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REVIEW ARTICLE

Genes, Brain and Behavior

5. Collaborative Study on the Genetics of Alcoholism: Functional genomics

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Abstract

Alcohol Use Disorder is a complex genetic disorder, involving genetic, neural, and environmental factors, and their interactions. The Collaborative Study on the Genetics of Alcoholism (COGA) has been investigating these factors and identified putative alcohol use disorder risk genes through genome-wide association studies. In this review, we describe advances made by COGA in elucidating the functional changes induced by alcohol use disorder risk genes using multimodal approaches with human cell lines and brain tissue. These studies involve investigating gene regulation in lymphoblastoid cells from COGA participants and in post-mortem brain tissues. High throughput reporter assays are being used to identify single nucleotide polymorphisms in which alternate alleles differ in driving gene expression. Specific single nucleotide polymorphisms (both

This is the fifth paper (The other papers in the series are: 1. Overview, 2. Sample and Clinical Data, 3. Brain Function, 4. Genetics) in a linked series of reviews.

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coding or noncoding) have been modeled using induced pluripotent stem cells derived from COGA participants to evaluate the effects of genetic variants on transcriptomics, neuronal excitability, synaptic physiology, and the response to ethanol in human neurons from individuals with and without alcohol use disorder. We provide a perspective on future studies, such as using polygenic risk scores and populations of induced pluripotent stem cell-derived neurons to identify signaling pathways related with responses to alcohol. Starting with genes or loci associated with alcohol use disorder, COGA has demonstrated that integration of multimodal data within COGA participants and functional studies can reveal mechanisms linking genomic variants with alcohol use disorder, and potential targets for future treatments.

KEYWORDS
alcohol use disorder (AUD), brain, gene expression, genomics, induced pluripotent stem cells, neuronal function

1 | INTRODUCTION

Recent advances in genetics, particularly large scale genome-wide association studies (GWAS), including several by the Collaborative Study on the Genetics of Alcoholism (COGA; see 4. Genetics in this issue) have identified loci associated with complex genetic disorders, including alcohol use disorders (AUD)[1–5] and other Substance Use Disorders (SUD).[6,7] While larger sample sizes have led to the identification of more loci and strengthened the confidence in putative loci, progress toward understanding the molecular mechanisms underlying AUD risk has been challenging.[8] The loci identified by GWAS as associated with increased risk for AUD or other SUD are often large and contain many genes and variants in linkage disequilibrium (LD; groups of variants within a locus are frequently inherited with each other). In some cases, the most significant variant(s) in a locus may not be the one(s) that contribute to risk for the trait. In addition, many of the variants are in noncoding regions, suggesting that gene regulation is a key mechanism. A major challenge to the field has been elucidating which variants and genes actually contribute to the risk for AUD.[8]

COGA has pursued the identification of the genetic mechanisms contributing to alcohol use disorder, through both genetic studies (see 4. Genetics in this issue) and functional molecular studies of gene expression, including screening for regulatory elements and studying effects of variations in identified loci in neuronal models. These studies were made possible not only by sharing key genomic data among COGA across its 10 sites and with external investigators, but also by the forward-looking decision made decades ago to cryopreserve lymphocytes from COGA participants, providing a unique source of cells from individuals with extensive phenotypic and genotypic data (see 2. Sample and Clinical Data, and 4. Genetics in this issue).

In this review, we describe how COGA has been conducting cellular and molecular studies to understand functional mechanisms linking genes and variants identified by GWAS to the risk for AUD. These include studies on gene regulation in lymphoblastoid cells from COGA participants with and without alcohol exposure, and in post-mortem brain tissues from individuals with or without AUD, combined with bioinformatic analyses (Figure 1). Molecular studies also use high throughput reporter assays (HTRA) to identify SNPs in which alternate alleles differ in driving gene expression. To evaluate variants with hypothesized function in the central nervous system (CNS), electrophysiology and gene expression studies are performed in neurons differentiated from induced pluripotent stem cells (iPSCs) derived from COGA participants (Figure 1). The overall goal is to exploit the robust genomic data, curated phenotypes, and banked cells from the individuals studied by COGA to identify mechanisms contributing to AUD.

2 | GENE EXPRESSION IN BRAIN

Many variants in loci identified by GWAS are in noncoding regions, and likely to drive differences in gene expression. Behavioral responses to alcohol ingestion as well as symptoms of dependence and withdrawal are likely mediated by the central nervous system, suggesting that gene expression analyses in selected brain regions could provide insights. In fact, a number of studies, including several by COGA,[9–13] have compared patterns of gene expression in brain regions of individuals with and without AUD, and examined the effects of alcohol on gene expression. Comparison of gene expression in post-mortem brain regions of individuals with and without AUD identifies differences that may be related to pre-existing genetic risk factors or to the effects of long-term exposure to high levels of alcohol, or both.

In a microarray study, McClintick and colleagues[9] compared gene expression in the hippocampus of 20 individuals with and 19 without AUD from the New South Wales Tissue Resource Centre. There were 639 named genes for which expression differed between those with and without AUD at a False Discovery Rate (FDR) ≤ 0.20. Although changes were small, 52% of those genes differed by at least 1.2-fold. Among these were the glucocorticoid receptor, the related FK506 binding protein 5 (*FKBP5*) and many metallothioneins. Pathways related to inflammation, hypoxia, and stress showed activation, while

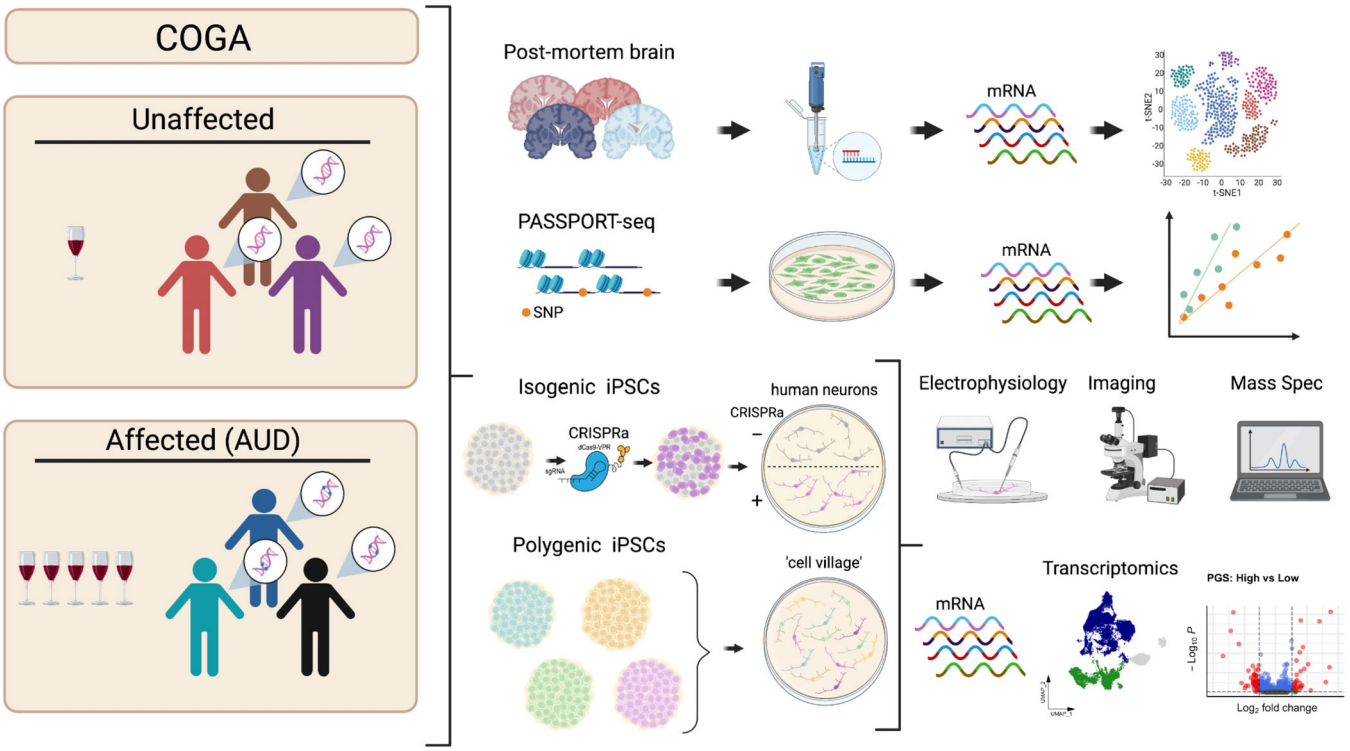

FIGURE 1 Summary of functional genomics strategies in COGA. COGA uses brain post-mortem tissue and lymphoblastoid cells from healthy (unaffected) and AUD-diagnosed (affected) COGA participants to study gene expression and its regulation by different sequencing strategies. Novel high throughput reporter assays such as PASSPORT-seq are applied to analyze genetic variants in which alternate alleles differ in driving gene expression. In parallel, COGA uses iPSCs derived from AUD affected and unaffected COGA participants and differentiates them into neurons. Genetically-defined human neurons allow evaluation of the functional consequences of AUD-associated variants with hypothesized effects in the central nervous system using transcriptomic and electrophysiological approaches. Isogenic iPSC-based strategies are used to model and functionally evaluate specific AUD-associated SNPs. Created with BioRender.com.

pathways that play roles in neurogenesis and myelination showed decreases in expression.[9]

Kapoor et al.[12] used RNA sequencing to examine gene expression in the pre-frontal cortex of individuals with and without AUD. In this study of 65 individuals with AUD and 73 without, 129 genes showed altered expression (FDR < 0.05). Differentially expressed genes were enriched for pathways related to interferon signaling and Growth Arrest and DNA Damage-inducible 45 (*GADD45*) signaling. Weighted gene co-expression network analysis identified two significant modules, one of which, enriched in genes related to calcium signaling pathways, opioid signaling, and nicotine responses, was significantly correlated with alcohol dependence, alcohol consumption, and AUDIT scores. A second module, also enriched for genetic associations with AUD and alcohol consumption, showed upregulation of pathways related to immune signaling.[12]

A later study that integrated multiomics data to fine-map associations with both AUD and drinks per week identified genes and individual SNPs that affect risk.[14] A meta-analysis of AUD GWAS summary statistics identified 10 independent loci containing 31 lead SNPs (1157 SNPs in total) in or near 79 genes. Many of the loci were shared with a GWAS of drinks per week.[15] The variants were enriched in promoter regions of genes expressed in the brain, particularly in early development. Integrating this with expression and metabolomics quantitative trait loci (eQTL and mQTL, respectively) data from brain identified 18 loci with 21 genes. Summary based Mendelian randomization nominated a single candidate causal gene at 16 of these loci. Many of the genes (e.g., *SPI1*, *MAPT*) have also been implicated in neuropsychiatric and neurodegenerative disorders. Interestingly, at one locus, two independent SNPs (i.e., not in LD) were identified, one was associated with AUD and the other with drinks per week. These findings are consistent with GWAS data that show only partial overlap in the genetics of these two phenotypes.[2–5,16] Key pathways included immune signaling, lipid metabolism, and alcohol metabolism.[14]

Current studies are using single nucleus RNA sequencing and ATAC-sequencing to measure gene expression and chromatin accessibility in many different types of cells within the brain (unpublished data). Because different cell types differ in gene expression, these studies should give us a much clearer understanding of functional differences between individuals with and without AUD.

3 | GENE EXPRESSION IN CULTURED CELLS

To dissect genetic risk-associated differences in gene expression from those caused by exposure to ethanol, McClintick et al.[10] examined the

effects of 24 h ethanol exposure (75 mM) on lymphoblastoid cell lines (LCLs). A paired design (each cell served as its own control in comparing ± ethanol) allowed detection of small differences between cells generated from COGA participants, 21 with and 21 without AUD. 99% of the genes detectably expressed in these LCLs are also expressed in brain. Differences between participants with and without AUD included 13 genes previously identified as associated with AUD or related traits, including *KCNA3*, *DICER1*, *ZNF415*, *CAT*, *SLC9A9*, and *PPARGC1B*. The ethanol exposure altered expression of 51% of the unique genes expressed in these cells, but the differences were modest. Genes affected included *ANK3*, *EPHB1*, *SLC1A1*, *SLC9A9*, *NRD1*, and *SH3BP5*, which were reported to be associated with AUD or related phenotypes in two GWAS.[17,18]

McClintick and colleagues[11] conducted an RNA sequencing study in which LCLs from COGA participants were treated with 75 mM ethanol for 48 h. Ethanol affected expression of 4456 of the 12,503 genes detectably expressed (at an FDR ≤ 0.05). Cells from individuals with and without AUD responded similarly to ethanol, although expression of genes in the cholesterol biosynthesis pathway, including the one encoding the rate-limiting enzyme HMGCR, was lower in participants with AUD. The affected genes fell into many pathways, including activation of NF-κB, neuroinflammation, IL6, IL2, IL8, and dendritic cell maturation pathways, consistent with increased signaling by NF-κB, TNF, IL1, IL4, IL18, TLR4, and LPS. Signaling by interferons A and B decreased, as did EIF2 signaling, phospholipase C signaling, and glycolysis.[11]

McClintick and colleagues[13] also studied the effects of chronic intermittent ethanol exposure (CIE; 4 h of 40 mM ethanol on four successive days, followed by 3 days without for 3 weeks; meant to reflect cycles of heavy drinking and abstinence) on a neuroblastoma cell line, SH-SY5Y. Again, ethanol changed gene expression: of 1498 genes at FDR < 0.20, half increased and half decreased, but the changes were relatively small; only 133 genes were altered at least 1.2-fold. Many of the altered genes were related to neuronal function (e.g., receptors, synthesis, degradation or transport of transmitters, channel subunits) or development (e.g., axon growth, synaptogenesis). CIE altered pathways related to neurogenesis and the plasticity of neurons, including axonal guidance, reelin signaling, synaptogenesis, dopamine signaling, and serotonin signaling. Ethanol also increased stress responses such as the unfolded protein response, and TGF-β and NF-κB signaling. Many genes involved in cholesterol biosynthesis were downregulated. Interestingly, 24 h withdrawal reduced most of the expression changes toward levels in unexposed cells.[13]

4 | HIGH-THROUGHPUT IDENTIFICATION OF SNPs AFFECTING GENE REGULATION

The loci identified by GWAS contain large numbers of variants, not all of which are known to be functional or related to the phenotype analyzed. High-throughput reporter assays (HTRAs) can systematically identify which SNPs have regulatory activity and which may differ between different cell types by comparing the effects of both alleles for each SNP on expression of reporter genes in relevant cell types. A novel technique, PASSPORT-seq,[19,20] allows functional testing of multiple 3′UTR variants in parallel (Figure 2). Initially, PASSPORT-seq was used to analyze all variants in the 3′UTR of 88 genes that showed expression differences in four brain regions between control and AUD participants.[20] Results demonstrated biased allelic expression of 53 SNPs in one neuroblastoma cell line (SH-SY5Y cells) and 130 in another (SK-N-BE (2) cells). Among these, the direction of allele bias in both SH-SY5Y and SK-N-BE (2) cell lines was the same in 25 loci. Eighteen of these 25 SNPs were in eQTLs as defined in the GTEx database,[21] 7 lay within binding sites of *ELAVL1* (ELAV-like RNA binding protein 1), 11 within the binding site of *PABPC1* (polyA-binding

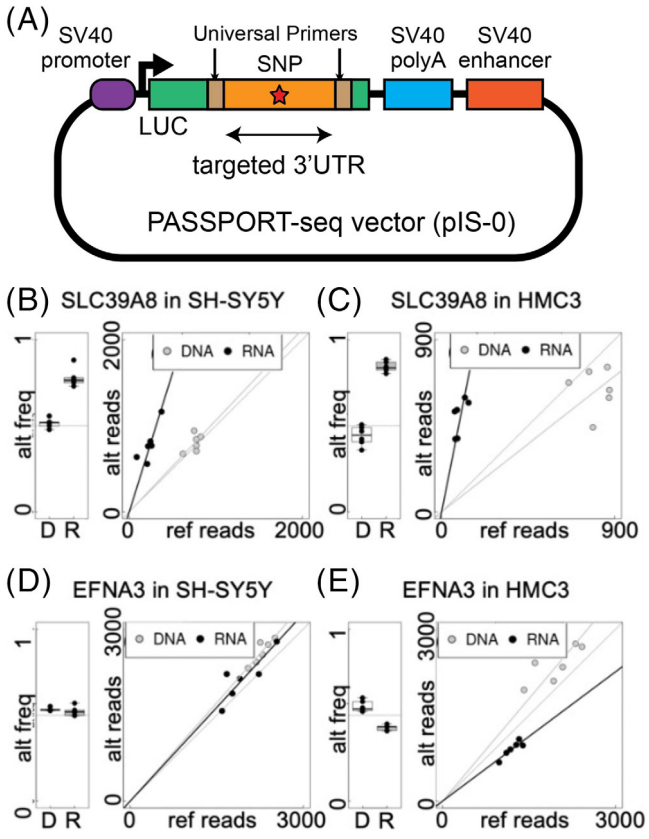

FIGURE 2 PASSPORT-seq assay for differential expression of alleles in 3′ untranslated regions. (A) PASSPORT-seq vector. 51 bp segments with the SNP to be tested in position 26 are inserted into the 3′ untranslated region of the luciferase gene. After transfecting human cells with pools of these constructs, RNA and DNA are extracted from the pooled cells and the amount of RNA expressed from each is compared with the amount of DNA. (B–E) Results of PASSPORT-seq assays. The number of unique reads of DNA and RNA from six replicate transfections of two different cell types is plotted, with alternate alleles on *Y* axis and reference alleles on *X* axis; on the left are the allele frequencies in the RNA (R) and DNA (D). (B, C) Effects of rs151371 variant in SH-SY5Y cells and HMC3 cells, respectively; both alleles showed biased allele expression. (D, E) Effects of rs2306124 in SH-SY5Y cells and HMC3 cells, respectively; this SNP demonstrated biased allele expression in HMC3 cells but not in SH-SY5Y cells.

protein cytoplasmic 1), and 4 SNPs alter the target regions of 13 miRNAs expressed in brain.[22] Another study, testing for the potential function of a set of variants that were identified in meta-analyses of alcohol-related traits in African American and European Americans, identified dozens of SNPs that reside in the binding sites of miRNAs and RNA-binding proteins, and are expression quantitative trait loci of genes including *KIF6*, *FRMD4A*, *CADM2*, *ADD2*, *PLK2*, and *GAS7*.[23] The PASSPORT-seq assay was recently expanded to test the impact on gene expression of 13,515 SNPs in 3′UTRs in both neuronal (SH-SY5Y) and microglial (HMC3) cell lines (in preparation). To complement the studies of 3′UTR variants, we have begun to examine the impacts of SNPs in putative enhancer regions on gene expression, using the STARR-seq approach.[24] The results of these HTRAs will then be used to predict which other SNPs should affect gene expression, and the predictions will be tested by additional HTRAs.

5 | IDENTIFYING MECHANISMS OF AUD-ASSOCIATED SNPs IN HUMAN NEURON MODELS

Human iPSC models provide a powerful approach for elucidating AUD etiology, permitting developmental and functional characterization of the effects of SNPs on cellular mechanisms, while retaining a participant's unique genetic background.[25,26] The NIAAA/COGA Sharing Repository possesses an extensive collection of cryopreserved lymphocytes and lymphoblastoid cells collected from COGA participants that have undergone substantial phenotypic and genotypic characterization, providing an essential tool for investigating mechanisms underlying AUD and related addiction phenotypes. Reprogrammed into iPSC, these cells can then be differentiated into neural progenitor cells (NPCs),[27] neurons,[28] astrocytes or oligodendrocytes.[29–31]

Several strategies may be used to study genome variation and specific genetic variants in iPSC-derived neurons. With one strategy, groups of participants may be selected from repository specimens using SNPs from GWAS data and specific clinical diagnoses or other phenotypic data. While retaining variable background genomes, this approach is limited by the relatively small number, by genetics standards, of iPSC lines that can be reasonably produced and cultured (generally 8–12 in most studies). Another strategy, incorporating genome editing, such as the CRISPR/Cas9 system, may be used to create isogenic pairs of cell lines, one with a variant and one without, but retaining identical genomic backgrounds. This approach is most appropriate for studying one or a few closely linked variants. But for studying complex, polygenic changes in the context of diverse genetic backgrounds, construction of isogenic lines is impractical due to the large number of targets, so participant selection may utilize a summary statistic such as polygenic risk score to focus on specific risk groups.

Several groups have previously used human iPSC-derived neuron cultures to study cellular phenotypes of individuals with AUD,[26,32] assessing the effects of acute or chronic ethanol exposure.[33–35] In a transcriptomic study of iPSC-derived forebrain-like neurons from healthy and alcohol-dependent individuals, Jensen and colleagues[35] found that sustained exposure (7 days) to 50 mM ethanol affects genes involved in cholesterol homeostasis, notch signaling, and cell cycle pathways. A different study showed that 7 days of 50 mM ethanol suppressed the NMDA-R response to acute ethanol in healthy neurons, while receptor subunit mRNA expression levels were only altered in neurons differentiated from individuals with alcohol dependence.[33] Longer exposure to the same ethanol concentrations (21 days) led to increased NMDA-R expression in iPSC-derived neurons from alcohol-dependent, but not from healthy individuals.[34] Both 24 h and 7-day exposure to 70 mM ethanol also activate the NLRP3 inflammasome pathway in iPSC-derived neural progenitors.[36]

COGA has also studied the effects of coding[37,38] and noncoding AUD-associated SNPs on neuronal function.[39] Studies in progress utilize combined data from GWAS summary statistics and phenotyping to generate polygenic risk scores (PGS) for participant selection in iPSC-based functional studies of AUD (unpublished data).

6 | FUNCTIONAL CONSEQUENCES OF NON-SYNONYMOUS SNPs ASSOCIATED WITH AUD

Early COGA studies focused on non-synonymous variants in single genes with high risk for AUD. A major focus of the AUD candidate gene literature has been on genes encoding alcohol-metabolizing enzymes, such as alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH).[40] The associations between AUD and SNPs in *ADH1B* and *ALDH2* represent some of the largest effect sizes in psychiatry. Studies of a non-synonymous variant in *ADH1B* (rs1229984), resulting in the replacement of Arginine (R48) with Histidine (H48), show that individuals with the minor allele demonstrate reduced risk for AUD.[40–42] It was hypothesized that dramatic change in enzyme activity (H48 oxidizes ethanol approximately 70- to 80-fold faster than R48) leads to transient elevation of acetaldehyde and development of aversive behavior associated with alcohol due to experience of severe flushing. Variations in *ALDH2* (rs671; E504K), the mitochondrial acetaldehyde dehydrogenase, that greatly reduce its activity and thereby increase acetaldehyde levels, were also associated with AUD.[40,43] Although *ADH1B* and *ALDH2* have by far the strongest known effects on risk for AUD, other candidate genes and heritable traits are known to predict AUD liability.

Initial COGA studies using iPSC-derived neurons focused on the *CHRNA5* gene, encoding the α5 subunit of the nicotinic acetylcholine receptor, with variants that have been associated with dependence on nicotine, alcohol and other substances. Results showed that the minor allele of rs16969968, encoding a D398N amino acid replacement, produced neurons that were more sensitive to stimulation with lower concentrations of nicotine but were rapidly desensitized, predicting an altered effect on reward pathways.[37] Another functional coding variant associated with AUD is a non-synonymous A118G (rs1799971; N40D) SNP in the *OPRM1* gene, which encodes the mu

opioid receptor (MOR) and has been also linked with an increased risk for drug addiction.[44–47] A118G is one of the most common SNPs in selected populations, affecting ~40% of Asian, ~16% of European and ~3% of African individuals.[48] This SNP encodes an amino acid substitution, replacing asparagine (N40) with aspartate (D40), thus removing a potential glycosylation site. Although the involvement of A118G in AUD has been controversial,[49–51] and there was no significant association with risk for alcoholism in a COGA family-based study,[52] its role in opioid dependence has been established in both mouse and human contexts. Studies found that human GABA inhibitory induced neurons (iNs) derived from iPSCs generated from individuals of European descent show stronger inhibition of synaptic transmission in D40, as compared with N40 neurons.[46] Similar changes in synaptic transmission were observed in CRISPR-edited, isogenic lines containing N40 or D40 variants, ruling out possible differences due to genetic backgrounds in different participants.[46]

To investigate the role of A118G in response to ethanol, the effects of acute and long-term ethanol exposure were examined. Previous studies in rodents showed that ethanol potentiates GABAergic transmission.[53] Acute application of 40 mM ethanol increased the frequency of spontaneous and miniature inhibitory postsynaptic currents (sIPSC and mIPSC) in N40-expressing inhibitory iNs, suggesting a presynaptic origin of GABA potentiation.[38] In addition, the increases of sIPSC and mIPSC were larger for major N40-harboring iNs, compared with neurons from D40 minor allele carriers. N40 iNs also exhibited a significant reduction of spontaneous action potential firing, consistent with pronounced GABA release. Conversely, a 10-day intermittent ethanol exposure (IEE), intended to mimic diurnal drinking patterns, produced stronger GABA potentiating responses exclusively in iNs harboring the minor D40 MOR allelic variant but not in iNs with the major allele, as was observed with acute ethanol exposure (Figure 3). Collectively, these studies demonstrate the contribution of non-synonymous variants to altered ethanol responses and provide a better mechanistic understanding of AUD pathology.

7 | FUNCTIONAL CONSEQUENCES OF A NONCODING AUD-ASSOCIATED SNP IN *KCNJ6*

Noncoding (intronic, synonymous, or intergenic) variants that may affect gene expression are likely to be greatly influenced by their specific genetic architecture.[25,54] Using iPSC lines made from participants with selected, GWAS-identified variants retains variable background genomes as well as noncoding species-specific sequences that could not yet be studied in model organisms.

A COGA GWAS study in families densely affected with AUD has shown genome-wide association with noncoding SNPs and a highly correlated imputed synonymous SNP in the *KCNJ6* gene and theta brain oscillations during a task measuring selective attention and response inhibition, a neural phenotype also associated with AUD.[55] A synonymous (rs702859) and two noncoding (rs702860 and rs2835872) SNPs were shown to affect neurodevelopmental trajectories of event-related theta oscillations with age and sex-specific effects[56] and to be associated with theta power during reward processing in COGA's prospective study of adolescents and young adults (Figure 4, Panels A and B)[57] (also see 3. Brain function in this issue). *KCNJ6* encodes the G protein-gated inwardly rectifying potassium channel subunit 2 (GIRK2) and plays a critical role in regulating the excitability of a variety of neuronal cell types.[58] In rodent studies, GIRK channels are implicated in alcohol-related behaviors.[58] In addition, GIRK channels can be directly potentiated by alcohol through interaction with a hydrophobic pocket in the channel.[59] It was unclear, however, how the noncoding *KCNJ6* variants might affect the GIRK channels and neuronal function and relate to AUD phenotype.

Popova and colleagues[39] generated NGN2-induced excitatory neurons from eight participants of European ancestry from the NIAAA/COGA cell repository: 4 with AUD-linked allelic variations in *KCNJ6* and 4 without, each haplotype selected for the presence (affected) or absence (unaffected) of AUD, respectively. Analysis of

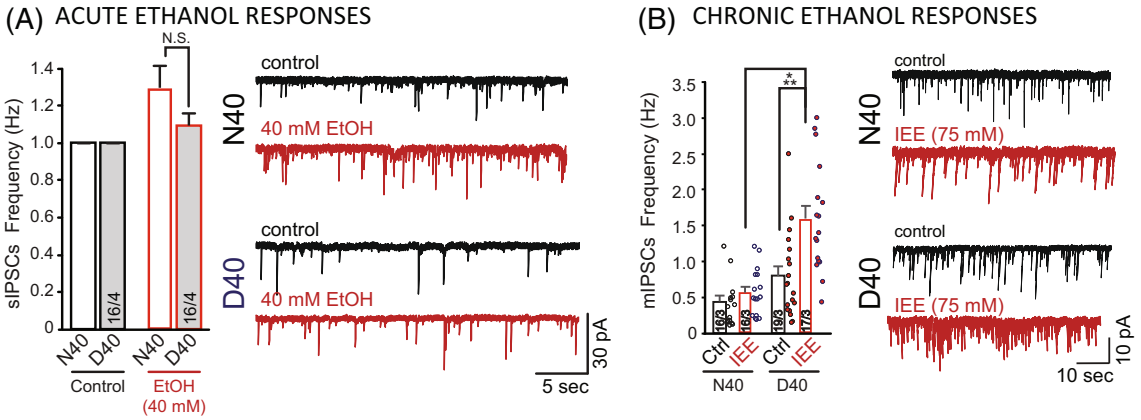

FIGURE 3 Functional consequences of non-synonymous SNPs in *OPRM1*. (A) Acute ethanol application causes an increase in the inhibitory tone (spontaneous inhibitory postsynaptic currents, iPSCs) in iPSC-derived inhibitory human neurons carrying N40 major allele. (B) Chronic ethanol treatment increases GABAergic transmission in inhibitory human neurons carrying D40 minor allele. Modified from Scarnati et al.[38]

these participants identified additional 19 SNPs in the 3′ UTR of *KCNJ6* in strong LD with the original three. Analysis focused on differences associated with this AUD-linked, noncoding *KCNJ6* haplotype in multiple genetic backgrounds.

Single-cell RNA sequencing (scRNAseq) was performed on groups of iPSC-derived neurons that were pooled and cultured in the same dish to reduce culture variability.[60] Comparing induced neurons from individuals with each haplotype, scRNAseq identified 1393 genes that were differentially expressed between affected and unaffected individuals, 797 up-regulated, and 596 down-regulated.[39] Gene ontology (GO) analysis of the up-regulated genes revealed changes in pathways associated with protein targeting within the cells, catabolic metabolism, and nonsense-mediated decay. Analysis of the downregulated genes predicted differences in pathways associated with nervous system development, axonal transport, and trans-synaptic signaling. Neurons from individuals with the alternative haplotype and AUD had significantly lower levels of *KCNJ6* expression, and ethanol exposure reversed these effects. Since most sequence variants in LD with the significant *KCNJ6* SNPs were in the 3′ UTR of the mRNA (21 out of 22 SNPs), it was hypothesized that differences in *KCNJ6* expression were due to differential mRNA stability, translation, or other post transcriptional processes.

At the cellular level, *NGN2*-induced excitatory neurons with the alternative AUD *KCNJ6* haplotype had a significantly higher density of neurites, accompanied by decreased GIRK2 levels (Figure 4, Panel C).[39] Fluorescence in situ hybridization (FISH) for *KCNJ6* also showed a decrease in mRNA, consistent with the scRNAseq results. Reduced GIRK2 expression was associated with increased excitability of the cells generated from individuals with AUD both at the level of individual neurons as well as at the network level (Figure 4, Panel C). A 7-day 20 mM alcohol exposure induced GIRK2 protein and mRNA expression, ameliorating differences in neuronal excitability (Figure 5, Panels A and B). *KCNJ6* overexpression alone replicated the effects of alcohol administration on neuronal excitability (Figure 5, Panel C).

It is tempting to compare these findings on induced human neurons, linking excitability with GIRK2 expression, with the original observations from COGA[55–57] that noncoding *KCNJ6* SNPs were associated with activation in theta power during attention, response inhibition and reward processing. However, these studies also raise additional mechanistic questions, such as how noncoding SNPs affect *KCNJ6* expression and how they might function in GABA and DA neurons. Many of these questions can be investigated using iPSC-derived neuron models in future studies.

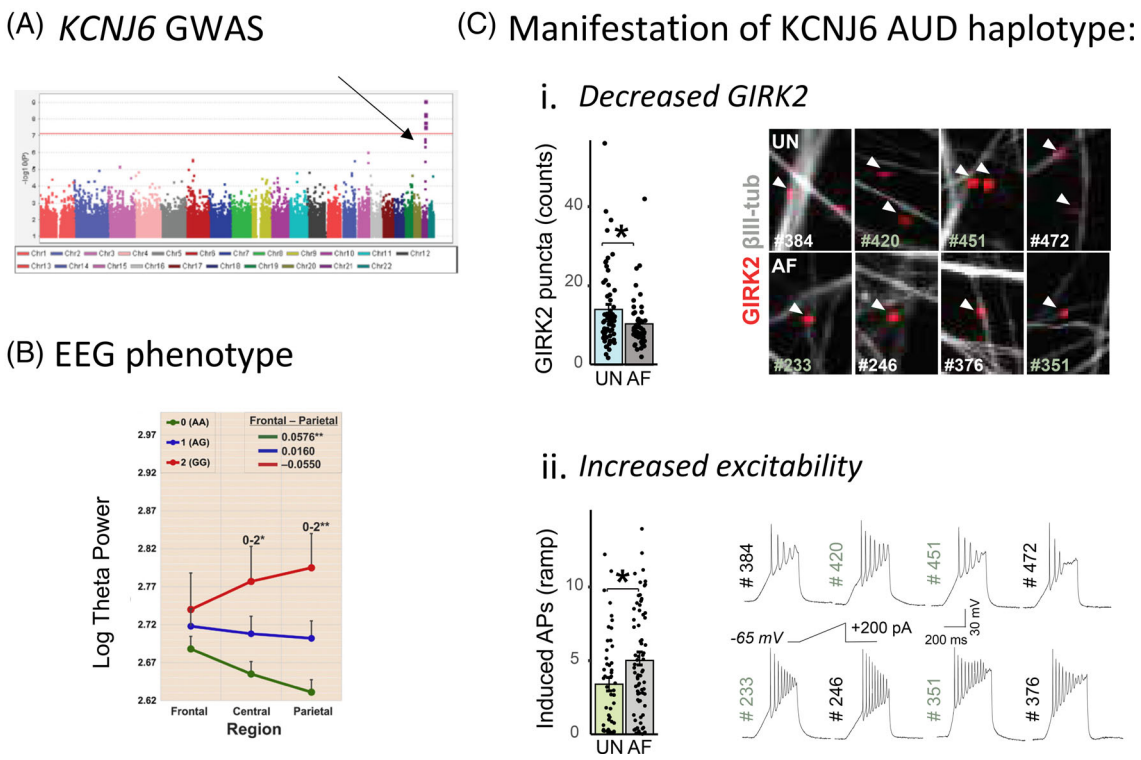

FIGURE 4 Functional consequences of a non-coding SNPs in *KCNJ6*. (A) Manhattan plot showing genome-wide association of frontal theta event-related oscillation with several SNPs, including a synonymous SNP, rs702859, in the *KCNJ6* gene on chromosome 21. (B) *KCNJ6* SNPs influence the magnitude and topography of ERO theta power during reward processing in a monetary gambling task. (C) Impact of *KCNJ6* haplotype (multiple linked SNPs) on iPSC-derived excitatory neurons: (i) GIRK2 expression is decreased in the affected individuals (AUD/*KCNJ6* minor alleles) as measured by puncta counts; (ii) neuronal excitability is increased in the affected individuals (AUD/*KCNJ6* minor alleles) as measured by number of induced action potentials. Modified from Kang et al.,[55] Kamarajan et al.,[57] and Popova et al.[39]

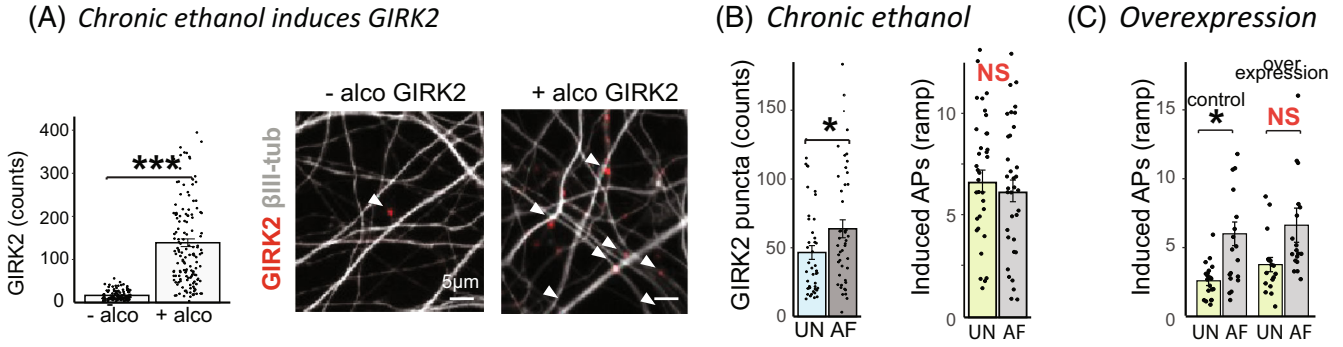

FIGURE 5 Ethanol-mediated responses in a context of non-coding SNPs in *KCNJ6*. (A) Chronic ethanol induces GIRK2 expression in iPSC-derived excitatory human neurons. (B) Chronic ethanol eliminates differences in GIRK2 expression and excitability between affected (AUD/*KCNJ6* minor alleles) and unaffected (not AUD/*KCNJ6* major alleles) iPSC-derived neurons. (C) *KCNJ6* overexpression mimics chronic ethanol responses. Modified from Popova et al.[39]

Since alcohol-exposed neuron cultures exhibited an increase in *KCNJ6* expression, artificially increasing *KCNJ6* expression permits investigation of regulated cellular mechanisms. Differences in *KCNJ6*/GIRK2 expression alone are predicted to be sufficient to alter the effects of alcohol on neuronal function, including spiking activity and associated energy utilization. For this approach, using an isogenic cell line strategy where variable genetic backgrounds are retained provides a complementary means of inquiry to SNP-specific findings. Current efforts in COGA aim to capitalize on lentiviral expression and CRISPRa technology to dissect *KCNJ6* influence on neuronal function, independently of the myriad factors involved in AUD.

CRISPR activation (CRISPRa) fuses a transcription activator domain to a "dead" Cas9 (lacking endonuclease activity), which is targeted to a selected promoter with a guide RNA and upregulates gene expression.[61] CRISPRa has been successfully implemented to investigate multiple eQTL gene variants associated with schizophrenia, resolving pre- and postsynaptic neuronal deficits together with genotype-dependent gene expression.[62] By utilizing endogenous regulatory mechanisms, CRISPRa is different from ectopic overexpression because of potential effects of UTRs on protein localization and trafficking. This is particularly pertinent for *KCNJ6* which has a long (19 kb) 3′UTR. Using CRISPRa, it will be possible to determine more precisely the effects of increased GIRK2 expression on neuronal function and the neuronal response to chronic ethanol exposure.

While iPSC-derived neuronal studies can reveal insights into the functional changes associated with genomic variants, there are some limitations inherent in the use of these models. Studies suggest that the transcriptomes of human neurons and astrocytes derived from iPSC are more similar to fetal than to mature adult cells.[63–65] In the study of *KCNJ6* variants, only a sub-cluster of individual cells expressed markers consistent with functional neurons.[39] Several strategies are likely to improve cell maturation, such as the optimization of the culture media composition,[66] the combination of transcription factor-based differentiation strategies with directed patterning,[67] and the use of longer term cultures.[68] The latter would be particularly suitable in the case of human iPSC-derived glutamatergic neurons, which have shown increased expression of NMDA glutamate receptors and consequent increased synaptic activity in older neuronal cultures.[67] Another effective strategy is the co-culture of neurons with supporting cells, such as human astrocytes or primary mouse astrocytes.[28,39] This co-culture approach promotes not only maturation, but also synchronization of neuronal activity.[68,69] In line with this, co-cultures of human iPSC-derived neurons and oligodendrocytes, which allow the study of neuron–glia cross-talk and myelination processes, led to the observation of neuronal alterations related to tuberous sclerosis complex that were not apparent in neuronal monocultures.[70] These issues show that iPSC-derived neurons, while invaluable for studying both coding and noncoding gene variants in the context of a human genetic background, should be optimized for the questions being asked and interpreted with care.

8 | COMBINED EFFECT OF MULTIPLE, AUD-RELATED GENETIC VARIANTS ON HUMAN NEURONAL FUNCTION: PGS FOR AUD

Although a small number of variants in ethanol metabolism genes have a large impact on risk for AUD,[40] the vast majority of variants are more common but do not exhibit substantial risk on their own; however, collectively multiple SNPs (i.e., polygenic) likely contribute to the genetic risk of AUD. The identification of these multiple variants will help predict the onset and development of individual risk for dependence.[71,72] A polygenic risk score (PGS) estimates the risk of an individual to develop a disorder based on contributions of multiple risk-associated variants throughout the genome[73] (also see 4. Genetics in this issue). A recent study by Page et al.[74] used a human iPSC approach to evaluate the combined functional effects of multiple schizophrenia-associated variants by comparing iPSC-derived cortical neurons from individuals affected by schizophrenia who had a high PGS for SCZ and neurotypical individuals with low PGS for SCZ. They observed differences in the function of sodium channels, excitability, and GABAergic neurotransmission,[74] suggesting that PGS captures some of the molecular mechanisms underlying schizophrenia.

PGS analysis has been used to evaluate risk for AUD both by others[16,75,76] and within COGA[77–82] (also see 4. Genetics in this issue). Current COGA studies are now evaluating participants distinguished by extreme high (>90%ile) or low (<10%ile) PGS for AUD, using iPSC-derived neurons to elucidate possible differences in transcription, neuronal physiology, and response to ethanol. The extensive clinical, neuropsychological, electrophysiological and genetic data available within COGA, in addition to lymphoid cells in the NIAAA/COGA Sharing Repository, can be used for analyses of mechanisms associated with PGS extremes, enabling a multidimensional assessment of AUD risk.

9 | CONCLUSION AND FUTURE DIRECTIONS

Future studies will benefit from incorporating emerging, novel techniques to further advance our understanding of the functional genomics contributing to the development and outcomes of AUD. Improvements in technologies that allow the analysis of multiple human brain samples or pooled cultures from multiple participants to reduce variability and costs will be key. HTRAs such as PASSPORT-seq have begun to model regulatory function of SNP variants in models of the appropriate cell types.[20,23] The high-throughput features of these techniques allow the functional evaluation of larger cohorts, enabling a population-level resolution of these studies. Functional validation of omics is essential to contextualize molecular findings in cellular mechanisms underlying AUD. Integration of proteome, transcriptome, and genome-wide association data is a powerful approach to identifying novel brain proteins underlying substance use disorders, such as smoking, cannabis use disorder, opioid use disorder, and alcohol use disorder.[14,83] Furthermore, region-specific proteomics of the prefrontal lobe and motor cortex revealed AUD-linked alterations in metabolic enzymes, as well as changes in cytoskeleton and excitotoxicity associated proteins.[84]

For future iPSC studies in AUD, mixed cultures of excitatory and inhibitory neurons co-cultured with human astrocytes can mimic connectivity patterns of the adult brain.[85] Three-dimensional brain organoids provide another platform for studying human neurons with physiological connections.[86–88] For example, organoids have been used to examine the effects of ethanol on neuronal development.[89–91] The use of genetically encoded indicators (GEIs) for population-based studies provides a complementary approach for high-throughput evaluation of activity.[92] Lastly, advanced proteomic approaches can be used to characterize iPSC-derived neurons in AUD, as has been done with Rett's disease[93] and schizophrenia.[94]

In summary, the rich collection of genotypic and phenotypic data that COGA has generated since its inception, together with the large repository of lymphoid cells and the myriad of functional approaches that are being applied in this multimodal project (Figure 1), make COGA uniquely positioned to unravel the genetic and molecular underpinnings underlying the risk for AUD (also see 1. Overview, in this issue). Our study of postmortem brain tissues has highlighted differences between individuals with and without a history of alcohol dependence, including pathways related to inflammation. The HTRAs have identified functional SNPs within loci associated with alcohol dependence. Specific SNPs identified by COGA or by external investigators, both coding and noncoding, have been modeled using iPSC-derived neurons from COGA participants for functional characterization. SNPs in *OPRM1* and *KCNJ6* impact the physiology of inhibitory (*OPRM1*) and excitatory (*KCNJ6*) neurons, respectively, highlighting a role for these neurons in AUD. In the future, AUD-associated variants identified by COGA will be modeled in iPSC-derived neurons using methods to selectively manipulate the expression of the gene of interest in a controlled genetic environment. In addition, PGS can be modeled in populations of iPSC-derived neurons to search for pathways that could be linked with responses to alcohol. These examples, starting with genes or loci associated with AUD, followed by the evaluation of their functional consequences in human neuronal models from COGA participants, and the correlation of those findings with the rich phenotypic data from the same participants, demonstrate that the deep integration of multimodal data within COGA enables functional studies to reveal mechanisms linking genomic variants with AUD.

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DATA AVAILABILITY STATEMENT

COGA data are available in dbGaP (phs000125, phs000763, phs000976, phs001208), or via an application to the National Institute on Alcohol Abuse and Alcoholism (https://www.niaaa.nih.gov/research/major-initiatives/collaborative-studies-genetics-alcoholism-coga-study) or a COGA investigator sponsored secondary analysis proposal.

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