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Gene expression differences associated with alcohol use disorder in human brain

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Excessive alcohol consumption is a leading cause of preventable death worldwide. To improve understanding of neurobiological mechanisms associated with alcohol use disorder (AUD) in humans, we compared gene expression data from deceased individuals with and without AUD across two addiction-relevant brain regions: the nucleus accumbens (NAc) and dorsolateral prefrontal cortex (DLPFC). Bulk RNA-seq data from NAc and DLPFC ($N \geq 50$ with AUD, ≥ 46 non-AUD) were analyzed for differential gene expression using modified negative binomial regression adjusting for technical and biological covariates. The region-level results were meta-analyzed with those from an independent dataset ($N_{NAc} = 28$ AUD, 29 non-AUD; $N_{PFC} = 66$ AUD, 77 non-AUD). We further tested for heritability enrichment of AUD-related phenotypes, gene co-expression networks, gene ontology enrichment, and drug repurposing. We identified 176 differentially expressed genes (DEGs; 12 in both regions, 78 in NAc only, 86 in DLPFC only) for AUD in our new dataset. After meta-analyzing with published data, we identified 476 AUD DEGs (25 in both regions, 29 in NAc only, 422 in PFC only). Of these DEGs, 17 were significant when looked up in GWAS of problematic alcohol use or drinks per week. Gene co-expression analysis showed both concordant and unique gene networks across brain regions. We also identified 29 and 436 drug compounds that target DEGs from our meta-analysis in NAc and PFC, respectively. This study identified robust AUD-associated DEGs, contributing novel neurobiological insights into AUD and highlighting genes targeted by known drug compounds, generating opportunity for drug repurposing to treat AUD.

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INTRODUCTION

Alcohol use disorder (AUD) affected approximately 28.6 million adults in 2021 in the United States [1], and there are 3 million deaths per year caused by harmful use of alcohol worldwide [2]. AUD has 50–60% heritability [3], and hundreds of genome-wide significant variants have been identified for alcohol dependence [4], problematic alcohol use [5–7], and/or consumption [7–9] (e.g., drinks per week). As a more complete picture is emerging for specific genetic variants underlying AUD and related phenotypes, the gene regulatory landscape associated with AUD remains largely unknown. Filling this critical gap will define potentially new neurobiological mechanisms associated with AUD and assist in the identification of possible new drug targets to treat AUD. By focusing on gene expression changes associated with AUD in human brain, this study identifies regulatory differences that may be driven by predisposing genetic variation or may be consequences of alcohol exposure; both improve our understanding of the neurobiological mechanisms that relate to AUD.

Our study compares data from AUD cases and non-AUD controls in two key brain regions involved in the addiction cycle: the nucleus accumbens (NAc), implicated in the binge/intoxication stage, and the dorsolateral prefrontal cortex (DLPFC), implicated in the preoccupation/anticipation stage [10]. Both brain regions are linked to reward pathways as components of the dopaminergic mesolimbic system [11]. Exposure to alcohol increases dopamine levels via effects on dopaminergic neurons that originate in the midbrain and project into forebrain regions with NAc and prefrontal cortex (PFC) being most relevant to reward processes and addiction [12, 13]. Disruption of the dopamine system in the NAc has been described as lying at the core of addiction [14]. Dopamine release into the NAc is regulated by the PFC [15] and PFC dysfunction is associated with impulsivity, compromised executive function, and increased engagement in risky behavior [16]. Thus, the NAc and PFC are distinct, yet interrelated brain regions with functions highly relevant to the molecular mechanisms of AUD.

A few prior studies of AUD-related bulk RNA-seq gene expression in human brain have been reported, providing initial evidence for differential gene expression (DGE) associated with AUD [17–21]. However, none of these studies assessed results across independent datasets. Kapoor et al. conducted the largest prior study ($N = 138$) with RNA-seq in PFC and identified 129 genes that showed significant altered expression (false discovery rate [FDR] < 0.05) between the 65 AUD cases and 73 non-AUD

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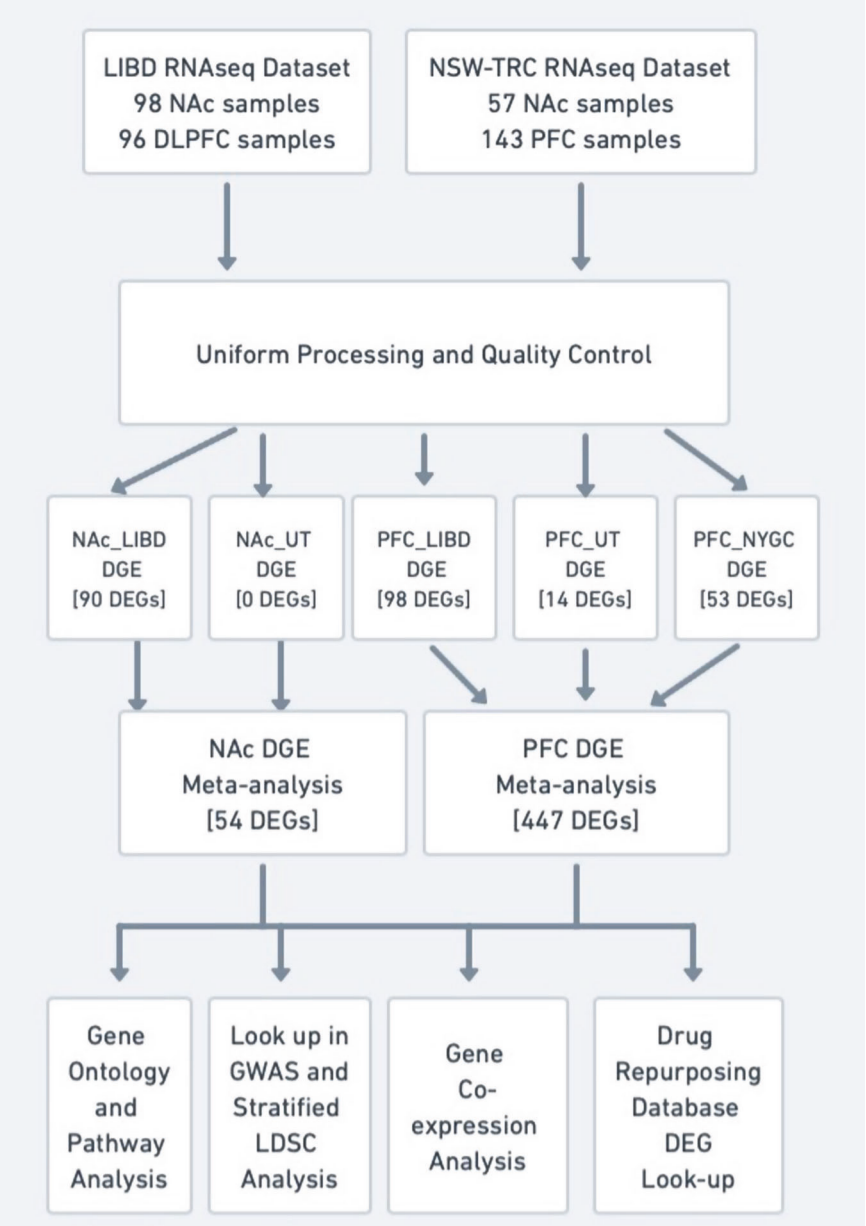

Fig. 1 Overview of analysis workflow.

controls [17]. A smaller subset with RNA-seq in NAc (N = 30 AUD, 30 non-AUD) showed 14 genes with significant altered expression [19]. Zillich et al. reported another dataset (N = 48 AUD, 51 non-AUD) with RNA-seq in three other brain regions and found significant evidence for DGE related to AUD in caudate nucleus (CN; 49 genes FDR < 0.05) and putamen (PUT; 1 gene FDR < 0.05), but no significant evidence in the ventral striatum (VS) [18].

In this study, we report a new dataset of 96 RNA-seq samples from NAc and 98 from DLPFC, which we meta-analyze with results from the Kapoor et al. dataset, after uniform reprocessing. Together, the increased sample size afforded by these independent NAc and PFC datasets improve statistical power to identify genes with robust evidence for DGE. We further evaluated overlap of differentially expressed genes (DEGs) between our study and Zillich et al. to compare DGE across brain regions, integrated DEGs with GWAS results to infer genetically driven DGE, and conducted gene ontology enrichment and gene co-expression analyses to explore potential mechanisms of DGE in AUD. We also looked up our AUD-associated DEGs in drug repurposing databases to identify existing pharmacotherapies that might target AUD.

MATERIALS & METHODS

A workflow overview of the datasets and analyses can be found in Fig. 1.

AUD cases and controls among human postmortem NAc and PFC samples

Postmortem human DLPFC (BA 46/9) and NAc tissues were obtained at autopsy from 122 subjects (61 AUD, 61 non-AUD) as part of the Lieber Institute for Brain Development (LIBD) Human Brain Repository [22]. AUD cases and controls were defined based on two or more lifetime DSM-5 symptoms within a 12-month period. Non-AUD controls had no lifetime history of DSM-5 AUD symptoms and postmortem ethanol toxicology of less than 0.06 g/dL. From the available samples in the LIBD brain bank, the sample was selected to maximize size and statistical power while

Table 1. Demographic information of samples in each dataset.

	LIBD						NSW-TRC					
	NAc_LIBD			PFC_LIBD			NAc_UT			PFC_UT			PFC_NYGC		
	Non-AUD (*N* = 47)	AUD (*N* = 51)	*P*-value	Non-AUD (*N* = 46)	AUD (*N* = 50)	*P*-value	Non-AUD (*N* = 29)	AUD (*N* = 28)	*P*-value	Non-AUD (*N* = 30)	AUD (*N* = 30)	*P*-value	Non-AUD (*N* = 47)	AUD (*N* = 36)	*P*-value
AgeDeath			0.496			0.873			0.760			1.000			0.864
Mean (SD)	53.7 (14.1)	51.8 (12.6)		52.2 (14.0)	52.6 (11.7)		57.8 (8.94)	57.0 (8.87)		57.5 (8.93)	57.5 (9.07)		53.5 (13.3)	54.0 (13.5)	
Sex			0.338			0.235			0.943			1.000			0.753
Male	35 (74.5%)	43 (84.3%)		32 (69.6%)	41 (82.0%)		23 (79.3%)	21 (75.0%)		23 (76.7%)	23 (76.7%)		39 (83.0%)	28 (77.8%)	
Female	12 (25.5%)	8 (15.7%)		14 (30.4%)	9 (18.0%)		6 (20.7%)	7 (25.0%)		7 (23.3%)	7 (23.3%)		8 (17.0%)	8 (22.2%)	
RIN			0.022			0.591			0.322			0.241			0.109
Mean (SD)	7.57 (0.672)	7.27 (0.576)		7.42 (0.576)	7.49 (0.636)		7.06 (0.653)	7.21 (0.544)		7.07 (0.645)	7.25 (0.550)		6.87 (1.23)	6.46 (1.09)	
PMI (hours)			0.551			0.890			0.252			0.272			0.015
Mean (SD)	25.6 (8.26)	26.6 (9.02)		27.4 (9.92)	27.7 (9.62)		31.2 (12.2)	35.4 (14.9)		30.7 (12.3)	34.6 (14.8)		24.0 (13.1)	32.4 (16.4)	
Smoking			0.570			1.000									
Case	24 (51.1%)	30 (58.8%)		24 (52.2%)	26 (52.0%)		18 (62.1%)	18 (64.3%)	0.249	18 (60.0%)	20 (66.7%)	0.412	8 (17.0%)	12 (33.3%)	0.042
Control	23 (48.9%)	21 (41.2%)		22 (47.8%)	24 (48.0%)		10 (34.5%)	6 (21.4%)		10 (33.3%)	6 (20.0%)		19 (40.4%)	6 (16.7%)	
Not Reported							1 (3.4%)	4 (14.3%)		2 (6.7%)	4 (13.3%)		20 (42.6%)	18 (50.0%)	
MDD			0.001			0.000									
No	25 (53.2%)	10 (19.6%)		26 (56.5%)	8 (16.0%)										
Yes	22 (46.8%)	41 (80.4%)		20 (43.5%)	42 (84.0%)										

minimizing potential confounders, so we additionally matched AUD cases and controls on major depressive disorder (MDD) and smoking status, both common comorbidities of AUD. Decedents with MDD were defined as those with a lifetime history of five or more DSM-5 MDD symptoms persisting for two weeks or longer. Smoking status was determined from cotinine or nicotine biomarker and next-of-kin reporting [23]. Further details regarding these samples and phenotype data were provided in White et al. [24]. RNA was extracted using LIBD's existing protocol [25, 26]. Illumina TruSeq Total RNA Stranded RiboZero Gold (Illumina Inc, San Diego, CA) was used for library prep. These samples are henceforth referred to as the NAc_LIBD and PFC_LIBD datasets.

The Kapoor et al. samples were obtained from the New South Wales Tissue Resource Center (NSW-TRC) and sequenced in two batches, one at the University of Texas at Austin (UT Austin) and the other at the New York Genome Center (NYGC). From both batches, fastq files for 143 prefrontal cortex (BA 8) samples and 58 NAc samples were transferred from UT Austin. The raw data is publicly available on NCBI (PRJNA551775, PRJNA530758, PRJNA781630). Phenotype and sequencing information was detailed in Kapoor et al. [17]. The samples that were processed at UT Austin are henceforth referred to as NAc_UT and PFC_UT. The samples processed at the NYGC are henceforth referred to as PFC_NYGC.

Given that BA 46/9 (LIBD) and BA 8 (NSW-TRC) are cytoarchitecturally adjacent and involved in similar brain functions, we chose to meta-analyze data from the two to maximize power and will refer to this region as PFC.

Bulk RNA-seq data processing and quality control (QC)

For all datasets, reads were trimmed and filtered using Trimmomatic [27] and transcripts were quantified using the GENCODE v40 (GRCh38) transcriptome with Salmon (v1.1.0) [28]. Gene-level quantification was done with the tximport R package (v1.14.2) [29]. QC metrics were calculated using MultiQC (v1.7) [30]. Samples were filtered using the following QC metrics, based on GTEx [31] and including additional important metrics, informed by our prior RNA-seq analyses [32] in LIBD, such as RNA Integrity Number (RIN) > 5, effective sequencing depth >10 million reads, post-Trimmomatic retained reads percentage >60%, mean read GC content between 35% and 65%, Salmon mapping percentage >30%, gene mapping percentage (combined intronic and exonic mapping) >80%, intergenic/genic mapping ratio <0.9, either Shannon index >the Shannon index IQR-based cutoff or transcriptome mapping percentage >50%, mitochondrial mapping percentage <10%, and ribosomal RNA mapping percentage <1%. After QC filtering, 98 samples (51 AUD, 47 Non-AUD) remained for NAc_LIBD and 96 (50 AUD, 46 Non-AUD) samples for PFC_LIBD (Table 1). Because the PFC samples from Kapoor et al. were sequenced at two different sites (UT Austin and NYGC) and had different sequencing depths, samples from each site were kept separate for analysis. After QC filtering, 57 (28 AUD, 29 non-AUD) NAc_UT samples remained, and all 143 (66 AUD, 77 Non-AUD) PFC samples remained (60 from PFC_UT, 83 from PFC_NYGC).

Cell type deconvolution

To estimate cell-type proportions for each dataset, the Bisque R package (v1.0.5) [33] was used along with single-cell references [34] for both NAc and DLPFC. Because both the NAc single cell reference and NAc_LIBD use data from LIBD, one sample coincidently overlapped. This sample was indicated to Bisque as overlapping. Proportions for macrophage, microglia, oligodendrocyte precursor cells (OPCs), astrocytes, GABAergic neurons, oligodendrocytes, and medium spiny neurons (MSNs) were estimated for NAc. Proportions for mural cells, macrophage, T-cells, microglia, OPCs, astrocytes, GABAergic neurons, excitatory neurons, and oligodendrocytes were estimated for PFC (Supplemental Fig. 1). Linear regression was used to test the association between predicted cell type proportions and AUD status,

accounting for MDD, smoking (current vs. not), age, sex, postmortem interval (PMI), and RIN for NAc_LIBD and PFC_LIBD and age, sex, PMI, and RIN for the NAc_UT, PFC_UT, and PFC_NYGC. No cell type proportion differences were significantly associated with AUD in any of the datasets (Supplementary Table 1). This motivated our decision to not include cell type proportion estimates in our DGE models.

Differential gene expression analysis

Genes were filtered for low expression using a cutoff of 10 counts in at least the number of samples that make up the smaller AUD status group for a given dataset (47 in NAc_LIBD, 46 in PFC-LIBD, 28 in NAc_UT, 30 in PFC_UT, 36 in PFC_NYGC). Surrogate variables (SVs) were calculated using the sva R package (v3.42.0) [35] to serve as proxies for known covariates and unmeasured technical and biological confounders. Additional known covariates such as age, sex, PMI, RIN, etc. were not included in the models as these covariates are highly correlated with the SVs and the SVs can potentially capture more information than the known covariates (i.e., both known covariates and other unmeasured sources of potential confounding). Maximizing percent variance explained with the fewest number of covariates was used for model selection. Each model had an r^2 value greater than 0.6 including model covariates other than AUD. We used the DESeq2 R package (v1.34.0) [36] to test for DGE using a generalized linear model with gene expression as the outcome variable. The following models were used for DGE analyses:

NAc_UT: expression ~ AUD + SV1 + … + SV9

NAc_LIBD: expression ~ AUD + MDD + smoking status (current vs. not) + SV1 + … + SV12

PFC_UT: expression ~ AUD + SV1 + … + SV10

PFC_NYGC: expression ~ AUD + SV1 + … + SV10

PFC_LIBD: expression ~ AUD + MDD + smoking status + SV1 + … + SV11

DEGs were assessed for potential sample outlier effects and removed based on Cook's distance using a threshold of greater than the 99th percentile of the F-distribution. A Benjamini–Hochberg FDR of 0.05 was used to declare statistically significant DGEs.

Differential gene expression meta-analyses

Meta-analyses of the individual dataset DGE results were performed for each brain region using the weighted Fisher's method in the metaPro R package (v1.5.5) [37], which combined DGE *p*-values for each gene across datasets, weighted by sample size of the dataset. Only genes tested for DGE across all datasets were considered for meta-analysis. An FDR of 0.05 was used to declare statistical significance. Genes that had discordant fold changes between datasets were removed after significance calling to increase cross-dataset consistency and robustness. The resulting meta-analysis DEGs were used in downstream analyses because of higher confidence in the validity of these results based on having increased statistical power from a larger sample size and uniform processing across datasets.

Lookup of meta-analysis DEGs in summary stats of other brain regions

To compare our results across brain regions, we looked up our meta-analysis DEGs in the publicly available summary statistics of the Zillich et al. [18] CN, VS, and PUT DGE analyses associated with AUD. This study had a sample size of 48 AUD cases and 51 controls. Bonferroni-corrected *p* values were used to declare significance, correcting for the number of meta-analysis genes looked up in each of the brain region summary statistics ($p < 0.05/48$ for NAc and $p < 0.05/401$ for PFC).

Gene ontology and pathway analysis

GSEA from the clusterProfiler R package (v4.2.2) [38] was used to detect functional enrichment of genes implicated in the DGE meta-analyses using the GO Molecular Function, Biological Process, and Cellular Component databases, and the KEGG Pathway database through the MSigDB R package [39] (v7.5.1). GSEA parameters of minGSSize = 15, maxGSSize = 500, and eps = 0 were used. Benjamini & Yekutieli FDR < 0.05 was used to declare a statistically significant term.

Lookup of DEGs in GWAS and stratified linkage disequilibrium score regression (LDSC)

We performed a look-up of DEGs in gene level summary statistics for problematic alcohol use [5] (gene level using H-MAGMA) and drinks per week [9, 40, 41] (gene level using MAGMA [42]) using Bonferroni corrected significance *p*-value < 0.05, correcting for the number of our meta-analysis DEGs that appear in the GWAS gene level summary statistics (50 genes in NAc and 423 genes in PFC). The full GWAS summary statistics of these two studies are publicly available [5, 43].

Partitioned heritability estimates and tests for enrichment of genetic loci associated with AUD-related phenotypes (drinks per week [9] and alcohol dependence [4]) constrained to DGE loci were conducted using stratified LDSC (v1.0.1) [44, 45]. The full GWAS summary statistics of these studies are publicly available [4, 43]. An annotation window of 100 kb from start and 100 kb from end of the meta-analysis significant DEGs was tested. A Bonferroni-corrected *p*-value threshold of 0.025 (0.05/2 phenotypes tested) was used to declare significance.

Gene co-expression analysis

The weighted gene co-expression network analysis (WGCNA) R package (v1.71) [46] was used to construct gene networks, with hierarchical clustering and dynamic tree-cutting to define modules for each dataset. The PFC_UT and PFC_NYGC were combined for this analysis to increase sample size. Covariates were accounted for using the removeBatchEffect function from the limma R package (v3.50.0) [47]. For NAc_LIBD and PFC_LIBD, MDD, smoking, sex, and age were used as covariates. For NAc_UT, PFC_UT and PFC_NYGC, batch and RIN were used as covariates, as done in Kapoor et al. Genes were filtered by including genes that had at least one count per million (CPM) in 50% of the samples. 16,625 genes were analyzed for NAc_LIBD; 16,255 genes were analyzed for PFC_LIBD; 17,058 genes were analyzed for the PFC_UT and PFC_NYGC combined dataset; and 15,891 genes were analyzed for NAc_UT. Soft power was set for each brain region and dataset where the scale-free topology fit index reached 0.90, which resulted in a power estimate of 16, 10, and 9 for NAc_LIBD, PFC_LIBD, and the combined NSW-TRC PFC datasets, respectively. Since the soft threshold step failed to converge for the NAc_UT dataset, that dataset was excluded from this analysis. Additional settings used were minimum module size = 30, cutting height = 0.25, deepSplit = 2, pamStage = FALSE, and verbose = 5. Modules were merged based on an eigengene (i.e., the first principal component of a module) correlation threshold of 0.75. We looked at module similarity across brain regions within the LIBD dataset by calculating the percentage of overlap between modules, defined as the number of overlapping genes divided by the size of the smaller of the two modules. We tested for association between module eigengenes and AUD status using a t-test.

Meta-analysis DEG look-up in drug repurposing databases

To identify the potential druggability of genes with AUD-related DGE, we used a drug repurposing tool [48], which leverages four different drug repurposing databases (Pharos, Open Targets, Therapeutic Target Database, and DrugBank) and a ranking system based on association statistics to provide a ranked list of drug compounds that target genes of interest. In this context, targeted means that the drug binds to the gene of interest, but the direction of the drug's effect on the targeted gene was not

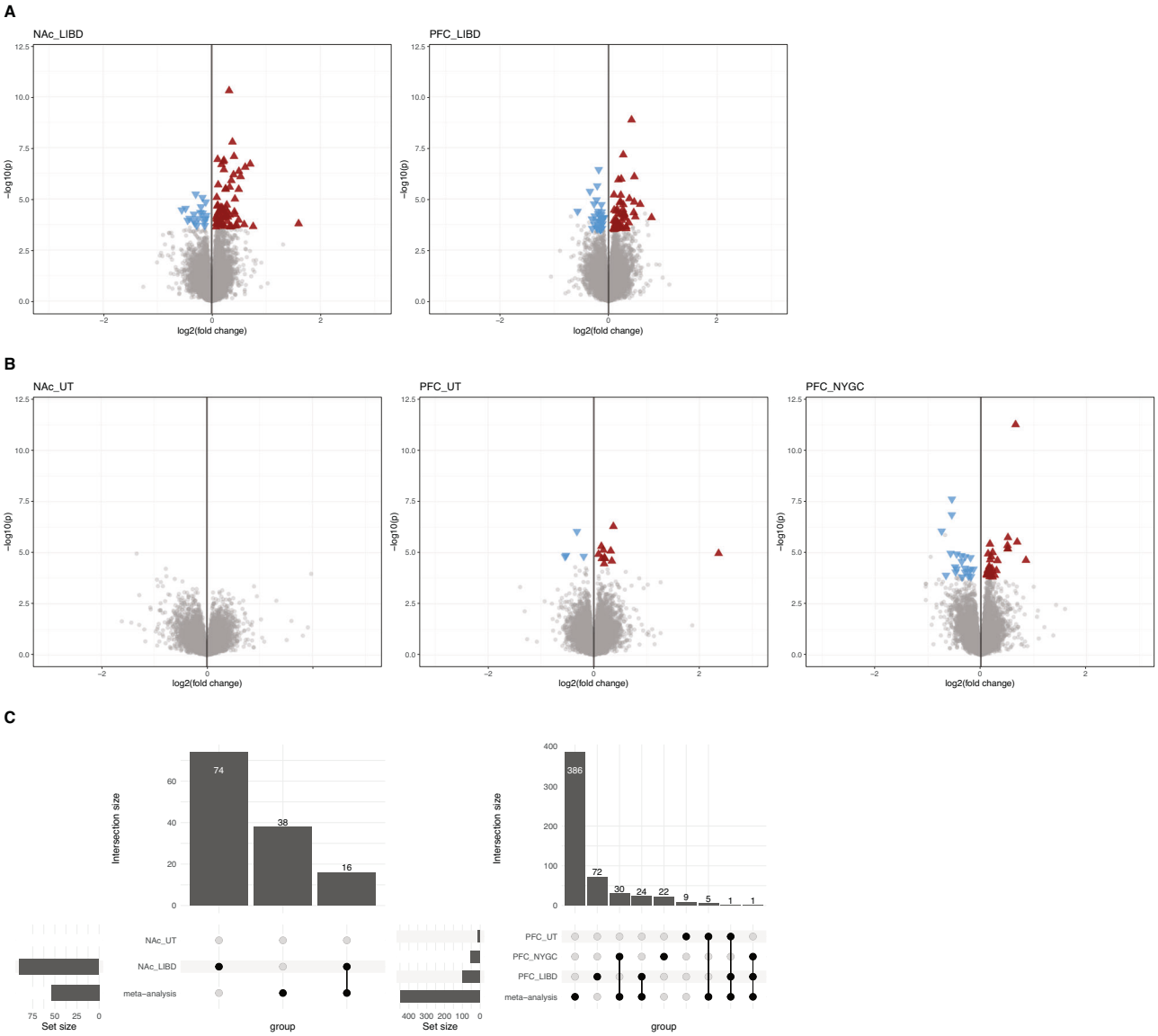

Fig. 2 Individual dataset and meta-analysis differential gene expression results. A Volcano plots of the differentially expressed genes (FDR < 0.05) by AUD status in the LIBD datasets. **B** Volcano plots of the differentially expressed genes (FDR < 0.05) by AUD status in the NSW-TRC datasets. For both (**A**) and (**B**), blue triangles represent significant downregulated genes and red triangles represent significantly upregulated genes. Genes not significantly differentially expressed are colored grey. **C** UpSet plots displaying the gene overlap count between the per-dataset DEGs and meta-analysis DEGs, by brain region.

available with the resources used and thus not considered. The lists of meta-analysis DEGs ranked by FDR-corrected p-values were used for the analysis. For NAc, all DEGs were used for the analysis. Given the large number of AUD-associated genes with differential expression in PFC, to focus on the most significant results, the top 100 DEGs for PFC were used for the analysis. To focus the results on drugs that have more specific targets, drugs in the top 10th percentile of gene target ratio were selected. The gene target ratio is calculated as the number of meta-analysis DEGs that a given drug targets divided by the total number of genes that the drug targets.

RESULTS

Differential gene expression results for each dataset

Each independent dataset was tested separately for DGE to account for batch effects. DGE analysis of 51 AUD and 47 non-AUD samples from the NAc_LIBD dataset resulted in 90 DEGs at FDR < 0.05 out of 20,690 genes tested. DGE analysis of 50 AUD and 46 non-AUD samples for the PFC_LIBD dataset resulted in 98 DEGs at FDR < 0.05 out of 20,298 genes tested (Fig. 2A, Supplementary Table 2). Twelve genes were overlapping between the significant results from NAc_LIBD and PFC_LIBD.

Uniform processing and QC (matching the processing and QC of NAc_LIBD and PFC_LIBD) of the NAc_UT resulted in 28 AUD and 29 non-AUD samples after QC filtering. For the PFC_UT, 30 AUD and 30 non-AUD samples were available for analysis. For PFC_NYGC, 36 AUD and 47 non-AUD samples were available for analysis. DGE analysis resulted in no DEGs out of 20,958 genes tested in the NAc_UT dataset. For the PFC_UT and PFC_NYGC, 14 and 53 DEGs had FDR < 0.05 (no overlapping DEGs) out of 21,224 and 20,762 genes tested, respectively (Fig. 2B).

DGE meta-analyses across datasets

Meta-analysis of 19,158 overlapping genes across the NAc samples resulted in 54 DEGs with FDR < 0.05 and matching effect

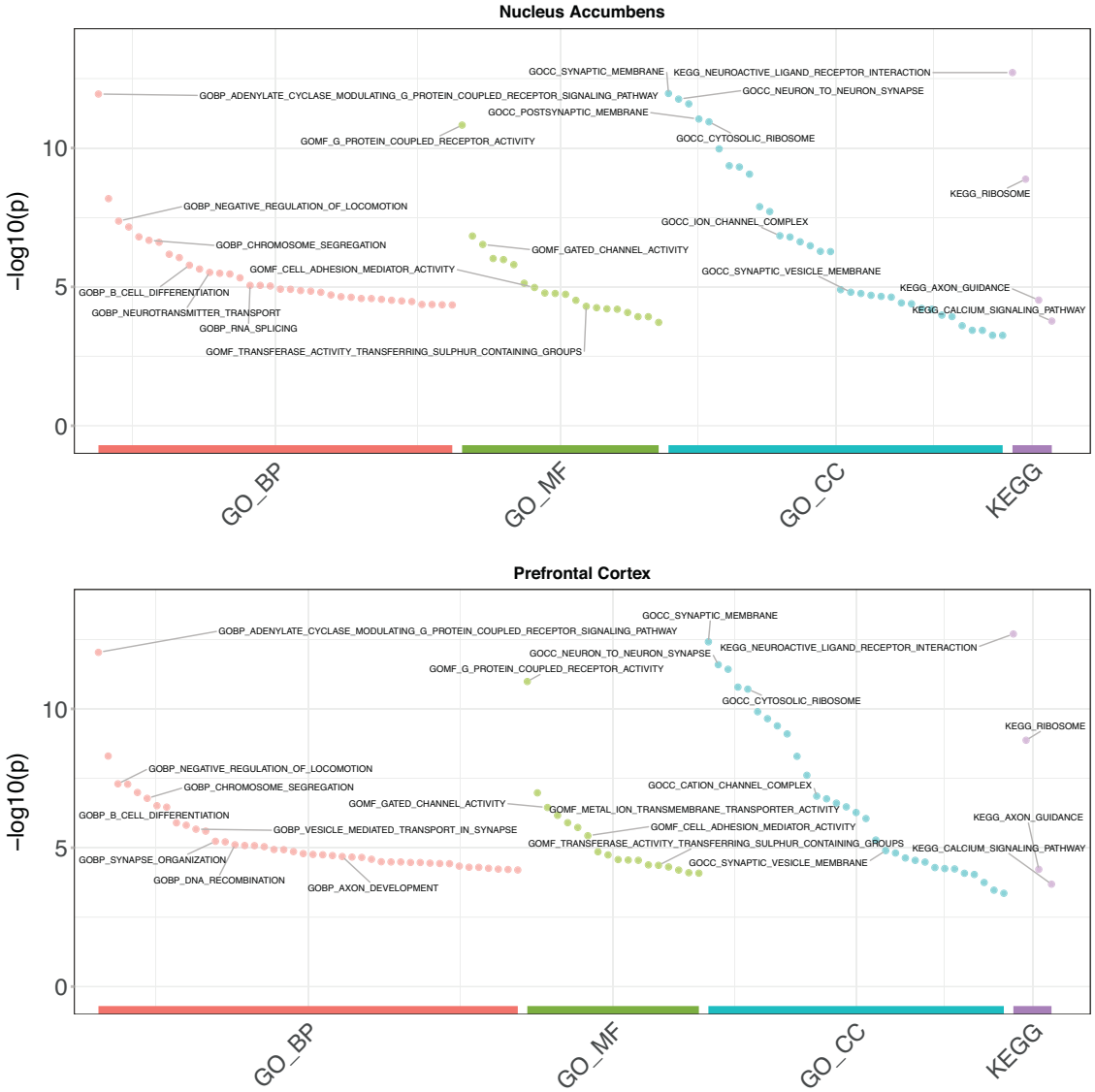

Fig. 3 GSEA significant (FDR < 0.05) terms plotted by *p*-value and database. Terms were clustered by semantic similarity. One term per cluster is labeled (term with lowest *p*-value).

directions (33 upregulated and 21 downregulated), with the top 5 DEGs genes being *ODC1*, *ZNF844*, *ARRDC3*, *FAM225A*, and *GUSBP11* (Supplemental Table 3). Meta-analysis of the 18,993 overlapping genes across the PFC samples resulted in 447 DEGs with FDR < 0.05 and matching effect directions (295 upregulated and 152 downregulated), with the top 5 DEGs genes being *TXNIP*, *ODC1*, *HMGN2*, *SLC16A9*, and *SLC16A6* (Supplemental Table 3). Twenty-five DEGs overlapped between FDR significant results for NAc and PFC. Overlap between individual studies and the meta-analyses by brain region are shown in Fig. 2C.

Lookup of meta-analysis DEGs in summary statistics of other brain regions

After Bonferroni correction for the NAc meta-analysis genes in the Zillich et al. summary statistics, *CSPP1* was the only gene significantly associated with AUD in CN; no NAc meta-analysis genes were associated with AUD in VS and PUT. After Bonferroni correction, no PFC meta-analysis significant genes were significantly associated with AUD in CN, VS, or PUT. The lack of overlap between NAc and PFC results with CN, VS, and PUT results suggests brain region specific-DGE associated with AUD or could be due to mismatch in statistical power from the smaller sample size in the Zillich et al. dataset compared to the meta-analyses.

Gene ontology and pathway analyses

To assess the functions and pathways of genes implicated in our DGE meta-analysis, we performed gene set enrichment analysis (GSEA) for meta-analysis results in each brain region, then used semantic similarity (Wang et al. algorithm [49]) to cluster the Gene Ontology (GO) GSEA results. GSEA for the NAc meta-analysis resulted in enrichment of 36 GO Biological Processes terms, 34 GO Cellular Component terms, 20 GO Molecular Function terms, and four KEGG pathways. GSEA for the PFC meta-analysis resulted in 44 significant GO Biological Processes terms, 31 significant GO Cellular Component terms, 18 significant Molecular Function terms, and four significant KEGG pathways (Fig. 3, Supplementary Table 4). 88 significant terms overlapped between the results from the two brain regions (94% of NAc significant terms and 91% of PFC significant terms).

GWAS overlap for genetically driven gene expression

To investigate the connection between genetic variation and DGE associated with AUD, we performed a lookup of our meta-analysis

significant DEGs (FDR < 0.05) in alcohol-related GWAS summary statistics and used stratified LD score regression to test enrichment of GWAS signals in meta-analysis DEGs. For the problematic alcohol use [5] GWAS, 50 NAc DEGs were tested, and one gene (*UGGT2*) was significantly associated after Bonferroni correction ($p < 0.05/50$); 424 PFC DEGs were tested, and six genes (*TCTA*, *TSPAN5*, *TLR6*, *EMX2OS*, *CA11*, and *SHISA5*) were significant ($p < 0.05/424$). For the drinks per week (DPW) GWAS [9], 41 NAc DEGs were tested, and two genes (*GBA2* and *GRM5*) were significant ($p < 0.05/41$); 379 PFC DEGs were tested, and nine genes (*HDAC7*, *HIPK3*, *ACVR2B*, *CDH18*, *TSPAN5*, *TTLL6*, *OLFML2A*, *SIRPA*, and *ATXN1L*) were significant ($p < 0.05/379$).

Testing the meta-analysis DEGs for enrichment in alcohol-related GWAS with fully available summary statistics, DPW [9] and alcohol dependence [4], using stratified LDSC resulted in enrichment (Bonferroni correction for two traits) for DPW in the PFC (Enrichment = 1.46, $p = 0.0131$) (Supplementary Table 5).

Gene co-expression analysis

WGCNA was used to explore the co-expression of genes within the datasets and test for module association with AUD. WGCNA generated 15 modules for the PFC_LIBD dataset, 14 modules for the NAc_LIBD dataset, and 22 modules for the PFC_UT and PFC_NYGC combined dataset (Supplementary Fig. 2, Supplementary Table 6). After FDR correction, no modules were associated with AUD. Module consistency across brain regions was tested by calculating the percentage of overlapping genes between modules. Comparing NAc_LIBD and PFC_LIBD modules, each module overlapped with at least one other module with a minimum of 39.2% and maximum of 97.8% shared genes (Supplementary Fig. 3), which supports prior evidence that there is both shared and region-specific co-expression across brain regions [50].

DEG look-up in drug repurposing databases

Using the Drug Repurposing Database tool [48] and our meta-analysis DEGs, 11 of the 54 genes with AUD-associated DGE in NAc were targeted by 29 drug compounds (Supplemental Table 7). Sixty-four of the top 100 genes with AUD-associated DGE in PFC were targeted by 436 drug compounds. To focus on targeted therapies, the 436 compounds were then subset to the 67 compounds in the top 10^{th} percentile of gene target ratio (ratio ≥0.33), calculated as the number of meta-analysis significant genes divided by the number of all genes targeted by the drug. This step was not conducted for the NAc results due to the smaller number of DEGs and identified compounds.

DISCUSSION

This study reports many newly identified DEGs associated with AUD, providing neurobiological insights into gene expression signatures of AUD that are shared across or specific to key brain regions for addiction. For NAc, there were no DEGs in the NAc_UT dataset when analyzed alone, but when uniformly processed and meta-analyzed with our new NAc_LIBD dataset, we identified 54 DEGs associated with AUD and with the same direction of effect across datasets. By uniformly processing and meta-analyzing our new PFC_LIBD dataset with the previously published PFC_UT and PFC_NYGC datasets, we increased the number of DEGs from 129 reported by Kapoor et al. to 447 DEGs associated with AUD in PFC. Overall, 25 genes were differentially expressed with AUD in both NAc and PFC. Unlike prior studies, independent datasets were combined via meta-analysis, enabling us to model the complexities of each dataset and take advantage of the combined sample size for more statistical power to detect AUD-associated DGE.

Of the 54 significantly expressed genes in the NAc meta-analysis, a lookup in the Zillich et al. summary statistics of VS, CN, and PUT regions resulted in one significant gene (*CSPP1*) that overlapped between our NAc results and the CN results. This may be due to the lack of statistical power due to smaller sample size in the Zillich et al. dataset. We do see initial evidence for cross-region DGE in relation to AUD with 25 DEGs overlapping between NAc and PFC meta-analyses, though many genes appear to have tissue-specific DGE. We also observed both shared patterns of brain region co-expression and region specificity through the cross-region WGCNA module overlap analysis.

The overlap of several meta-analysis DEGs in alcohol behavior GWAS suggests that at least some of the AUD-associated DGE may be genetically driven. By conducting a stratified LDSC analysis, we tested for enrichment of alcohol-related genetic loci in and around DEGs for AUD. Significant enrichment between the DPW summary statistics and PFC DEGs further supports a genetic component to the DEGs. These results together suggest that some of the DGE result from genetic predisposition, although further exploration, for example through eQTL analysis, is needed.

Many of the top DEGs and DEGs that overlapped with GWAS results were previously reported to play a role in AUD or other psychiatric disorders. For example, *ODC1*, which was significant in both NAc and PFC meta-analyses and is associated with cell proliferation of neural progenitor cells, was reported to be downregulated in psychiatric phenotypes by loss of function variants. *TXNIP*, a top DEG in the PFC, was upregulated in mice PFC in schizophrenia-like states [51, 52] and implicated in astrocytic glucose hypometabolism in depressive state rats [53]. Differential expression of *HMGN2*, a top DEG in the PFC, was reported for schizophrenia-associated DGE in peripheral blood mononuclear cells [54]. *SLC16* was a top DEG in the PFC, and belongs to a family of solute carrier transporters, many of which have reported roles in neuropsychiatric disorders, such as *SLC6* in depression and post-traumatic stress disorder [55]. *TSPAN5*, a DEG in the PFC and significant in both PAU and DPW GWAS, is downregulated by acamprosate [56], one of only three FDA-approved treatments for AUD. *TLR6*, a Toll-Like Receptor (TLR), was a DEG in the PFC and significantly associated in problematic alcohol use GWAS. Dysregulation of TLRs, which are directly involved in the regulation of inflammatory reactions, has been reported in AUD due to an inflammatory response to excessive alcohol consumption [57]. *CDH18*, a DEG in the PFC and significantly associated in DPW GWAS, and other cadherin pathway genes have been associated with schizophrenia, bipolar disorder and MDD through GWAS [58]. These converging associations could indicate a shared genetic, and ultimately transcriptomic, risk for AUD and some psychiatric diseases, a hypothesis supported by shared genetic correlations [59]. While several of our DEGs have known associations with AUD and other psychiatric disorders via GWAS and studies in model organisms, many of the DEGs are novel to the AUD phenotype, and their roles in AUD merit further investigation.

Due to NAc and PFC's known roles in reward processing, we investigated if any of the meta-analysis significant genes were observed in the Gene Ontology Biological Processes dopamine receptor signaling pathway gene set (GO:0007212) [60, 61]. Of the 38 genes in this pathway, two were significant DEGs in our meta-analyses. *CALY* in the PFC meta-analysis and *GNA11* in the NAc meta-analysis. *CALY* encodes calcyon protein, which interacts with D1 dopamine receptor. *CALY* was upregulated in individuals with AUD from our PFC meta-analysis and was similarly reported for being unregulated in PFC of individuals with schizophrenia [62]. The protein produced by *GNA11* is the alpha subunit for the G_{11} protein [63] but a role of this gene in psychiatric disease has not been identified. While we see differential expression associated with AUD in two genes in this pathway, it is reasonable to not see major enrichment of DEGs in this pathway as AUD impacts many biological processes, not just dopamine response, as shown by our GSEA results.

Since our GSEA analyses produced 94 significant terms and pathways in NAc and 97 significant terms and pathways for PFC, a

full discussion of these results is not feasible here. Both brain region results have the same top seven significant terms and pathways, all related to brain activity: neuroactive ligand receptor interaction, synaptic membrane, neuron to neuron synapse, intrinsic component of synaptic membrane, postsynaptic membrane, cytosolic ribosome, and adenylate cyclase-modulating G protein-coupled receptor signaling pathway. With 88 overlapping terms and pathways between the brain region results, this analysis suggests that AUD is implicated in similar pathways and networks in NAc and PFC regions. Further interpretation and follow-up experiments of these results is warranted.

By integrating our DGE results with drug repurposing databases, we identified many FDA-approved drug compounds that target the DEGs in both NAc and PFC. Some of the listed compounds are already used for AUD treatment, such as clomethiazole, which targets the GABA receptors and is used for alcohol withdrawal treatment [64], and acamprosate, which targets *GRM5* and is used for treatment of alcohol dependence. *GRM5* also overlapped between our NAc DGE results and the DPW GWAS, and is also targeted by cinacalcet, rufinamide, and glutamic acid, which are novel candidate drugs to treat AUD with support from both GWAS and differential expression. Another gene with overlapping support from both DGE and GWAS was *GBA2*, which is targeted by miglustat and used for treatment of Gaucher's Disease [65]. We identified some compounds already used for AUD treatment, lending confidence to our approach, however, most of the compounds that we identified are used for purposes unrelated to AUD and merit further investigation as potential novel therapeutics for AUD.

This study comes with limitations. The data were limited to individuals of European ancestry, so results warrant testing in other datasets of diverse ancestries to assess their generalizability. Though this is the largest combined sample size of human NAc and PFC to date (N up to 239), even larger sample sizes across diverse populations and brain regions that play a role in the addiction cycle are needed. This study is also based around lifetime history of AUD, so some gene expression could have returned to control levels over time if drinking was reduced or eliminated prior to death, biasing the results for those genes towards the null. No estimated cell type proportions in any of the datasets were significantly associated with AUD, though we were limited by the small reference sample sizes available for deconvolution, with only one study sample overlapping with the single nuclei RNA-seq reference data. Having a small reference dataset and minimal overlap between study and reference can lead to inaccurate estimation. Since the estimation only reflects the proportions seen in the single-nuclei reference dataset, using a larger single nuclei dataset in the future, with greater overlap with study samples will help improve estimations.

Future work to expand our findings could include further analysis of the drug repurposing results, GSEA results, or new analyses of our bulk RNA-seq datasets through differential splice variation. Future investigation of the drug repurposing results including additional experimentation to understand the effects on gene expression and pharmacologic potential of these drugs is essential prior to their use for AUD as a clinical indication. The significant GSEA terms from our analyses, which provide insights into potential pathways and mechanisms implicated in AUD, can be further understood using mechanistic and functional experimentation. Evidence of splice variation by AUD has been previously reported for the NSW-TRC samples [19] but has not been analyzed for the LIBD samples. Future analyses of splice variation in the LIBD samples and subsequent meta-analyses with NSW-TRC results may further improve understanding of the relationships between AUD and transcription in the brain.

This work is the first meta-analysis of DGE associated with AUD in NAc and PFC human brain regions. Meta-analysis of DGE results from two independent studies using uniform processing and analysis helped identify more robust results than from one study alone. Many identified top DEGs are known to be involved in AUD and other psychiatric disorders, while others are novel. Overlap of several DEGs with alcohol use-related GWAS and enrichment in GWAS of DPW suggests that these DGE findings may have a genetic component. By using a drug repurposing tool, we were able to identify pharmacotherapies that have already been used for AUD as well as many novel candidate pharmacotherapies for AUD. In sum, this work expands existing knowledge and provides new insights into the neurobiology of AUD and generates new opportunities for further research.

DATA AVAILABILITY

Bulk RNA-seq data generated from this study has been deposited on GEO (GSE253155) and full summary statistics for analyses are provided as supplement tables.

CODE AVAILABILITY

Code used for analyses is available upon request.

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AUTHOR CONTRIBUTIONS

DBH, LJB, EOJ, RDM Conceived and designed the study. Formal analysis was done by CDW. DBH, LJB, EOJ, JDW supervised the study. Investigation was done by SH, RT, JHS, and TMH. Methodology was done by CDW, DBH, LJB, EOJ, JDW, MSM, BCQ, AD-S, RDM, and BTW. All authors took part in the writing and editing process.

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COMPETING INTERESTS

The following authors declare no conflict of interest: CDW, JDW, MSM, BCQ, SH, RT, JHS, AD-S, TMH, RDM, BTW, EOJ, and DBH. JEK is a member of a drug monitoring committee for an antipsychotic drug trial for Merck. LJB is listed as an inventor on U.S. Patent 8,080,371, 'Markers for Addiction' covering the use of certain SNPs in determining the diagnosis, prognosis, and treatment of addiction.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Informed consent for brain donation was obtained by LIBD from the deceased's next of kin, in compliance with local and federal protocols (refer to PMC6808324), and this study was conducted in accordance with all relevant guidelines and regulations for the use of postmortem human brain tissue.

ADDITIONAL INFORMATION

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