ARTICLE

Alcohol reverses the effects of KCNJ6 (GIRK2) noncoding variants on excitability of human glutamatergic neurons

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INTRODUCTION

Alcohol use disorder (AUD) is a heritable ($h^2 = 0.49$) condition characterized by an impaired ability to stop or control alcohol use despite adverse social, occupational, or health consequences [1]. An estimated 14.1 million American adults (ages 18+) were diagnosed with AUD in 2019 and the numbers continue to grow, particularly due to the COVID-19 pandemic [2]. Even though several evidence-based treatments are available for AUD [3–5], there is a high discrepancy in treatment outcomes, suggesting the existence of a variety of traits in those diagnosed with AUD [6, 7]. A family-based, genome-wide association study (GWAS) of a frontal theta event-related oscillations (ERO) phenotype identified an association with several single nucleotide polymorphisms (SNPs), including a synonymous SNP, rs702859, in the KCNJ6 gene on chromosome 21 [8]. More recent studies concluded that this polymorphism influences the magnitude and topography of ERO theta power during reward processing in a monetary gambling task, reflecting a genetic link to neuronal circuits [9, 10]. Two other SNPs (rs702860 and rs2835872) within KCNJ6 were linked with the ERO endophenotype and AUD, but are noncoding, either intronic or within the 3′ untranslated region (3′ UTR), respectively. Additional studies identified various genetic loci correlating with selected EEG results but all consistently include alcohol dependence [11, 12]. To understand how these SNPs might influence these phenotypes, it is essential to characterize the pathways and mechanisms by which genetic risk unfolds at molecular, cellular and network resolutions. Furthermore, since the KCNJ6 SNPs are noncoding or synonymous, the optimal strategy would incorporate human genetic backgrounds of actual subjects to preserve potential effects of noncoding sequences.

Synonymous and noncoding single nucleotide polymorphisms (SNPs) in the KCNJ6 gene, encoding G protein-gated inwardly rectifying potassium channel subunit 2 (GIRK2), have been linked with increased electroencephalographic frontal theta event-related oscillations (ERO) in subjects diagnosed with alcohol use disorder (AUD). To identify molecular and cellular mechanisms while retaining the appropriate genetic background, we generated induced excitatory glutamatergic neurons (iN) from iPSCs derived from four AUD-diagnosed subjects with KCNJ6 variants (“Affected: AF”) and four control subjects without variants (“Unaffected: UN”). Neurons were analyzed for changes in gene expression, morphology, excitability and physiological properties. Single-cell RNA sequencing suggests that KCNJ6 AF variant neurons have altered patterns of synaptic transmission and cell projection morphogenesis. Results confirm that AF neurons express lower levels of GIRK2, have greater neurite area, and elevated excitability. Interestingly, exposure to intoxicating concentrations of ethanol induces GIRK2 expression and reverses functional effects in AF neurons. Ectopic overexpression of GIRK2 alone mimics the effect of ethanol to normalize induced excitability. We conclude that KCNJ6 variants decrease GIRK2 expression and increase excitability and that this effect can be minimized or reduced with ethanol.

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SPRINGER NATURE
KCNJ6 encodes the G protein-gated inwardly rectifying potassium channel subunit 2 (GIRK2), which, when assembled into a GIRK channel, plays a role in regulating cell excitability [13]. The inward rectifying properties of GIRK are based on the ability to conduct potassium ions into the cell more easily than out, leading to a reduction in excitability [14, 15]. GIRK function in neurons is associated with activation of G protein-coupled receptors (GPCRs), which leads to dissociation of Gβγ subunits from the G protein complex and binding to the channel [16]. In addition, this family of GIRK channels can be potentiated by ethanol concentrations (20–50 mM) relevant to human alcohol usage through direct interaction with a hydrophobic pocket on the channel [17, 18]. Studies in model organisms also reveal the importance of GIRK function in AUD: mice lacking GIRK2 demonstrate reduced ethanol analgesia, greater ethanol-stimulated activity in open-field tests, increased self-administration of ethanol, and failure to develop a conditioned place preference for ethanol [19–21]. Thus, GIRK channels not only modulate the excitability of the neurons but also play an essential role in regulating responses to alcohol.

Recent studies have employed the use of subject-specific induced stem cell technology to model, characterize and elucidate mechanisms underlying various types of addiction disorders including AUD [22–25]. The generation of specific subtypes of cultured human neurons enables the study of formerly inaccessible functional properties using standard neuroscience methods [22, 24–26]. The contribution of various KCNJ6 SNPs was found to affect a variety of properties associated with neuronal function or sensitivity to drugs of abuse, suggesting targets for potential therapeutic interventions. Associating specific genetic variants with mechanisms contributing to addictive behaviors may produce more effective therapies tailored to individual genotypes.

We hypothesize that noncoding SNP variants in KCNJ6 alter neuronal excitability, potentially contributing directly or indirectly to network-level endophenotypes such as ERO. Furthermore, since ethanol interacts directly with GIRK2, we predict that excitability will be modulated by ethanol. To test these hypotheses, we evaluated a human neuron model system that includes variant genotypes, so that specific intergenic or noncoding sequences as well as genetic backgrounds influencing these functions would be preserved. Therefore, we selected subjects from the NIAAA/COGA Sharing Repository, four with the ERO-associated allelic variant in KCNJ6 and the presence of diagnosed alcohol dependence and four without the variant and unaffected for AUD and produced eight iPSC lines. iPSCs were reprogrammed into excitatory human neurons (iN) to investigate the potential contribution of the KCNJ6 SNPs to AUD diagnosis and the ERO power endophenotype (Fig. 1A). We found that neurons from KCNJ6 variant AUD-affected individuals demonstrated initial transcriptomic and morpho-physiological differences, mostly affecting excitability of the cells, which were paralleled by differences in GIRK2 expression levels. Ethanol exposure, conversely, induced GIRK2 expression, ameliorating differences in excitability. Moreover, by overexpressing KCNJ6 we replicated effects of ethanol on neuronal excitability. The results promote a better understanding of the association between genetic variants and brain-wide changes affecting AUD risk and can potentially be used for development of personalized interventions.

**METHODS**

**Generation of human iPS cells and glutamatergic neurons (iN)**

Subjects were selected from the NIAAA/COGA Sharing Repository of the Collaborative Study on the Genetics of Alcoholism (COGA) project with characteristics summarized in Table 1. Selection criteria consisted of KCNJ6 SNP genotype, DSM-IV diagnosis of AUD, sex, and availability of frozen lymphocytes in the repository. All subjects were of European ancestry. See Supplementary Methods for details. The previously-described protocol for generating glutamatergic human induced neurons (iN) [27–29] was modified. To obtain cultures with enhanced levels of spontaneous activity, we minimized the time of Ngn2 induction to reduce alternate cell identities [30] and fed cultures with a low-molarity medium for at least 30 days post-induction [31] as described in the Supplementary Methods. Cultures of iN typically exhibited resting membrane potentials averaging —40 mV with the presence of synaptic markers, spontaneous action potentials, and synaptic activity.

**Intermittent ethanol exposure (IEE)**

Since ethanol evaporates during culture incubation (Supplementary Fig. 7), we added ethanol to 20 mM and then replenished half the medium daily with 40 mM ethanol. Ethanol concentrations were measured using an AM1 Alcohol Analyzer (Analogix Instruments, Ltd.) [25].

**RNA sequencing**

For bulk RNAseq, individual iN cultures were harvested in lysis buffer and column purified (Quick-RNA kit, Zymo). RNA was sent to Novogene, Inc., for sequencing. Analysis is described in the Supplementary Methods. RNAseq data have been deposited with the NIH GEO repository (accession number GSE196491). For single-cell RNA sequencing (scRNAseq), iN cultures were treated for 7 days using IEE [25] starting 21 days after plating onto glia. At 28 days, iN were dissociated using trypLE Express (Thermo Fisher) for 5 min at 37 °C and then processed and analyzed as described in the Supplementary Methods. These data have been deposited with the NIH GEO archive, accession number GSE203530.

**Electrophysiology**

Analyses of iN used whole-cell patch-clamp electrophysiology as previously described [24, 25] with details provided in the Supplementary Methods. Drugs were applied through a perfusion system in the following concentrations: 160 nM ML297 or 20 mM ethanol. Clamp software (pClamp 11; Molecular Devices) was used for analysis of recordings.

**Immunocytochemistry and confocal imaging**

iN cultures were fixed for 30 min in ice cold methanol and permeabilized using 0.2% Triton X-100 in PBS for 15 min at room temperature. Cells were then incubated in blocking buffer (5% BSA with 5% normal goat serum in PBS) for 1 h at room temperature and then incubated with primary antibodies diluted in blocking buffer overnight at 4 °C, washed with PBS three times, and incubated with secondary antibodies for 1 h at room temperature. Confocal imaging was performed using a Zeiss LSM700. Primary antibodies used: rabbit anti-GIRK2 (Alomone labs, APC-006, 1:400), mouse anti-βIII-tub (BioLegend, MMS-435P, 1:1000), chicken anti-MAP2 (Millipore AB5543, 1:1000), mouse anti-Sym1 (SYSV, 106-011, 1:200), mouse anti-PSD 95 (SYSV, 124-011, 1:2000), mouse anti-mCherry (Thermofisher Scientific, M11217, 1:100).

**Fluorescent in situ hybridization (FISH)**

Fluorescent, single molecule in situ hybridization was performed with the RNAscope® Multiplex Fluorescent Detection Kit v2 (Advanced Cell Diagnostics, ACD) following the manufacturer’s instructions and details provided in the Supplementary Methods.

**Image cytometry**

Image analysis was performed using the Fiji/ImageJ image analysis program [32] as described in the Supplementary Methods. ImageJ scripts can be downloaded from the repository at [https://github.com/ramt604/imagej](https://github.com/ramt604/imagej). The person performing analysis was blinded to genotype or line information.

**Lentiviruses**

FSW-H-Syn-GCaMP6f assembly was described previously [33]. FUGW-KCNJ6-mCherry was assembled using lentiviral backbone from FUGW (AddGene #14883). KCNJ6 coding sequence amplified from human neuron cDNA (forward primer: agcaggggagaggg, reverse primer: ggaggaga-gaagggtg), and mCherry with a nuclear localization signal from pMLe-nMCherry (a gift from Dr. Kelvin Kwan). During construction, the GIRK2 protein-coding sequence was tagged with a 3xHA tag and linked to mCherry with a T2A “self-cleaving” element.

**Calcium imaging in iPSC-derived iN populations**

For assessing neuronal excitability, iN co-cultured with mouse glia on Matrigel™ coated 10 mm glass coverslips were transduced with lentiviruses encoding the GCaMP6f fluorescent protein.
expressing hSyn-GCaMP6f at least 2 weeks prior to imaging to ensure robust expression. All fluorescence imaging experiments were performed in ~50 DIV (days in vitro) iNs. Details of the protocol and analysis are found in the Supplementary Methods.

**Statistics**

Parameters that were sampled in cells from multiple microscopic fields and from multiple cell lines were fit to a linear mixed-effects model using the lme4 package in R [34]. The model included the cell line identifier and sex.
A Tukey post-hoc test identified pairwise differences in two-factor models (e.g., genotype and ethanol). Electrophysiology results were modeled using generalized estimating equations (GEE) with the cell line as grouping identifier using the geepack R package [35]. Where appropriate, ANOVA or Student’s t test was used as indicated in figure legends. For RNAseq, pseudo-bulk data (single-cell reads pooled by subject identifier) were modeled in DESeq2 [36], testing first by likelihood ratio testing (LRT) over all groups, and then pairwise comparisons were evaluated using Wald tests. After Benjamini-Hochberg multiple measurements correction, a false discovery rate of 5% was set as a threshold for significance. Numbers of replicate cells and/or fields per cell line and genotype used for statistical testing are listed in Supplementary Table 6.

RESULTS

Gene expression predicts functional differences between KCNJ6 haplotypes

To investigate the role of KCNJ6 gene variants in neuronal function and ethanol response we selected eight subjects of European ancestry from the COGA cell repository, with contrasting KCNJ6 SNPs and the presence or absence of alcohol dependence (Table 1). Since multiple SNP genotypes were used, we label groups as AUD “affected” (AF) or “unaffected” (UN) for simplicity, since in this set of subjects diagnosis correlates with SNP haplotype. However, the focus of this study is the KCNJ6 haplotype.

To confirm and extend SNP genotypes, iN cultures were harvested for bulk RNAseq analysis. To identify isoforms, RNAseq data identified only the longer KCNJ6 isoform ENST00000609713 (Fig. 1B), which includes a 3’ exon of 18,112 nucleotides primarily consisting of 3’UTR. We identified 19 additional 3’UTR SNPs linked with the initial three (Supplementary Table 1; red lines in Fig. 1B), constituting a haplotype of 22 variants (synonymous and noncoding) within the expressed isoform of KCNJ6 mRNA, all in a region of linkage disequilibrium (LD; Supplementary Fig. 1C). There are no nonsynonymous variants, therefore, this haplotype is predicted to alter KCNJ6 mRNA stability, translation, or other post-transcriptional processes.

Previous studies suggested that iN cultures may be heterogeneous, potentially confounding a bulk RNAseq analysis [30]. To enable analysis of iNs, we used single-cell RNAseq (scRNAseq) of pooled neurons from multiple cell lines, a strategy known as a “cell village” [37]. Based on preliminary results indicating differences in excitability, we cultured each haplotype separately to avoid potential secondary effects, combining cells within each group in equivalent proportions. Following maturation (~30 days after induction), dissociated cells were processed to generate scRNAseq libraries. Mapping cells by t-distributed stochastic neighbor embedding (tSNE), a distinct cluster of cells coordinately expressed several markers consistent with neuron physiological function (Fig. 1C and Supplementary Fig. 2E), including synaptophysin (SYP), voltage-gated sodium channel (SCN3A), glutamate transporter (SLC17A6), and both NMDA (GRIN2A, GRIN2B, GRIA2, GRIA4) and kainate (GRIK2) glutamate receptors. This cluster also expressed both KCNJ6, encoding GIRK2, and KCNJ3, encoding GIRK1, which are required to form heterotetrameric, functional channels [14, 38]. Detection of mRNA in individual cells by scRNAseq underestimates expression, so the cluster identified as neurons likely expresses markers more uniformly than observed here. We conclude that this cluster of cells expresses components required for physiological activity, and that other cells, expressing subsets of these neuronal markers, are alternate products of induction, which we label as “transitional” neurons (Fig. 1C), since reprogramming is not identical to differentiation [39, 40]. Therefore, we focused gene expression analysis on the phenotypically neuronal subset of cells.

Table 1. Subjects selected for iPSC.

| Group | ID   | Sex | DSMIV Dx | rs702859 | rs702860 | rs2835872 |
|-------|------|-----|----------|----------|----------|-----------|
| AF    | 376  | M   | Affected | GG       | GG       | AA        |
| 246   | F    | Affected | GG  | GG       | AA        |
| 351   | F    | Affected | GG  | GG       | AA        |
| UN    | 472  | M   | Unaffected | AA      | AA       | GG        |
| 384   | M    | Unaffected | AA  | AA       | GG        |
| 451   | F    | Unaffected | AA  | AA       | GG        |
| 420   | F    | Unaffected | AA  | AA       | GG        |

The ID is a three-digit code that is not traceable to the subject identity. Genotypes for listed SNPs were obtained from GWAS studies [8]. SNP genotypes were confirmed by iPSC genomic DNA PCR and Sanger sequencing.

Given the differences in gene expression and partial reversal of KCNJ6 downregulation, we evaluated detailed neuronal phenotypes
in UN and AF iN cultures, including GIRK2 expression, neuronal morphology, and physiological response. Since GIRK2 immunocytochemistry had not been reported in cultured human neurons, we validated detection using mouse primary cortical cultures [42] (Fig. 2A, B). Lentiviral-transduced GIRK2 overexpression increased levels of immunostaining about 3.5-fold (Fig. 2C, mCherry + cells are KCNJ6 lentiviral-transduced). Importantly, silencing KCNJ6 with shRNAs or frameshift knockout by CRISPR/Cas9 eliminated GIRK2

**Fig. 2** Validation of GIRK2 expression and function in human induced neurons. A Representative confocal images of GIRK2 immunoreactivity in mouse cortical neurons. Arrowheads indicate locations of GIRK2-staining puncta, with an example punctum enlarged in the inset, overlapping or adjacent to JIII-tubulin-positive processes. We observed two cellular expression patterns—one where the entire neuron is decorated with GIRK2 antibody (Supplementary Fig. 4), or another where GIRK2 expression is relatively faint and observed mostly on neuronal processes, shown here. B GIRK2 expression patterns in human induced neurons (iNs), showing representative confocal images from line 420. Inset shows two adjacent puncta. GIRK2 immunoreactivity matched a pattern of process-selective expression in mouse (A and Supplementary Fig. 4A), where GIRK2 was detected as relatively small (~0.5 µm diameter) puncta scattered primarily along the processes. Localization of GIRK2 immunoreactivity in human iN did not directly colocalize with synaptic vesicle marker VGLUT2 or synaptic marker Syn1 (Supplementary Fig. 5), but instead was found most frequently adjacent to synapses but overlapping the shafts of the JIII tubulin-positive processes, and less so on MAP2 positive processes (Figs. 2D.a, 3C, E). Cultured neurons express JIII tubulin throughout the cell, but not as strongly in axonal processes [69]. We previously found that processes in human iN cells stained for ankyrin G, identifying the axonal initial segment, which similarly lacked JIII-tubulin [70]. Detection of GIRK2 primarily on JIII-tubulin+/MAP2 processes, therefore, suggests pre-axonal, and likely presynaptic, localization.

C Following infection of iN cultures with lentivirus expressing both KCNJ6 and mCherry, large numbers of GIRK2+ puncta are seen in representative images (line 420). D Evaluation of GIRK2 function in iNs, (a) quantification of GIRK2 expression on MAP2+ vs. JIII-tubulin+ neuronal processes. GIRK2 is more abundant on JIII-tubulin processes (p = 0.0006, one-tailed Student’s t test, n = 15 cells per group, cell line 420). (b) Basal levels (upper pie plot) of the GIRK current in iNs as percent of neurons responding with hyperpolarization to the selective GIRK activator (160 nM ML297); compared with responding percentage when GIRK2 is overexpressed (lower pie chart). (c) Representative image of iN overexpressing GIRK2, as confirmed with mCherry fluorescence. (d) Representative traces of induced action potential firing before and after GIRK activation, demonstrating the contribution of GIRK function to cell excitability. (e) Representative trace of spontaneous postsynaptic current (sEPSCs) recordings during ML297 (160 nM) GIRK activator wash-in, demonstrating a shift of 7 mV holding current (amplifier-dependent compensation of GIRK-mediated membrane hyperpolarization). (f) Quantification of neuronal excitability at baseline and following GIRK activation with 160 nM ML297 (p = 0.015, paired Student’s t test, n = 9 cells before/after ML297, cell line 376).
immunoreactivity (Supplementary Fig. 4B, C), which further confirms antibody specificity.

To confirm that GIRK2 expression contributes to potassium channel function, we treated iN cultures with ML297, a selective activator of the GIRK1/GIRK2 heterotetramer complex [43, 44]. Results show that the magnitude and frequency of native (basal) currents observed in human iN were relatively small (Fig. 2D,b), with only 6.8% of the neurons responding to a shift in membrane potential holding current (~10 pA). However, cells overexpressing GIRK2 (identified by mCherry co-expression; Fig. 2D,c) exhibited an increased frequency of response (GIRK currents) to 30% of the cells after ML297 addition (Fig. 2D,b) without a change in magnitude (Fig. 2D,e). Importantly, GIRK activation affected excitability of the neurons by shifting resting membrane potential to more negative values (Fig. 2D,d), affecting the ability of neurons to fire APs when induced (Fig. 2D,f). We conclude that increased KCNJ6 expression affects neuronal excitability by altering GIRK channel activity.

**KCNJ6 haplotype alters morphology and membrane excitability**

To identify neuronal properties affected by KCNJ6 haplotype, we focused on three aspects: morphology, expression of GIRK2, and physiological properties. A detailed analysis of neuronal morphology is essential to rule out differences affecting excitability [45]. Most measures of basic neuronal morphology did not exhibit differences by haplotype, including soma size, circularity, and solidity, which describe the most fundamental aspects of neuron shape (Fig. 3B,a–c). However, neurite area, as determined by βIII-tubulin-staining, reflecting the number and/or branching of neurites per cell, increased in the AF group (p = 0.018; Fig. 3B,d), with example images from individual cell lines in Fig. 3C. Results are plotted for each cell line or aggregated by KCNJ6 haplotype. As predicted by KCNJ6 mRNA expression (Fig. 1J), GIRK2 immunoreactivity was decreased in the AF group (Fig. 3D), measured by puncta counts (p = 0.0012; Fig. 3D,a), puncta circularity (p = 0.007; Fig. 3D,c), and solidity (p = 0.037; Fig. 3D,d) but not puncta size (Fig. 3D,b). No difference was found by sex. These results demonstrate an overall decrease in GIRK2 in neuronal processes in the AF (KCNJ6 variant allele) group.

Reduced GIRK2 expression is predicted to affect the excitability of neurons. As expected, we found no differences in membrane capacitance, membrane resistance, spontaneous excitatory post-synaptic potential frequency (sePSs) frequencies or amplitudes, or spontaneous action potential (AP) firing (Fig. 3F). However, we observed increased excitability in the AF group (p = 1.2 × 10^{-5}; Fig. 3G). Less current injection, using a ramp technique, was required for the AF group to shift the resting membrane potential (RMP) of neurons held at −65 mV, producing more AP firing, compared with the UN group (p = 2.0 × 10^{-9}; Fig. 3G,d). Therefore, the KCNJ6 variant haplotype group (AF) exhibited increased neurite area, reduced neurite GIRK2 expression, and greater excitability. Other neuronal properties were unchanged, suggesting that overall neuronal differentiation status was similar.

Since cultures are heterogeneous (Fig. 1C), we wished to evaluate larger sample sizes. Therefore, we used calcium imaging to assess spontaneous and glutamate-stimulated firing. We selected four cell lines for these studies (AF: 233 and 246, and UN: 420 and 472) and used the viral-transduced, genetically-encoded calcium indicator GCaMP6f as a proxy for neuronal activity [46]. Example images show consistent expression in all cell lines (Fig. 4A). Cultures were treated with repeated pulses of 10 μM glutamate (each followed by wash-out with ACSF), and pulses of 50 μM glutamate to elicit transient increases in fluorescence, indicating receptor-induced excitability, followed by pulses of 18 mM KCl, indicating cellular excitability (Fig. 4B–D). Neurons from both UN and AF groups had heterogeneous spiking patterns, with >50% of the neurons remaining inactive during the baseline period or after stimulation with glutamate or KCl. This is consistent with scRNAseq results indicating that only ~23% of the iN cells express genetic markers for neuronal activity (Fig. 1C). Increased spontaneous and induced spiking (>1–2 spikes) was apparent in the AF group when viewing individual cell responses in a raster plot (Fig. 4B, C). Spiking frequencies increased 2.4-fold in baseline conditions (Fig. 4E; p = 1.1 × 10^{-9}) and 1.3-fold following glutamate stimulation (Fig. 4F; p = 1.6 × 10^{-5}) in the AF group, but no difference was found in KC1-induced activity (Fig. 4G; p = 0.85), confirming the above results from individual neurons. When examining the AF or UN neurons individually, we also observed higher spontaneous and glutamate-elicted excitability for lines 233 and 246 when compared to line 472, which was found to be the least active spontaneously or under glutamate stimulation among the four studied (Supplementary Fig. 6). Overall, cells from the AF KCNJ6 variant haplotype group were more excitable, with no difference in basal physiological properties (i.e., KC1-induced activity). Furthermore, not only did the spike rate increase per cell (bar plots, Spikes/ROI/min), but we also observed increases in the proportion of cells in a field that responded (pie plots). These results not only confirm findings of differences in excitability, but also demonstrate that the difference in the AF iN is found not only individual cell activity but also in the frequency of detecting active cells within a population.

**Ethanol exposure eliminates differences in properties affected by KCNJ6 haplotype**

Analysis of KCNJ6 mRNA expression in selected neurons (Fig. 1C) not only detected decreased basal levels in the AF group, but also demonstrated increased expression following ethanol treatment. This predicts that increasing GIRK2 expression in the AF group following ethanol treatment would eliminate differences from the UN group in morphology, expression, and physiology. To control ethanol dosage in culture, we utilized an intermittent ethanol exposure (IEE) paradigm as in previous studies [25]. Ethanol was replenished daily to account for loss by evaporation, producing mean concentrations of 15.4 ± 1.16 mM (Supplementary Fig. 7). The half-life of ethanol is 14.5 h (with a 95% CI of 13.5 to 15.6 h), so over 24 h the concentration would drop to ~5–8 mM before replenishment. We targeted a peak concentration of 20 mM ethanol, which is similar to a blood alcohol concentration (BAC) of 0.08% (17 mM), the legal limit for intoxication. We also selected this concentration based on concentration-dependent interactions of GIRK2 with ethanol [17]. After 7 days of IEE, we evaluated neuronal morphological and functional properties. Focusing on parameters that were different by genotype without IEE (Figs. 3, 4), we found that ethanol eliminated the differences in total neurite area (p = 0.98, Fig. 5A,d), GIRK2 puncta counts (p = 0.46, Fig. 5B,a), excitation following current injection (p = 0.32, Fig. 5E,a), step- (p = 0.48, Fig. 5E,b), and ramp- (p = 0.95, Fig. 5E,d) induced APs. Membrane capacitance was slightly but significantly reduced in the IEE AF group (p = 9.6 × 10^{-9}, Fig. 5D,a). Other measurements were unchanged, again indicating that the cultured neurons exhibited similar differentiation properties. Results are consistent with the interpretation that neurite and excitability differences correlate with reduced GIRK2 expression, and that ethanol increases expression of GIRK2, reversing these effects.

To confirm that both KCNJ6 haplotype and ethanol exposure affect GIRK2 expression, we evaluated GIRK2 immunocytochemistry and KCNJ6 mRNA in neurons using fluorescent in situ hybridization (FISH). GIRK2 increased following IEE (p = 1.0 × 10^{-20}; Fig. 5G, H). By probing KCNJ6 fluorescent puncta colocalized with MAP2 immunocytochemistry, we confirmed that neurons from AF individuals had lower levels of KCNJ6 mRNA compared to UN neurons (Fig. 5J,a, p = 8.2 × 10^{-3}). We also found that the proportion of MAP2+ neurons expressing KCNJ6 was reduced in AF compared with UN (50.1% vs. 64.7%, Fig. 5J,b), and
Fig. 3  Impact of AUD-associated KCNJ6 haplotype on neuronal properties. A Principles of morphological analysis of induced neurons: (a) Neurite area was the total TuJ1+ (βIII-tubulin+) staining area outside the cell soma. (b) Solidity is the area of the soma divided by its convex hull area. (c) Soma size was the area of the MAP2+ cell body. (d) Circularity compared the perimeter to the area. B Morphometry of iNs from KCNJ6 haplotype variant and affected (AF, cyan) or unaffected (UN, gray) individuals. Results are summed by group (left) or plotted individually by cell line (right), with subjects identified by line number (see Table 1—females identified with gray numbers). Individual cells are plotted as dots with the bar showing the mean, with error bars indicating the standard error of the mean (SEM). No significant differences were found in (a) soma size, (b) circularity, or (c) soma solidity, but (d) total neurite area was increased in the AF group (p = 0.018). C Representative images of iNs from individual lines, with arrows identifying individual GIRK2 puncta (red) localized on βIII-tubulin+ processes (gray). D GIRK2 expression was decreased in the AF as measured by puncta counts (a, p = 0.0012), circularity (c, p = 0.007), or solidity (d, p = 0.037), while there was no difference in puncta size (b). E Representative images of individual GIRK2 puncta (red) localized on βIII-tubulin+ processes (gray). F Electrophysiological analysis of passive neuronal properties, showing no difference in (a) membrane capacitance (b) membrane resistance, or (c) spontaneous EPSCs frequency. G Electrophysiological analysis of induced neuronal properties. (a) Quantification of current required to shift resting membrane potential to −65 mV in pA: difference by group p = 1.2 × 10−5; (b) quantification of maximum number of action potentials (APs) induced with the “step” protocol, p = 0.086; (c) representative traces of APs induced with the “step” protocol; (d) quantification of number of action potentials (APs) induced with the “ramp” protocol, p = 2.0 × 10−9; (e) representative traces of APs induced with the “ramp” protocol. A generalized linear model was used to evaluate group differences for morphometry and GIRK2 expression, and generalized estimation equations was used for electrophysiology results. Numbers of cell lines and replicates for each experiment are shown in Supplementary Table 6.
the AF neurons had a greater proportion of cells with low levels of expression (Fig. 5J.c) with no detectable High or Very High (>9 puncta/cell) expression. Differences in expression were found by haplotypic in somatic regions of neurons (Fig. 5J.f, $p = 5.5 \times 10^{-3}$) but not in non-somatic processes (Fig. 5J.g, $p = 0.23$). Ethanol increased KCNJ6 mRNA levels in both groups (Fig. 5J.a-e), eliminating the difference between genotypes. Interestingly, we also observed that the proportions of AF neurons expressing KCNJ6 was increased (8.3%, Fig. 5J.b) by ethanol and was paralleled by a reduction in number of low-expressing cells and increased appearance of high-expressing neurons (Fig. 5J.c). Results indicate that variant KCNJ6 haplotype leads to reduced KCNJ6 expression, which is consistent with RNA sequencing and immunocytochemical results, and that ethanol increases KCNJ6 expression, eliminating differences between haplotype groups.

**GIRK2 overexpression mimics IEE in human neurons with KCNJ6 variants**

To test if ethanol-induced GIRK2 expression could underlie the elimination of differences in neuronal properties associated with the KCNJ6 haplotype, we compared ethanol treatment with virus transduced KCNJ6 overexpression. Since ethanol has been shown to potentiate GIRK2-containing channels, we used a single treatment with 20 mM ethanol followed by fixation 24 h later. As expected, neither ethanol nor GIRK2 overexpression exhibited differences in passive neuronal properties (not shown). However, while current-induced activity increased in control cultures ($p = 6.8 \times 10^{-6}$, Fig. 6C), no significant difference was found in cultures exposed to 24 h ethanol ($p = 0.74$) and GIRK2 overexpression ($p = 0.23$). Similarly, while control cultures had increased ramp-induced APs ($p = 5.9 \times 10^{-4}$; Fig. 6A) neither GIRK2 overexpression ($p = 0.14$) or ethanol ($p = 0.10$) were significantly different. As a control, GIRK2 puncta count increased in a sample cell line after overexpression (line 376, $p = 0.04$, Fig. 6D) without an increase in circularity or solidity (not shown). These results indicate that the increased excitability in KCNJ6 minor allele haplotype cells is due to reduced expression of GIRK2 protein and this effect is at least partially ameliorated or reversed by exposure to doses of ethanol found in human brain after moderate to heavy daily drinking.

**DISCUSSION**

This study focused on the effect of an AUD- and endophenotype-linked KCNJ6 haplotype on neuronal function, with a goal of identifying mechanisms that could eventually drive therapeutic strategies. To retain the appropriate genetic background for
individuals with AUD, we selected subjects from the NIAAA/COGA Sharing Repository, using both KCNJ6 haplotype and alcohol dependence as criteria (Table 1). By preparing iPSC from these subjects and inducing them to model excitatory neurons, we identified differences in KCNJ6 mRNA levels and GIRK2 immunoreactivity in individuals with variant KCNJ6 haplotypes. Gene expression differences predicted effects on neuronal signaling including synaptic function. The KCNJ6 haplotype was also associated with differences in neuron projection area and membrane excitability, but not in other measures of neuronal differentiation. Surprisingly, we found that ethanol exposure led to increased KCNJ6 mRNA and GIRK2 expression, paralleled by reduced or eliminated morphological and physiological differences. Reversal of KCNJ6 haplotype effects by ethanol could also be mimicked by overexpressing GIRK2. These results demonstrate that genetic variants enhancing risk of AUD, even if they do not alter protein sequence, can trigger neuronal mechanisms at the cellular level mirroring or predicting endophenotypes, here ERO, linked with AUD behavior.

**Noncoding KCNJ6 polymorphisms in AUD**

Most GWAS SNPs map to noncoding regions of the genome and 3.7% are found in the UTRs [47, 48]. The 3′UTRs of transcripts expressed in brain tend to be longer than those expressed in other tissues and can be involved in post-transcriptional regulation of transcript abundance by affecting RNA stability, translation and/or localization [49, 50]. Annotation of KCNJ6 identifies two transcripts, one with a short 3′ UTR (1926 bp; ENST00000645093) and one with a substantially longer 3′ UTR (18,112 bp; ENST00000609713). RNAseq coverage analysis in human iPSC detects only the longer isoform, although it is possible that a relatively small portion could be the shorter isoform, but the uniformly distributed coverage (Fig. 1B) does not indicate this. This longer transcript is orthologous to a 16-kb transcript found in rat...
Fig. 5  Ethanol treatment reduced KCNJ6 haplotype differences in iN excitability and GIRK2 expression. A Morphological analysis of IEE iNs generated from affected and unaffected individuals, showing no difference in: (a) neuronal soma size ($p = 0.32$); (b) soma circularity ($p = 0.54$); (c) soma solidity ($p = 0.92$); and (d) total neurite area ($p = 0.98$). B There was no difference in GIRK2 expression in the IEE AF group iNs compared with UN by (a) puncta counts ($p = 0.46$), (b) puncta size ($p = 0.36$), (c) puncta circularity ($p = 0.052$), or (d) solidity ($p = 0.47$). C Representative images of individual GIRK2 puncta (red) localized on III-Tubulin-positive processes (gray) for each cell line. D Electrophysiological analysis of passive neuronal properties in IEE iNs, showing (a) a small decrease in AF membrane capacitance ($p = 9.6 \times 10^{-7}$), but no difference in (b) membrane resistance ($p = 0.63$), (c) spontaneous EPSCs frequency ($p = 0.32$), or (d) spontaneous EPSCs amplitude ($p = 0.19$). E Representative sEPSCs traces for each cell line. (f) The AF group exhibited no change in resting membrane potential after IEE ($p = 0.79$). F Electrophysiological analysis of active neuronal properties found no difference in IEE iNs for (a) current required to shift resting membrane potential to $-65$ mV ($p = 0.32$), (b) maximum number of action potentials (APs) induced with the "step" protocol ($p = 0.48$), with (c) representative traces of APs induced with the "step" protocol, (d) number of action potentials (APs) induced with the "ramp" protocol ($p = 0.95$), with (e) representative traces of APs induced with the "ramp" protocol. F Representative images of individual lines of iNs, exposed to 7d of IEE, marked with arrows pointing to individual GIRK2 puncta (red) localized on III-Tubulin-positive processes (gray). G Summarized results from all lines showing differences in GIRK2 expression levels before and after 7 days of 20 mM IEE with ethanol (EtOH; $p = 1.0 \times 10^{-6}$). H Representative images of individual GIRK2 puncta (red) localized on III-Tubulin-positive processes (gray) prior and following 7 days 20 mM IEE with ethanol. Sample images are from line 246.1 Representative images of FISH detection of KCNJ6 mRNA for each cell line. J Quantification of FISH. (a) The number of KCNJ6 puncta normalized to the number of cells in an image shows decreased expression in control AF compared with UN ($p = 8.2 \times 10^{-3}$) and increased expression following IEE ($p = 8.2 \times 10^{-3}$; using Tukey’s pairwise comparisons). (b) The percentage of KCNJ6-expressing MAP2+ cells substantially increase in the (c) AF group but not in the (b) UN group. Numbers of KCNJ6 puncta were analyzed by expression levels per cell, as recommended by the FISH manufacturer in (d) UN or (e) AF cells. (f) KCNJ6 puncta within the neuronal soma show increases following IEE in both UN and AF groups ($p = 0.006$ for genotype, Tukey’s post-hoc for UN, $p = 0.01$, for AF, $p = 0.01$). (g) A similar analysis of non-somatic puncta, presumably within neurites, showed no differences following IEE between genotypes ($p = 0.23$).

Fig. 6  GIRK2 overexpression mimics ethanol response. A Current required to shift resting membrane potential to $-65$ mV, in untreated (control, $p = 5.9 \times 10^{-10}$), lentiviral KCNJ6 overexpression (over, $p = 0.73$), or 1 d 20 mM EtOH ($p = 0.75$) cultures. B Representative traces of APs induced with the "ramp" protocol. C Quantification of maximum number of action potentials (APs) induced with "ramp" protocol, control ($p = 6.8 \times 10^{-6}$), overexpression ($p = 0.014$), or 1 d 20 mM EtOH ($p = 0.10$). D quantification of GIRK2 puncta after overexpression (line 376, one-tailed Student’s t test $p = 0.04$).

brain [51], which includes multiple AU-rich elements, which may affect mRNA stability and could be affected by KCNJ6 allelic variation. We conclude that the vast majority of KCNJ6 mRNA is consistent with having the extended 3′ UTR, with the potential for variant SNPs to affect mRNA stability or translation efficiency.

In addition to the three synonymous and intronic SNPs used to select subjects (Table 1), examination of variants in the long 3′ UTR by RNAseq alignment predicts linkage of 19 additional SNPs (Fig. 1B; Supplementary Table 1). None of the 3′ UTR SNPs could be mapped uniquely to known regulatory sequences such as predicted microRNA target sites. That is, some SNPs are predicted to destroy or add predicted targeting sites, but all were found in multiple locations within the 3′ UTR, not only where they might be altered by a SNP. It is possible, however, that altered microRNA targeting of one or more sites among many in the 3′ UTR could play a role in regulating the stability or translation of the variant KCNJ6 mRNA, but no clear candidates could be identified for testing.

However, results demonstrate that the noncoding haplotype alters expression of GIRK2 protein and KCNJ6 mRNA. In from subjects with the homozygous variant KCNJ6 haplotype exhibited lower expression of GIRK2 by immunocytchemistry (Fig. 3D), but while the difference in neuronal mRNA levels as detected by scRNAseq analysis matched this trend, it did not reach significance ($p = 0.0508$; Fig. 1C). FISH analysis, however, confirmed that KCNJ6 mRNA was reduced in the AF group (Fig. 5J). KCNJ6 mRNA was also detected in fewer MAP2+ cells and at lower levels per cell (Fig. 5J, b, c). The results of counting GIRK2 puncta and evaluating KCNJ6 mRNA by scRNAseq and FISH support a diminished expression of GIRK2 in neurons from the affected group. Secondary effects of KCNJ6 mRNA and GIRK2 protein regulation include many predictors of altered neuronal connectivity and signaling. Genes that were reduced in the AF group (GO:BP Down in Fig. 1E; Supplementary Fig. 3B) point to changes in synaptic transmission and projection morphology. Since we focused on KCNJ6 haplotype differences in cells without ethanol, we did not expect to match findings in, for example, human AUD brains [52], which found prominent effects in astrocytes and microglia.

Surprisingly, ethanol exposure increased expression of both GIRK2 immunoreactivity and KCNJ6 mRNA. Following 7 days of IEE,
GIRK2 puncta in the AF group increased to levels significantly higher than the UN group (Fig. 5B). Similarly, KCN6 mRNA levels were shown to be increased by scRNAseq results (Fig. 1C) and FISH analysis (Fig. 5J). While previous studies identified a binding site for ethanol in a defined pocket of GIRK2 [53], suggesting that it could affect protein stabilization, this could not explain the increased mRNA level. There is no indication that ethanol or KCN6 haplotype altered translation of KCN6 mRNA to neuronal processes (Fig. 5J.d, e). Ethanol has been found to affect microRNA expression patterns that regulate mRNA [49, 54–59]. It is intriguing to speculate that ethanol might increase expression of GIRK2 through this type of mechanism, with polymorphisms in the 3′UTR serving as the modulator.

**GIRK2 in the context of alcohol dependence**

GIRK channels play an essential role in maintaining the excitability of neurons. For example, in Trisomy 21 individuals, whose cells contain an additional copy of KCN6, neuronal activity was shown to be reduced due to excessive channel activity [60–62]. On the other hand, loss of GIRK2 activity in knockout mice increases susceptibility to induced seizures [20, 21]. We detected the presence of GIRK channel function in glutamatergic iNs using the ML297 agonist of GIRK1/2 heterotetramers, showing that activation led to a reduction in the frequency of action potentials induced by depolarization (Fig. 2D). However, only 6.7% of neurons responded to ML297, but overexpression of GIRK2 increased the proportion of responding neurons to 30% (Fig. 2D). We therefore chose to focus on evaluating neuronal excitability as an indirect response to changes in GIRK function.

By identifying the correlation between GIRK2 expression levels and altered GIRK2-mediated function, we predict that neuronal excitability will be affected by KCN6 variants and alcohol exposure. While GIRK channels play relatively small role in maintaining neuronal homeostasis, modulating GIRK activity to alter cell excitability is predicted to play a critical role. Additionally, downstream effectors of the G protein-mediated signaling pathways presumably regulate neuronal function indirectly [14, 15, 17, 19]. GIRK channels are implicated in several other disorders with abnormal neuronal excitability, including epilepsy, suggesting that they have therapeutic potential [19–21, 60–63].

**Potential limitations**

Factors beyond KCN6 haplotype may contribute to these interpretations. It is possible that the sex of individual neurons might contribute to the results. However, in nearly all parameters tested, there was no difference by sex. scRNAseq identified only 6 genes as different by sex and 4 of these are encoded by sex chromosomes. Basal morphology (Fig. 3B, D) was unaffected by sex, but membrane capacitance (Fig. 3F.a, p = 2.6 × 10⁻¹⁰), induced APs (step, Fig. 3Gb, p = 0.012), and ethanol-treated membrane capacitance (Fig. 5Da, p = 3.1 × 10⁻⁸) all reached significance, although it is difficult to interpret how sex interacts with neuronal function in this context. Finally, we chose to focus on homozygous haplotypes and extreme AUD diagnoses to enhance contrasts, however, additional studies might examine if heterozygotes would exhibit gene dosage effects or be rescued by the presence of the major alleles, similar to endophenotype studies with the original subjects [8].

**From genes to behavior**

In this study, we found that the variant KCN6 haplotype affects excitability of neurons (Figs. 3, 4E). In Ca²⁺ imaging experiments, concomitant with enhanced excitability in neurons from affected individuals following stimulation with glutamate pulses, we observed an overall increase in basal activity of the neuronal population (Fig. 4F). This is reminiscent of the original observation linking KCN6 variants with an EEG endophenotype, where Kamarajan et al. observed that individuals with alcohol dependence and the KCN6 haplotype had an ERO theta power that varied as a function of the KCN6 haplotype in both loss and gain conditions [10]. There is an extensive human literature linking impulsivity to alcohol use problems [64–67]. Impulsivity is elevated in offspring who are at high risk for substance use disorders and may be a reflection of a genetic vulnerability for substance use problems [68]. Excitability in individual neurons and particularly in a culture dish of neurons is several levels of complexity removed from EEG patterns in brain. However, our study points to a mechanistic underpinning of how heritable risk traits are likely to play a role in development of unique physiological response to alcohol in the brain.

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AUTHOR CONTRIBUTIONS

DP, IG-R, PAS, and RPH conceived the project. DP, IG-R, MMY, PZ, ADM, IP, AJ, KYK, and RPH performed experiments. DP, IG-R, IP, JES, PAS, and RPH performed statistical analyses. CK, XX DBC, BP, SK, DMD, AG, HJE, and JAT characterized and identified subjects for analysis and provided critical feedback throughout the project. DP, IG-R, PAS, and RPH prepared the paper with critical evaluation from JES, HJE, JAT, and ZPP.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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