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Using human stem cells as a model system to understand the neural mechanisms of alcohol use disorders: current status and outlook

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Abstract

Alcohol use disorders (AUDs), which include alcohol abuse and dependence, are among the most common types of neuropsychiatric disorders in the United States (U.S.). Approximately 14% of the U.S. population is affected in a single year, thus placing a tremendous burden on individuals from all socioeconomic backgrounds. Animal models have been pivotal in revealing the basic mechanisms of how alcohol impacts neuronal function, yet there are currently limited effective therapies developed based on these studies. This is mainly due to a limited understanding of the exact cellular and molecular mechanisms underlying AUDs in humans, which leads to a lack of targeted therapeutics. Furthermore, compounding factors including genetic background, gene copy number variants, single nucleotide polymorphisms (SNP) as well as environmental and social factors that affect and promote the development of AUDs are complex and heterogeneous. Recent developments in stem cell biology, especially the human induced pluripotent stem (iPS) cell development and differentiation technologies, has provided us a unique opportunity to model neuropsychiatric disorders like AUDs in a manner that is highly complementary to animal studies, but that maintains fidelity with complex human genetic contexts. Patient-specific neuronal cells derived from iPS cells can then be used for drug discovery and precision medicine, e.g. for pathway-directed development in alcoholism. Here, we review recent work employing iPS cell technology to model and elucidate the genetic, molecular and cellular mechanisms of AUDs in a human neuronal background and provide our perspective on future development in this direction.

Keywords

Alcohol Use Disorder (AUD); Induced Pluripotent Stem (iPS) Cell; Induced Neuronal cell (iN); Central Nervous System (CNS); Human Disease Modeling

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Excessive consumption of alcohol in spite of harmful consequences is clinically recognized as an alcohol use disorder (AUD). This can be further classified into a chronic relapsing brain disorder that is characterized by habitual alcohol consumption, loss of control over alcohol intake and an undesirable emotional state when not drinking. AUDs are among the most common pathologies that impact the central nervous system (CNS) (Grant et al., 2015). It is estimated that 16 million Americans (~ 14%) are afflicted with an AUD (<https://www.niaaa.nih.gov/alcohol-health/overview-alcohol-consumption/alcohol-use-disorders2015>). The risk of developing an AUD is influenced by both genetic and environmental factors and/or social factors. Furthermore, alcohol abuse and dependence are not only common but also potentially lethal due to intensification of a variety of additional medical and psychiatric conditions, thus ultimately shortening the lifespan of addicts by approximately a decade. In addition, fetal exposure to alcohol causes Fetal Alcohol Spectrum Disorders (FASDs), which has been shown to cause long-term cognitive impairment and brain defects (Mattson, Crocker, & Nguyen, 2011; Riley, Infante, & Warren, 2011). Current treatments for alcoholism exhibit varying levels of success, which is at least partially due to an incomplete understanding of the etiology and the pathophysiology, especially the molecular pathways, cellular and synaptic mechanisms that lead to AUDs in human. Moreover, varying success with treatments can also be rationalized as due to the genetic diversity between individuals, including genetic background, gene copy number variants, single nucleotide polymorphisms (SNPs) as well as environmental and/or social factors. This may explain treatment responders versus non-responders but provides another layer of complexity when developing effective therapeutics for AUDs.

In spite of its high prevalence and societal consequences, and the vast amount of research conducted, the molecular mechanism(s) and pathophysiology of AUDs in the human CNS remains undetermined. Most of the molecular and mechanistic investigations have been carried out using animal models, which may not fully recapitulate the pathogenesis and developmental processes of the human disease state (Akhtar, 2015; Grobin, Matthews, Devaud, & Morrow, 1998; Kumar et al., 2009; Weiner & Valenzuela, 2006) and obtaining primary human neurons from patients is inherently difficult. The majority of studies on the human brain are conducted on post-mortem tissue, which usually correlates to the end-stage of the disorder, and is not suitable to conduct functional assays on synaptic transmission and neuronal physiology; or based on brain imaging studies which provide macroscopic data which lacks cellular and molecular level information (Goldman, 2016). An ideal model system permits the study and comparison of mature neurons from healthy and affected patients. How do we understand the pathophysiology of human AUDs, if animal models do not accurately model the human disease state?

Human adult somatic cells can be reprogrammed to create induced pluripotent stem (iPS) cells

The pluripotent cells that reside in the inner cell mass (ICM) of the early embryo, termed embryonic stem (ES) cells, have the remarkable ability to give rise to the body of the embryo proper. These cells have the unique capability to proliferate indefinitely while retaining an unrestricted developmental potential (Pera, Reubinoff, & Trounson, 2000).

Nevertheless, ES cell research is not only ethically and politically controversial but is also limited in capturing the genetic heterogeneity that contributes to human disease (Flake & Zanjani, 1997; Strong, Farrugia, & Rebull, 2009). So, to investigate the genetic heterogeneity that contributes to human disease is it possible to convert adult cell types into an embryonic state? Forced co-expression of critical transcription factors (TFs) that are turned on in pluripotent cells of the early embryo (Oct4, Sox2, Klf4 and c-Myc) can reprogram somatic cells into self-propagating iPS (induced pluripotent stem) cells (Takahashi & Yamanaka, 2006). The advent of iPS cell lines provided an integral tool for the understanding and elucidation of human neurodevelopmental, neurodegenerative and neuropsychiatric diseases, in a human neuronal context.

Diseases of the brain can be caused by both hereditary and environmental factors, both of which modulate neuronal development and function (van Loo & Martens, 2007). With recent advances in neuronal differentiation technology using iPS cells, it is possible to study the neurodevelopment and neuropathology of AUDs in humans *ex vivo*. Reprogramming is reliant on the efficient delivery and expression of (TFs) into a number of somatic cell types (Um, 2012). The main differences in reprogramming arise from the cell types, species and delivery method. Dermal fibroblasts are the most commonly used donor somatic cells for reprogramming (González, Boué, & Izpisua Belmonte, 2011; Raab, Klingenstein, Liebau, & Linta, 2014). Fibroblasts contain many attractive characteristics that make them ideal for reprogramming, including the ease of cultivation in culture and low methylation levels of the promoter regions for genes expressed in the early embryo, such as *OCT4* and *NANOG*, thus granting the appropriate TFs access to the promoter (Raab et al., 2014). Within ~ 5 weeks in culture, a small percentage (~ 1%) of the initial somatic cells are reprogrammed to pluripotent stem cells, which are an infinite source of starting material from which several defined cell types may be generated and investigated (Kim, Choi, Choi, & Do, 2011). Patient-derived iPS cell lines can then be used to model human neurological disease by differentiating them into tissue-specific cells germane to the disease of interest. To comprehensively model human neurodegenerative and neuropsychiatric disorders, many efforts have been committed to generating defined neuronal cell types from iPS and ES cells (Marchetto, Brennand, Boyer, & Gage, 2011). However, iPS cells may not fully recapitulate the epigenetic landscape of the individuals (Mertens, Paquola, et al., 2015), but direct-differentiated induced neuronal (iN) cells by ectopic expression of neuronal lineage TFs (Pang et al., 2011) can then serve to better elucidate the molecular mechanisms underlying neurological disorders.

Recent advances in stem cell biology and iPS cells provide an essential platform to model the cellular and molecular underpinnings of human neurological diseases *in vitro* that was until now impossible. The advent of human iPS cell research has yielded new clinical strategies for their use in regenerative therapy of damaged tissue and organs (Pei, Xu, Zhuang, Tse, & Esteban, 2010). Several patient-specific iPS cells have been produced to model various neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Bahmad et al., 2017; Marchetto et al., 2011). In addition, iPS cells have also been used to model and understand the molecular mechanisms underlying substance abuse phenotypes (Oni et al., 2016) including AUDs (Figure 1). To better illustrate the power of human iPS cell disease modeling, we will first discuss the use of iPS cells in

two highly prevalent neuropsychiatric disorders, schizophrenia (SCZ) and bipolar disorder (BD).

Modeling Neuropsychiatric Disease Using iPS Cells (A Perspective on SCZ and BD)

Lessons from human stem cell models of SCZ

The neuropsychiatric disorder SCZ is a devastating mental disorder that impacts more than 21 million individuals globally (van Os & Kapur, 2009). SCZ was among the first neuropsychiatric disorders whose pathophysiology was modeled using patient derived iPS cells (Brennand et al., 2011; Chiang et al., 2011). These studies assayed connectivity, synapse number, spine density and expression of glutamatergic receptors in iPS cell derived neurons. Interestingly, application of the dopamine antagonist loxapine, but not other structurally similar antipsychotics, during the final three weeks of differentiation resulted in an elevation of neuronal connectivity in iPS-cell derived neurons from idiopathic SCZ patients. This suggests drug screening in SCZ human neurons can possibly identify specific compounds for its treatment. On the other hand, iPS cell disease modeling from SCZ subjects with defined genetic factors have also been established. For example, iPS cells with mutations in Disrupted in Schizophrenia 1 (*DISC1*), which encodes a protein involved in neurite outgrowth and cortical development (Wen et al., 2014; Ye et al., 2017), have been generated. Neurons derived from iPS cells harboring *DISC1* mutations had synaptic defects, and genes that correlate to synaptic transmission and development were also improperly regulated (Soliman, Aboharb, Zeltner, & Studer, 2017). The power of patient derived iPS cells in modeling SCZ is quite clear from this study for two main reasons: 1) 90 of the identified genes that were dysregulated in neurons carrying the *DISC1* mutation had been previously linked to mental disorders such as SCZ. 2) Gene editing was used to correct the *DISC1* mutation upon which disease phenotypes were reversed, indicating that the effects observed on cellular phenotype, as well as gene expression, was a direct consequence of the patient derived *DISC1* mutation. This illustrates how patient derived iPS cells can be used to understand the precise cellular mechanisms caused by a mutation linked to a certain disease.

Lessons from human stem cell models of BD

More recently, iPS cell technology has been employed to model mood disorders, such as BD, an extremely prevalent neuropsychiatric illness. (Bavamian et al., 2015; Chen et al., 2014; Kim et al., 2015; Madison et al., 2015; Mertens, Wang, et al., 2015; Stern et al., 2017). Approximately > 1% of the worldwide population suffers from BD (Angst, 1998; Hirschfeld, 2003; Merikangas et al., 2007) and ~ 15% of the affected individuals commit suicide when no appropriate treatment is administered (Sharma & Markar, 1994). These shocking statistics led BD to become the top disease of morbidity and a major contributor to a decline in employee productivity, as ranked by the World Health Organization (Dusetzina et al., 2012). Although there is a plethora of evidence indicating BD is highly penetrable, the pathophysiology of BD still remains elusive (Manji & Lenox, 2000). The heritable nature of BD cannot be traced to a single gene, and it has proven to be a daunting task to develop new effective therapeutics.

Postmortem neuropathological studies uncovered many changes in the brains of subjects with BD and findings from BD animal models seemed to be consistent (Martinowich, Schloesser, & Manji, 2009). These changes include, hyperactivity in protein kinase A and C (PKA and PKC) pathways (Bezchlibnyk & Young, 2002; Chang, Li, & Warsh, 2003; H. Wang & Friedman, 2001), reduced glial cell number (Andreazza & Young, 2014) and perturbations in neurotransmission (Berk et al., 2007; Du, Quiroz, Yuan, Zarate, & Manji, 2004; Mahmood & Silverstone, 2001; Scarr, Pavey, Sundram, MacKinnon, & Dean, 2003). However, what causes these changes in a BD patient remains undetermined, making it difficult to understand the pathophysiology of BD. One widely prescribed therapy for BD is lithium, however, only a subpopulation of the BD patients responds to lithium while others are unresponsive. To accurately and comprehensively model BD, a recent study using an iPS cell model for human BD reported the cellular phenotypes of hippocampal dentate-gyrus like human neuronal cells from manic type I BD patient fibroblasts (Mertens, Wang, et al., 2015). Electrophysiology and Ca^{2+} imaging revealed hyperactive action potential firing in BD derived neuronal cells compared to that of controls, which was attenuated with lithium administration. Intriguingly, a decrease in action potential bursting was only observed in human neuronal cells derived from patients who also responded to lithium treatment clinically. This study indicates that neuronal hyperactivity is a BD phenotype, and the BD model created is directly linked with the manic clinical symptoms seen in patients with BD. Mechanistically, the lithium mediated reduction in neuronal hyperexcitability can be explained by alterations in gene expression; specifically, 560 genes were differentially affected in lithium responsive neurons compared to only 40 genes variably expressed in neurons that had no response to lithium application. This study (Mertens, Wang, et al., 2015) provided us with a unique example of disease relevant phenotypes isolated in the dish that are directly translatable to the clinic. Furthermore, this study gives direct evidence that cohorts of patients exhibiting similar clinical phenotypes may exhibit differential response to therapeutics based on that individual's unique genomic landscape. From a clinical standpoint, iPS cell technology can not only test the efficacy and mechanism of action for widely prescribed medications but also allows developing novel drug candidates to discover new therapies proactively in a species-relevant model system prior to their use in the clinic (Figure 1).

Current Work Attempting to Use iPS Cell Technology to Model AUDs

Alcoholism is a condition composed of chronic remittance and relapse. AUDs, like other neuropsychiatric disorders, have multifaceted etiology and pathophysiology, these include complex genetic and epigenetic components, environmental and social factors (Moussas, Christodoulou, & Douzenis, 2009). There is a fourfold increase in alcohol-dependence risk in relatives of known alcoholics, and monozygotic twins of alcohol-dependent individuals have a greater predisposition for an AUD than dizygotic twins (Basiaux et al., 2001; Lykouras, Moussas, & Botsis, 2004). Interestingly, children adopted into an alcoholic household have the same fourfold increase in the risk for developing an AUD, as do offspring from the same parents (Lykouras et al., 2004; Nolen-Hoeksema & Hilt, 2006). This ultimately means there is a higher risk to develop an AUD if the person has alcoholic parents, which could be explained by genetics or an active component to alcohol abuse

(heritability accounts for ~ 40–60% of the risk to develop an AUD). However, the environment cannot be ignored which is illustrated by individuals adopted into an alcoholic household having the same risk of developing an AUD as those born to alcoholic parents. It would be naïve to think that it is either one or the other, but rather it is more likely that genetic and environmental factors together interact to dictate the occurrence and severity of alcohol dependence.

In fact, the most prominent SNPs that have been linked to alcohol abuse, identified using genome wide association studies (GWAS), have been mapped to genes encoding liver enzymes, specifically alcohol dehydrogenase and aldehyde dehydrogenase (Tawa, Hall, & Lohoff, 2016). However, there are also a number of studies implicating genes that modulate synaptic function such as *OPRM1*, *CHRNA5* and *GABRA2* to alcohol abuse as well, suggesting that AUDs have a key neuropsychiatric component that has not been thoroughly investigated (Berrettini, 2013; Hoehe et al., 2000; D. Li et al., 2014; Lubke, Stephens, Lessem, Hewitt, & Ehringer, 2012). Moreover, it is more likely that a given individual with an AUD carries SNPs located in several genes, or even in non-coding regions, where each individual variant exerts a small effect on AUD risk but the additive sum of these variants together yields a significant impact (Mayfield, Harris, & Schuckit, 2008). It is also plausible that effects seen from specific SNPs vary from patient to patient due to each individual's unique genetic background. This suggests that the risk factors for the development of an AUD are quite complex and necessitate the use of a technology capable of modeling both its complex genetic underpinnings. Furthermore, human pluripotent stem cells enable the use of gene editing technologies to more directly isolate the precise cellular phenotypes of a SNP.

In addition to etiological components characterizing AUDs, *i.e.* a certain genetic variant that predisposes an individual to excessive alcohol consumption, there are also more pathophysiological components. Pathophysiological effects include the cellular, molecular and synaptic phenotypes produced upon acute alcohol administration. There is compelling evidence to suggest that alcohol primarily targets the synapse (Lovinger & Roberto, 2013). In fact, excitatory N-methyl-D-aspartate (NMDA) and inhibitory gamma aminobutyric acid type-A (GABA_A) ligand-gated ionotropic receptors are two of alcohol's main targets. Furthermore, alcohol has been shown to act as a potent epigenetic modulator impacting cellular metabolism and differentiation. Specifically, neuroinflammation and oxidative stress (damage) of the mitochondria and cellular proteins are believed to add to the progression of neurological disorders that are caused by alcohol abuse (Alfonso-Loeches, Ureña-Peralta, Morillo-Bargues, Oliver-De La Cruz, & Guerri, 2014; De Filippis et al., 2016). Numerous studies using both *in vitro* and *in vivo* animal models, as well as postmortem human brain tissue have laid the groundwork for most of the theories explaining the molecular underpinnings of AUDs. However, animal models do not completely mimic disease initiation and progression in humans. Furthermore, post-mortem brain tissue does not permit assaying the physiological effects of alcohol exposure, specifically the impact of alcohol on human neuronal function through electrophysiology. To circumvent the use of animal and post-mortem brain models, pluripotent cells can be generated from somatic tissue of living donors suffering from AUDs, and can ultimately be differentiated into neural cell types harboring a patient-derived specific mutation.

It is important to briefly discuss the concentrations of alcohol employed in most studies aiming to model AUDs using animal models. Although alcohol is not a particularly potent drug, it exerts dramatic depressive effects on the CNS which do not manifest until an individual's blood alcohol concentration is rather high (5–10 mM) (Katzung, 2001). Furthermore, mutations in enzymes responsible for alcohol breakdown can explain inter-individual variability in alcohol concentration and metabolism in the blood (Edenberg, 2007). Thus, it is nearly impossible to precisely model the variable drinking habits and changing blood alcohol concentrations of an AUD individual (Dolganiuc & Szabo, 2009) and in animal models. The majority of studies performed in rodent models use a 50 mM alcohol concentration (Lieberman, Levine, Kranzler, Abreu, & Covault, 2012), which in more practical terms corresponds to a blood alcohol concentration of 0.24 g/L. With the normal legal driving limit for blood alcohol concentration being 0.08 g/L a dose of 50 mM alcohol would equate to $\sim 3\times$ the legal limit for operating a vehicle in the United States (Lieberman, Kranzler, Levine, & Covault, 2017). However, studies have also been conducted using alcohol concentrations that are lower (~ 10 mM) and higher (~ 70 mM). A 10 mM alcohol concentration is used to model aspects of social drinking behaviors (Rewal et al., 2012; Sundstrom-Poromaa et al., 2002; J. Wang et al., 2016; Wei, Faria, & Mody, 2004), while a concentration of 70 mM would correlate more to the blood alcohol concentration of an extremely heavy drinker in human (Olson, Smith, Kloss, Ho, & Apple, 2013). Concentrations above 100 mM have been found to directly cause cytotoxic effects on cells (Baker & Kramer, 1999; L. N. Li, Margolis, & Hoffman, 1991). The concentration used is critical when setting up your experimental design and would ultimately depend on the type of question(s) being asked. Finally, in addition to concentration, time dependent administration also needs to be considered. Most studies assay acute (24 hour) versus chronic (~ 7 days) alcohol exposure (Lowery-Gionta, Marcinkiewicz, & Kash, 2015; Mandrekar, Bala, Catalano, Kodys, & Szabo, 2009). An acute exposure to alcohol can be used to mimic binge drinking, while a more chronic exposure recapitulates mild, reoccurring alcohol use. Taken together, it is important to consider these factors when designing experiments to answer specific (focused) types of questions regarding AUDs. In addition, are the toxic effects of alcohol direct or indirect?

Alcohol's deleterious impact on neuronal function is most likely caused by its oxidation product acetaldehyde (Wang et al., 2000), and accumulation of DNA damage can be caused by this highly reactive alcohol break down product. The cell has a mechanism to combat acetaldehydes deleterious effects, and this is by enzymatically converting acetaldehyde to acetone, which is mediated by aldehyde dehydrogenase 2 (*ALDH2*) (Lai et al., 2014). The importance of this enzyme is illustrated in the ~ 540 million people that carry a polymorphism in *ALDH2*, where alcohol consumption in these individuals promotes an aversive response and their risk of developing esophageal cancer increases (Yokoyama & Omori, 2003). Furthermore, $\sim 8\%$ of the world's population, specifically individuals of East Asian ancestry, have an inherited genetic variant in *ALDH2*. This immediately raises the question: Are stem cells (specifically neural stem cells) sensitive to accumulating DNA damage when exposed to alcohol, due to acetaldehyde accumulation? Interestingly, a recent study (Garaycochea et al., 2018) showed that alcohol-derived aldehydes negatively impact the genomic stability of hematopoietic stem cells (HSCs) the cells that renew blood. DNA

damage is normally combatted via surveillance and repair mechanisms, where the primary line of defense is provided by ALDH2-mediated detoxification. These data may not be limited to explaining how alcohol impacts HSCs but may also provide some insight into neural stem cell genomic instability caused by alcohol exposure. Furthermore, in this study, (Garaycochea et al., 2018) created a method to determine the mutational landscape of individual HSCs, and applying this technology to map the mutational landscape of neural stem cells can help researchers understand how alcohol affects differentiation into mature neurons.

In addition, given the fact that *ALDH* isoforms are key enzymes of alcohol metabolism (Edenberg, 2007), it is not surprising that they serve as the first line of defense in the brain from alcohol abuse and acetaldehyde toxicity. Mature astrocytes are known to express high levels of ALDH isoforms, making them active defenders of neurons from the detrimental effects of alcohol consumption. Thus, SNPs in ALDH enzymes and their role in astrocyte function must be considered for their neuroprotective role in the brain. Recent advances have enabled the generation of astrocytes from human iPS cells through multiple approaches involving either a chemically defined culture medium (Shaltouki, Peng, Liu, Rao, & Zeng, 2013) or a neural progenitor cell intermediate (TCW et al., 2017). These human iPS cell derived astrocytes have gene expression, morphological and functional characteristics which mimic that of primary astrocytes (Shaltouki et al., 2013; TCW et al., 2017). Given the reliable platforms for generating human astrocytes from patient derived stem cells, their use in modeling the role of SNPs in ALDH enzymes such as ALDH2 would yield new insight into the effects of alcohol induced toxicity and neuronal dysfunction that may ultimately contribute to the progression of AUDs. Thus, the use of disease relevant cells derived from human stem cells enables the study of multiple aspects of AUD pathophysiology ranging from alcohol concentration, time of alcohol exposure, genetics, environment and alcohol metabolism.

Alcohol's impact on NMDA receptor function in human neurons

Studies performed assaying NMDA receptor function and measuring specific receptor subtype mRNA expression levels, reveals the power of using human iPS cells as a model to investigate the biological impact of alcohol on human neural cell types. The acute and chronic effects of alcohol exposure on human iPS-cell derived neural cells was investigated, specifically assaying NMDA receptor function and receptor subunit mRNA expression levels (Lieberman et al., 2012). Importantly, the responses were found to be similar to data obtained from animal models. Acute administration of alcohol (50 mM) significantly attenuated the peak amplitude of NMDA receptor mediated currents as seen with whole-cell voltage clamp recordings in iPS cell-generated neurons. Interestingly, the authors observed no attenuating effect on NMDA response if alcohol is administered chronically (50 mM alcohol applied each day for 7 days) prior to measuring acute re-exposure, in iPS cell-derived human neurons. These data, taken together, suggest that tolerance to attenuation of the NMDA response to acute alcohol exposure can be accomplished. Next, NMDA subunit mRNA expression was analyzed for the genes *GRIN1* (NR1 subunit), *GRIN2A* (NR2A subunit), *GRIN2B* (NR2B subunit) and *GRIN2D* (NR2D subunit) in human iPS cell-derived neural cultures from alcoholic and non-alcoholic donors in response to 0 or 50 mM chronic

alcohol treatment. Neural cultures produced from alcoholic donors displayed a significant increase for *GRIN1* (and a trend towards significance for *GRIN2B*) mRNA levels in response to chronic alcohol exposure. The work described above, (Lieberman et al., 2012), suggests that human iPS cell-derived neural cells could potentially provide scientists with a powerful tool to model and assay the molecular underpinnings that alter NMDA receptor function and subunit expression to acute and chronic alcohol exposure, which still to this day are not completely understood.

Alcohol's impact on GABA_A receptor function in human neurons

Accumulating evidence suggests that GABA is a major player in dictating many of the behavioral effects of alcohol, including anxiolysis, motor incoordination, sedation, withdrawal phenotypes and alcohol preference (Lieberman et al., 2012). The associations between GABA_A and alcohol were first speculated due to the parallels between the behavioral effects of alcohol and allosteric modulators of GABA_A (barbiturates and benzodiazepines) (Grobin et al., 1998; Kumar et al., 2009; Weiner & Valenzuela, 2006). In addition, data from animal models suggest that the behavioral phenotypes of acute alcohol exposure can be influenced by co-application of GABA_A agonists or antagonists. Electrophysiological recordings in several brain regions, from animal models, show a potentiation of GABA_A receptor response following exposure to alcohol (Aguayo, 1990; Jia, Chandra, Homanics, & Harrison, 2008; Nie et al., 2004; Nie, Madamba, & Siggins, 2000; Yeh & Kolb, 1997). However, recordings conducted using human iPS cell-derived neuronal cells contradict findings obtained from animal models (Lieberman et al., 2017). There was no observed strengthening of the GABA_A response following acute exposure to alcohol (50 mM) (Lieberman et al., 2012). This is highly suggestive of species specificity of GABA_A receptor function, further potentiating the importance of human neuronal models in understanding the synaptic role of alcohol. Alcohol's impact on GABA_A receptor function seems to be quite complex, and the sensitivity of receptor response to alcohol could be a key determinant of the type of effect seen. Receptor subunit stoichiometry and regulation of subunit expression in response to chronic alcohol exposure are believed to influence addiction characteristics, such as alcohol withdrawal and tolerance (Olsen & Sieghart, 2009). Work performed in both *in vivo* animal models and *in vitro* cultured animal cells revealed that chronic alcohol exposure does in fact change GABA_A receptor subunit gene expression (Cagetti, Liang, Spigelman, & Olsen, 2003; L. L. Devaud, Smith, Grayson, & Morrow, 1995), specifically an increase in GABA_A $\alpha 4$ (*GABRA4*) subunit and a decrease in expression of the $\alpha 1$ (*GABRA1*) subunit. Furthermore, studies performed using post-mortem human brain tissue found variations in $\gamma 2$ (*GABRG2*) (Enoch et al., 2012) and δ (*GABRD*) (Bhandage et al., 2014) subunit expression. To properly determine the impact of alcohol on GABA_A receptor function, Lieberman *et al.* generated iPS cell-derived neural cultures from both alcoholic and non-alcoholic donors and investigated the expression and function of GABA_A receptor subunits in response to acute and chronic alcohol exposure.

Modeling the effect of alcohol exposure *in vitro* on human subject-derived neuronal cells is beneficial in that it captures the role of human genetic variability in influencing both the acute alcohol response as well as the development and progression of the human disease state in a disease relevant system (Lieberman et al., 2017). Lieberman *et al.* detected an

increase in mRNA expression levels of *GABRA1*, *GABRG2* and *GABRD* in iPS cell derived neuronal cells following chronic alcohol exposure (21 days at a concentration of 50 mM). *GABRA1* and *GABRG2* were increased in both control and AUD donors, while *GABRD* was only upregulated in AUD cell lines. However, work performed using *in vitro* and *in vivo* animal models have reported variable results in GABA_A receptor subunit expression levels following chronic alcohol exposure. Contradictions in data between animal and human models could be due to differences in cell-type and/or brain regions. Whole-cell patch clamp recordings were used to assess the effect of alcohol exposure on GABA_A evoked currents, but no effect was observed on the electrophysiological properties measured. Taken together, the study described above illustrates the multifaceted and heterogeneous nature of AUDs, which further supports the use of patient derived iPS cells to accurately model the pathophysiology of human disease.

Alcohol's impact on innate neuroinflammation in human neurons

Interference of cellular metabolism and differentiation as a result of epigenetic disruption is a major consequence of alcohol exposure (De Filippis et al., 2016). Progression of neurological disorders initiated by alcohol abuse are exacerbated by neuroinflammation and oxidative damage of cellular proteins and mitochondria (Haorah et al., 2008). The ability for alcohol to initiate an innate immune-like response was investigated in the CNS using patient derived iPS cells, to generate neural cell types specific to the same individual (De Filippis et al., 2016). Pathogenesis of AUDs were modeled in such way that allowed the researchers to study alcohol's effects on pluripotency (iPS cells), neurogenesis (neural progenitor cells, NPCs) and differentiation (post-mitotic neurons) (De Filippis et al., 2016).

The proliferation capacity of patient-derived iPS cells and NPCs were unaffected by either acute (24 hours) or prolonged (7 days) exposure to alcohol (70 mM). However, alcohol exposure was observed to prime an innate immune-like response via activation of NLRP3 (NOD-like receptor family pyrin domain containing 3) damage sensing inflammasome pathway. Activation of the inflammasome pathway led to an accumulation of microtubule-associated protein 1A/1B-light chain 3⁺ (LCB3⁺) autophagic puncta, as well as a deficiency in the distribution of lysosomes and mitochondria. This is in agreement with previous work describing cross talk between the inflammasome pathway and mitochondrial/lysosomal machinery (Salminen, Kaarniranta, & Kauppinen, 2012). In addition, progenitor cells exposed to alcohol prior to differentiation resulted in a significant decrease in the generation of mature neurons, when compared to an untreated isogenic control. This is an extremely important finding when analyzed in the context of fetal alcohol exposure. It has been shown that *in utero* alcohol exposure disrupts the development of the embryo and fetus, and we believe that the use of iPS cells may have uncovered the impact of alcohol on proper differentiation. Furthermore, it is known that reactive oxygen species (ROS) are generated during the process of alcohol metabolism (Wu & Cederbaum, 2009), and the inflammasome pathway has been implicated as a factor in a signaling response to a double challenge (Cullen, Kearney, Clancy, & Martin, 2015). This raises the possibility that alcohol induced activation of the inflammasome pathway in iPS cells and NPCs would cause an increased sensitivity to a second "hit" or insult. This is precisely the case where a second insult in combination with alcohol pre-exposure enhances the innate immune response, resulting in

an increase of neuroinflammation. These data suggest that alcohol exposure in iPS cells and NPCs causes an increased sensitivity to ROS, and this could potentially contribute to the pathophysiology of neurogenesis in humans. Taken together, neuronal inflammation may add to the pathophysiology of AUDs via the induction of the inflammasome pathway.

Advantages and Disadvantages for iPS Cells in Modeling Human Neuropsychiatric and Neurological Disorders

Enormous effort has been devoted to generation of defined subtypes of neural cells from iPS cells (as well as ES cells). Neural cells made from patient-specific iPS cells can better elucidate the molecular mechanisms, which underlie the pathophysiology of many neurological disorders and disease, due to the ability to capture the role of genetic variants in disease etiology. In addition, reprogramming of AUD-iPS cells is not limited to terminal differentiation to only neurons. In fact, expression of proper developmental cues can drive differentiation to other cell types as well. For example, functionally active mature hepatocyte-like cells have been generated from human iPS cells to better understand the effects of alcohol on liver biology and function (Tian, Prasad, & Jang, 2016). The field of addiction research (specifically AUDs) can potentially benefit from an *in vitro* human model system (Lieberman et al., 2017, 2012; Oni et al., 2016) because it will allow studies to mitigate an individual's genetic and/or epigenetic complexity. These benefits include: **1)** Reprogramming technology provides a tool to assay functional effects on human neural tissue of alleles that have been previously found to be associated with a risk of alcohol dependence. **2)** iPS cell derived neural cells can provide a model to compare differences between individuals, specifically allowing variations in molecular and physiological responses to both acute and chronic alcohol exposure to be studied. **3)** Gene function can be studied with regard to genetic variation between individuals. And finally, **4)** this will allow studies to investigate how differing effects of alcohol exposure can be observed in at-risk subjects.

Importantly, cross species analyses could be conducted using human iPS cell model as well as animal models. An example to illustrate this point comes from a study performed in adult rodent models where a downregulation of the *GABRA1* gene was observed in response to alcohol exposure (Leslie L. Devaud, Smith, Grayson, & Morrow, 1995; Kumar et al., 2009; Mhatre & Ticku, 1992). Conversely, an increase in expression of *GABRA1* was seen in a human neural cell model, following alcohol administration (Lieberman et al., 2017). This raises the question: Does an animal (rodent) model sufficiently mimic all aspects of human disease? Animal models of brain disorders birthed our general understanding of brain illnesses, but the belief is they don't accurately recapitulate human neuropathologies. Furthermore, post-mortem brain tissue mainly mimics the end-stage of a neurological disease or disorder, and functional physiological analysis (electrophysiology) cannot be performed on these neurons. Ultimately, iPS cell technology has the potential to become a cell-replacement therapy for many neurological diseases that currently have no cure; however there remains risk of generating several unanticipated outcomes in human application (i.e. tumor formation).

Use of human neuronal cells derived from iPS cells to examine gene expression profiles may only model the transcriptome of embryonic or early stage immature neurons. It is possible to also have variable expression patterns of certain loci located within a specific chromosome, which could hinder the number and certain types of transcripts that can be assayed for expression changes. Furthermore, it is imperative to drive differentiation of iPS cells to a specific cell type (excitatory versus inhibitory) that is appropriate for the hypothesis being tested. However, we argued above that the beauty of reprogramming technology is the ability to model disease using patient-derived iPS cells to accurately model human neuropathology. Nevertheless, the following points need to be considered to model AUDs using human neuronal cells: **1)** Does forcing a neuronal lineage to a certain cellular subtype (excitatory or inhibitory) accurately model the environment that human neurons reside in? **2)** iPS cell technology is expensive and time consuming and for this reason some studies are limited to testing a single concentration of drug because of the increasing financial strain assaying many different concentrations would bring. **3)** The effects of a drug or molecule may require additional factor(s) in order to fully produce a phenotype. Therefore, when interpreting iPS cell data from drug studies it is important to keep in mind that neural cultures produced from iPS cells may lack endogenous factors required to achieve an effect from a substance or molecule. **4)** As with any *in vitro* study, patient-derived neural cells are not in their native environment. **5)** Variations in *in vitro* culture conditions or even well-to-well variability may influence cellular phenotypes (i.e. gene expression patterns). Finally, **6)** although the genetic sequence variations in neural cells made from alcohol dependent subjects are most likely preserved in the iPS cells, the somatic cells have a characteristic epigenetic profile that gives specific cell types a certain identity, i.e. heterogeneous. For example, skin fibroblasts collected from different parts of the body actually have different epigenetic landscapes represented by variations in transcriptomes.

Reprogramming relies on the expression of certain TFs in order for the original somatic tissue to acquire pluripotency. However, directing somatic cells to a pluripotent state could alter the epigenetic character of the original donor cells (Hochedlinger & Plath, 2009; Lunyak & Rosenfeld, 2008; Meissner, 2010; Pasque, Jullien, Miyamoto, Halley-Stott, & Gurdon, 2011; Spivakov & Fisher, 2007). Thus, the environmental effects of alcohol exposure in alcoholic individuals are not likely to be maintained during reprogramming (i.e. epigenetics) (Table 1). Interestingly, a recent study reported that iN cells but not neurons derived from iPS cells maintained age related epigenetic marks (Mertens, Paquola, et al., 2015). The iN cells are generated by ectopic expression of proneuronal differentiation specific TFs (Pang et al., 2011; Vierbuchen et al., 2010) that allows generation of subtype specific human neuronal cells including excitatory (Zhang et al., 2013), inhibitory (Yang et al., 2017) and dopaminergic (Fantuzzo et al., 2017) flavors. Thus, iN cell models may provide a viable model system that potentially maintains the epigenetic modifications contributing to the disease.

Concluding Remarks and Future Perspectives

The glaring potential of reprogramming technology resides in the future application for precision medicine including therapeutics for AUDs (Figure 1). iPS cell technology allows access to patient-specific cells for several distinct purposes: **1)** In the situation where the

function of the disease associated genetic mutation is in question, genome editing can be used to repair the “disordered” genetic components in iPS cells, differentiate these cells into the affected neuronal subtype(s) and be used to study the impact of specific genetic mutations; 2) In the long run, when more mechanistic insights have gained and more technologies have been developed, cell replacement therapy with “healthy” neurons could potentially be possible and current stage animal studies have shown this approach to be viable (Kriks et al., 2011; Yang et al., 2016). And finally, 3) directed differentiation of the patient-specific iPS cells into defined neuronal subtypes can be utilized for functional readouts, thus offering a novel platform for modeling the genetic (or epigenetic) contribution to familial or sporadic AUDs (Soliman et al., 2017). Alcohol abuse contributes to mortality and cancer development, on a global scale. AUDs are extremely complex and composed of both pathophysiological and etiological effects, and might often intertwine, which makes accuracy and precision in disease modeling essential. A clear elucidation of the molecular and cellular mechanisms specific to a subject with an AUD potentially will provide effective interventions for their clinical manifestations, or maybe preventions of the morbidity. Human stem cell technology allows pivotal biological questions to be asked in a human neuronal context. For example, how does a genetic variant that predisposes an individual to develop an AUD fit into a certain molecular pathway(s)? AUDs like all neuropsychiatric disorders, often impact many brain regions and cellular subtypes, which are often difficult to study especially in an *in vitro* model system. However, recent advances in 3D organoid models (Dutta, Heo, & Clevers, 2017), as well as grafting human neuronal or glial tissue into mouse brains and then follow their behavioral modifications (Han et al., 2013), potentially will offer new insights into the etiology of AUDs.

To address the complexity of human neural circuitry modeling, a multicompartamental microfluidic culturing systems have been developed, which allows different subtypes of human neurons to be cultured in different compartments, while permitting synaptic connectivity (Fantuzzo et al., 2017). Moreover, this system allows imaging as well as functional analysis. This system can potentially be used to dissect the underlying molecular, cellular, synaptic and circuit modifications that are involved in AUDs. A more complex neural circuitry model to study basic biological and clinical applications is the *in vitro* organoid model (Simian & Bissell, 2016). Organoids can be defined as self-organizing 3D structures, which can be generated from both pluripotent stem cells (ES and iPS cells) and adult stem cells, to recapitulate the *in vivo* development and differentiation characteristics of the host mammalian tissue (Dutta et al., 2017). A combination of 3D organoid technology with patient-derived iPS cell reprogramming techniques, may permit patient-derived organoids to aid as a bridge to shorten the glaring gap between disease modeling in the context of a human and animal models. However, the 3D organoids are devoid of their native microenvironment, which normally consists of stromal cells, muscle, blood vessels and immune cells (Dutta et al., 2017) and the human neurons in culture are probably still in early developmental stages, but certainly can be used for studying the impact of alcohol on this mini-brain structure, i.e. to model the pathophysiology of FASDs. Recent advancements utilizing the brain organoid to model human disorders or disease, from Zika viral (ZIKV) infection of human neurons (Qian, Nguyen, Jacob, Song, & Ming, 2017; Salick, Wells, Eggan, & Kaykas, 2017) to neuropsychiatric disorders (Quadrato, Brown, & Arlotta, 2016)

to neurodegenerative disorders (Brawner, Xu, Liu, & Jiang, 2017), have yielded very important insights on human neuronal biology and pathophysiology. Nevertheless, modeling complex disorders like AUDs using neurocircuitry models holds the promise not only to provide great insights for understanding the underlying mechanism of AUDs but also provides a viable system for high throughput screening of effective drugs.

Some neuropsychiatric disorders often develop over time and their pathophysiology is often complex and may not benefit from *in vitro* disease modeling. Rodent brains with grafted human neurons allow the study of multifaceted and shifting environmental settings on patient derived human iPS cells. When undifferentiated human ES cells were injected directly into the brain ventricles of fetal mice this resulted in the production of human neurons and glia (Muotri, Nakashima, Toni, Sandler, & Gage, 2005). Amazingly, human adult neural stem cells survive, migrate and express the appropriate differentiation markers used to identify neurons and oligodendrocytes, following long-term engraftment in a mouse model for spinal cord injury (Cummings et al., 2005; Guzman et al., 2007). In fact, neonatal mice grafted with human glial progenitor cells yield adult mice whose brains are chimeric for human glial cells (Goldman, Nedergaard, & Windrem, 2015; Windrem et al., 2014) and SCZ glial chimeric mice have broad behavioral and sleep abnormalities (Windrem et al., 2017). This approach is extremely intriguing, we speculate that human glial or neurons derived from iPS cells derived from subjects with AUDs, when grafted into mouse brains which permits longer term chronic exposure to alcohol *in vivo*, allows behavioral and metabolic assessments of the animals as well as morphological, functional and genomic evaluations of grafted human neurons under the influence of alcohol. This potentially can generate a novel hypothesis for the elucidation of the pathophysiology as well as the etiology of AUDs.

Utilizing stem cell biology to model AUDs is still in its nascent stage however it holds great potential to uncover the genetic, molecular, cellular and circuitry mechanisms underlying the etiology and pathophysiology of AUDs. Recent developments modeling human neuropsychiatric or neurological disorders provided guidelines on how we can potentially harness this fascinating technology to devise effective individualized therapeutics or preventions for those who suffer or are susceptible to suffer from AUDs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

In this review, we summarize recent work employing iPS cell technology to model and elucidate the genetic, molecular and cellular mechanisms of AUDs in a human neuronal context and provide our perspective on future development in this direction.

Figure 1. Applications for iPS Cells in Modeling Alcohol Use Disorders (AUDs): From Disease Modeling to Precision Medicine

Somatic tissue (mainly fibroblasts) can be obtained from patients that have been clinically diagnosed to have an AUD. Fibroblasts are then reprogrammed to an embryonic state through coexpression of the OSKM factors (Oct4, Sox2, Klf4 and c-Myc) to produce induced pluripotent stem (iPS) cells. iPS cells can then be differentiated into neurons. Conversely, it is possible to convert fibroblasts, via forced expression of specific transcription factors (Brn2, Ascl1 and Myt1l), into functional induced neuronal (iN) cells (Pang et al., 2011). iPS-derived patient specific neurons can be used for modeling AUDs to understand underlying mechanisms driving pathology. In addition, assays can be employed to assess the cytotoxicity of small molecules being tested for potential drug development.

iPS cells generated from a patient carrying a known genetic variant linked to AUDs can be edited using genetic engineering prior to terminal neuronal differentiation. The “fixed” human neurons can then be used in applications for precision medicine such as transplantation/regenerative medicine. It is also possible to model the effects of ethanol on various stages of iPS cell development, self-propagation and differentiation (*see pink boxes*). The diagram above illustrates how iPS cells provide an upgraded model system for AUD disease modeling, high-throughput drug screening and cytotoxicity assays.

Table 1

Some pros and cons of different approaches employed to model human disease

Type of Disease Model	PROS	CONS
<p>Animal Model</p>	<ul style="list-style-type: none"> • Can address questions taking an <i>in vivo</i> approach. Whole organism studies including behavioral assessments. • Protocols are well established and standardized • Can be genetically modified 	<ul style="list-style-type: none"> • Do not fully model the disease state as they present in the human system. • Availability of a model for every disease • Some ethical concerns
<p>Human Embryonic Stem (hES) Cells</p>	<ul style="list-style-type: none"> • Unlimited capacity to self-renew in culture • Broad developmental potential • Accurately and realistically models human disease states • Studies can be performed on patient specific cells • Gene editing possible 	<ul style="list-style-type: none"> • Acquisition of hES cells is difficult • Major ethical concerns • Phenotypes may not be fully developed
<p>Induced Pluripotent Stem (iPS) Cells</p>	<ul style="list-style-type: none"> • Unlimited capacity to self-renew in culture • Pluripotent • Accurately and realistically models human disease states • Studies can be performed on patient specific cells • Gene editing possible 	<ul style="list-style-type: none"> • Phenotypes may not be fully developed • Forced expression of specific transcription factors required for reprogramming could confound experimental conclusions