Original Article

MOLECULAR METABOLISM

Genome-wide association study for circulating FGF21 in patients with alcohol use disorder: Molecular links between the *SNHG16* locus and catecholamine metabolism

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ABSTRACT

Objective: Alcohol consumption can increase circulating levels of fibroblast growth factor 21 (FGF21). The effects of FGF21 in the central nervous system are associated with the regulation of catecholamines, neurotransmitters that play a crucial role in reward pathways. This study aims to identify genetic variants associated with FGF21 levels and evaluate their functional role in alcohol use disorder (AUD).

Methods: We performed a genome-wide association study (GWAS) using DNA samples from 442 AUD subjects recruited from the Mayo Clinic Center for the Individualized Treatment of Alcoholism Study. Plasma FGF21 levels were measured using Olink proximity extension immunoassays. Alcohol consumption at time of entry into the study was measured using the self-reported timeline followback method. Functional genomic studies were performed using HepG2 cells and induced pluripotent stem cell (iPSC)-derived brain organoids.

Results: Plasma FGF21 levels were positively correlated with recent alcohol consumption and gamma-glutamyl transferase levels, a commonly used marker for heavy alcohol use. One variant, rs9914222, located 5' of *SNHG16* on chromosome 17 was associated with plasma FGF21 levels ($p = 4.60E-09$). This variant was also associated with AUD risk (β: −3.23; *p*:0.0004). The rs9914222 SNP is an eQTL for *SNHG16* in several brain regions, i.e., the variant genotype was associated with decreased expression of SNHG16. The variant genotype for the rs9914222 SNP was also associated with higher plasma FGF21 levels. Knockdown of *SNHG16* in HepG2 cells resulted in increased FGF21 concentrations and decreased expression and enzyme activity for COMT, an enzyme that plays a key role in catecholamine metabolism. Finally, we demonstrated that ethanol significantly induced FGF21, dopamine, norepinephrine, and epinephrine concentrations in iPSC-derived brain organoids.

Conclusions: GWAS for FGF21 revealed a *SNHG16* genetic variant associated with FGF21 levels which are associated with recent alcohol consumption. Our data suggest that *SNHG16* can regulate FGF21 concentrations and decrease COMT expression and enzyme activity which, in turn, have implications for the regulation of catecholamines.

(The ClinicalTrials.gov Identifier: NCT00662571)

Keywords Alcohol use disorder; FGF21; GWAS; Catecholamine metabolism

1. INTRODUCTION

Alcohol use disorder (AUD) is the most prevalent substance use disorder worldwide [1]. Acamprosate, naltrexone, and disulfiram are Food and Drug Administration (FDA) approved for the pharmacotherapy of AUD in the United States. However, growing evidence has prompted the development of fibroblast growth factor 21 (FGF21) based therapy as a potential treatment for AUD [2—4]. FGF21 is a metabolic hormone with multiple functions, including signaling to the hypothalamus's paraventricular nucleus to decrease sugar and alcohol intake [2,5].

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Abbreviations: GWAS, Genome-wide association study; AUD, Alcohol use disorder; FGF21, Fibroblast growth factor 21; iPSC, Induced pluripotent stem cell; PBMCs, Peripheral blood mononuclear cells; EBs, Embryonic bodies; UPLC, Targeted Metabolomics Using Ultraperformance Liquid Chromatography; ELISA, Enzyme linked immunosorbent assay; TLFB, Timeline followback; Q—Q, Quantile—quantile; COMT, Catechol O-methyltransferase; TH, Tyrosine hydroxylase; DDC, Dopa decarboxylase; DBH, Dopamine β hydroxylase; PNMT, Phenylethanolamine-N-methyltransferase

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However, the half-life of FGF21 is only about 0.5–2 hours [6]. FGF21 is highly expressed in the liver, but it also crosses the blood–brain barrier. The bulk of literature on this subject has raised the possibility that FGF21 administration might affect alcohol consumption and reward behaviors [3]. FGF21 levels can also be induced by ethanol and are associated with recent alcohol consumption [4,7]. The effects of FGF21 in the central nervous system are associated, at least in part, with the regulation of catecholamines, neurotransmitters that play a crucial role in reward pathways [2]. We performed a genome-wide association study (GWAS) to identify genetic variants associated with FGF21 levels and investigated their functional role in AUD.

The Mayo Clinic Center for the Individualized Treatment of Alcoholism recruited 442 AUD subjects with clinical data, and DNA samples were obtained prior to treatment [8]. We designed the present study to apply our established pharmaco-omics-informed genomics research strategy to identify genetic variants that might contribute to the concentrations of plasma FGF21 and its functional role in AUD. This study is similar in design to previous pharmacogenomic studies that we have successfully performed identifying selective serotonin reuptake inhibitor treatment response in patients with major depressive disorder (9–11) [24] and acamprosate treatment response in patients with AUD [9,10].

The series of studies described subsequently highlight that plasma FGF21 levels are positively associated with recent alcohol use in patients with AUD. Specifically, our GWAS for plasma FGF21 levels identified a genome-wide significant signal (rs9914222) 5′ of the *SNHG16* gene (p:4.60E-09). This SNP has also been associated with AUD risk in a recent large GWAS (β: −3.23; $p = 0.0004$). Ethanol could down-regulate SNHG16, an oncogenic long non-coding RNA identified by our GWAS for plasma FGF21 levels in AUD patients. Knockdown of SNHG16 could increase FGF21 concentrations which, in turn, regulate COMT activity as well as the release of dopamine, epinephrine, and norepinephrine. This also raisess the possibility that SNHG16, which appears to play a functional role in regulating circulating FGF21 concentration, might also contribute to catecholamine metabolism through a novel pharmacogenomic mechanism.

2. METHODS

2.1. Study participants

Previously, the Mayo Clinic Center for the Individualized Treatment of Alcoholism Study (ClinicalTrials.gov Identifier: NCT00662571) recruited 442 participants with AUD [11,12]. All subjects whose biological samples were included in this study gave their consent to participate in the study and publish the study results in a peer-reviewed journal. We conducted this study under protocols (reference IRB numbers: 07–007204 and 20–000372) reviewed and approved by the Mayo Clinic Institutional Review Board.

2.2. Quantification of plasma FGF21 levels

Plasma FGF21 levels were measured using Olink proximity extension immunoassays. The Olink assays were performed using 88 samples/plate. All ten plates were run in a single batch. Data were normalized to standard plasma controls using a standard Olink workflow to produce relative protein abundance information. Data are expressed as normalized protein expression (NPX) in arbitrary units on a log2 scale to normalize the data and minimize intra-assay and inter-assay variation. NPX reflects protein concentration but does not reflect the exact concentrations in the plasma samples. A high NPX value corresponds to a high protein concentration.

2.3. Genome-wide association study (GWAS)

The genomic dataset was derived from the Mayo Clinic Center for the Individualized Treatment of Alcoholism Study, as described previously [8]. Genome-wide associations between genetic variants and plasma FGF21 levels were examined using linear regression, adjusted for sex, age, and study site. R version 3.6.3 and PLINK 1.9 were used to perform these analyses.

2.4. Generation of iPSCs and brain organoids

We used peripheral blood mononuclear cells (PBMCs) for iPSC reprogramming with the CytoTune-iPS 2.0 Sendai Reprogramming Kit (A16518, Thermo Fisher Scientific). All patient-derived iPSCs had normal karyotypes and expressed pluripotency markers. These cell lines were regularly verified to be free from mycoplasma infection. We then differentiated patient-derived iPSCs into brain organoids [13]. Briefly, pre-patterned floating embryonic bodies were cultured with small molecules and proteins, including 1x N2 supplement (Invitrogen, Grand Island, NY), 1x NEAA and 1x Glutamax, 100 nM LDN-193189, 3 μM CHIR99021, 2 μM purmorphamine, SHH 100 ng/mL, FGF8 100 ng/mL (Selleckchem, Carlsbad, CA) for 4 days. On days 5 and 6, the culture medium consisted of DMEM/F12 medium supplemented with 1x N2 supplement, and 1x Glutamax, 100 nM LDN-193189, 3 μM CHIR99021, 2 μM purmorphamine, SHH 100 ng/mL, FGF8 100 ng/mL. On day 7, the organoids were cultured in DMEM/F12 medium supplemented with 1x N2 supplement, 1x NEAA, 1x Glutamax, 100 nM LDN-193189, and 3 μM CHIR99021. From day 14 onward, organoids were cultured in a 12 well-plate using a bioreactor with neurobasal medium supplemented with 1x B27 supplement, 1x NEAA, and 1x Glutamax (Invitrogen, Grand Island, NY), 20 ng/mL BDNF (Peprotech, Rocky Hill, NJ), 20 ng/mL GDNF, 0.2 mM L-Ascorbic Acid (Sigma–Aldrich, St Louis, MO), and 0.5 mM cAMP [13]. Drug treatment was conducted on days 83–90 of brain organoid differentiation, and the culture medium was changed every day.

2.5. COMT enzyme activity assay

COMT enzyme activity was determined by measuring the amount of carbon 14 (^{14}C) incorporated into the non-methylated substrate 3,4-Dihydroxybenzoic acid (DBA). COMT catalyzed the methylation of DBA using S-adenosyl-L-(methyl 14C)-methionine as the methyl donor. The assay has been described in detail elsewhere [14,15]. Resin was used to remove calcium in the protein samples because calcium inhibits COMT [14,15]. Briefly, each reaction (300 uL) included 50 μg of protein, 2 μM of ^{14}C–S-Adenosylmethionine (SAM), 1 mM magnesium chloride, 4.2 mM Dithiothreitol, 1 mM 3,4 Dihydroxybenzoic acid, 20 μM SAM, and 0.5M Tris–HCl buffer, pH 7.6. Chemicals and compounds were purchased from Sigma (St. Louis, MO, USA). Samples were incubated in a shaker water bath for 40 min at 37 °C. Blank samples for the assay included all reagents but DBA. The reaction was terminated by adding 100 uL of 1.0N HCl to each assay tube. Organic solvent extraction was used to separate the radioactive product, the methylated catechol from the free radioactive cosubstrate by adding 5 mL of toluene: 3-methyl butanol, 2:1 (v/v) to each tube, followed by vortexing for 10 s and centrifugation at 700g for 10 min. After centrifugation, 3.5 mL of the organic phase were added to counting vials containing 1 mL Ultima Gold scintillation cocktail (Perkin Elmer Life Sciences, Boston, MA, USA). Radioactivity was measured using a Beckmann LS 6500 liquid scintillation counter (Ramsey, MN, USA). The radioligand binding assays were performed in duplicate in at least three independent experiments.

2.6. Targeted metabolomics using ultraperformance liquid chromatography (UPLC)—tandem mass spectrometry

Protein extraction was performed using iPSC-derived brain organoids. Protein (5 mg) was assayed for neurotransmitters using the neuromodulator panel in the Metabolomics Core Facility at the Mayo Clinic. High resolution separation was performed using an Acquity UPLC system with the injection of 1 mL of derivatized solution using a UPLC BEH C18 column (Waters Corp). Mass detection was performed using a TSQ Ultra Quantum running in ESI positive mode [16,17]. A 10-point standard curve was generated from the calibration standard solution, and the standard curve was used to quantify concentrations of neurotransmitters.

2.7. Enzyme linked immunosorbent assay (ELISA) for FGF21

We measured FGF21 levels in the iPSC-derived brain organoids and HepG2 cell lysates with an FGF21 ELISA kit (EK0994, Boster Biological Technology, Pleasanton, CA) in accordance with the manufacturer's instructions. Specifically, we used 5 mg of protein for ELISA. Using the calibration standard solution, we made an 8-point standard concentration curve to quantify FGF21 concentrations. We performed ELISA in duplicate and read absorbances on a microplate reader at 450 nm.

2.8. Drug treatment

We treated cells with ethanol (EtOH) (25 mM). This concentration is considered physiologically relevant for EtOH use, with 25 mM EtOH being slightly higher than the 0.08% blood alcohol concentration often used as a measure of intoxication [18]. The concentrations of naltrexone (30 nM) [19], acamprosate (5 μM) [19], nalmefene (70 nM) [20], baclofen (1 μM) [21], topiramate (5 μM) [22], and gabapentin (30 μM) [23] used to conduct these experiments were selected to fall within the range of blood drug concentrations in patients taking standard clinical doses of these drugs. Cells were treated with drugs for 7 days, with a daily medium change.

2.9. Immunofluorescent analysis and confocal microscopy

We used paraformaldehyde (4%) to fix cells at room temperature for 15 min. We then washed the cells with PBS and permeabilized them with 0.2% Triton X-100 in PBS. Blocking solution consisted of normal donkey serum (3%) in PBS. Slides were then incubated with the primary antibody in 5% BSA overnight (Table A.1). The secondary antibody was incubated at room temperature for an hour. We used Antifade mounting media with dapi to stain the cell nuclei (VECTOR Laboratory, Burlingame, CA, USA). We used fluorescence microscopy (Olympus, FV1200) to visualize the slides.

2.10. Data and statistical analysis

We used R Statistical Software (version 4.0.2; R Foundation for Statistical Computing) and GraphPad Prism Software v7 to perform Statistical analysis. We used the Shapiro—Wilk test to test data distribution. When the datasets were not normally distributed, unpaired t-test or Mann—Whitney U test were performed. Real-time PCR and COMT enzyme activity results were analyzed using paired *t*-tests. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Plasma FGF21 levels are associated with alcohol consumption

We set out to 1) measure plasma FGF21 levels using the Olink proteomic platform, 2) apply our established pharmaco-omics-informed genomics research strategy to identify genetic variants that might associate with levels of FGF21, and 3) functionally and mechanistically pursue "signals" coming from that GWAS. This research strategy has been used repeatedly to generate and test genomic and pharmacogenomic hypotheses and has proven to be a powerful research tool for identifying novel biology [9,24—26]. Specifically, we previously recruited 442 subjects with AUD for an acamprosate clinical trial (Table 1) [27]. Alcohol consumption was measured by self-report using Timeline Followback (TLFB). Plasma FGF21 levels were assayed using the OLINK inflammation platform. Our data showed that plasma FGF21 levels were positively associated with recent alcohol consumption i.e., the number of drinking days, heavy drinking days, and total drinks (Figure 1A—C, and Figure A.1). For example, recent alcohol consumption within 90 days prior to blood sampling was positively correlated with plasma FGF21 levels. In addition, plasma FGF21 levels were positively correlated with gamma-glutamyl transferase (GGT) levels which are commonly used as a marker for heavy alcohol use (Figure 1D). Those observations indicated that recent alcohol use was clearly associated with plasma FGF21 levels. As a result, we next applied a systematic approach to study genetic variants that might be associated with plasma FGF21 levels (Figure 2A).

3.2. GWAS for plasma FGF21 levels

We used FGF21 levels as a quantitative trait for a GWAS to identify genetic variants that might be associated with the levels of plasma FGF21 (Figure 2A). Quantile—quantile (Q—Q) and Manhattan plots of the GWAS for plasma FGF21 levels in our AUD patients are shown in Figure 2B—C. The Manhattan plot shows a genome-wide significant SNP cluster on chromosome 17 with the lowest *p*-value (*p*:4.60E-09) for the rs9914222 SNP (Figure 2C—D). The minor allele frequency (T) for the rs9914222 SNP was 18.8% in our European-American AUD patients, consistent with the 20% value reported for European populations by the 1000 Genomes Project (Figure 2D) [28]. This intronic SNP is located within a gene cluster that includes the *PRCD*, *CYGB, and SNHG16* genes (Figure 2D). This minor allele (T) of this variant has also been moderately associated in an independent GWAS with a decreased risk of AUD ($n = 38{,}686$, $\beta = -3.23$; $p = 0.0004$) (Table A.2) [29]. The variant genotype for the rs9914222 SNP was associated with higher plasma FGF21 concentrations which are associated with greater baseline drinking (Figure 3A). In parallel, we found that the rs9914222 SNP was an eQTL for *SNHG16*, a long non-coding RNA, in several brain regions (Figure 3B, and Table A.3). Specifically, the variant genotype was associated with lower expression of *SNHG16*. As a result, our subsequent functional

Table 1 — Clinical and demographic characteristics of study subjects.

	Subjects (n = 442)	
	Mean	SD or %
Age (years)	42.1	11.8
Sex: male	287	64.9%
Baseline PHQ-9 Score	9.4	6.1
Baseline GADS Score	9.0	5.9
Baseline PACS Score	13.38	8.0
Base line GGT	69.4	98.3
Alcohol consumption measure		
(TLFB 90 days prior to enrollment)		
Total drinks	562.2	506.5
Number of drinking days	45.8	26.9
Number of heavy drinking days	41.5	26.9
Number of max drinks per drinking day	17.2	11.5
Average drinks per drinking day	11.8	7.7
Average drinks per drinking week	43.6	39.4
Average drinks per drinking month	186.8	169

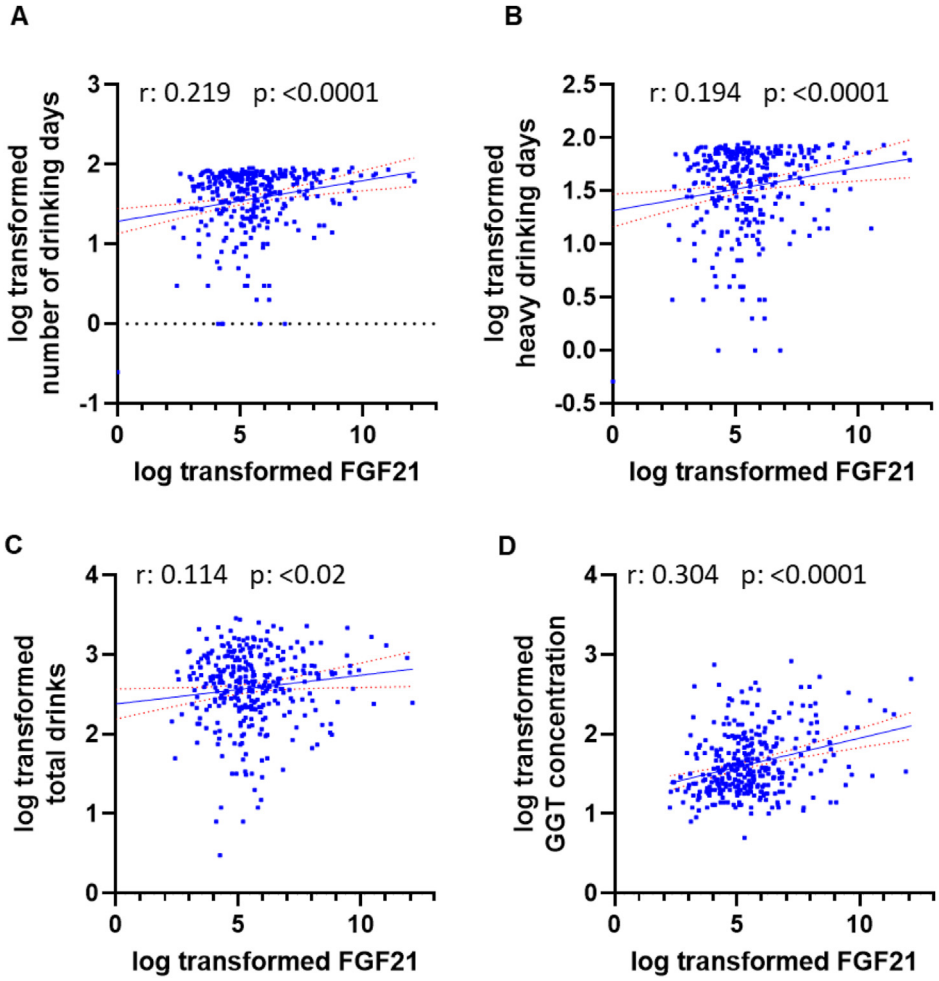

Figure 1: Plasma FGF21 concentrations were associated with recent alcohol use phenotypes. Plasma FGF21 levels were positively correlated with recent alcohol use as determined by TLFB 90 days prior to blood collection i.e. **(A)** the number of drinking days, **(B)** the number of heavy drinking days, and **(C)** total drinks. **(D)** Plasma FGF21 levels were also positively correlated with blood levels of gamma-glutamyl transferase (GGT) which is commonly used as a marker for heavy alcohol use.

genomic studies focused on the possible molecular links among the *SNHG16* locus, FGF21 plasma levels, and AUD.

3.3. *SNHG16* plays a role in catecholamine metabolism

FGF21 is a liver enzyme reported to play a protective role in ethanol-associated liver injury by activating catecholamines [30,31]. We began by testing the effect of *SNHG16* on FGF21 using HepG2 cells. Knockdown of SNHG16 resulted in a significant increase in FGF21 concentrations (Figure 4A). In addition, when SNHG16 was knocked down to 20% of its baseline level, the expression of the catecholamine metabolic enzyme, catechol O-methyltransferase (COMT) decreased by approximately 80%. COMT is a neurotransmitter and drug methyltransferase that catalyzes the methylation of all catecholamine neurotransmitters [32]. We next assayed COMT activity before and after the knockdown of SNHG16 using a radiochemical assay, the most sensitive method for measuring COMT activity [14,15,33,34]. As expected, COMT enzyme activity was significantly reduced after knockdown of SNHG16 (Figure 4A). Strikingly, we observed similar results when we treated the cells with ethanol (Figure 4B). Specifically, FGF21 concentrations were elevated after ethanol exposure, but both SNHG16 and COMT expression were downregulated in the presence of ethanol, results compatible with the decrease in COMT enzyme activity that we observed (Figure 4B). COMT is a key enzyme that catalyzes the metabolism of catecholamines, i.e. dopamine, norepinephrine, and epinephrine [32,35], all of which play essential roles in the reward system. Therefore, we generated iPSC-derived brain organoids to explore the effects of ethanol on concentrations of FGF21 and catecholamines (Figure 5A) in these organoids. Consistently, FGF21 was ethanol inducible in iPSC-derived brain organoids (Figure 5B). In parallel, the concentrations of neurotransmitters, including dopamine, norepinephrine, and epinephrine, were significantly increased in response to ethanol treatment (Figure 5B). In addition, SNHG16 and a series of crucial catecholamine biosynthetic and metabolic enzymes encoding tyrosine hydroxylase (TH), COMT, dopa decarboxylase (DDC), dopamine β hydroxylase (DBH), and phenylethanolamine-N-methyltransferase (PNMT) were ethanol responsive (Figure 5C—D). We should point out that the expression of *CYGB* is below detectible levels in both liver cells and iPSC-derived brain organoids, whereas PRCD did not have a significant impact on FGF21 concentrations or COMT activity (Figure A.2.). As a result, the genomic studies described subsequently will focus on *SNHG16* and genes involved in the catecholamine biosynthetic and metabolic pathways.

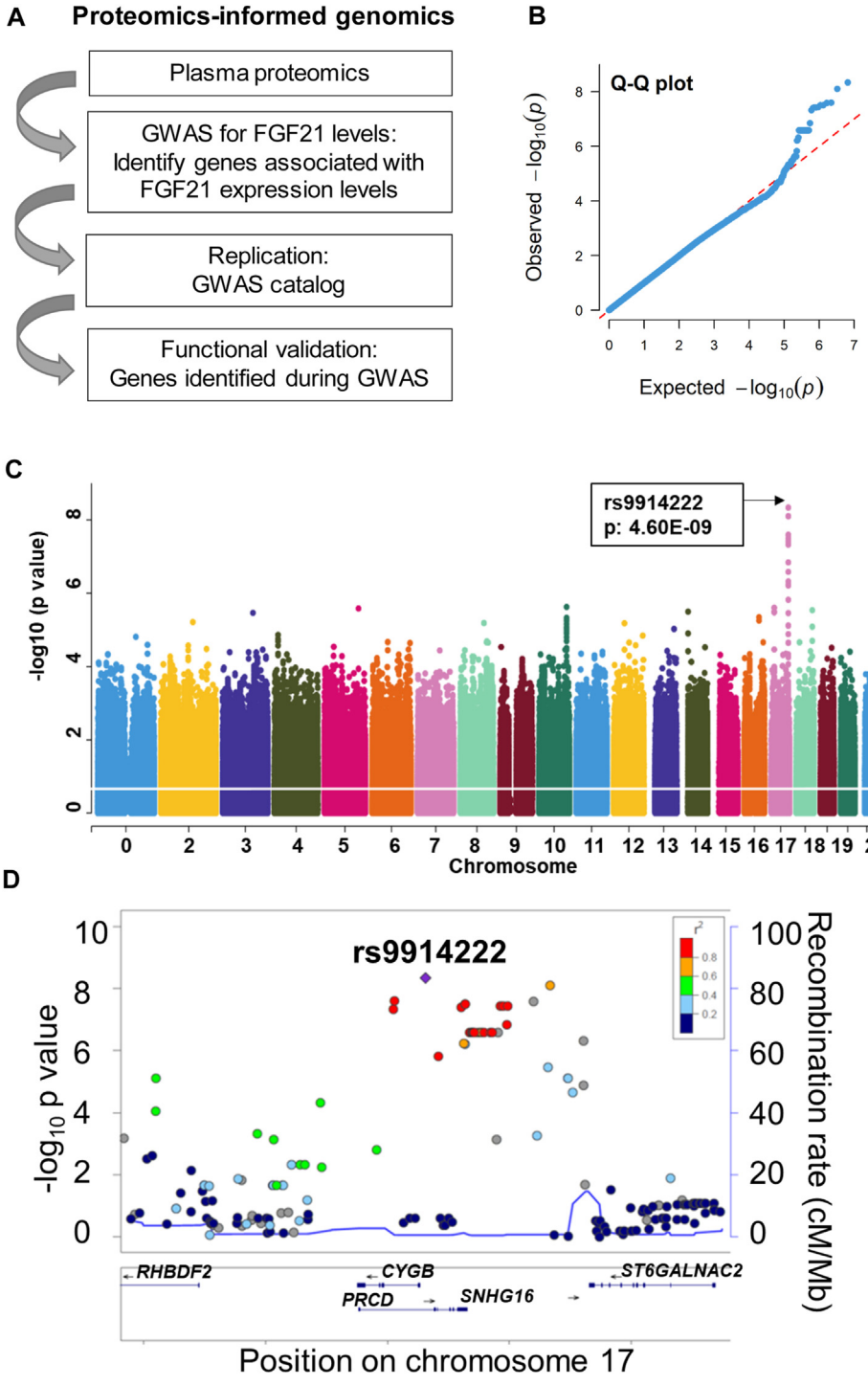

Figure 2: **(A)** Schematic outline of our proteomics-informed genomics research strategy. **(B–C)** Q–Q plot and Manhattan plot for GWAS of plasma FGF21 levels. **(D)** The locus zoom plot displays that the top SNPs on chromosome 17 map within a gene cluster: *CYGB*, *PRCD* and *SNHG16*. The SNP most highly associated with plasma FGF21 levels in patients with AUD was rs9914222 (*p:* 4.6E-09).

3.4. Pharmacologic manipulation of SNHG16 expression and catecholamine metabolism

Previous studies have raised the possibility that FGF21 administration could affect alcohol consumption and reward behaviors [3]. FGF21 levels can be induced by ethanol and, as mentioned previously, are associated with recent alcohol consumption [4,7] (Figure 6A). Our results highlight that plasma FGF21 levels are positively associated with recent alcohol use in patients with AUD. Our GWAS for plasma FGF21 levels identified a genome-wide significant signal (rs9914222) 5′ of *SNHG16* (*p*:4.60E-09) that was an eQTL for expression of this long non-coding RNA. This SNP has also been associated with AUD risk [29]. *SNHG16* is an ethanol responsive gene that can influence FGF21

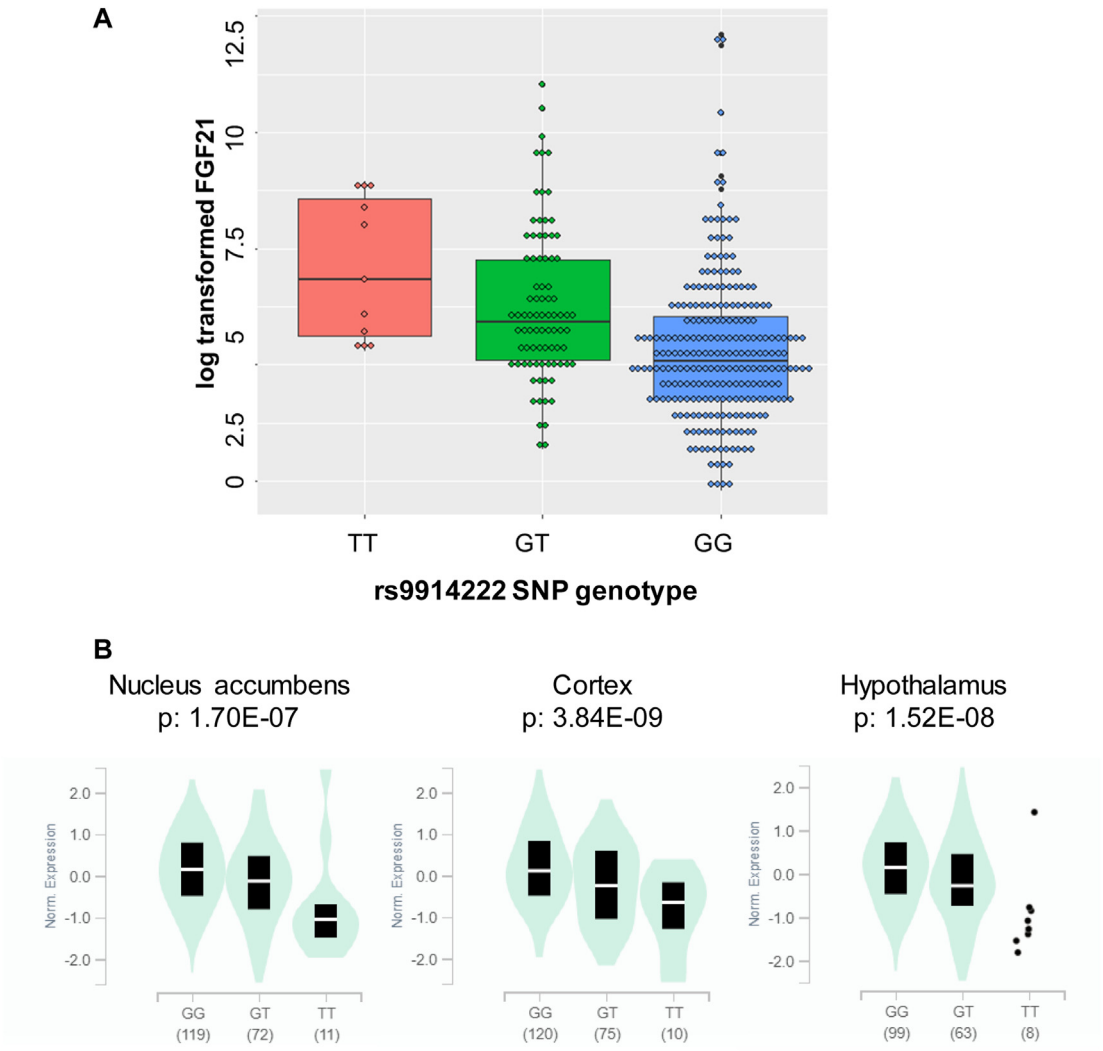

Figure 3: Biological functions of the rs9914222 SNP. **(A)** SNP-dependent plasma FGF21 levels in patients with AUD demonstrating that rs9914222 is an eQTL. **(B)** rs9914222 is associated with SNHG16 mRNA expression in several brain regions. https://gtexportal.org/home/snp/rs9914222.

concentrations, which, in turn, regulate COMT activity and the release of catecholamines (see Figure 6A). This series of observations provide functional insight into molecular links between the *SNHG16* locus, FGF21 concentrations, and catecholamine metabolism. We then took advantage of our iPSC-derived brain organoids and exposed them to drugs that are either FDA approved or have been used "off-label" in the pharmacotherapy of AUD. Strikingly, we found that acamprosate, nalmefene, and topiramate could reduce mRNA expression of SNHG16 in iPSC-derived brain organoids (Figure 6B). Even more striking, TH, COMT, DDC, DBH, and PNMT all play critical roles in catecholamine biosynthesis or metabolism and alter their mRNA expression after drug treatment (Figure 6C—G). These observations potentially open the way to pharmacologic manipulation of the expression of SNHG16, which in turn could have significant implications for FGF21, catecholamines, and alcohol response phenotypes.

4. DISCUSSION

AUD is the most common substance use disorder worldwide, and 13.9—29.1% of adults in the United States suffer from AUD during their lifetime [36]. The etiology of AUD is multifactorial. Currently, three drugs, acamprosate, naltrexone, and disulfiram, have received FDA approval for the pharmacotherapy of AUD in the United States. However, a growing body of evidence suggests a potential role for FGF21 as a novel treatment target for AUD [4,7]. We designed the present study to apply GWAS to identify genetic variants that might be associated with plasma FGF21 levels and to study their possible functional role in AUD. Specifically, we applied a pharmaco-omics-informed genomic strategy during which we utilized plasma samples from 442 AUD subjects enrolled in the Mayo Clinic Center for the Individualized Treatment of Alcoholism Study to assay circulating FGF21. We then performed a GWAS to identify novel genes that might influence FGF21 concentrations (Table A.4) and determine whether molecular mechanisms identified in the periphery might also play an important role in neurotransmitter function in iPSC-derived brain organoids.

Our study showed that plasma FGF21 concentrations were associated with higher alcohol consumption in patients with AUD either 30 days or 90 days prior to blood collection (Figure A.1). Blood GGT levels were also positively correlated with FGF21 levels (Figure 1D). In line with these observations, a significant positive correlation of serum FGF21

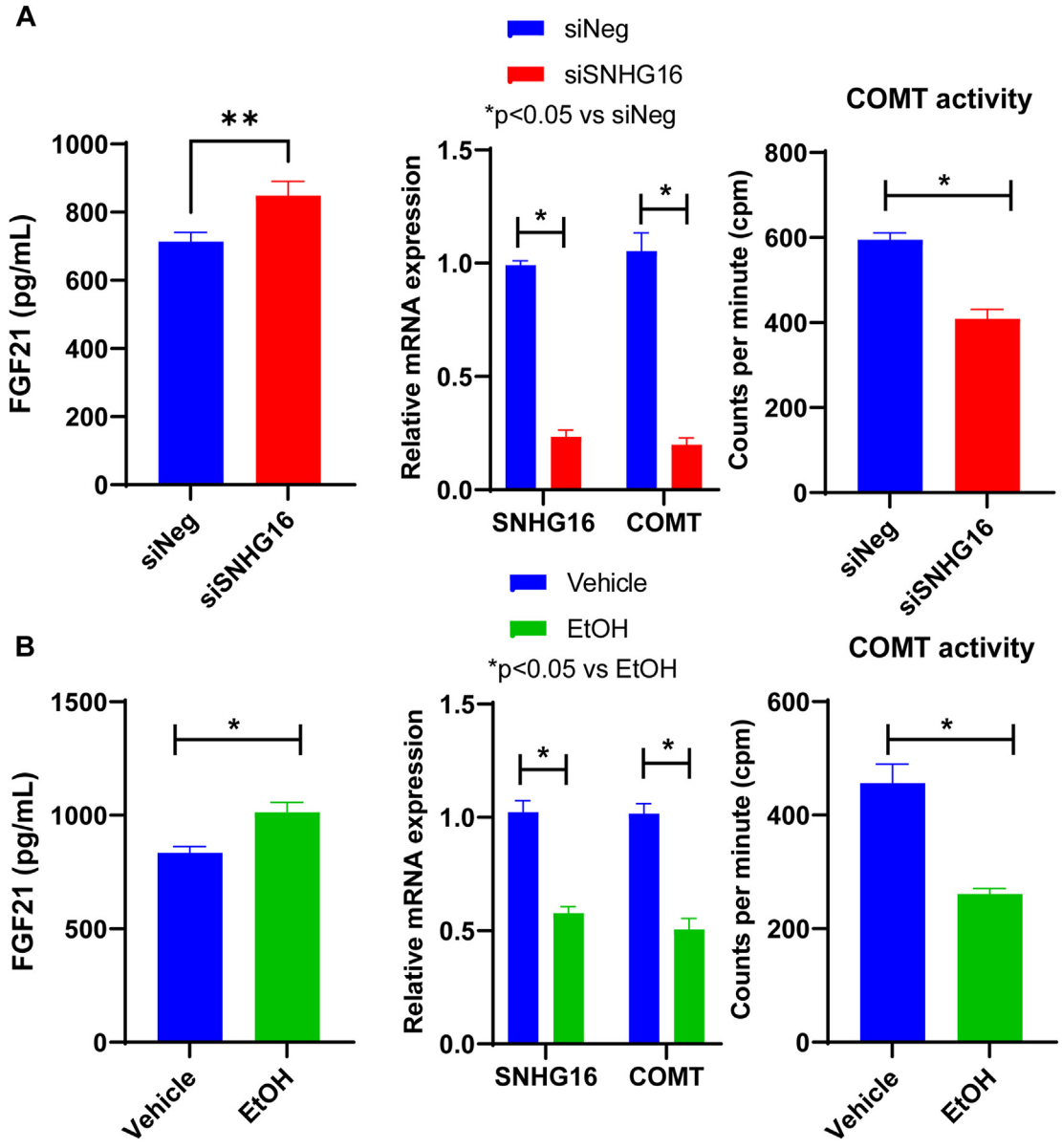

Figure 4: Biological functions of the *SNHG16* gene. **(A)** FGF21 concentration was measured after the knockdown of SNHG16 using siRNA in HepG2 cells. Relative mRNA expression of SNHG16 and COMT was determined after the knockdown of SNHG16. COMT enzyme activity was also measured after the knockdown of SNHG16 in HepG2 cells. At least three independent experiments were performed. $*p < 0.05$. **(B)** FGF21 concentration was determined before and after ethanol treatment of HepG2 cells. Relative mRNA expression of SNHG16 and COMT was determined in response to ethanol treatment. COMT enzyme activity was then measured using HepG2 cells treated with ethanol. *A p value ≤ 0.05 was considered statistically significant (two tailed paired t test). Three independent experiments were performed. All values are mean $\pm$ S.E.M.

and GGT levels was reported in a Chinese study cohort (n = 353) [37]. We performed a GWAS with plasma FGF21 concentrations as a biological and quantitative trait to identify genetic variants associated with variations in FGF21 concentrations that might have implications for AUD. It should be pointed out that the application of genomics alone has often been disappointing in neuropsychiatric research. Unlike cancer or other diseases for which we have "objective biochemical measures" to assist with diagnosing or evaluating treatment response, many phenotypes in psychiatry are not yet closely linked to the underlying biology. As a result, the inclusion of other omics data may offer a more comprehensive view of overall molecular variation that contributes to individual differences in response to drug therapy or disease susceptibility than would genomics alone. This research strategy has limitations, but it has already helped us understand the underlying biology of several neuropsychiatric diseases [9,10,24].

Our GWAS for plasma FGF21 levels identified a genome-wide significant signal (rs9914222) (p:4.60E-09) within a gene cluster that included CYGB, PRCD and SNHG16 (Figure 2D). The variant genotype for the rs9914222 SNP was associated with elevated plasma FGF21 levels. Furthermore, plasma FGF21 levels were positively correlated with recent alcohol consumption and with levels of plasma GGT, a commonly used marker for heavy alcohol use (Figure 1). Several studies had already reported the correlation of FGF21 levels and alcohol consumption, however, the causal pathway remains to be determined [3,7,30]. The rs9914222 SNP was an eQTL for SNHG16 in several brain regions. In an attempt to understand the possible role of

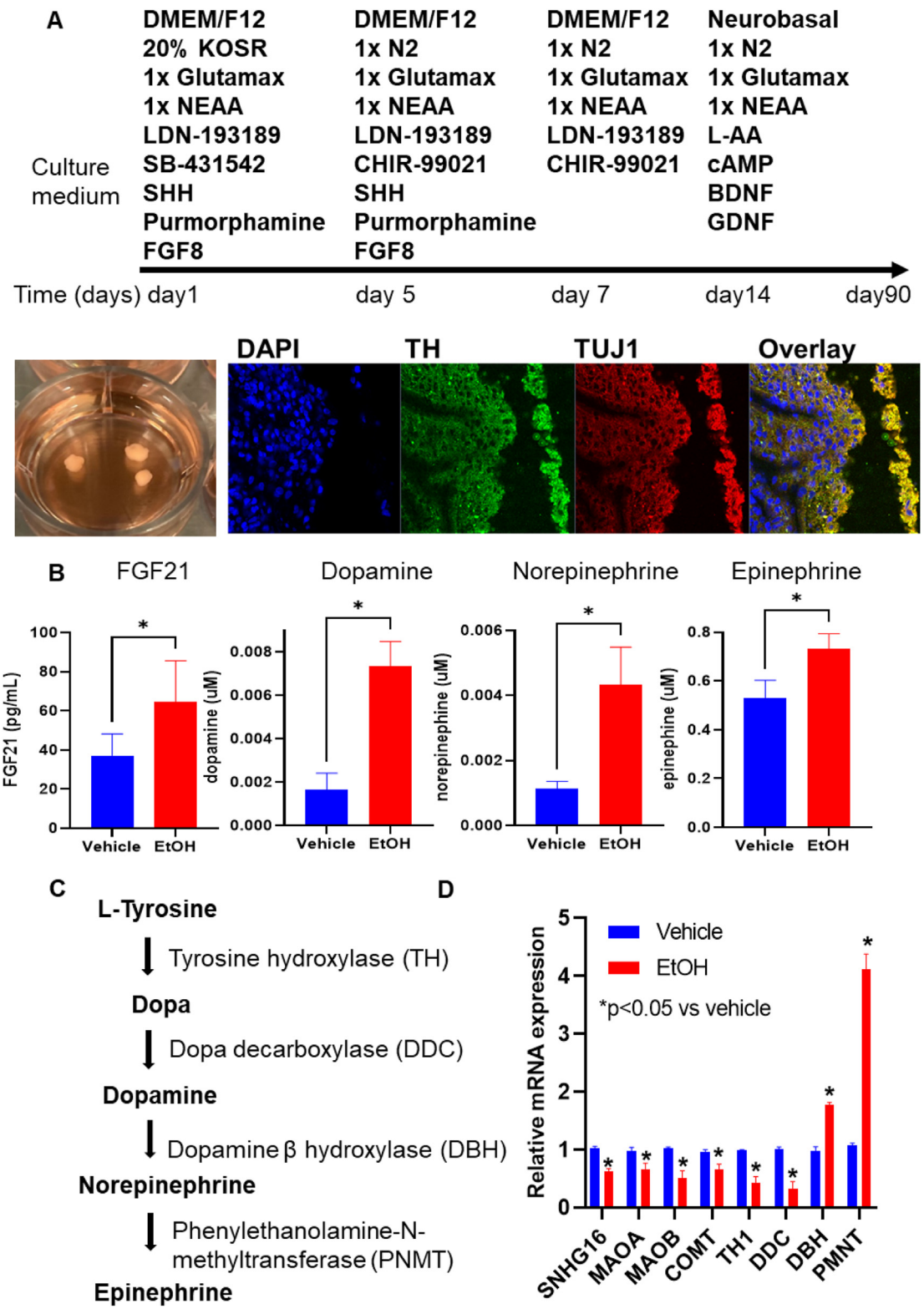

Figure 5: Ethanol induced FGF21 in iPSC-derived brain organoids and activated the release of catecholamines. **(A)** A schematic outline of procedures used during the differentiation of iPSC-derived brain organoids. The panel below the schematic displays representative examples of staining for tyrosin hydroxylase (TH), and Neuron-specific class III beta-tubulin (TUJ1). **(B)** the effect of ethanol on FGF21 concentration was measure using ELISA. Dopamine, norepinephrine, and epinephrine concentrations were measured using UPLC−Tandem Mass Spectrometry. **(C)** A schematic outline of the catecholamine biosynthesis pathway. **(D)** mRNA expression of SNHG16, MAOA, MAOB, COMT, TH, COMT, DDC, DBH, and PNMT in response to ethanol treatment (25 mM) for 7 days. Realtime PCR experiments were performed in iPSC-derived brain organoids (n = 3). The expression of these genes was determined after exposure to drug for 7 days. *A p value $\leq$ 0.05 was considered statistically significant (two tailed paired t test). All values shown are mean $\pm$ S.E.M.

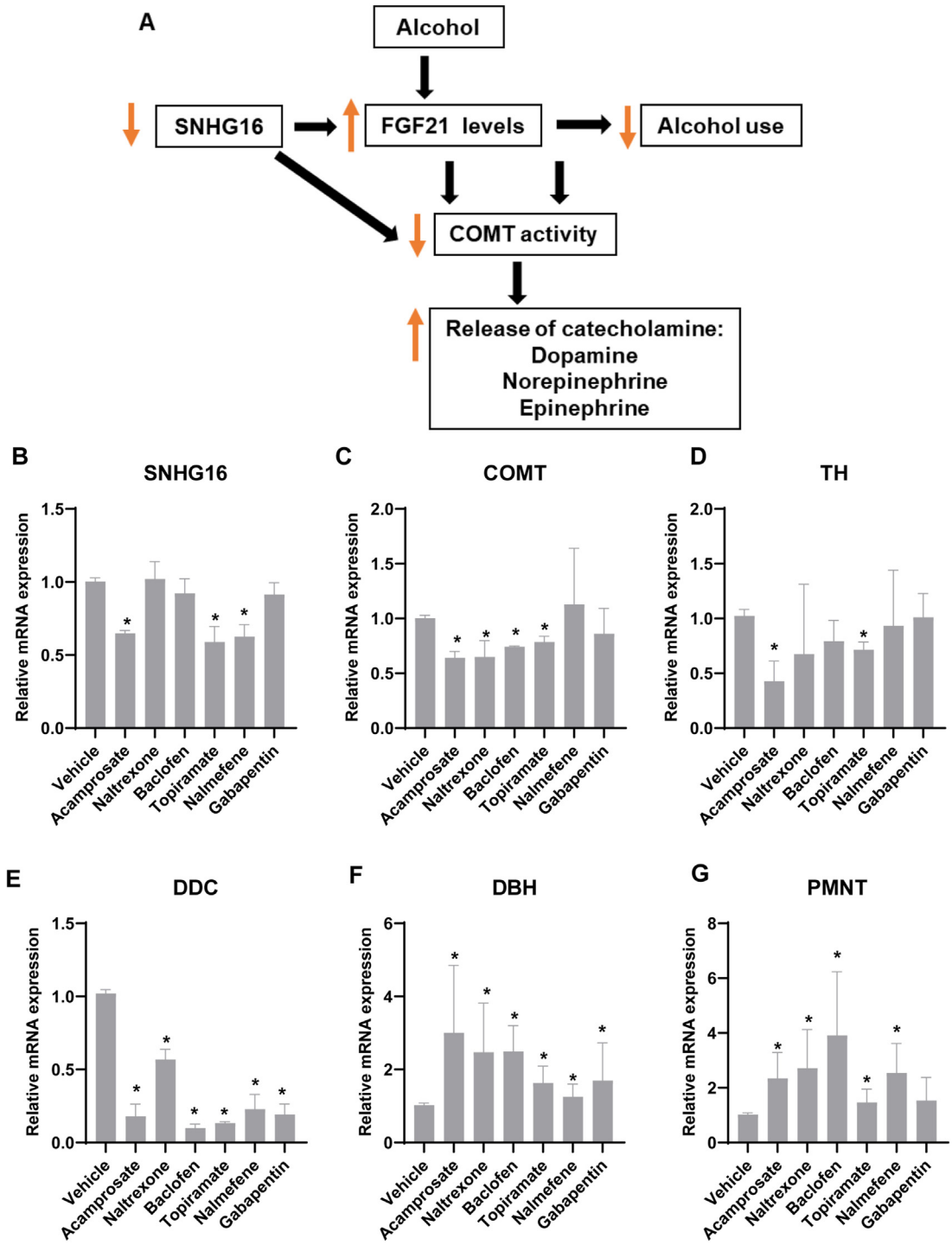

Figure 6: Gene expression of iPSC-derived brain organoids. **(A)** Schematic diagram illustrating the effects of ethanol on FGF21 which have implications for alcohol use. Specifically, FGF21 can be induced by ethanol. Our GWAS for plasma FGF21 showed that a SNP located 5′ of *SNHG16* is associated with AUD. *SNHG16* could regulate COMT expression and activity, which play a role in catecholamine metabolism. Finally, we determined that ethanol induced both FGF21 and catecholamines, including dopamine, norepinephrine and epinephrine using iPSC-derived brain organoids. **(B–G)** mRNA expression of SNHG16, TH, COMT, DDC, DBH, PNMT in response to drug treatment. Realtime PCR experiments were performed in iPSC-derived brain organoids (n = 3). The expression of those genes was determined after exposure to drugs for 7 days. *A p value ≤ 0.05 was considered statistically significant (two tailed paired t test). All values are mean $\pm$ S.E.M.

all three genes mapping to this region in the regulation of FGF21 concentration, we first knocked down each of these three genes separately and determined the concentration of FGF21 in HepG2 cells. Knockdown of SNHG16 resulted in increased FGF21 concentrations and decreased COMT expression and enzyme activity which play a key role in catecholamine metabolism (Figure 4A). However, CYGB expression is very low in the HepG2 cell line (ie CT value $>$ 35 during qPCR) and iPSC-derived brain cells such as neurons, astrocytes, and

brain organoids. In addition, based on the GTEx database, CYGB is not highly expressed in the liver or in the brain. As a result, CYGB was not included for further functional studies. Both PCRD and SNHG16 were expressed in HepG2 cells and iPSC-derived brain organoids. However, PRCD expression did not have a significant impact on FGF21 concentrations in HepG2 cells (Figure A.2.). Furthermore, PRCD mRNA expression was not significantly changed in response to ethanol treatment in both HepG2 cells and iPSC-derived brain organoids (Figure A.2.).

A recent study suggested that dopamine and serotonin concentrations in human cerebrospinal fluid (CSF) sample were positively correlated with CSF FGF21 concentrations which, in turn, were associated with impulsivity [38]. Our study demonstrated that FGF21 levels were positively correlated with recent alcohol use in our cohort of AUD patients. We also showed that FGF21 can be induced by ethanol in iPSC-derived brain organoids. In parallel, dopamine, norepinephrine, and epinephrine concentrations were also significantly increased as determined by UPLC–tandem mass spectrometry (Figure 5B). SNHG16 has been reported to be an oncogenic long non-coding RNA [39]. Our data strongly suggest that *SNHG16* can regulate FGF21 concentrations and decrease COMT expression and enzyme activity, which, in turn, have implications for regulating catecholamines. In an attempt to identify drugs that could be used to manipulate the gene expression of SNHG16 and—as a result—its downstream effect on catecholaminergic pathways, we found that acamprosate, an FDA approved drug for AUD therapy, could decrease SHNG16 expression to ~60% of its baseline level, which in turn altered the mRNA expression of *COMT, DDC* and *DBH* in iPSC-derived brain organoids. This series of observations, taken as a whole, serves to emphasize a possible relationship between *SNHG16*, FGF21 concentrations and catecholamine metabolism. It also raises the possibility that *SNHG16* could be a regulator of catecholamine metabolism and, as a result, that the SNHG16-FGF21 axis could be a potential pharmaceutical target for the treatment of AUD.

It should be emphasized that additional functional genomic studies will be required in order to validate the signals from our GWAS. Our patient-derived iPSC cell model system is unique because **1**) these cells still retain each patient's unique genetic background, and **2**) we will be able to recapitulate the development of human brain tissue. The utilization of patient-derived iPSC-derived brain organoids as a cell model system may make it possible to advance our understanding of disease pathophysiology and drug action. However, our study also has limitations. As is true of all *in vitro* cell model systems, iPSC-derived brain organoids have limitations. The iPSC-derived brain organoids are composed of multiple brain cell types, including neurons, oligodendrocytes, astrocytes, and microglia, and the cell composition varies over time during differentiation. It should also be emphasized that the concentrations of drugs used in our cell culture studies were within the range of clinical use concentrations [19–23,40]. Obviously, further study will be needed to perform full dose–response curves and a time course to verify optional treatment conditions. In addition, the effects of alcohol can vary widely based on time of exposure and EtOH concentration. However, we used 25 mM EtOH in our cell culture system, a level that is considered physiologically relevant, which is only slightly higher than the 0.08% blood alcohol concentration (BAC) that is thought to indicate legal intoxication in most states in the United States. Finally, even considering these limitations, the results reported here represent an important step in the process of obtaining functional insight into molecular links between the *SNHG16* genetic locus and catecholamine metabolism.

Several studies have reported that genetic variants in *FGF21* might have implications for food and drug craving, alcohol preference and metabolic traits [5,41,42]. For example, rs838133 (a synonymous variant) is one of the most well-studied SNPs in *FGF21* [42]. It has been report that homozygous genotype for the minor allele of the rs838133 SNP are associated with elevated FGF21 protein levels and FGF21 serum levels in patients with metabolic associated fatty liver disease but not in healthy controls [43]. However, other studies reported no significant association between rs838133 and FGF21 expression [5]. This might be, at least in part, because the effect size is small and fails to be detected as an eQTL [5]. In addition, the minor allele of the rs838133 SNP was associated with sugar and alcohol preference in a UK Biobank study with 451,000 individuals of European ancestry [2,5]. We should point out that rs838133 was not associated with plasma FGF21 level in our AUD patients (p: 0.93). In addition, the Psychiatric Genomics Consortium's Substance Use Disorders working group has published a GWAS of alcohol dependence and rs838133 was not associated with alcohol dependence (p:0.08 n = 20,364) [29]. A recent GWAS of FGF21 using serum samples from 4201 subjects in the Taiwan Biobank did not identify genome-wide significant signals. However, our GWAS of plasma FGF21 levels was conducted in AUD patients, and we identified genome-wide significant signals, suggesting genetic variants that might have implications for FGF21 concentration. However, the effects of genetic variants on FGF21 could be disease-specific or even ethnic-specific.

In conclusion, our data raise the possibility that *SNHG16*, which appears to play a functional role in regulating plasma FGF21 concentrations, might also contribute to catecholamine metabolism through a novel pharmacogenomic mechanism. This series of studies demonstrates that "multiple omics," i.e. the union of genomics, proteomics, and functional genomics, may helps us move beyond "biomarkers" to novel hypotheses of the molecular mechanisms underlying alcohol consumption and molecular links between the *SNHG16* locus and catecholamine metabolism.

AUTHOR CONTRIBUTIONS

M. Ho, and R. Weinshilboum wrote the manuscript; M. Ho, and R.Weinshilboum designed the research; M. Ho, C. Zhang, I. Moon and L. Wei, performed the research; M. Ho, C. Zhang, J. Biernacka, L. Wei and B. Coombes analyzed the data; C. Zhang, J. Biernacka and L. Hu contributed analytical tools; M. Skime, D. Choi, M. Frye, T. Oesterle, V. Karpyak, K. Schmidt, K. Gliske, J. Braughton, Q. Ngo, C. Skillon, and M. Seppala supported the patient recruitment effort. All authors have given final approval of the version to be published.

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CONFLICT OF INTEREST

Dr. Weinshilboum is a co-founder and stockholder in OneOme LLC, a pharmacogenomics decision-support company. Dr. Choi is a scientific advisory board member for Peptron Inc. Dr. Frye reports Grant Support from Assurex Health, Mayo Foundation, and Medibio. All other authors have no conflicts to declare.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2022.101534.

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