA<sub>A</sub> genes and GABRD (chr1p36, encoding the GABA<sub>A</sub> δ subunit), or RBFOX3 (chr17q25, encoding NeuN).

Cluster analysis identified two groups among the 36 neural cell lines: 17 in a low-expression Cluster 1 and 19 in a high-expression Cluster 2. There was a significant association between cluster membership and GABRA2 rs279858 genotype ($\chi^2= 9.9$, df=2, p=0.007): Cluster 1 cell lines had a greater frequency of C alleles than those in Cluster 2.
Figure 3.1. *GABRA2* RNA Expression by rs279858 Genotype

(A) qPCR data from 36 iPS-derived neural cell lines reveals a significant effect of *GABRA2* rs279858 genotype on *GABRA2* RNA levels. Expression was significantly lower in rs279858 C-allele carriers than in lines from T/T individuals. (B) There was no difference in RNA expression of *RBFOX3* (NeuN) as a function of rs279858 genotype. (*p* < 0.05)
Table 3.1. Correlations Among Chromosome 4p12 GABA<sub>A</sub> Subunit mRNA Expression Levels

<table>
<thead>
<tr>
<th></th>
<th>GABRG1</th>
<th>GABRA2</th>
<th>GABRA4</th>
<th>GABRB1</th>
<th>GABRD</th>
<th>RBFOX3</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABRG1</td>
<td>1</td>
<td>.865**</td>
<td>.727**</td>
<td>.781**</td>
<td>.137</td>
<td>-.165</td>
</tr>
<tr>
<td>GABRA2</td>
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<td>1</td>
<td>.699**</td>
<td>.713**</td>
<td>.109</td>
<td>-.098</td>
</tr>
<tr>
<td>GABRA4</td>
<td>.727**</td>
<td>.699**</td>
<td>1</td>
<td>.748**</td>
<td>.105</td>
<td>-.061</td>
</tr>
<tr>
<td>GABRB1</td>
<td>.781**</td>
<td>.713**</td>
<td>.748**</td>
<td>1</td>
<td>.118</td>
<td>.033</td>
</tr>
<tr>
<td>GABRD</td>
<td>.137</td>
<td>.109</td>
<td>.105</td>
<td>.118</td>
<td>1</td>
<td>.132</td>
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<tr>
<td>RBFOX3</td>
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<td>-.098</td>
<td>-.061</td>
<td>.033</td>
<td>.132</td>
<td>1</td>
</tr>
</tbody>
</table>

** p<0.01 (Pearson Correlation, 2-tailed)
(0.76 vs. 0.34; \( \chi^2 = 12.9, \text{df}=1, p=0.003 \)) (Table 3.2). Scatter plots comparing RNA levels for \textit{GABRA2} to those for \textit{GABRG1}, \textit{GABRA4}, or \textit{GABRB1} RNA, Figure 3.2, illustrate group differences in chr4p12 GABA\(_A\) subunit gene expression levels between the two clusters (Figure 3.2 A-C). Mixed-effects models identified significant differences in RNA expression between Cluster 1 and Cluster 2 for each of the chr4p12 GABA\(_A\) genes (Figure 3.3 A-D) (\textit{GABRG1} F=44.6, df=1,34, \( p<0.001 \), \textit{GABRA2} F=44.5, df=1,34, \( p<0.001 \), \textit{GABRA4} F=45.9, df=1,34, \( p<0.001 \), \textit{GABRB1} F=82.7, df=1,34, \( p<0.001 \)). There was no between-cluster difference in RNA levels for \textit{GABRD} (Figure 3.3E; F=0.34, df=1,30, \( p=0.86 \)) or \textit{RBFOX3} (Figure 3.3F; F=0.41, df=1,29, \( p=0.53 \)).

**RNA Sequencing**

RNA-Seq data were available from other projects for 10 of the iPS-derived neural cell lines. Sequence data for biological replicates from 6 of these lines were also available (i.e. a subset of iPSC lines were differentiated into neural cells on 2 distinct occasions). Including biological replicates, RNA-Seq data were available for 16 mature neural cultures derived from 10 iPSC lines.

RNA-Seq results confirmed the qPCR findings of lower expression of chr4p12 GABA\(_A\) genes in neural cell cultures from Cluster 1 vs. Cluster 2 (Figure 3.4B 2-5) (\textit{GABRG1} F=22.2, df=1,7.6, \( p=0.002 \); \textit{GABRA2} F=7.7, df=1,8.2, \( p=0.024 \); \textit{GABRA4} F=15.7, df=1,7.8, \( p=0.004 \); \textit{GABRB1} F=6.0, df=1,8.5, \( p=0.039 \)). There was no significant difference (\( p>0.50 \)) in the expression of genes encoding GABA\(_A\) subunits located on chromosomes 5q34 (\textit{GABRG2}, \textit{GABRB2}), 15q12 (\textit{GABRB3}), or 1p36 (\textit{GABRD}) (Figure 3.4C 1-4), or in the expression of the cation-chloride transporters NKCC1 (encoded by \textit{SLC12A2}) and KCC2 (encoded by
Table 3.2. *GABRA2* rs279858 Genotype by Chromosome 4p12 GABA<sub>A</sub> Gene Expression Levels

<table>
<thead>
<tr>
<th>Cluster #</th>
<th>Genotype Count (Frequency)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Allele Count (Frequency)&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>C/C 10 (0.59)  C/T 6 (0.35)  T/T 1 (0.06)</td>
<td>C 26 (0.76)  T 8 (0.24)</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4 (0.21)  5 (0.26)  10 (0.53)</td>
<td>13 (0.34)  25 (0.66)</td>
</tr>
</tbody>
</table>

<sup>a</sup> cluster x genotype $\chi^2 = 9.9$, df=2, p=0.007

<sup>b</sup> cluster x allele $\chi^2 = 12.9$, df=1, p=0.003
Figure 3.2. Clustering of Cell Lines by Chromosome 4p12 GABA_A Expression in Differentiated Neural Cells

Scatterplots of qPCR RNA levels contrasting (A) *GABRA2* vs. *GABRG1*, (B) *GABRA2* vs. *GABRA4*, and (C) *GABRA2* vs. *GABRB1* RNAs for 36 iPS-derived neural cell lines. RNA levels for Cluster 1 cultures (x symbol) were lower for all 4 genes than those for Cluster 2 cultures (gray circles).
RNA levels for chr4p12 GABA_{A} genes measured by qPCR were significantly lower in Cluster 1 cell lines (n=17) than in Cluster 2 cell lines (n=19): (A) GABRG1, (B) GABRA2, (C) GABRA4, and (D) GABRB1. There were no significant differences in RNA levels between clusters for (E) GABRD or (F) RBFOX3 (NeuN). (** p<0.001)
SLC12A5) (Figure 3.4C 5-6), which regulate the intracellular chloride concentration during development and determine whether GABA is an inhibitory or excitatory neurotransmitter (Stein et al., 2004). Similarly, with the exception of GABRA2 and GABRB1 genes, there were no significant differences between Cluster 1 and Cluster 2 neural cultures in the level of RNA for a panel of 74 neural patterning genes used by other investigators (Brennand et al., 2014) to compare developmental characteristics of iPSC-derived neural cultures (supplemental Table 1). These results suggest that the two clusters had a similar degree of neuronal maturation and forebrain lineage features. Interestingly, there was no difference in expression of the two genes directly adjacent to the chr4p12 GABA\textsubscript{A} cluster, GNPDA2 and COMMD8 (Figure 3.4B 1 and 6), located ∼1.3 Mb and 25 kb from GABRG1 and GABRB1, respectively (Figure 3.4A). Taken together, these findings strongly suggest that genetic variation in the GABRA2 AD-associated haplotype block influences developmental regulation of the entire cluster of chr4p12 GABA\textsubscript{A} subunit genes in this iPSC-derived neural culture model. Further, our findings were replicable across multiple neural differentiations of individual iPSC lines, as there were no differences observed in GABA\textsubscript{A} expression patterns from the iPSC-derived neural cells differentiated on different dates.

**Lack of Association of Genotype and GABA\textsubscript{A} Expression in Adult Post-Mortem Brain**

We examined the expression of chr4p12 GABA\textsubscript{A} genes in a set of 60 adult post-mortem Caucasian brain samples using a whole genome microarray gene expression dataset [http://braincloud.jhmi.edu; (Colantuoni et al., 2011)], merged with SNP array data for each sample (dbGap dataset phs000417.v2.c1). Because rs279858 was not available in the SNP dataset for these samples, we examined 4p12 GABA\textsubscript{A} gene expression as a function
Figure 3.4. RNA-Seq Confirms qPCR Results and Delineates Differences in Expression of Chromosome 4p12 GABA<sub>A</sub> Genes

RNA-seq data from 16 iPSC-derived neural cell lines generated from 10 donor subjects. (A) Schematic depicting the orientation of the chr4p12 GABA<sub>A</sub> cluster and adjacent genes. Arrows indicate the direction of transcription, with the approximate distance shown between each gene. (B) There were significant differences in expression between neural cell lines in Cluster 1 and Cluster 2 for the chr4p12 GABA<sub>A</sub> genes GABRG1; GABRA2; GABRA4; GABRB1, but not for the genes directly adjacent to the chr4p12 GABA<sub>A</sub> cluster GNPDA2, and COMMD8. (C) GABA<sub>A</sub> genes located on other chromosomes did not differ in expression between neural cell lines in Cluster 1 and Cluster 2 (GABRG2, GABRB2, GABRB3, GABRD). Genes encoding cation-chloride transporter proteins also did not differ between Cluster 1 and Cluster 2 (SLC12A2 and SLC12A5). (* p<0.05; ** p<0.005)
of the GABRA2 intron 4 SNP, rs279856 located 3.3 kb from rs279858 and in near-complete linkage disequilibrium with it ($r^2=0.91$) in Caucasians. There was no significant relationship of rs279856 genotype with GABRA2 RNA expression ($F=0.67$, df=2,57, $p=0.52$). Similar results were obtained examining expression data for a collection of post-mortem adult brain samples from 134 subjects of European descent [http://braineac.org/; UK Brain Expression Consortium, (Trabzuni et al., 2011)]. There was no association of rs279858 genotype with GABRA2 RNA expression in either frontal cortex ($F=0.43$, df=2,124, $p=0.65$) or hippocampus ($F=1.25$, df=2,119, $p=0.29$).

**Complex Genetic Regulation of the Chromosome 4p12 GABA_A Cluster**

When we examined the within-cluster expression of GABRA2 as a function of rs279858 genotype in the set of 36 iPSC-derived neural cultures, we found that expression did not differ by genotype among cell lines within Cluster 1 or 2 (Figure 3.5). The lack of a gene-dose effect was not unexpected in Cluster 1, given the very low expression phenotype and small variability between lines (Figure 3.2). In contrast, we expected that an autosomal Mendelian cis- or trans-acting effect of a rs279858-linked functional variant on RNA levels would be observable in Cluster 2 cultures because GABRA2 expression was readily detectable with significant between-sample variation in Cluster 2 lines.

A potential alternative genetic model more consistent with these results would include a cis-acting genetic locus influencing expression of the chr4p12 GABA_A gene cluster paired with a random, mitotically-stable, allelic expression bias. In this model, a stochastic clonal allelic expression bias for the chromosome carrying a cis-acting repressive functional variant linked with the rs279858*C-allele would determine Cluster 1 vs. 2
membership for a given iPSC line. Support for a model that includes allelic expression bias in this region was obtained using RT-qPCR and TaqMan allele-specific probes to examine neural cell RNA from 16 Cluster 2 cell lines heterozygous for exonic SNPs in at least one of the chr4p12 GABA\(_A\) genes (\textit{GABRG1, GABRA2, GABRA4} and \textit{GABRB1}). We observed allelic imbalance [a 50\% or greater increase in RNA from one of the two alleles, i.e. >±0.5ΔC\(_T\) (Serre et al., 2008, Jeffries et al., 2012)] in chr4p12 GABA\(_A\) RNA expression compared to fibroblast genomic DNA in 7 of 16 lines (44\%). In contrast, 0 of 11 lines (0\%) heterozygous for an exonic SNP in the \textit{AKR1C3} gene (chr10p15) showed an imbalance in expression of the two alleles (Fisher’s exact test, \(\text{p}=0.02\)). Of the lines that were heterozygous at two or more chr4p12 GABA\(_A\) genes, 6 of 8 lines displayed concordant allelic expression across markers (i.e., either a greater than ±0.5ΔC\(_T\) difference for each gene marker or no bias for each gene). Both of the lines that were discordant over the interval displayed allelic bias in 3 of 4 GABA\(_A\) genes, but with the most distal gene (\textit{GABRG1} in one line, \textit{GABRB1} in the other line) showing less than a 0.5ΔC\(_T\) difference. In the case of donor 727 (rs279858*T/T genotype), neural cells differentiated from two independent iPSC clones (727-6 and 727-8) had allelic bias for opposite chromosomes over this region (Figure 3.6). This is consistent with a random, mitotically-stable clonal event occurring at the time of iPSC generation producing complementary allelic bias in the chr4p12 GABA\(_A\) genomic interval for the 2 independent iPSC lines from this subject.

**Discussion**

iPSC technologies provide novel model systems to examine the molecular and cellular effects of disease-associated genetic variation using disease-relevant cell types not
Figure 3.5. Expression of GABRA2 RNA by rs279858 Genotype in Cluster 1 vs. Cluster 2 Neural Cell Lines

Cluster 1 iPSC-derived neural cell lines had lower levels of GABRA2 RNA and a greater number of C-allele carriers than Cluster 2 lines, but levels of GABRA2 RNA did not show a rs279858 allele dose effect between cell lines within each cluster.
Figure 3.6. Random Allelic Expression Bias of Chromosome 4p12 GABA\textsubscript{A} Genes in independently derived iPSC-neural cultures from a Cluster 2 Subject

Allele biased gene expression in mature neural cultures generated from two independently derived iPSC lines (727-6 and 727-8) from a Cluster 2 donor homozygous for the T-allele at GABRA2 rs279858 but heterozygous at exonic SNPs in the other three chr4p12 GABA\textsubscript{A} genes (rs976156 GABRG1 exon 3 Thr88Thr, rs7660336 GABRA4 3UTR, and rs10028945 GABRB1 3UTR). An opposite pattern of allelic bias (ΔCT between FAM and VIC probes) was observed comparing lines 727-6 and 727-8 for each of the heterozygous SNPs in (A) GABRG1, (B) GABRA4, and (C) GABRB1. Expression of opposite alleles for each gene in the two lines spanning the 4p12 GABA\textsubscript{A} gene region indicates the presence of a random process during iPSC induction that can produce bias in expression from either the maternal or paternal chromosome.
otherwise readily available. In the current study, we generated neural cultures from 36 iPSC lines derived from 21 donor subjects characterized by rs279858, a synonymous T-to-C polymorphism in exon 5 of GABRA2. Previous research has shown associations between the rs279858*C allele and increased risk for AD (Ittiwut et al., 2012, Enoch et al., 2009a, Lappalainen et al., 2005, Bauer et al., 2007, Fehr et al., 2006, Soyka et al., 2008, Li et al., 2014), marijuana and illicit drug dependence (Agrawal et al., 2006), anxiety (Enoch et al., 2006), and differences in the risk of relapse or likelihood of drinking following treatment for substance abuse (Bauer et al., 2012, Bauer et al., 2007). Despite these multiple behavioral associations of GABRA2 variation in humans, the cellular and molecular components underlying the associations are not understood.

We found that relative levels of RNA for the chr4p12 GABA<sub>A</sub> subunit genes GABRG1, GABRA2, GABRA4, and GABRB1 were significantly inter-correlated in neural cultures derived from iPSC lines. RNA levels for these GABA<sub>A</sub> subunits identified distinct low-expression and high-expression clusters of cell lines. The low-expression cluster had a higher frequency (0.76 vs. 0.34) of AD-associated GABRA2 rs279858*C alleles than the high-expression cluster. Examination of next-generation RNA–Seq data available for 10 neural cell lines allowed us to extend our analysis to include the expression of GABA<sub>A</sub> genes located on multiple chromosomes. RNA-Seq data confirmed our qPCR results showing low expression of chr4p12 GABA<sub>A</sub> genes in cell lines that comprise Cluster 1, and extended our findings by showing that GABA<sub>A</sub> subunit genes located on other chromosomes showed no difference in expression as a function of expression cluster. Furthermore, transcripts of the GNPDA2 (glucosamine-6-phosphate deaminase 2) and COMMD8 (COMM domain-containing protein 8) genes that flank the chromosome 4 GABA<sub>A</sub> cluster were readily detected in
samples in which the four GABA<sub>A</sub> genes were minimally expressed. Together, these RNA-Seq and qPCR results suggest that the effects of the AD-associated GABRA2 polymorphism on gene regulation are specific for the ∼1.4 Mb region containing the chr4p12 GABA<sub>A</sub> gene cluster.

Our results suggest that the GABRA2 haplotype block containing rs279858 harbors a functional polymorphism with regulatory effects that influence the expression of all four GABA<sub>A</sub>-subunit genes on chr4p12 in parallel. These results are consistent with other work suggesting the presence of multi-gene locus control elements within the 4p12 and 5q34 GABA<sub>A</sub> subunit gene clusters. In two independently derived mouse GABRA6 knockout lines, in which neomycin insertion cassettes were used to target exon 8 of GABRA6, expression of the two adjacent genes, GABRA1 and GABRB2, was reduced in the forebrain, suggesting the presence of a locus control element in the chromosome 5q34 GABA<sub>A</sub> gene cluster (Uusi-Oukari et al., 2000). In addition, human and primate studies provide evidence of a coordinated developmental switch in the relative expression of GABA<sub>A</sub> subunits (Fillman et al., 2010, Hashimoto et al., 2009).

The lack of association of rs279858 with the expression of chr4p12 GABA<sub>A</sub> genes in adult post-mortem brain suggests that genetic variation in this region may increase risk for substance-related disease via developmental mechanisms. However, more research is needed to show a developmental influence of chr4p12 variation in vivo. Evidence of genetic variation influencing development but contributing to disease risk in adulthood has been described for the serotonin transporter-linked polymorphic region, 5-HTTLPR of SLC6A4. Carriers of the 5-HTTLPR S-allele are more susceptible to anxiety and depression (Caspi et al., 2010), have reduced functional and white matter connectivity of the amygdala
and anterior cingulate cortex (Pacheco et al., 2009, Pezawas et al., 2005) and show enhanced amygdala activation to fear stimuli (reviewed in (Munafo et al., 2008). In rodents, anxiety- and depression-like behavior in adult mice were only seen when the serotonin transporter was disrupted early in development (Ansorge et al., 2004), suggesting a developmental effect of SLC6A4 genotype on susceptibility to anxiety, depression, and regional brain connectivity in adulthood.

GABA_A receptors play key roles in cortical development. Early in development, GABA_A receptors are excitatory due to a high intracellular chloride concentration and provide the main excitatory drive onto immature neural cells (Lee et al., 2005). This early excitatory function is important for normal corticogenesis, cell proliferation, and synaptic formation (Wang and Kriegstein, 2009). Because GABA_A subunit stoichiometry affects channel properties (Olsen and Sieghart, 2009), we speculate that the genotype-associated differences in GABA_A subunit expression may impact GABA signaling effects on cellular connectivity during development. Reported associations of GABRA2 genetic variation with EEG beta frequency oscillations (Edenberg et al., 2004, Lydall et al., 2011), increased activation of the insula (Villafuerte et al., 2012) and nucleus accumbens (Heitzeg et al., 2014) during reward anticipation, and differences in the activation of reward pathways by alcohol cues (Kareken et al., 2010) are consistent with a developmental model of the effects of the AD-associated GABRA2 genetic variant rather than an alteration in ligand binding.

Analysis of RNA expression by RT-qPCR produced two unexpected observations. First, although the rs279858*C-allele was enriched in low expression Cluster 1 cell lines (Table 1), the within-cluster expression of chr4p12 GABA_A genes did not differ by GABRA2 genotype (Figure 3.5). Therefore, the genotypic association with expression level
illustrated in Figure 3.1A reflects the greater frequency of rs279858*C-allele carriers in Cluster 1 vs. Cluster 2 (0.94 vs. 0.47), suggesting that the genetic effect linked to rs279858 strongly determines Cluster membership. The absence of an rs279858 genotype effect within Cluster 2 cell lines suggests that a simple Mendelian genetic model is not sufficient. One model consistent with our results includes a cis-acting genetic locus (in linkage disequilibrium with rs279858) that influences the expression of the chr4p12 GABA\(_A\) gene cluster together with a recently discovered phenomenon of random clonal allelic-biased expression of a subset of autosomal non-imprinted genes (Chess, 2012). Specifically, we hypothesize that a random stochastic process occurring during the reprogramming of individual fibroblasts to generate iPSC clones results in random allelic bias for the expression of chr4p12 GABA\(_A\) genes. In this model, iPSC allelic expression bias for the chromosome carrying a cis-acting repressive functional variant linked with the rs279858*C-allele would determine Cluster membership for a given iPSC line.

Autosomal random stochastic inactivation was initially described for a group of immune and nervous system gene families (see Chess, 2012 for review) including immunoglobulins, interleukin receptors, odorant receptors, and neural protocadherin genes. More recent genomic studies have revealed that random mono-allelic expression is widespread and involves a much larger number of autosomal genes (5-20% in mammalian cells) (Gimelbrant et al., 2007, Serre et al., 2008). Mitotically stable, random mono-allelic expression has been demonstrated in clonal lymphoblastoid cell lines (Gimelbrant et al., 2007, Serre et al., 2008), in clonal groups of cells in placental tissue (Gimelbrant et al., 2007), in iPSCs and iPSC-derived neural cultures (Lin et al., 2012), and in human clonal neural stem cells derived from fetal brain and spinal cord (Jeffries et al., 2012). This
process typically does not result in exclusively mono-allelic expression but rather clonal lines expressing either one or both alleles at loci showing random clonal allelic bias potential (Gimelbrant et al., 2007, Jeffries et al., 2012). Functional outcomes of allelic bias in the expression of cell surface proteins include the potential for increasing the cellular diversity of immune and neural cells.

A second unexpected observation was the discordance in cluster assignment for pairs of neural cell lines derived from 4 individual donors. The 36 iPSC lines examined included 2 independent iPSC clones from each of 15 donor subjects. Paired lines from 11 donors (9 rs279858 homozygotes and 2 heterozygotes) were concordant (i.e., both lines from a given donor were grouped in the same Cluster), while paired lines from 4 subjects (1 rs279858*T/T, 1 C/C, and 2 T/C) were discordant. We speculate that the AD-associated rs279858*C-allele is in moderate but not complete linkage disequilibrium with an as-yet-identified repressive functional variant, and that iPSC-derived rs279858 homozygote neural cells that are discordant in chr4p12 GABA_A gene expression are in fact heterozygous for this unidentified functional variant. Allelic bias could then reveal the cis-effect of the functional repressive C-allele linked variant in heterozygotes. Our results demonstrating a modest allelic bias in expression of the entire chr4p12 GABA_A gene region (e.g., Figure 3.6) suggests that allelic bias may partially explain the complex genetic regulatory features in this region.

In summary, we found correlated gene expression of four genes encoding GABA_A receptor subunits located in a 1.4 Mb region of chr4p12, with GABA_A gene expression levels significantly associated with a tag-SNP for this region that has been linked to AD. The endophenotype of low mRNA expression for the chr4p12 GABA_A gene cluster in this iPSC in
vitro model may be a more useful marker than the diagnosis of AD to identify functional polymorphisms in this region. Our results suggest that such functional variants may produce behavioral effects relevant to AD via effects on GABA\textsubscript{A} gene expression in the developing nervous system. This work supports the utility of iPSCs as a model system to explore previously unknown molecular effects of genetic variation associated with risk for neuropsychiatric disease. Limitations of such models include the need to examine multiple donor and iPSC lines to address inter- and intra-cell line variability and the limited ability of cultures to replicate the prolonged and complex development of the human nervous system \textit{in vivo}. Finally, our finding that nearly half of iPSC lines produce neural cultures with strikingly low levels of expression of the chr4p12 GABA\textsubscript{A} gene cluster may be of interest to other investigators using this model system to examine neuropsychiatric conditions, particularly if only a limited number of disease and control donor lines are compared.
Supplemental Table 3.1. Comparison of RNA levels for neural patterning genes in Cluster 1 vs. Cluster 2 neural cell cultures.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Patterning identity</th>
<th>RPKM (mean)</th>
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<td>Cluster 2 (n=10)</td>
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74 neural patterning genes from Brennand et al., 2014 for comparing developmental and lineage similarity for groups of iPSC-derived neural cells.
Chapter 4.

*FKBP5* Genotype Interacts with Early Life Trauma to Moderate Heavy Drinking in

College Students

The following chapter is a duplicate version of a manuscript currently under review: Lieberman, R., Armeli, S., Scott, D.M., Kranzler, H.R., Tennen, H., and Covault, J. American Journal of Medical Genetics Part B: Neuropsychiatric Genetics

Abstract

Alcohol use disorder (AUD) is debilitating and costly. Identification and better understanding of risk factors influencing the development of AUD remain a research priority. Although early life exposure to trauma increases the risk of adulthood psychiatric disorders, including AUD, many individuals exposed to early life trauma do not develop psychopathology. Underlying genetic factors may contribute to differential sensitivity to trauma experienced in childhood. The hypothalamic-pituitary-adrenal (HPA) axis is susceptible to long-lasting changes in function following childhood trauma. Functional genetic variation within *FKBP5*, a gene encoding a modulator of HPA axis function, is associated with the development of psychiatric symptoms in adulthood, particularly among individuals exposed to trauma early in life. In the current study, we examined interactions between self-reported early life trauma, past-year life stress, and a single nucleotide polymorphism (rs1360780) in *FKBP5* on heavy alcohol consumption in a sample of 1845 college students from two university settings. Although we found no effect of early life trauma on heavy drinking in rs1360780*T*-allele carriers, rs1360780*C* homozygotes exposed to early life trauma had a lower probability of heavy drinking (p < 0.01). The absence of an interaction between current life stress and *FKBP5* genotype on heavy
drinking (p = 0.52) suggests that there exists a developmental period of susceptibility to stress that is moderated by FKB5 genotype. These findings implicate interactive effects of early life trauma and FKB5 genetic variation on heavy drinking.

Introduction

Alcohol use disorder (AUD) is a complex psychiatric disorder affecting 14% of the U.S. population during a one-year period (Grant et al., 2015). Identifying factors that influence the development of AUD would be beneficial in the identification of at-risk individuals and potentially in the treatment of those with AUDs. A meta-analysis of 124 studies showed that abuse and neglect experienced during childhood are significantly associated with depressive disorders, substance use, suicide attempts, and risky sexual behavior (Norman et al., 2012). Early life stress is also associated with greater alcohol intake and abuse (Enoch, 2011), which predispose to alcohol-related health problems and the development of AUD (Kranzler et al., 1990, Dawson and Archer, 1993).

Data from the National Comorbidity Survey indicate that childhood adversity including childhood maltreatment, trauma, and stressful life events such as divorce or death of a family member are reported by 45% of subjects with childhood-onset and 26-32% of individuals with adult-onset psychiatric illness (Green et al., 2010). Yet many individuals exposed to early trauma/stress do not develop psychopathology. Genetic variation is likely one contributor to between-person sensitivity or resilience to adverse outcomes following trauma. Exposure to childhood stressful events can influence reactivity of the hypothalamic-pituitary-adrenal (HPA) axis, effects that can persist into adulthood, as
shown by dysregulated release of the glucocorticoid cortisol to acute stress in adults with a history of physical or emotional adversity (Lovallo et al., 2012).

Termination of the HPA axis stress response is in part regulated via the glucocorticoid receptor (GR), a ligand-activated transcription factor located in the cytosol with a high affinity for glucocorticoids (Schatzberg et al., 2014, de Kloet et al., 2005). The FK506 binding protein (FKBP5), a glucocorticoid receptor chaperone encoded by FKB5, has been implicated in the pathophysiology of HPA axis abnormalities associated with traumatic events. FKBP5 protein functions to down regulate GR activity by reducing GR binding affinity for cortisol and inhibiting GR translocation into the nucleus (Sinclair et al., 2013, Grad and Picard, 2007, Stechschulte and Sanchez, 2011). Hormone-activated GR binding at regulatory sites in the FKB5 gene leads to an upregulation of FKB5 mRNA expression, creating an ultra-short feedback loop reducing GR signaling and prolonging the HPA axis response (Binder, 2009). Polymorphisms in a 100-kb haplotype block in FKB5 have been associated with risk of several stress-related conditions, including post-traumatic stress disorder (PTSD) (Binder et al., 2008), anxiety, depression (Binder et al., 2004, Lavebratt et al., 2010), addictive disorders (Levran et al., 2014, Huang et al., 2014, Jensen et al., 2015) and aggressive or violent behavior (Bevilacqua et al., 2012). The most widely studied tag SNP for this haplotype block, rs1360780 is located in an enhancer region adjacent to a GR binding site and has been shown to have functional effects using reporter gene assays (Klengel et al., 2013). The minor T-allele of the rs1360780 polymorphism has been associated with risk of trauma-related psychiatric symptoms (Zannas and Binder 2014) and greater FKB5 mRNA and protein levels (Binder et al., 2004, Binder, 2009), which are thought to contribute to an altered HPA axis regulation. The
association of *FKBP5* genotype and several stress-related psychiatric symptoms depends on an interaction of genotype with exposure to stressful life events (Zannas and Binder 2014). *FKBP5* rs1360780 genotype interacts with early life but not adult trauma in predicting PTSD symptom severity (Binder et al., 2008, Xie et al., 2010, Boscarino et al., 2012), while *FKPB5* genotype interactions with both early and lifetime stress have been reported for depression and suicide (Roy et al., 2010, Appel et al., 2011, Zimmermann et al., 2011). In the setting of childhood adversity, rs1360780 is associated with epigenetic effects including altered chromatin looping and DNA methylation patterns in *FKBP5* (Klengel et al., 2013), changes that alter the expression of the *FKBP5* gene. Interactions between rs1360780 genotype and self-reported childhood emotional neglect predicted greater threat-related amygdala reactivity in adolescents (White et al., 2012) and adults (Holz et al., 2014), suggesting that *FKBP5* genotype × environment interactions may lead to developmental changes in neural circuits.

Importantly, the specific effects of *FKBP5* genotype and its interaction with stress may vary by developmental age. For example, in a study of 310 infants, the rs1360780 minor T-allele was associated with greater cortisol reactivity to minor stress (Luijk et al., 2010). However, no main effect of genotype was observed on salivary cortisol levels in a sample of 368 healthy adults in response to the Trier Social Stress Test (TSST) (Mahon et al., 2013). In contrast, among young adults homozygous for the rs1360780*C* allele, those with greater childhood adversity showed an attenuated cortisol response to the TSST. In this study, cortisol response to the TSST in T-allele carriers did not differ as a function of childhood adversity (Buchmann et al., 2014), which suggests that the *C/C* genotype confers
protective effects on the stress-activated cortisol response in adults with a history of childhood adversity.

In the present study, we investigated the association of heavy drinking in a large sample of African-American and Caucasian college students based on self-reported early life trauma, current life stress, and a well characterized single nucleotide polymorphism (SNP), rs1360780, in the FKB5 gene.

Methods

Participants and procedure

Undergraduate students were recruited over a 4-year period (2008-2011) at a Historically Black College and University (HBCU) and a New England Public University (NEPU) through the psychology research pool and campus-wide broadcast emails and flyers inviting students to participate in a study about daily experiences and health-related behavior. Only students who reported drinking alcohol at least twice in the past month and had not received treatment for alcohol use were eligible. Participants were compensated for their participation after giving written, informed consent to participate in the protocol, which was approved by the institutional review boards at each university.

Participants at both universities first completed a web-based baseline survey that included various demographic questions, an inventory of traumatic life experiences prior to age 6 and negative life events in the past year. Participants also provided saliva samples for genotyping. Approximately two weeks after completing the baseline survey, students accessed a secure website each day from 2:30–7:00 PM for 30 days to complete a brief survey. This time window was selected to coincide with most undergraduate students' naturally occurring end of the school day, but before typical evening activities began.
(including drinking). Relevant to our study, participants were asked each day to report on their alcohol consumption for the past evening (i.e., after the previous day’s survey) and for the current day. If a daily survey was not completed, the participant was reminded by email to complete the next day’s survey. Further, if participants failed to complete a daily survey, during their next login, the server queried them about their drinking during missed intervals lasting up to 3 days.

The baseline sample at the HBCU consisted of 741 students of which 53% were female and 96% were self-identified African Americans (or of African descent). Three-hundred three individuals were excluded from analysis because they did not complete at least 15 daily diary entries or had missing data in the relevant baseline measures, resulting in a final sample of 438 students (58% female and 97% of African descent) for analysis with a mean age of 20.0 years ($SD = 1.6$). Approximately half of the students (48%) were freshmen or sophomores. Excluded participants were more likely to be male, $\chi^2(1) = 13.41, p < 0.001$. The final sample did not differ from excluded individuals on age, $t(735) = 1.02, p = 0.31$.

The baseline NEPU sample consisted of 1815 students, 78.0 of which were self-identified European Americans, 11.0% Asians, 4.5% African Americans, 4.0% Hispanic/Latinos and 2.8% misc./other. Exclusion of Asian and misc./other students resulted in a sample of 1600. An additional 190 participants were excluded because they did not complete at least 15 daily diary entries and 3 additional students had missing data in the relevant baseline measures, resulting in 1407 participants eligible for analysis. The final NEPU sample was 55% female; had a mean age of 19.2 years ($SD = 1.4$); was comprised mostly of freshmen or sophomores (74%), and was predominantly Caucasian.
[91% (n = 1278)], with small percentages of African Americans [4.9% (n = 69)] and Hispanics/Latinos [4.3% (n = 60)]. Participants excluded from this sample were more likely to be male, $\chi^2(1) = 23.7, p < 0.001$, and or racial/ethnic minorities, $\chi^2(2) = 7.0, p = 0.03$. The final sample did not differ from excluded individuals on age, $t(1595) = .008, p = 0.99$. Across both universities, we had a final sample of 1845 (54% female).

**Measures**

*Alcohol use* was measured each day by asking participants how many standard alcoholic drinks they consumed the previous night (from 0 to 15, in one-drink increments, with an option for >15) as well as the number of drinks, if any, they had consumed on the day of the diary report up to the time of logging their response. Students were reminded each day that a standard drink was defined as one 12-oz can or bottle of beer or wine cooler, one 5-oz glass of wine, or a 1.5-oz measure of liquor straight or in a mixed drink. If participants missed a daily survey, they were queried about drinking levels on the missed day(s) on the next occasion that they logged into the system (before they completed that day's survey); 16.4% and 10.7% of the daily records were backfilled in the HBCU and NEPU samples, respectively. We created full-day records by combining daytime and previous night reports from consecutive days. Lagging values from subsequent day reports allowed us to use only 29 of the reporting days. The number of drinks was summed across daytime and evening drinking and was converted into a binary heavy drinking indicator [4+ drinks for women and 5+ drinks for men (NIAAA, 2004)].

*Early life trauma* (ELT) was measured using the Traumatic Events Screening Inventory (TESI)-Adult Screening version 3.2 (Ford et al., 2000). The TESI has 18 items that assess various types of trauma, with a total of nine trauma categories (i.e.,
accident/illness/disaster, traumatic loss/separation, traumatic physical victimization, traumatic sexual victimization, traumatic emotional victimization, traumatic domestic violence victimization, witnessed trauma, traumatic war victimization, and other traumatic event). Participants indicated (yes/no) as to whether the events occurred before age 6, between ages 6 and 17, above age 18 and within the past year. We created a binary variable reflecting the occurrence of at least one early life traumatic event prior to age 6.

Past year stressful life events were measured using the Life Events Scale for Students (LESS; Linden, 1984). We used the 25 unambiguously negative items selected by Covault et al. (2007). Students were asked which of the events occurred in the past year; we created a composite by summing the number of endorsed events.

Genotyping

DNA was extracted from saliva samples using the Oragene DNA salivary extraction kit (DNA Genotek, Kanata, Ontario, Canada) per the manufacturer’s instructions. The FKB5 SNP rs1360780 was genotyped using an Applied Biosystems TaqMan Assay On-Demand probe and primer set (C_8852038_10) with Universal Master Mix II (Life Technologies, Carlsbad, CA) per the manufacturer’s instructions. We used 10μL PCR amplification reactions containing 1μL DNA that were run in 96-well plates with the following PCR conditions: 95° for 10 minutes, followed by 40 cycles of 95° for 15 seconds and 60° for 60 seconds. Post-PCR fluorescent plate reads were carried out on an Applied Biosystems 7500 instrument and analyzed using Applied Biosystems TaqMan Genotyper software. 10% of the samples were repeated with complete concordance. Genotypes for each racial/ethnic group were in Hardy Weinberg equilibrium (p = 0.93; 0.26 and 0.39 respectively for EA, AA and Hispanic groups). The minor allele frequency (MAF) was
higher in the AA subject group (0.38; \( c^2 = 19.6, \text{df}=2, \ p < 0.001 \)) compared with the EA (0.31) or Hispanic (0.29) groups consistent with MAF reported in the 1000 genomes data set for this SNP (European ancestry 0.29 and African ancestry 0.39). The 2-level genotype counts (rs1360780*C homozygotes vs. rs1360708*T carrier) were as follows: European American C/C = 611, T-allele carrier = 668; African American C/C = 184, T-allele carrier = 317; Hispanic/Latino; C/C = 34, T-allele carrier = 31.

**Data analysis**

We used generalized estimating equations to model the additive and interactive effects of early-life trauma, past-year negative life events, and the main and interactive effects of *FKBP5* rs1360780 genotype and either early-life trauma or past-year negative life events to predict heavy drinking days. Given the binary nature of the outcome variable (heavy drinking day: present/absent), we specified a logit link and binomial error distribution. Predictors were entered in 2 blocks: main effects and 2-way interactions. Values shown in the tables reflect effects for the block of entry (i.e., without the subsequent blocks included). *FKBP5* rs1360780 genotype was collapsed into binary predictors (minor T-allele carrier vs. C/C individuals). Data from both universities were combined into one sample. We controlled for school (0 = HBCU, 1 = NEPU), weekly variation in drinking by including a weekday-weekend contrast (weekdays = 0, weekend = 1), ethnicity (with 2 dummy codes comparing African descent and Latino/Hispanic with Caucasian [the reference group coded 0]), age, and year in school (with 3 dummy codes comparing each year with freshmen [the reference group coded 0]).

**Results**

Descriptive statistics
We had daily drinking reports on 12,489 days (28.5 days per person) in the HBCU sample and 39,906 days (28.4 days per person) in the NEPU sample. Overall, we had 52,395 person-days for analysis nested within 1845 participants. Drinking was reported on 25.8% and 20.6% of the days and heavy drinking on 11.6% and 13.1% of the days in the HBCU and NEPU samples, respectively. Early-life traumatic events were reported by 35.5% and 21.4% of participants in the HBCU and NEPU samples, respectively. The mean number of stressful life events in the past year was 5.66 (SD = 3.65) in the HBCU sample and 3.94 (SD = 2.74) in the NEPU sample.

*Models predicting heavy drinking days*

Table 4.1 shows the results for the logistic regression model predicting heavy drinking days. In block 1 neither ELT nor *FKBP5* genotype were associated with heavy drinking, but recent life stress was a significant predictor, with higher levels of past-year negative events associated with a greater probability of heavy drinking. In block 2, only the interaction between *FKBP5* genotype and ELT was significant. Follow-up probing of the interaction indicated a non-significant effect of early life trauma on heavy drinking in *FKBP5* rs1360780 T-allele carriers (B = -0.23, SE = 0.14, p = 0.51, OR = 1.25). In contrast, ELT had a significant effect on heavy drinking in *FKBP5* rs1360780 C/C participants, such that C/C participants reporting a positive history of early life trauma had a lower frequency of heavy drinking (B = -0.29, SE = 0.15, p = 0.003, OR = 0.747) (Figure 4.1). It should be noted that the school and race contrasts were highly correlated. However, examination of variance inflation factors (VIF) for these predictors indicated that they were not problematic, i.e., the VIF values were below 6, which is less than the commonly used cut-off
Table 4.1. Model Predicting Heavy Drinking Days

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<td>0.085</td>
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<td>0.147</td>
<td>0.481</td>
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<tr>
<td>Junior</td>
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<td>0.335</td>
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<td>0.504</td>
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<tr>
<td>Beyond senior</td>
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<td>&lt;.001</td>
<td>0.605</td>
<td>1.674</td>
<td>3.124</td>
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<td>&lt;.001</td>
<td>-0.542</td>
<td>-0.309</td>
<td>0.653</td>
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<tr>
<td>Negative life events (NLE)</td>
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<td>0.010</td>
<td>&lt;.001</td>
<td>0.024</td>
<td>0.062</td>
<td>1.044</td>
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<td>0.071</td>
<td>.074</td>
<td>-0.267</td>
<td>0.012</td>
<td>0.881</td>
</tr>
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<td>0.058</td>
<td>.918</td>
<td>-0.120</td>
<td>0.108</td>
<td>0.994</td>
</tr>
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<tr>
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<td>0.244</td>
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<td>0.012</td>
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Note. B = change in log of odds for a unit change in predictor. Early trauma 0 = no, 1 = yes; FKBP5: 0 = CC genotype, 1 = T-allele carrier; Sex: 0 = males, 1 = females; Weekend -1 = weekday, 1 = weekend.
Daily self-reports of alcohol consumption in a diverse sample of 1845 college students revealed no significant effect of early life trauma on the probability of heavy drinking in carriers of the *FKBP5* rs1360780*T*-allele (B = -0.23, SE = 0.14, p = 0.51, OR = 1.25). In contrast, a significant effect of early life trauma on the probability of heavy drinking was observed in *FKBP5* rs1360780*C/C* genotype individuals (B = -0.29, SE = 0.15, p = 0.003, OR = 0.747). (**p < 0.01, NS = not significant**)

Figure 4.1. Interactive Effect of *FKBP5* rs1360780 and Early Life Trauma in Predicting Heavy Drinking
of 10 (Chatterjee, 2000). More importantly, removal of these contrasts did not alter the interaction findings of interest.

We also ran several supplemental analyses. First, we augmented the model to examine whether the \textit{FKBP5} genotype \times ELT interaction varied by university. To do this, we entered the 2-way interactions among university, ELT and genotype along with the 3-way university \times genotype \times ELT. None of these interactions was significant (all p’s > 0.50). We then estimated a similar model to evaluate the moderating effect of sex (instead of university); again, none of 2-way or 3-way interactions was significant (all p’s > 0.20)

\textbf{Discussion}

In the current study we examined heavy drinking, a risk factor for alcohol-related health problems and the development of alcohol use disorder (Kranzler et al., 1990, Dawson and Archer, 1993), in 1845 participants from two universities as a function of \textit{FKBP5} rs1360780 genotype. We found no main effect of \textit{FKBP5} rs1360780 genotype on the probability of heavy drinking but a significant interaction effect of \textit{FKBP5} genotype and early-life trauma on the probability of heavy drinking in students of African or European ancestry. In contrast, we did not see an interaction of genotype with the number of past-year negative life events in predicting heavy drinking. These findings are in agreement with prior research reporting no main effect of \textit{FKBP5} genotype, but a genotype \times early-life trauma interaction effect on the subsequent occurrence of psychiatric symptoms (Zannas and Binder, 2014). Our results complement previous findings in relation to gene by environment effects of \textit{FKBP5} and early-life trauma and expand them to include a link between these factors and heavy drinking in young adults.
Previous work has implicated the *FKBP5* rs1360780 minor T-allele as a risk allele for psychiatric symptoms in individuals exposed to early life trauma. Consistent with the direction of allelic effects previously reported, we observed that T-allele carriers reported more heavy drinking days in the setting of early life trauma than C-allele homozygotes, while in the absence of early life trauma the frequency of heavy drinking did not differ by rs1360780 genotype. This interaction observed did not reflect a greater frequency of heavy drinking among T-allele carriers with a history of childhood trauma, but a lower frequency of heavy drinking days among C-allele homozygotes who had been exposed to childhood trauma. This result suggests that individuals with the C/C genotype who experience early adversity may be protected from heavy drinking as young adults.

Resilience, defined as an individual’s ability to adapt successfully to adversity, is an active process involving changes at the molecular level that result in normalization of biological and behavioral functions in the setting of stress (Russo et al., 2012). Resilience develops in approximately 10-25% of maltreated children (Walsh et al., 2010). The biological mechanisms leading to resilience in some children but not others exposed to early-life trauma are not understood but likely involve interactions between the type and timing of trauma/adversity and genetic variation, epigenetic responses, social supports and psychological factors. Related to our finding, Buchmann et al. (2014) examined the interaction between *FKBP5* rs1360780 genotype and early life trauma on cortisol increases following the Trier Social Stress Test (TSST) in healthy young adults. They found that a history of early life trauma interacted with the *FKBP5* rs1360780*C/C* genotype to moderate TSST-induced increases in cortisol levels (Buchmann et al., 2014). This group also reported that higher levels of self-reported childhood emotional neglect were
associated with reduced threat-related amygdala reactivity in rs1360780*C/C homozygotes (Holz et al., 2014). Interestingly, polymorphisms in the HPA axis-related gene encoding the corticotropin releasing hormone receptor 1 (CRHRI) have been shown to be protective with respect to effects of early-life trauma on the development of depression in adulthood (Bradley et al., 2008, Polanczyk et al., 2009). Similarly, functional genetic variation in the NPY gene, which encodes the anxiolytic neuropeptide Y, has been implicated in resiliency to stress (Zhou et al., 2008), and higher plasma levels of neuropeptide Y promote resilience to PTSD in combat-exposed veterans (Yehuda et al., 2006). Neuropeptide Y has also been implicated as protective from greater alcohol consumption in stress-exposed primates (Lindell et al., 2010). Although the drinking motives potentially linking the interactive effects of early-life stress and FKBP5 genotype on the frequency of heavy drinking in college students are not clear, the emerging literature on the effects of FKBP5 genotype suggest that they may relate to developmental effects of early-life stress x genotype interactions on hormonal stress response regulation (Buchmann et al., 2014) and/or developmental effects on neural systems related to threat/stress reactivity (Holz et al., 2014, White et al., 2012).

Strengths of the current study include: i) a large sample that was diverse in gender, race/ethnicity, and socioeconomic background, ii) the self-reporting of alcohol consumption near its real-time occurrence, thus limiting recall error and bias, and iii) the examination of heavy drinking in college students, which is a public health concern. Weaknesses of the study include: i) the retrospective self-reporting of both early-life trauma and past-year life stress via questionnaire and ii) the lack of distinction between the proportion of heavy drinking related to stress vs. social enhancement or emotion-
related drinking. Because the frequency of stress-related drinking was not well defined, it is unclear whether \textit{FKBP5} genotype interacts with early-life trauma to produce differences in coping-related drinking or social-related activities in the college student sample examined.

In conclusion, we found that the \textit{FKBP5} rs1360780*C/C genotype interacts with self-reported history of early-life trauma to predict fewer days of heavy alcohol consumption in young adult college students. To our knowledge, this is the first study to implicate interactions between early life trauma and variation in \textit{FKBP5} with alcohol consumption. Importantly, these results are consistent with the assignment of risk and protective alleles at this locus with prior reports identifying interactions of rs1360780 with childhood trauma on risk of PTSD and depression.
Chapter 5.

Examining FKBP5 mRNA Expression in Human iPSC-Derived Neural Cells

The following chapter is a duplicate version of a manuscript to be submitted in 2015.

Lieberman, R., Kranzler, H. R., and Covault, J.

Abstract:

The transcriptional activity of the glucocorticoid receptor (GR, encoded by *NR3C1*) is regulated by molecular chaperones including FK506 Binding Protein 51 (FKBP5, encoded by *FKBP5*). In peripheral blood leukocytes, *FKBP5* mRNA expression is upregulated following activation of the GR via its binding to GR response elements (GRE) located in promoter and intronic regions of the *FKBP5* gene, creating a short feedback loop. The single nucleotide polymorphism rs1360780 in one *FKBP5* intronic GRE has been associated with psychiatric illness and functional molecular effects on protein and mRNA expression, chromatin structure, and DNA methylation. However, examination of the molecular effects of rs1360780 has largely been limited to easily accessible peripheral cells, and may not reflect activity in neural cells. To examine *FKBP5* mRNA expression following GR activation in human neural cells, we utilized induced pluripotent stem cells (iPSCs) derived from 20 subjects. Following differentiation into forebrain-lineage mixed neural cultures, cells were exposed to 1 μM dexamethasone for 6 hr and mRNA expression of *FKBP5* and *NR3C1* was analyzed using qPCR. Results from the iPSC-derived neural cells were compared with those from a subset of 15 fibroblast lines. We found no significant change in mRNA expression of either *FKBP5* or *NR3C1* in iPSC-derived neural cultures following dexamethasone treatment. However, there were significant changes in *FKBP5*
and NR3C1 in fibroblasts, mimicking previous findings in peripheral blood-derived cells. There was no effect of FKB5 rs1360780 genotype on FKB5 mRNA expression in neural cells or fibroblasts. These results suggest that iPSC-derived forebrain-lineage neural cells are not a suitable cell model for researchers wanting to examine relationships between GR activation, FKB5 expression, and genetic variation in human neural cells in vitro, and raise the question whether results seen in non-neural cells are broadly representative of glucocorticoid effects on FKB5 expression in neural cells.

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis response to stress requires an interaction of many signaling molecules to function properly. Dysregulation of the stress response can be damaging, potentially increasing vulnerability to disease, particularly if the affected individual is genetically predisposed (De Kloet, 2004). Indeed, differences in response to stress have been observed in individuals with a variety of psychiatric disorders including schizophrenia, bipolar disorder, depression, post-traumatic stress disorder, and alcohol use disorder, among others. However, the molecular mechanisms that underlie dysregulation of the stress response, particularly those in the human brain, remain unclear.

The glucocorticoid receptor (GR) is a cytosolic transcription factor activated by the stress hormone cortisol (de Kloet et al., 2005, Schatzberg et al., 2014). Following cortisol binding, the GR translocates into the cell nucleus and functions as a regulator of transcription by binding to specific DNA sequences in promoter or enhancer regions called GR response elements (GREs) (de Kloet et al., 2005, Binder, 2009, Grad and Picard, 2007). GR translocation is a complex process involving a variety of molecular chaperones and co-
chaperones including the FK506 binding protein 51 (FKBP5), which is encoded by the
FKBP5 gene. FKBP5 is part of a protein heteromeric-complex that represses the
transcriptional activity of the GR by reducing cortisol binding affinity and inhibiting GR
translocation to the nucleus (Sinclair et al., 2013, Grad and Picard, 2007, Stechschulte and
Sanchez, 2011). Following GR activation, FKB5 mRNA is upregulated via GR binding to
GREs located in promoter and intronic regions of FKB5. Increased levels of FKB5 protein
inhibit GR activity, creating a short negative feedback loop whereby GR activation down
regulates GR function (Binder, 2009, Zannas and Binder, 2014a).

Polymorphisms within a 100 kb haplotype block in FKB5 that is tagged by the
rs1360780 single nucleotide polymorphism (SNP) have been linked to a number of stress-
related psychiatric conditions including PTSD, anxiety, depression, and addictive disorders
(Binder et al., 2008, Klengel et al., 2013, Lavebratt et al., 2010, Binder et al., 2004, Kang et
al., 2012, Roy et al., 2010, Levran et al., 2014, Jensen et al., 2014, Huang et al., 2014). For
PTSD, childhood trauma, but not adult trauma, interacts with FKB5 genotype to predict
symptoms, while for other stress-related disorders FKB5 genotype interactions have been
reported with both childhood and lifetime stressors (Zannas and Binder, 2014a). These
genetic variants may lead to differential expression of FKB5 mRNA and protein by altering
chromatin structure and DNA methylation (Binder et al., 2004, Klengel et al., 2013, Binder,
2009), which may have functional long-lasting consequences for an individual’s response
to stress. For example, in a study of 310 infants, carriers of the FKB5 rs1360780 minor T-
allele had increased cortisol reactivity to minor stress (Luijk et al., 2010). However, no
genotype effect was observed on salivary cortisol levels in a sample of 368 healthy adults
using the trier Social Stress Test (Mahon et al., 2013), suggesting that genotype effects on
stress response may vary during development. *In vitro* evidence for developmental state-specific regulation of *FKBP5* comes from examination of human hippocampal progenitor cells, where dexamethasone exposure during the proliferation/differentiation phase, but not during the post-proliferation/differentiation phase, induced long lasting DNA demethylation of *FKBP5* (Klengel et al., 2013). However, studies examining *FKBP5* mRNA expression following GR activation or as a function of genotype have largely relied on easily accessible peripheral blood cells, immortalized human cell lines, or animal models, suggesting expression studies using human neural cells will be of importance, since gene expression profiles in peripheral cells are weakly correlated with expression in the central nervous system (Sullivan et al., 2006). To our knowledge no study has examined the effect of dexamethasone exposure on *FKBP5* induction in genetically characterized human neural cells, which supports the utility of an *in vitro* human iPSC-derived neural cell model system.

The purpose of this study was to examine gene expression changes in iPSC-derived human neural cells *in vitro* following acute GR activation with the synthetic glucocorticoid dexamethasone. We reprogrammed fibroblasts generated from 20 donor subjects into induced pluripotent stem cells (Takahashi and Yamanaka, 2006, Takahashi et al., 2007) and, using an established protocol to differentiate stem cells into a forebrain lineage, examined the effects of a 6 hr dexamethasone exposure on mRNA expression of *FKBP5* and the GR gene *NR3C1* in human neural cells. Gene expression changes following acute dexamethasone treatment were compared between iPSC-derived neural cultures and a subset of 15 donor subjects’ fibroblasts.

**Materials and Methods**
**Human iPSCs**

iPSCs were generated from fibroblasts obtained using a skin punch biopsy of the inner, upper arm from 20 participants enrolled in either a laboratory study examining effects of alcohol or a clinical treatment study at the UCONN Health Center (UCHC, Farmington, CT) (Kranzler et al., 2014a, Milivojevic et al., 2014). Biopsy samples were minced and cultured in Dulbecco's modified eagles medium (DMEM, Life Technologies) supplemented with 20% fetal bovine serum (FBS, Life Technologies), 1x non-essential amino acids (Life Technologies) and 1x penicillin/streptomycin (Life Technologies). Fibroblast cultures were expanded and passaged using trypsin (Life Technologies) prior to being frozen or sent for reprogramming.

The UCHC Stem Cell Core (Farmington, CT) reprogrammed fibroblasts to pluripotency using either a retrovirus to express five factors (OCT4, SOX2, KLF4, c-MYC, and LIN28) (N=13) or a sendai virus to express four factors (OCT4, SOX2, KLF4, and c-MYC) (N=7). Two to four weeks after viral transduction, multiple pluripotent stem cell colonies for each subject were selected and expanded as individual clones. Twenty-seven iPSC lines generated from the 20 donor subjects were used for the current experiments, with 2 clones examined from seven donor subjects for whom data from the two clones was averaged and plotted as a single point along with those from the other 13 subjects.

As previously described (Lieberman et al., 2012), iPSCs were cultured on a feeder layer of irradiated mouse embryonic fibroblasts using human embryonic stem cell media containing DMEM with F12 (DMEM/F12, 1:1 ratio, Life Technologies) supplemented with 20% Knockout Serum Replacer (Life Technologies), 1x non-essential amino acids, 1 mM L-glutamine (Life Technologies), 0.1 mM β-mercaptoethanol (MP Biomedicals, Solon, OH),
and 4 ng/mL of basic fibroblast growth factor (bFGF, Millipore). iPSCs were monitored daily and colonies exhibiting spontaneous differentiation were manually removed. Media was fully replaced daily and cells were cultured for 7 days, or to confluency, before being passaged using 1 mg/mL Dispace (Life technologies) in DMEM/F12.

**iPSC Neural Differentiation**

iPSCs were differentiated into neural cell cultures as previously described (Lieberman et al., 2012) using an established protocol developed by the WiCell Institute for the differentiation of human embryonic stem cells into neural cells of a forebrain lineage (#SOP-CH-207, REV A, www.wicell.org, Madison, WI). Briefly, we used an “Embryoid Body” (EB)-based protocol wherein iPSC colonies are removed from the feeder layer substrate and cultured in suspension before going through a neural induction phase to generate primitive neuroepithelial cells. Following 3 weeks of culture in neural induction media, cells were dissociated and cultured in 24-well plates on Matrigel (BD Biosciences, Bedford, MA) coated glass coverslips in neural differentiation media containing the neural growth factors B27 supplement (Life Technologies), laminin (Sigma-Aldrich), brain-derived neurotrophic factor (BDNF, Peprotech, Rocky Hill, NJ), glial-derived neurotrophic factor (GDNF, Peprotech), and insulin-like growth factor 1 (IGF-1, Peprotech). All cells were incubated at 37°C in 5% CO₂.

**Fibroblast Culture**

Fibroblasts available from 15 of the 20 donor subjects were thawed from liquid nitrogen storage and plated into 12-well culture dishes in fibroblast media containing
DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies) and 1x non-essential amino acids (Life Technologies). Cells were cultured to confluency for 10-14 days with full media changes twice per week.

**Immunocytochemistry**

Six to eight weeks after being plated onto glass coverslips, neural cultures were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and permeabilized using 0.2% triton X-100 (Sigma-Aldrich) in PBS for 10 min. Following a 1-hr block using 5% donkey serum (Jackson ImmunoResearch, West Grove, PA), cultures were incubated overnight at 4°C with the following primary antibodies diluted in 5% donkey serum in PBS: mouse anti-beta III-tubulin (1:500, Covance, Dedham, MA), mouse anti-GFAP (1:500, Millipore, Brillerica, CA), rabbit anti-MAP2 (1:500, Millipore), and rabbit anti-TBR1 (a forebrain glutamatergic neuromarker; 1:1000, ProteinTech Group, Chicago, IL) incubated overnight with the addition of 0.1% triton X-100. Cells were then incubated at room temporate for 45 min in donkey anti-mouse alexa flour 594 (1:1000, Life Technologies) and donkey anti-rabbit alexa flour 488 (1:1000, Life Technologies) secondary antibodies diluted in 3% donkey serum in PBS for visualization.

**Acute Dexamethasone Exposure**

Six to seven week-old neural cells and ≈95% confluent fibroblast primary cultures were exposed to 1 μM dexamethasone (Sigma-Aldrich) for 6 hr. This concentration of dexamethasone has previously been shown to increase expression of *FKBP5* in human peripheral blood mononuclear cells (Vermeer et al., 2004). Dexamethasone was
reconstituted to a concentration of 100 mM in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and diluted to experimental concentrations in either neural differentiation media for use with neural cells or in fibroblast media for use with fibroblasts. On the day of the experiment, plates of cells were removed from the incubator and 100% of the media was aspirated and replaced with 500 μL of neural differentiation media or 1 mL of fibroblast media containing either vehicle (DMSO) or dexamethasone. For neural cells, 4-6 wells of a 24-well plate were used per condition. For fibroblasts, 3 wells of a 12-well plate were used per condition. Cells were returned to the incubator during the 6 hr exposure.

**Seven-Day Dexamethasone Exposure**

Neural cell cultures differentiated from 10 cell lines (N=4 rs1360780*C homozygotes and N=6 T-allele carrier) derived from seven donor subjects were exposed to a priming dose of 100nM dexamethasone two weeks after neural plating. On the first day of treatment, media was aspirated and fresh neural differentiation media containing 100nM dexamethasone was added to the neural cell cultures. Dexamethasone-containing media was fully replaced after three days for an additional four days. Following seven days of treatment, media was fully aspirated and replaced with normal neural differentiation media for an addition three weeks, after which neural cells were acutely exposed to neural differentiation media containing 1μM dexamethasone or DMSO as described above. For this pilot experiment, two separate iPSC clones were examined from 3 donor subjects.

*Quantitative Polymerase Chain Reaction (qPCR)*
Following 6 hr dexamethasone exposure, RNA was extracted from the neural cell and fibroblast cultures using TRIzol reagent (Life Technologies) according to the manufacturer’s protocol. RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Pittsburgh, PA) and 50 µL of cDNA was synthesized from 2 µg of RNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies) per the manufacturer’s protocol. Additionally, the RNA samples for all subjects underwent the same preparation without the addition of reverse transcriptase to test for potential DNA contamination.

cDNA was analyzed by qPCR using an Applied Biosystems 7500 instrument (Life Technologies) and FAM-labeled TaqMAN Assay on Demand (Life technologies) probe and primer sets for NR3C1 (Hs00353740_m1) and FKBP5 (Hs01561006_m1). Expression of these genes was normalized to the VIC-labeled housekeeping gene GUSB (beta-glucuronidase, 4326320E, Life Technologies). cDNA synthesized from RNA extracted from each individual well was plated in triplicate onto an Optical 96-well plate (Life technologies) and 20 µL reactions were carried out using Gene Expression Master Mix (Life Technologies) per the manufacturer’s protocol, with the addition of 0.1% bovine serum albumin (BSA, Life Technologies).

PCR cycles were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. A standard curve consisting of a 4-level serial dilution of 100%, 50%, 25%, and 12.5% cDNA from neural cells differentiated from one subject was added to each plate to determine relative mRNA expression between all subjects and across different qPCR plates. Data shown in graphs are displayed as mRNA expression normalized to the
housekeeping gene *GUSB* where a unit of 1 is equivalent to the same abundance of the target gene relative to *GUSB* in the reference RNA sample.

**Statistical Analysis**

Gene expression was analyzed for statistical significance via One-Way ANOVA with a Bonferroni post-test comparing selected cell type and treatment condition using GraphPad Prism software (V5.0f for Mac, GraphPad Software, San Diego, CA, [www.graphpad.com](http://www.graphpad.com)). Statistical significance was identified as *p*<0.05. Post-hoc testing compared drug treatment within cell type, basal differences in gene expression between neural cells and fibroblasts, and between cell type and drug treatment as a function of rs1360780 C/C vs. T-carrier genotype.

**Results**

*mRNA Expression of FKBP5 and NR3C1 is Altered in Fibroblasts but not in iPSC-Derived Neural Cultures Following Dexamethasone Treatment*

iPSCs derived from the 20 subjects were differentiated into mixed neural cultures containing MAP2, Beta III-Tubulin, and TBR1-positive neurons and GFAP-positive astrocytes (Figure 5.1A-B). Acute dexamethasone treatment did not significantly alter mRNA expression of either *FKBP5* (post-hoc test, *p*>0.05, Figure 5.2A) or *NR3C1* (pot-hoc test, *p*>0.05, Figure 5.2B) in iPSC-derived neural cultures.

Fibroblasts used for iPSC reprogramming were available from 15 of the 20 donor subjects. Acute dexamethasone treatment significantly increased expression of *FKBP5* (post-hoc test, *t*=12.1, *p*<0.0001, Figure 5.2A) and decreased expression of *NR3C1* (post-
hoc test, \( t=2.6, \ p<0.05 \), Figure 5.2B) in fibroblast cultures. Comparing expression levels between neural cells and fibroblasts revealed a significantly higher level of \( NR3C1 \) mRNA in fibroblasts at baseline (post-hoc test, \( t=18.2, \ p<0.0001 \), Figure 5.2B) with no significant difference observed for baseline levels of \( FKB5 \) (post-hoc test, \( p>0.05 \), Figure 5.2A).

**FKBP5 mRNA Expression in Neural Cells and Fibroblasts Does Not Differ by rs1360780 Genotype**

The 20 neural cell lines examined consisted of 7 rs1360780*C homozygotes and 13 rs1360780*T-allele carriers (12 C/T and 1 T/T). No significant effect of dexamethasone was observed in neural cells derived from donor subjects of either genotype (post-hoc test, \( p>0.05 \), Figure 5.3), and no significant difference in basal expression was observed (post-hoc test, \( p>0.05 \)).

The 15 fibroblast lines examined included 6 rs1360780*C homozygotes and 9 rs1360780*T-allele carriers (8 C/T and 1 T/T). Exposure to 1 \( \mu \)M dexamethasone significantly upregulated \( FKB5 \) mRNA expression in fibroblasts from both C/C (post-hoc test, \( t=7.4, \ p<0.0001 \)) and T-allele carriers (post-hoc test, \( t=9.3, \ p<0.0001 \)) (Figure 5.3). No significant difference was observed between C/C and T-allele carrier genotypes in the basal expression of \( FKB5 \) (post-hoc test, \( p>0.05 \)) or its induction by dexamethasone exposure (post-hoc test, \( p>0.05 \)).
Figure 5.1. iPSCs Differentiate into Mixed Neural Cultures

7 weeks post-plating iPSC-derived neural cultures contain a mixture of neurons and glia. (A) Neural cultures contain MAP2-positive neurons (green) and GFAP-positive astrocytes (red). (B) The deep layer forebrain nuclear marker TBR1 (green) is seen co-localizing in the nucleus of neurons marked by the neural marker Beta III-Tubulin (red). Nuclei are labeled with DAPI (blue). Scale bars represent 20μm.
Figure 5.2. *FKBP5* and *NR3C1* mRNA Expression in Fibroblasts and iPSC-Derived Neural Cultures Following 6 Hr Dexamethasone Exposure

(A) A significant effect of dexamethasone exposure was observed on *FKBP5* mRNA expression in fibroblasts (post-hoc test, t=12.1, p<0.001) but not iPSC-derived neural cells (post-hoc test, p>0.05) (B) A significant effect of dexamethasone exposure was observed on *NR3C1* mRNA expression in fibroblasts (post-hoc test, t=2.6, p<0.05) but not in iPSC-derived neural cells (post-hoc test, p>0.05). Comparison of basal expression in fibroblasts vs. iPSC-derived neural cells revealed significantly lower *NR3C1* mRNA expression in iPSC-derived neural cell cultures (post-hoc test, t=18.2, p<0.0001). (****p<0.0001, ***p<0.001, *p<0.05)
Figure 5.3. Genetic Variation in *FKBP5* Does Not Associate with Differences in *FKBP5* mRNA Induction Following Dexamethasone Exposure

FKBP5 Expression by rs1360780 Genotype in Neural Cells and Fibroblasts

Dexamethasone did not significantly increase *FKBP5* mRNA expression in iPSC-derived neural cells derived from either rs1360780 genotype (C/C; post-hoc test, p>0.05, T-carrier; post-hoc test, p>0.05). A significant upregulation of *FKBP5* mRNA was observed in fibroblasts derived from donor subjects of both rs1360780 genotype (C/C; post-hoc test, t=7.4, p<0.0001, T-carrier; post-hoc test, t=9.3, p<0.0001). No significant difference was observed between rs1360780 genotype in the induction of *FKBP5* mRNA following dexamethasone treatment in fibroblasts (post-hoc test, p>0.05). (**p<0.0001, ***p<0.001, *p<0.05)
Early Exposure to Dexamethasone does not Induce Responsiveness to Acute Dexamethasone in Neural Cells by rs1360780 Genotype

In view of a prior report identifying an FKBP5 rs1360780 genotype interaction on long-lasting DNA de-methylation with early exposure to dexamethasone using a neural cell line (Klengel et al., 2013), in an exploratory fashion we examined whether iPSC-derived neural cells from rs1360780 C/C vs. T-allele carriers would become responsive to dexamethasone following exposure to a priming dose of 100nM dexamethasone at an earlier time point of cell maturation. A subset of 10 iPSC-derived neural cell lines (4 rs1360780*C homozygotes, and 6 T-allele carriers) were exposed to 100nM dexamethasone for seven days, following which cells were cultured in normal neural media for three-weeks prior to a 6 hr 1μM dexamethasone challenge (Figure 5.4A). We chose to expose the neural cultures at two-weeks due to the morphological characteristics of the culture, in which cultures are generally sparse while multiple neurites projecting from plated cell clusters can be observed with migrating cells along the processes. Treatment with 100nM dexamethasone at week two post-plating did not induce changes in FKBP5 mRNA expression following 6 hr 1μM dexamethasone challenge at six-weeks in neural cells derived from either rs1360780 C/C (post-hoc test, p>0.5) or T-allele carrier (post-hoc test, p>0.05) donor subjects (Figure 5.4B).

Discussion

The differentiation of iPSCs enables researchers to examine difficult-to-obtain human cell types *in vitro*. In the current study, we examined the effect of acute exposure to 1 μM dexamethasone on mRNA expression of FKBP5 and NR3C1 in 27 human neural cell
Figure 5.4. Developmental Pre-Treatment with Dexamethasone Does Not Activate FKBP5 mRNA Expression in Neural Cells Following Subsequent Acute Dexamethasone Exposure

(A) A subset of 10 iPSC-derived neural cell lines from 4 rs1360780*C homozygotes and 6 rs1360780*T-allele carriers were pre-treated with 100nM dexamethasone at two-weeks post-plating for seven days. (B) Following a subsequent 21-day washout, acute challenge with 1μM dexamethasone for 6 hrs did not significantly change FKBP5 mRNA expression levels in neural cells derived from either rs1360780*C homozygotes (post-hoc test, *p*>0.05) or T-allele carriers (post-hoc test *p*>0.05).
lines differentiated from iPSCs that were derived from 20 donor subjects. This concentration of dexamethasone had previously been shown to induce FKB5 expression in human peripheral blood mononuclear cells (Vermeer et al., 2004), a cell type chosen in many studies of the effects of dexamethasone on gene expression due to its sensitivity to glucocorticoids. However, differences in the sensitivity to glucocorticoids can vary across cell types (Ebrecht et al., 2000) and little research has examined the effect of dexamethasone treatment in neural cells, particularly those from human donors.

We found that the expression of FKB5 mRNA did not significantly change in human iPSC-derived neural cells following dexamethasone treatment, which contrasts findings using peripheral blood-derived cells reporting large increases following dexamethasone stimulation, including a 10-fold upregulation in whole blood (Menke et al., 2012) and 15-fold increases in T- and B-lymphocytes (Ebrecht et al., 2000). Interestingly, we did observe a significant upregulation of FKB5 mRNA following dexamethasone treatment in human fibroblasts. Comparing results from previous studies to our current data suggests that FKB5 mRNA expression is induced less strongly in neural tissue than in peripheral cells following GR activation. Support for this argument comes from examination of the SH-SY5Y human neuroblastoma cell line, in which gene expression for FKB5 increased by \( \approx 25\% \) following exposure to cortisol (Tatro et al., 2009), a finding that was mimicked in the mouse hippocampal HT-22 cell line, which showed a \( \approx 50\% \) increase in FKB5 mRNA expression following a 6 hr corticosterone exposure, with a peak increase of \( \approx 250\% \) after three days (Lee et al., 2010). Additional rodent studies report that regulation of FKB5 mRNA expression varies by brain region, with the highest basal expression found in the hippocampal CA1 and dentate gyrus regions. Increases in FKB5 mRNA expression
resulting from stimulation with dexamethasone or acute stress ranged from approximately 50-200% and differed by brain region, with larger increases in *FKBP5* mRNA expression found in the central amygdala and paraventricular nucleus than in the hippocampal CA1 and dentate gyrus regions, and no change in expression observed in the CA2 and CA3 regions (Scharf et al., 2011). *FKBP5* expression was also increased in the prefrontal cortex of DBA2/J mice following an acute alcohol injection, which increased in a dose-response fashion, but was not increased in the ventral tegmental area or nucleus accumbens (Kerns et al., 2005, Costin et al., 2013), further suggesting brain region-specific regulation. It is possible that we did not observe a significant change in *FKBP5* mRNA expression following dexamethasone exposure due to the forebrain-type neural differentiation protocol used. Future research might aim to differentiate human iPSCs into other neural cell types, such as hippocampal dentate gyrus-like neural cells (Mertens et al., 2015), in attempts to elucidate mechanisms that lead to differences in *FKBP5* mRNA induction following GR activation that vary by brain region.

Post-mortem analysis of human brain gene expression has implicated *FKBP5* in psychiatric disorders. *FKBP5* mRNA levels are over 2-fold higher in the hippocampus of alcoholic individuals (McClintick et al., 2013), significantly elevated in the dorsolateral prefrontal cortex of individuals with bipolar disorder and schizophrenia (Sinclair et al., 2013), and significantly lower in the amygdala of suicide victims (Perez-Ortiz et al., 2013). At the genetic level, SNPs spanning promoter and intronic regions of the gene have been associated with anxiety and depression (Binder et al., 2004, Kang et al., 2012), post-traumatic stress disorder (Binder et al., 2008, Klengel et al., 2013), and most recently with severity of alcohol withdrawal symptoms (Huang et al., 2014) and negative effects of
nicotine (Jensen et al., 2014). At the molecular level, FKB5 genetic variation is associated with altered chromatin structure and differences in methylation in traumatized individuals (Klengel et al., 2013), leading to “high induction” and “low induction” alleles where FKB5 expression is upregulated more in trauma-exposed carriers of the rs1360780 T-allele (Binder, 2009). However, conclusive evidence for an FKB5 risk genotype moderating FKB5 expression is lacking, and to our knowledge is limited to a few studies. Using peripheral blood mononuclear cells, Binder et al. (2004) found no significant difference in FKB5 mRNA expression, but significantly higher FKB5 protein levels in rs1360780 T-allele homozygotes (Binder et al., 2004). In post-mortem human brain from controls and subjects with bipolar disorder or schizophrenia, a significant effect of rs4713916, a SNP located in the promoter region of FKB5 and in moderate linkage disequilibrium with rs1360780 (D’=0.85), on basal FKB5 protein expression was found in homozygotes of the minor T-allele (Sinclair et al., 2013). Perhaps most relevant to our findings due to the examination of expression following GR activation, Hoehne et al. (2014) found that psychosocial stress increased FKB5 mRNA expression in peripheral blood cells of rs1360780 C-allele homozygotes, but T-allele carries showed a blunted induction of FKB5 mRNA that did not differ from unstressed controls (Hoehne et al., 2014). It may be that FKB5 genotype effects on gene expression following dexamethasone stimulation can only be seen in the setting of developmental stress occurring at particular time points, as human hippocampal progenitor cells treated with dexamethasone during the early proliferation/differentiation phase, but not later in the post-proliferation/differentiating phase, exhibited long-lasting de-methylation of FKB5 DNA (Klengel et al., 2013). However, this study did not look at the effect of dexamethasone treatment at these two
time points on *FKBP5* mRNA expression and was limited to the use of a single genetically uncharacterized neural progenitor cell line, whereas human iPSC technologies allow for the examination of neural cells from multiple genetically characterized donor subjects. In the current study, we found no effect of *FKBP5* rs1360780 genotype on induction of *FKBP5* following acute dexamethasone exposure in either human iPSC-derived neural cells or fibroblasts, or in neural cells pre-treated with dexamethasone at an earlier stage of development. A limitation of our study is that our T-allele carrier group contained only a single rs1360780 T-allele homozygote, which could have obscured an association between genotype and *FKBP5* gene expression if dexamethasone response differences are limited to T/T individuals.

In summary, we found that acute dexamethasone treatment significantly increased levels of *FKBP5* mRNA in fibroblasts, but not iPSC-derived forebrain-lineage neural cells. No significant effect of *FKBP5* rs1360780 genotype on *FKBP5* mRNA expression following dexamethasone treatment was observed in either fibroblasts or iPSC-derived neural cells, or in neural cells pre-treated with a priming dose of dexamethasone earlier in cell maturation. This work supports the potential of using iPSC neural differentiation to examine effects of genetic variation and drug exposure on mRNA expression in human neural cells, but suggests that future research aimed at exploring stress-response genes using iPSC technologies may wish to identify differentiation methods that generate neural cell types that express GR at higher levels and are more sensitive to GR activation than the default forebrain lineage. This work further suggests that changes in *FKBP5* gene expression following dexamethasone exposure observed in human peripheral cells may not
adequately reflect the gene expression effects in human neural cells derived from the same subjects *in vitro*
Chapter 6.

Discussion and Future Directions

The overall goal of this dissertation was to examine the utility of iPSCs in the study of alcohol use disorder. We started by examining the effects of acute and chronic alcohol exposure on neural cell cultures derived from alcoholics and non-alcoholics (Chapter 2). Next we used iPSCs to examine the molecular phenotype of a single nucleotide polymorphism in the GABA_α2 subunit gene (\(GABRA2\)) associated with development of alcohol use disorder in neural cells derived from genetically characterized donor subjects (Chapter 3). Finally, we identify a novel gene x environment interaction in the \(FKBP5\) gene that moderates heavy drinking in large, diverse human sample (chapter 4) and further examine the molecular characteristics of \(FKBP5\) expression by genotype and following stimulation with the synthetic glucocorticoid dexamethasone in iPSC-derived human neural cells (chapter 5), as previous work examining regulation of \(FKBP5\) expression has been conducted only in rodent and human peripherally-derived cells.

Psychiatric disorders have complex genetic influences and are difficult to model both \textit{in vivo} and \textit{in vitro}. The advent of human iPSC technologies promises to revolutionize psychiatric research by making previously unobtainable human neural cells accessible for the study of molecular phenotypes associated with psychotic illness and the exploration of novel pharmacological agents for treatment. In order to characterize the validity of using neural cells derived from iPSCs to study the biology of alcohol use disorder, we examined whether they recapitulate some of the previous findings associated with the disorder that were originally observed in animal or human post-mortem samples. Our initial study in chapter 2 examined the acute and chronic effects of alcohol on NMDA receptor
electrophysiological responses and gene expression and found that changes observed in response to alcohol treatment did in fact replicate findings reported previously in other model systems. We found that NMDA receptor-mediated depolarization was attenuated by acute alcohol and that following chronic exposure, mRNA expression of NMDA receptor subunits were significantly upregulated in neural cells derived from alcohol donor subjects. We chose to examine alcohol effect on the NMDA receptor gene expression because prior work in animal and human post-mortem models had established it as a target for alcohol’s actions (Nagy, 2008b), and the primary goal of the first study was to validate iPSCs as a tool to study AUD. Additional studies examining alcohol’s effect on the NMDA receptor in human iPSC-derived neural cells could include exploring post-translational and epigenetic modifications that occur in response to acute and chronic alcohol, as acute alcohol has been shown to induce phosphorylation of the NR2B subunit (Yaka et al., 2003a), and long lasting changes in methylation have been found in the GRIN2B gene following chronic exposure (Marutha Ravindran and Ticku, 2005) in rodent models. As GABA_A receptors are also a primary target of alcohol, we examined the effect of acute and chronic alcohol exposure on GABA-mediated currents and subunit gene expression. No effect of acute or chronic alcohol was observed on the GABA_A receptor; results that are discussed in detail later. Interestingly, while examining GABA_A subunit gene expression following chronic alcohol exposure, we noticed large variability in the basal mRNA levels of genes encoding certain subunits. This eventually led to the findings presented in chapter 3.

iPSCs offer a powerful tool to study the neurogenetics of psychiatric disorders with the ability to differentiate neural cells from genetically characterized donor subjects. In chapter 3 we examined in vitro molecular correlates of a common polymorphism in the
GABRA2 gene (rs279858, T- to C-SNP) where the C-allele has been replicated in numerous studies to be associated with the development of AUD. In chapter 3 we found that mRNA expression of GABRA2 was lower in carriers of the AUD-risk C-allele using qPCR on RNA extracted from 36 iPSC-derived neural cell lines. Furthermore, as GABRA2 resides within a cluster of four GABA\(\alpha\) genes on chromosome 4p12, examination of the adjacent genes revealed that mRNA expression of all four genes was significantly correlated, such the low expression of one gene predicted low expression of all four genes, which was significantly associated with the AUD-risk C-allele in GABRA2. Low expression of the chromosome 4p12 GABA\(\alpha\) cluster was replicated using RNA sequencing, which also revealed that expression of GABA\(\alpha\) gene residing on other chromosomes, neural genes encoding ion transporters, and the neuron-specific transcription factor NEUN did not differ between the low-expression and high-expressing cell lines, which importantly suggests that the differences in chromosome 4p12 GABA\(\alpha\) gene expression were not due to the cells being of a different developmental state. This finding is challenging to relate to the human condition because no genotype association with expression level was found in adult human post-mortem brain samples. However, our finding can be related to reports indicating that GABRA2 genotype is associated with differences in brain oscillations measured by EEG (Edenberg et al., 2004), and in activation of brain regions to reward anticipation and drug cues measured using fMRI (Villafuerte et al., 2012, Heitzeg et al., 2014, Kareken et al., 2010), such that the molecular effect of the polymorphism that influences risk of AUD might be via developmental mechanisms affecting brain connectivity related to genotype effects on GABA\(\alpha\) expression during development rather than due to alterations in ligand binding or channel function between genotypes in adulthood. Although in its current state these
findings present challenges in being relatable to development of AUD in humans, they could lead to advances in our understanding of the disorder through additional related studies. For example, future work could use this molecular phenotype as a marker to identify a functional polymorphism linked to rs279858 responsible for the low expression of the chromosome 4p12 GABA_A genes in iPSC-derived neural cells. Furthermore, researchers could use the molecular phenotype to identify genetic variation that associates with AUD across a wider population, considering that the marker we used to stratify cell lines (rs279858 in exon 5 of GABRA2, which is synonymous polymorphism) is associated with AUD in Caucasians but not African Americans (Covault et al., 2008). Finally, one can imagine that the yet-to-be identified variant that leads to the low expression phenotype in our neural cultures could be used as a pharmacogenetic marker for the effects of medications designed to treat AUD, in hopes to identify patients that are more likely to respond to pharmacological intervention.

Identification of novel genetic markers that associate with hazardous drinking is an important component in the prevention of AUD, while understanding the molecular correlates of these genetic markers could aid in clinical treatment. In chapter 4 we examined the association of a polymorphism within the FKBP5 gene (rs1360780) which encodes the glucocorticoid chaperone FK506 binding protein 51 on hazardous alcohol use in a large, diverse college student sample. This gene has been extensively examined in other stress-related psychiatric conditions inducing PTSD, depression, and anxiety. We found that FKBP5 rs1360780 genotype interacted with self reported early life trauma to moderate the frequency of heavy drinking, a risk factor for the development of AUD. This is the first time that a polymorphism in this gene has been associated with alcohol
consumption. Importantly, in chapter 5, we utilized iPSC neural differentiation to further explore the molecular characteristics of \textit{FKBP5} and the effect of rs1360780 genotype on \textit{FKBP5} expression in neural tissue. Previous \textit{in vitro} studies on \textit{FKBP5} gene expression, both as a function of stimulation with dexamethasone and rs1360780 genotype, have largely relied on peripheral blood cells due to their ease of generation and responsiveness to glucocorticoids. One limitation of using peripheral blood cells is that gene expression changes, either following drug exposure or by genotype, may not accurately reflect occurrences in neural cells. Despite this limitation, many have proposed, perhaps erroneously, that \textit{FKBP5} expression is upregulated in the brain following glucocorticoid receptor activation, and that rs1360780 genotype causes differential regulation by glucocorticoids of the \textit{FKBP5} gene. Binder (2009) has acknowledged this limitation, stating “so far all published data supporting the induction of \textit{FKBP5} by glucocorticoids have been in non-brain tissues” (Binder, 2009). This highlights a promising use for iPSC technology, in that genotype-specific neural cultures can be generated and examined \textit{in vitro} to answer questions such as are the effects of dexamethasone consistent in peripheral cells and neural cells? We set out to answer this question by doing a simple experiment utilizing neural cells from 20 donor subjects characterized by their \textit{FKBP5} rs1360780 genotype. We found no significant induction of \textit{FKBP5} following acute stimulation with dexamethasone at concentrations reported to induce expression in peripheral blood cells (Vermeer et al., 2004). Furthermore, no effect of \textit{FKBP5} genotype was observed on basal or stimulated expression levels. Perhaps most interesting, \textit{FKBP5} expression in subject-matched fibroblasts were responsive to dexamethasone treatment, suggesting that tissue-specific regulation of \textit{FKBP5} may exist. It could be argued that this is the most significant aspect of
this study, in that we examined two tissue types derived from the same donor subject. Future work may wish to explore additional cell types, considering that expression levels of \textit{FKBP5} differ by brain region in rodent models (Scharf et al., 2011), it may be that \textit{FKBP5} mRNA expression is more sensitive to the effects of dexamethasone in some neural subtypes compared to others. Additionally, it would be interesting to compare and contrast epigenetic changes following acute and chronic treatment with dexamethasone, as long lasting de-methylation has been reported in dexamethasone treated human cell lines (Klengel et al., 2013), which could be explored in various iPSC-derived neural subtypes.

One of the areas that our iPSC system did not model prior rodent students was the potentiation of GABA\textsubscript{A} receptor function by acute alcohol and a regulation of GABA\textsubscript{A} subunit expression following chronic alcohol, which have been some of the more replicated findings in rodent models (Kumar et al., 2009). In our experiments, iPSC-derived neural cells failed to replicate findings that GABA\textsubscript{A} receptor-mediated currents are potentiated by acute alcohol, in both the original report (chapter 2), which examined the effect of acute 50mM alcohol on GABA-evoked currents in a pilot experiment consisting of only 4 cells derived from one subject, and more recently in a larger sample of 47 neurons derived from 7 donor subjects (Figure 6.1). Although we failed to see an effect of acute alcohol on GABA\textsubscript{A} receptor currents, it is important to note that results from prior reports suggest that not all neurons show GABA\textsubscript{A} modulation by alcohol, with GABA\textsubscript{A} receptor currents being potentiated by alcohol in only a subset of neurons (20-70\%) (Signore and Yeh, 2000, Aguayo, 1990). This result that may be in part be due to brain-region specific sensitivity of GABA\textsubscript{A} receptors to alcohol (Weiner and Valenzuela, 2006), which may be influenced by differences in the diversity of subunits expressed between brain regions, since subunit
No effect of acute alcohol exposure (50mM) was observed on GABA-evoked currents in mature iPSC-derived neural cells differentiated from 7 donor subjects. Each dot represents when GABA was applied via puff application, which was repeated every 30 seconds, and alcohol was perfused into the recording chamber following a 5-minute baseline.
stoichiometry is a major determinant of the GABA_A receptor's physiological properties and pharmacological targets (Rudolph et al., 2001, Sieghart and Sperk, 2002). That alcohol differentially modulates GABA_A receptors by brain region due to subunit composition of the receptor leads to an interesting idea for future work. Since iPSCs were first introduced in 2006 and 2007, numerous protocol have been utilized to differentiate various cell types, including cultures enriched for dopamine, serotonin, and GABAergic neurons, among others. Considering the results presented in Chapter 2, it might be useful to compare and contrast the effect of acute alcohol on GABA_A and NMDA receptor currents in multiple neural cell types differentiated from a single donor subject. One might expect that the proportion of responsive cells to acute alcohol will differ between regional neural cell types. Results from a study such as this could then be used to compare responsive neural cell types between donor subject phenotype (i.e. non-alcoholic vs. alcoholic) or between carriers of AUD-risk genotypes to explore potential differences in response to acute alcohol.

Modulation of GABA_A receptor gene expression following chronic alcohol is one of the most robust, replicated findings in studies examining rodent and human post-mortem brain samples (Kumar et al., 2009). Therefore, we examined the effect of 7-day alcohol exposure on expression of three GABA_A subunit genes, GABRA1, GABRG2, and GABRD, in our iPSC-derived neural cells in an attempt to further validate the utility of iPSC-based models to study AUD. We originally included examination of the GABRA2 and GABRA4 genes, but the large effect of rs279858 genotype-associated differences in expression of these genes (Chapter 3 and discussed above) obscured alcohol effects. Using neural cultures derived from 12 subjects (7 non-alcoholics, 5 alcoholics) we found no significant change in mRNA
Figure 6.2. No Effect of Chronic Alcohol on GABA\textsubscript{A} Subunit mRNA Expression

7-day exposure to 50mM alcohol does not induce changes in expression of genes encoding GABA\textsubscript{A} receptor the (A) $\alpha_1$ (GABRA1), (B) $\gamma_2$ (GABRG2), or (C) $\delta$ (GABRD) subunits in neural cells derived from either non-alcohol (white bars) or alcoholic (black bars) donor subjects.
expression of these three genes following 7-day alcohol exposure (Figure 6.2). There are numerous possibilities as to why GABA<sub>A</sub> receptor gene expression was not modulated in our neural cultures. One can speculate that the GABA<sub>A</sub> receptors in the forebrain-type neural cells that we differentiate are not susceptible to the effects of alcohol, as subunit composition moderates alcohol’s effects (Wallner et al., 2006), and that differentiation into neural cultures enriched for other neural subtypes might evoke significant expression differences following chronic alcohol. Furthermore, it may be that the concentration and duration of alcohol exposure that we used (50mM for 7 days) was inadequate to induce adaptive changes in these genes. Finally, it may be that our iPSC-derived neural cultures are lacking the necessary transmitters that are targets of alcohol inducing adaptive changes in GABA<sub>A</sub> gene expression.

In neural cells, alcohol exposure induces the synthesis of GABA<sub>A</sub>-modulating neurosteroids (Barbaccia et al., 1999, Tokuda et al., 2011), which can acutely modulate GABA<sub>A</sub> receptor activity (Uchida et al., 2002, Sanna et al., 2004) while chronic neurosteroid exposure can induce changes in GABA<sub>A</sub> receptor gene expression (Gulinello et al., 2001, Follesa et al., 2004) that are similar the modulatory effects of acute and chronic alcohol exposure on GABA<sub>A</sub> receptors, suggesting that alcohol’s effects on the GABA<sub>A</sub> receptor are mediated through neurosteroids rather than via a direct interaction with the receptor (Morrow et al., 1999). Preliminary work supports the idea that our iPSC-derived neural cells are deficient in basal GABA<sub>A</sub>-modulating neurosteroids. Gas chromatography/ mass spectrometry (GC/MS) was performed on iPSC-derived neural cell conditioned media and revealed no detectable levels of GABA<sub>A</sub>-modulating neurosteroids (androsterone, androstanediol, and allopregnanolone). Interestingly, the cultures do possess functional
enzymes that synthesize these neurosteroids. When we spiked in the labeled precursors D5-androstenedione and C13-testosterone, after a brief 4-hour incubation period we were able to detect the downstream metabolites androsterone and androstanediol, indicating that our cultures contain active 5α-reductase, 3α-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase (Figure 6.3). An intriguing possibility is that we were unable to detect changes in both the acute actions of alcohol on GABA-evoked currents and the chronic effects on GABA\textsubscript{A} compensatory gene expression due to a lack of precursor neurosteroids in the culture, and that by adding precursors to the media we may be able to observe effects of alcohol at GABA\textsubscript{A} receptors. To explore this possibility, we incubated mature neural cells from one subject for 3-4 hours with the neurosteroid precursor pregnenolone, which is a precursor of GABA\textsubscript{A}-modulating neurosteroids androsterone, androstane-di, and allopregnanolone, prior to electrophysiological recordings examining the acute effect of alcohol on GABA-evoked currents. As described previously, no potentiating effect of acute alcohol was observed on GABA\textsubscript{A} receptor-mediated currents in untreated cells. However, a ≈30% potentiation of GABA-evoked responses to acute alcohol was observed when the cells were pretreated with the neurosteroid precursor, an effect that was not observed with alcohol was co-perfused with dutasteride, which inhibits neurosteroid synthesis by blocking the key enzyme 5α-reductase (Figure 6.4). To explore neurosteroid/alcohol interactions on GABA\textsubscript{A} receptor gene expression after chronic exposure, mature neural cells from two subjects were treated with 3.16nM of the neuroactive precursor steroid pregnenolone, 50mM alcohol, and a combination of the two for 7-days. Similar to our previous findings, no effect of alcohol was observed on GABA\textsubscript{A} subunit mRNA. Furthermore, no effect was observed when the cells were treated with
Figure 6.3. Synthesis of Neuroactive Steroids Following Administration of Labeled Precursor Steroids D5-Androstenedione and C13-Testosterone in IPSC-Derived Neural Cells

Following a 4-hour incubation with labeled precursor steroids in mature iPSC-derived neural cultures, we were able to detect downstream neuroactive metabolites Androsterone and Androstenediol using GC/MS. Neither Androsterone nor Androstenediol could be detected in neural cell media that was not incubated with precursor steroids (data not shown). Neurosteroid synthesis pathway adapted from Milivojevic et al. (2011).
As an exploratory experiment, neural cells derived from one control subject were used to examine the effect of acute alcohol on GABA-evoked currents following 3-4 hour neurosteroid precursor treatment. The same experimental design was used as seen in Figure 6.1. As discussed previously, no effect of acute alcohol was observed on GABA-evoked currents. Bath application of 50mM alcohol induced a modest potentiation of GABA-evoked current when neural cultures were incubated for 3-4 hours prior to recording with pregnenolone. This potentiation was not observed when alcohol was co-applied with the drug dutasteride, which inhibits neurosteroid synthesis.
Figure 6.5. Chronic Exposure to Alcohol + Pregnenolone Does Not Induce Changes in GABA_A Subunit mRNA Expression

Mature neural cultures from two donor subjects were used to examine whether chronic 7-day treatment with a 3.16nM pregnenolone or a combination of 3.16nM pregnenolone +50mM alcohol regulated expression of genes encoding GABA_A receptor subunits. As discussed previously, no effect of alcohol was observed on (A) GABRA1, (B) GABRG2, and (C) GABRD mRNA expression following 7-days of alcohol exposure (solid gray bars). No effect of pregnenolone (hashed bars) or a combination of pregnenolone +alcohol (gray hashed bars) regulated expression of these subunits.
pregnenolone, or a combination of alcohol + pregnenolone (Figure 6.5). A limitation of this study includes lack of information about the metabolic half-life of pregnenolone in cell culture. While limited conclusions can be drawn from examining alcohol/neurosteroid interactions in neural cells from only a few subjects, it is important to highlight the possibility that the neural media that is used for the prolonged culture of these cells may be deficient in certain compounds necessary for observing full neural development, replication of in vivo physiology, or the effects of drugs/alcohol. In support of this, a recent report examined popular neural differentiation media and found that it did not contain physiologically-relevant concentrations of chemical compounds, and actually inhibited neuronal activity and network development (Bardy et al., 2015). The group tested a neural medium with concentrations of salts, amino acids, and vitamins that more closely resembles human physiologic composition and reported increased number of synaptically active neurons. Future work examining the effect of alcohol exposure will benefit from these advances in defining optimal neural cell media to explore whether differences in GABA\textsubscript{A} receptor function and gene expression can be observed in this in vitro model system.

One benefit of human iPSCs as a model system to study AUD is the potential to identify novel targets of alcohol that may lead to novel and potentially more efficacious treatments. One method to identify novel genes that are regulated by alcohol is by examining expression of the entire transcriptome using tools such as microarray and RNA sequencing. To date, many studies have used large-scale gene expression profiling to study AUD, including examination of basal transcript level in human post-mortem brain derived from alcoholics vs. controls (McClintick et al., 2013, Liu et al., 2006) and from alcohol
preferring vs. non-preferring rats (Liang et al., 2010, Edenberg et al., 2005), which have also been used to examine the effects of chronic alcohol self-administration (Rodd et al., 2008). A limitation of post-mortem studies includes not being able to decipher whether significant gene expression differences were present at baseline or after years of alcoholism, while the rodent studies are limited in their relatedness to the human condition. Therefore, RNA sequencing of alcohol-exposed human iPSC-derived neural cells may provide useful insights into gene expression changes following alcohol exposure in human neural cells. Since gene expression changes following alcohol exposure could be identified by examining sham-treated vs. alcohol-treated cells derived from the same subject, contrasts could also be made between cells derived from alcoholics vs. non-alcoholics. We have currently sequenced neural cells exposed to 50mM alcohol for 7 days, as well as the paired sham condition, from a total of 10 donor subjects consisting of 5 non-alcoholic and 5-alcoholic donor cell lines, and are in the process of analyzing the results. To our knowledge, this will be the first time transcriptome profiles will be generated following alcohol exposure of human iPSC-derived neural cells from phenotypically characterized donors. Preliminary findings indicate that basal expression levels between neural cells derived from non-alcoholics and alcoholics are highly correlated, and exposure to alcohol for 7 days causes a greater number of significantly regulated genes in the neural cells derived from non-alcoholic donor subjects (Figure 6.6). The only similar study reported to date used peripherally derived lymphoblastoid cells from 21 controls and 21 alcoholics that were exposed to alcohol for 24 hours before microarray analysis. This study found that alcohol exposure caused many small changes in gene expression, and the genes that differed between controls and alcoholics clustered into functional groups including integrin
RNA Sequencing was used to examine the effects of 7-day alcohol exposure on gene expression in mature neural cells derived from 5 non-alcoholic and 5 alcoholic. Donor subjects. (A) Basal gene expression was highly correlated between donor subject phenotype across the entire transcriptome. (B and C) More genes were significantly regulated in neural cells derived from control subjects following chronic 7-day alcohol treatment using paired t-test. Genes that were significantly regulated were grouped into bins based on fold-change in expression.
signaling, ERK/MAPK signaling, and Rho family GTPase signaling (McClintick et al., 2014). However, because gene expression profiles in peripheral cells are weakly correlated with the expression in the central nervous system (Sullivan et al., 2006), we anticipate that our sequencing results using human neural cells may be more relevant to the in vivo condition.

The results reported in this dissertation highlight some of the potential benefits of using iPSCs in the study of AUD. An additional benefit of using iPSCs is the ability to genetically modify mutations through the use of TALEN or CRISPR technologies, which could be of interest in our studies if a functional mutation is discovered that leads to reduced chromosome 4p12 GABA_A gene cluster expression. Furthermore, iPSCs have the potential to be used in the study of pharmacogenetics of candidate AUD treatments. For example, topiramate’s efficacy in reducing heavy drinking was found to be moderated by a polymorphism in a gene encoding the GluK1 subunit of the kainate receptor subunit (GRIK1, rs2832407), which itself is a target of topiramate (Kranzler et al., 2014a). iPSCs could be generated from donor subjects characterized at this marker (rs2382407) and differentiated into neural cell types to examine the molecular effects of topiramate between genotypes. Challenges of utilizing iPSC technology include the generally limited understanding we currently have of induced stem cells and neural differentiation. The field is continuing to explore and discover the optimal conditions needed for iPSC-derived neural cells to best recapitulate in vivo characteristics, for example the derivation of new culture mediums that facilitate neural connectivity (Bardy et al., 2015). Additionally, research is continuing to gain insights into the transcriptome and epigenetic landscapes of human iPSCs compared to embryonic stem cells and parent tissues (Bar-Nur et al., 2011,
Choi et al., 2015), which may lead to iPSC derivation and differentiation protocols that better mimic the human \textit{in vivo} condition.
Chapter 7.

Conclusion

AUD is a complex, heterogeneous, devastating disorder that is prevalent in modern society. Through decades of research examining the actions of alcohol, we have gained a significant understanding into its mechanisms of action. Despite this, prevention and treatment of AUD is lacking. Better model systems to explore in vitro actions of alcohol and molecular effects of AUD-associated genetic polymorphisms may lead to novel understandings of the disorder and in turn more efficacious treatment options. The studies presented here have examined the use of human induced pluripotent stem cell differentiation as a model for AUD research. Although many challenges are presented with the use of iPS cells in the study of complex psychiatric disorders, we conclude that human iPSCs provide an adequate substrate for future researchers to utilize in the examination of drug and genotype effects that may influence risk. Through the use of subject-specific cells, we have the potential to gain novel insights into the development of AUD, leading to better preventative measures and therapeutics for affected individuals. In summary, iPS cell technologies hold great promise for the future considering that their application in the field of psychiatry is in its infancy.


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