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Alcohol Use Disorder–Associated DNA Methylation in the Nucleus Accumbens and Dorsolateral Prefrontal Cortex

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ABSTRACT

BACKGROUND: Alcohol use disorder (AUD) has a profound public health impact. However, understanding of the molecular mechanisms that underlie the development and progression of AUD remains limited. Here, we investigated AUD-associated DNA methylation changes within and across 2 addiction-relevant brain regions, the nucleus accumbens and dorsolateral prefrontal cortex.

METHODS: Illumina HumanMethylation EPIC array data from 119 decedents (61 cases, 58 controls) were analyzed using robust linear regression with adjustment for technical and biological variables. Associations were characterized using integrative analyses of public annotation data and published genetic and epigenetic studies. We also tested for brain region–shared and brain region–specific associations using mixed-effects modeling and assessed implications of these results using public gene expression data from human brain.

RESULTS: At a false discovery rate of $\leq$.05, we identified 105 unique AUD-associated CpGs (annotated to 120 genes) within and across brain regions. AUD-associated CpGs were enriched in histone marks that tag active promoters, and our strongest signals were specific to a single brain region. Some concordance was found between our results and those of earlier published alcohol use or dependence methylation studies. Of the 120 genes, 23 overlapped with previous genetic associations for substance use behaviors, some of which also overlapped with previous addiction-related methylation studies.

CONCLUSIONS: Our findings identify AUD-associated methylation signals and provide evidence of overlap with previous genetic and methylation studies. These signals may constitute predisposing genetic differences or robust methylation changes associated with AUD, although more work is needed to further disentangle the mechanisms that underlie these associations and their implications for AUD.

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Alcohol use disorder (AUD) affected approximately 28.6 million adults in the United States in 2021 (1). Given the profound impact that AUD has on individuals and society, research on AUD-relevant brain regions is critical for understanding the mechanisms that underlie its development and progression.

Epigenetic modifications, particularly DNA methylation (DNAm), play a central role in regulating gene expression and are key to the interplay between genetic variation and environmental influences (2). Variation in DNAm associated with alcohol use may be driven by underlying predisposing genetic risk or may be a consequence of alcohol use that could help explain potentially reversible neurological changes that stem from excessive consumption (3). To disentangle these 2 scenarios, robust associations between DNAm, AUD, and genotype must be identified and replicated across independent datasets. This study, which is focused on associations between DNAm and AUD, presents an epigenome-wide association study (EWAS) of AUD in the nucleus accumbens (NAc) and dorsolateral prefrontal cortex (DLPFC) of postmortem human brains. The NAc is involved in motivation, pleasure, and reward/reinforcement learning and is primarily implicated in the binge/intoxication stage of addiction, with a secondary role in the withdrawal/negative affect stage (4–6). The DLPFC controls inhibition of impulsive responses, cognitive flexibility, and planning and is involved in the preoccupation/anticipation stage of addiction (4).

All previous EWASs of alcohol-related behaviors in postmortem human brain had sample sizes of 46 to 96, with AUD-associated differential DNAm found in the cortex (7–12) and basal ganglia (12), which respectively encompass the DLPFC and NAc plus nearby regions that are also implicated in addiction neurobiology (4–6). No overlapping sites have been reported across these studies, which is not surprising given the highly context-specific nature of DNAm, the different analytic strategies used, and the small sample sizes.

In the current study, we examined epigenome-wide AUD-associated DNAm in the brain using the largest sample size to

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date, 119 decedents that do not overlap with those included in previous publications. Given the correspondence across brain regions tested and the large sample size relative to previous alcohol EWASs, we also tested for consistency of results across this and previous studies. Lastly, with DNAm assayed from the NAc and DLPFC of the same decedents, we also shed light on AUD-associated DNAm that is shared across and unique to these brain regions, which, to our knowledge, has not been explored previously.

METHODS AND MATERIALS

Human Postmortem NAc and DLPFC Samples

Postmortem human NAc and DLPFC (Brodmann area [BA] 46/9) tissues were obtained at autopsy by the Lieber Institute for Brain Development Human Brain Repository (13–16). Psychiatric narratives were independently reviewed by two board-certified psychiatrists to determine lifetime psychiatric conditions based on DSM-5 criteria. AUD cases were defined as decedents with a lifetime history of two or more AUD symptoms within a 12-month period, with or without positive postmortem ethanol toxicology. AUD control decedents had no lifetime history of AUD symptoms and postmortem ethanol toxicology of $<$0.06 g/dL. Detailed information on typical alcohol consumption patterns during life was not available in the psychiatric narratives used for diagnoses. We matched (17) AUD cases and controls based on age, sex, smoking status [based on cotinine or nicotine biomarker and next-of-kin reporting (13)], major depression diagnosis (MDD), and other drug indicators (Table 1). MDD status and related drug indicators were included in the matching criteria because of the common comorbidity of AUD and MDD, which we chose to represent in the data to maximize sample size. Decedents with MDD had a lifetime history of 5 or more MDD symptoms that persisted for 2 weeks or longer. All decedents included were White because there were few decedents from other race/ethnicity groups who met the inclusion criteria at the time of sample construction. To further minimize confounding, only decedents who were 25 years old or older without detectable opioid history were included. Decedents who met DSM-5 criteria for another substance use or psychiatric disorder except for AUD and MDD were excluded.

DNAm Data Processing

DNA was extracted from the NAc and DLPFC following Lieber's protocol (18,19). DNAm was measured using the Illumina HumanMethylation EPIC array, with data processing conducted using minfi (20). Probes that failed standard quality control (Table S1 in the Supplement) were excluded before quantile normalization. No samples were excluded based on low call rate (detection $p > .01$ for $>$1% of probes), mismatched sex, or methylated versus unmethylated values $<$10. One assay chip produced outliers based on DNAm-derived principal components, which led to the removal of 5 NAc and 3 DLPFC samples. CpGs were annotated to hg38 genomic locations from Zhou *et al.* (21). After quality control, 769,135

Table 1. Description of Samples Used for Analysis

Variable	Overall, N = 119[a]	AUD Status		p Value[b]
		Non-AUD, n = 61[a]	AUD, n = 58[a]	
Age at Death, Years	52 (13)	53 (13)	52 (12)	.7
Sex				.4
Female	24 (20%)	14 (23%)	10 (17%)	
Male	95 (80%)	47 (77%)	48 (83%)	
Smoking				.4
Current	63 (53%)	30 (49%)	33 (57%)	
Not current	56 (47%)	31 (51%)	25 (43%)	
MDD				<.001
No	41 (34%)	31 (51%)	10 (17%)	
Yes	78 (66%)	30 (49%)	48 (83%)	
Toxicology for Ethanol ≥0.06 g/dL				<.001
Negative	81 (68%)	61 (100%)	20 (34%)	
Positive	38 (32%)	0 (0%)	38 (66%)	
Manner of Death				.003
Natural	62 (52%)	40 (66%)	22 (38%)	
Not natural (accident, homicide, suicide)	56 (47%)	21 (34%)	35 (60%)	
Unknown	1 (1%)	0 (0%)	1 (2%)	
Postmortem Interval, Hours	27 (9)	27 (9)	28 (10)	.7
Tissue				.6
DLPFC + NAc	113 (95%)	57 (93%)	56 (97%)	
DLPFC only	4 (3.4%)	2 (3.3%)	2 (3.4%)	
NAc only	2 (1.7%)	2 (3.3%)	0 (0%)	

AUD, alcohol use disorder; DLPFC, dorsolateral prefrontal cortex; MDD, major depression diagnosis; NAc, nucleus accumbens.

[a]Values are presented as mean (SD) for continuous variables and n (%) for categorical variables.

[b]Wilcoxon rank-sum test, Pearson's χ^2 test, or Fisher's exact test.

CpGs were analyzed for 115 NAc samples and 767,700 CpGs for 117 DLPFC samples (Table 1).

AUD EWAS Analyses

We compared methylation M-values in AUD cases and controls using linear regression with robust standard errors (22–24). Analyses were adjusted for MDD status, age at death, sex, smoking (current vs. not), 5 DNAm-derived principal components, sample plate (P1 vs. P2, P1 vs. P3), and row position on methylation chip (rows 1–4 vs. 5–8). For both brain regions, principal components calculated from the DNAm data were included to correct for technical artifacts and cellular heterogeneity because DNAm cell type composition references were unavailable for the NAc. Results were corrected for inflation using the BACON empirical null distribution method (25), and lambda values were calculated on BACON-corrected p values. A cross-region meta-analysis of BACON-adjusted p values for 766,095 CpGs common to both brain regions was conducted using METAL, with correction for sample overlap via METAL's adjustment of study weights based on estimated covariance between z scores (26). Effect similarity across brain regions was assessed using I^2 heterogeneity statistics. Significance was assessed using a false discovery rate (FDR)–corrected $p \leq .05$ (27). The sensitivity analyses, enrichment tests, and concordance assessments described below used these results.

We also identified differentially methylated regions (DMRs) using the DLPFC, NAc, and cross-region meta-analysis results via the ENmix comb-p function (seed = 0.05) (28,29). DMRs that contained at least 2 CpGs were considered significant at a Sidak p value $\leq .05$. DMRs were annotated to genes with the nearest transcription start site using the annotatePeak function (30), with the TxDb.Hsapiens.UCSC.hg38.knownGene (31) and org.Hs.eg.db (32) packages.

Sensitivity Analyses for Postmortem Ethanol Toxicology and Manner of Death

To assess whether significant AUD-associated CpGs showed differential methylation related to recent alcohol exposure, we used robust linear regression of methylation M-values on ethanol toxicology status ($\geq$0.06 g/dL vs. $<$0.06 g/dL) in AUD cases. This analysis adjusted for postmortem interval (hours) and all covariates from the AUD case-control EWASs.

We also tested whether adjusting for manner of death affected AUD associations for significant CpGs. To do this, we repeated the robust linear regression from our primary analyses with an additional covariate of natural versus not natural (accident, homicide, suicide) manner of death. Significance was assessed using an FDR-adjusted $p \leq .05$ for both sensitivity analyses.

Enrichment Tests

Gene-set enrichment for known pathways was conducted using functions from missMethyl, which corrects for the number of probes per gene (33) and multigene associated probes on the Illumina arrays (34). From each EWAS (NAc, DLPFC, cross-region meta-analysis), we supplied gsameth with the list of significant CpGs, all tested CpGs, and gene sets for enrichment testing. CpG-to-gene(s) mapping used Zhou *et al.* (21) annotations, with gene symbols converted to Entrez ids using the AnnotationDbi (35) and org.Hs.eg.db (32) packages.

Two-sided Fisher's exact tests were used to assess enrichment of AUD-associated probes in CpG (islands, shelves, and shores) and genic (promoters, 5′ untranslated regions [UTRs], exons, introns, and 3′ UTRs) contexts, with locations for these contexts sourced from the annotatr (36) package and the LOLA (37) package used for intersecting positions. LOLA was also used to test for enrichment of significant CpG positions with chromatin immunoprecipitation sequencing histone modification sites from brain using the Roadmap Epigenomics Consortium epigenomes of non-diseased decedents (Table S2 in the Supplement) (38).

Concordance With Published EWASs of AUD and Alcohol Consumption

We performed several comparisons between our results and results of previous EWASs of AUD or alcohol consumption to capture both probe-level and broader agreement. Selected EWASs had large sample sizes ($n \geq 46$ for brain and $n > 5000$ for blood) and results reported by probe or coordinates. Three studies tested for associations with alcohol abuse, dependence, or use disorder in brain tissues (10–12), and 2 studies tested for associations with alcohol consumption in blood (39,40). For CpGs tested in at least one of our EWAS analyses and identified as significant in a previous study or among the top 20 CpGs if no significant results were reported, we declared "look-up" statistical significance based on FDR $\leq .05$, calculated from the p values of the intersecting CpGs.

Second, we received full summary statistics from Zillich *et al.*'s EWAS of AUD in 5 brain regions (12) and Clark *et al.*'s BA 10 whole genome methylation and hydroxymethylation AUD-association study (11). For each, we tested for enrichment of the top 1% of p values from our study in the top 1% of p values from the previous publication using the enrichmentAnalysis function in the shiftR package, with 10,000 permutations (41). This threshold balanced including too few sites, which could reduce power, and including too many sites, which may lead to false positive results.

Lastly, the consistencies in technology (Illumina EPIC) and statistical framework (linear regression of M-values) across studies enabled us to conduct a sample size–weighted meta-analysis across the brain regions that were shared with Zillich *et al.*'s study. Clark *et al.*'s results were not included in this analysis because of differences in methylation technology and association analysis. The cross-study meta-analysis was performed on 636,087 intersecting CpGs for DLPFC/BA 9 and 673,083 CpGs for the NAc/ventral striatum (VS). Effect similarity was assessed using I^2 heterogeneity statistics, and significance was determined at FDR $\leq .05$.

Concordance With Genome-Wide Association Study Results for Alcohol Behaviors

We employed stratified linkage disequilibrium score regression (42,43) to estimate partitioned heritability and assess enrichment of alcohol-associated genetic loci within 5, 10, 100, 250, and 500 kb windows around significant CpGs from our NAc, DLPFC, and cross-region meta-analysis results. We utilized

genome-wide association study (GWAS) summary statistics for drinks per week (44) and DSM-IV alcohol dependence (45) in individuals of European ancestry, with linkage disequilibrium calculated from the 1000 Genomes Project phase 3 "EUR" reference (46). We also used gwasrapidd (47) to identify addiction-related associations reported in the GWAS catalog (48) for genes annotated to significant CpGs in our study.

Linear Mixed-Effects Modeling of AUD-Associated DNAm Across Brain Regions

We conducted the cross-region meta-analysis to identify CpGs with consistent effect sizes across the 2 brain regions. CpGs that were significant in either the NAc or DLPFC EWAS, but not in the cross-region meta-analysis, may reflect different methylation levels in the 2 regions or varying AUD associations across them. To explicitly test these possibilities, we used linear mixed-effects modeling to assess differential DNAm by brain region, AUD status, and their interaction. In this model, CpGs associated with AUD status likely have similar associations across the NAc and DLPFC, while those associated with brain region may have different methylation levels across the two. CpGs associated with the AUD by brain region interaction may have different strengths or directions of association with AUD depending on the brain region (i.e., brain region–specific effects). Using these results, we also identified DMRs for AUD, brain region, and their interaction using comb-p, as above.

Expression Profiling Across Brain Regions Using the Allen Human Brain Atlas

To investigate gene expression patterns near brain region–shared and brain region–specific AUD-associated DMRs, we utilized microarray data from the middle frontal gyrus (MFG), which contains the DLPFC, and the NAc (labeled Acb) from 6 postmortem brains in the Allen Human Brain Atlas (AHBA) (Table S3 in the Supplement) (49). For each microarray probe targeting a gene or its synonym annotated to a significant DMR from our linear mixed-effects modeling, we conducted paired *t* tests to compare expression levels across the 2 brain regions. We employed a bootstrap sampling approach with 1000 iterations, selecting one replicate per brain region from each donor in each iteration. The null hypothesis of no difference in expression across brain regions was rejected if the median bootstrapped *p* value was $\leq$.05.

RESULTS

EWAS of AUD

Figure 1 shows a flowchart of our data, analyses, and results. We identified 53 and 31 CpGs that were significantly associated with AUD in the NAc (λ = 1.03) and DLPFC (λ = 1.02), which were annotated to 65 and 36 genes, respectively. Our cross-region meta-analysis identified 31 AUD-associated CpGs (λ = 1.03), with 10 CpGs overlapping with either the NAc or DLPFC EWAS, although none were significant in all 3 analyses (Figure 2). Altogether, the EWAS analyses identified 105 CpGs associated with AUD, annotated to 120 unique genes, which we carried forward as our primary results (Table S4). We also identified 99 non-overlapping DMRs (annotated to 97 genes) across the DLPFC, NAc, and cross-region meta-analysis (Table S4).

Sensitivity Analyses for Recent Alcohol Exposure and Manner of Death

We tested whether recent alcohol exposure in decedents with AUD influenced methylation among the 105 AUD-associated CpGs (Table S5). One CpG in the NAc (cg15747423; *UST*) and one in the DLPFC (cg25985151; *DNAI1*) were significantly (FDR $\leq$ .05) associated with ethanol toxicity. These results suggest that our DNAm-AUD associations are largely robust to acute alcohol exposure around the time of death.

All CpGs remained significant when adjusting for manner of death (Table S5), with a high correlation of *z* scores between the adjusted and unadjusted results (*r* = 0.97–0.99) (Figure S1 in the Supplement). This suggests that the manner of death was not a confounder for our significant CpGs. In both tissues, manner of death was moderately correlated with MDD (*r* = 0.6 and 0.61) and somewhat correlated with AUD (*r* = 0.4 and 0.42) and smoking (*r* = −0.35 and −0.39) (Figure S2 in the Supplement), indicating that our original model likely accounted for some variation in DNAm due to the manner of death.

Enrichment Tests

We tested Kyoto Encyclopedia of Genes and Genomes and Gene Ontology pathway databases for enrichment using the 120 genes annotated for our AUD-associated CpGs. No pathways had an FDR $\leq$ .05 (Table S6). For CpG and genic locations, AUD-associated CpGs in the NAc were depleted in intergenic regions and enriched in islands, promoters, and 5′ UTRs (Figure 3A, B and Table S7). No CpG location-based enrichment/depletion was identified for the DLPFC AUD-associated CpGs. AUD-associated CpGs from the cross-region meta-analysis were enriched in promoters, 5′ UTRs, and exons (Figure 3B and Table S7). In brain-derived Roadmap Epigenomics epigenomes, significant CpGs from the NAc EWAS and cross-region meta-analysis were enriched in H3K27ac, H3K9ac, and H3K4me3 marks (Figure S3B, C; Table S7).

Concordance With Published EWAS of AUD and Alcohol Consumption

We compared our results to 5 published EWASs of AUD or consumption performed in brain or blood (Table S8). Dugué *et al.* tested for associations with alcohol consumption in blood (*N* = 5606) (39). Of 1237 CpGs available in our study of their 1415 significant CpGs, 3 reached look-up significance in our NAc EWAS: cg03474926 (*RALGDS*), cg24678869 (*DENND4B*), and cg04162032 (*LYPD8*). Ten genes were annotated to significant CpGs in both our study and Dugué *et al.*, although for different probes (Figure 4). Lohoff *et al.* also tested for alcohol consumption associations in blood (*N* = 8161) (40). While we did not identify any significant look-up results for their 2463 associated CpGs that were also available in our study, 14 genes were annotated to significant probes in both Lohoff *et al.* and our results, with 7 genes also overlapping with Dugué *et al.* (*YARS1*, *RABGGTB*, *TRA2B*, *RREB1*, *RALGDS*, *CDH23*, and *ANKRD11*) (Figure 4).

Of the 3 studies in brain tissues (10–12), cg00402668 (intergenic) reached look-up significance in the DLPFC EWAS

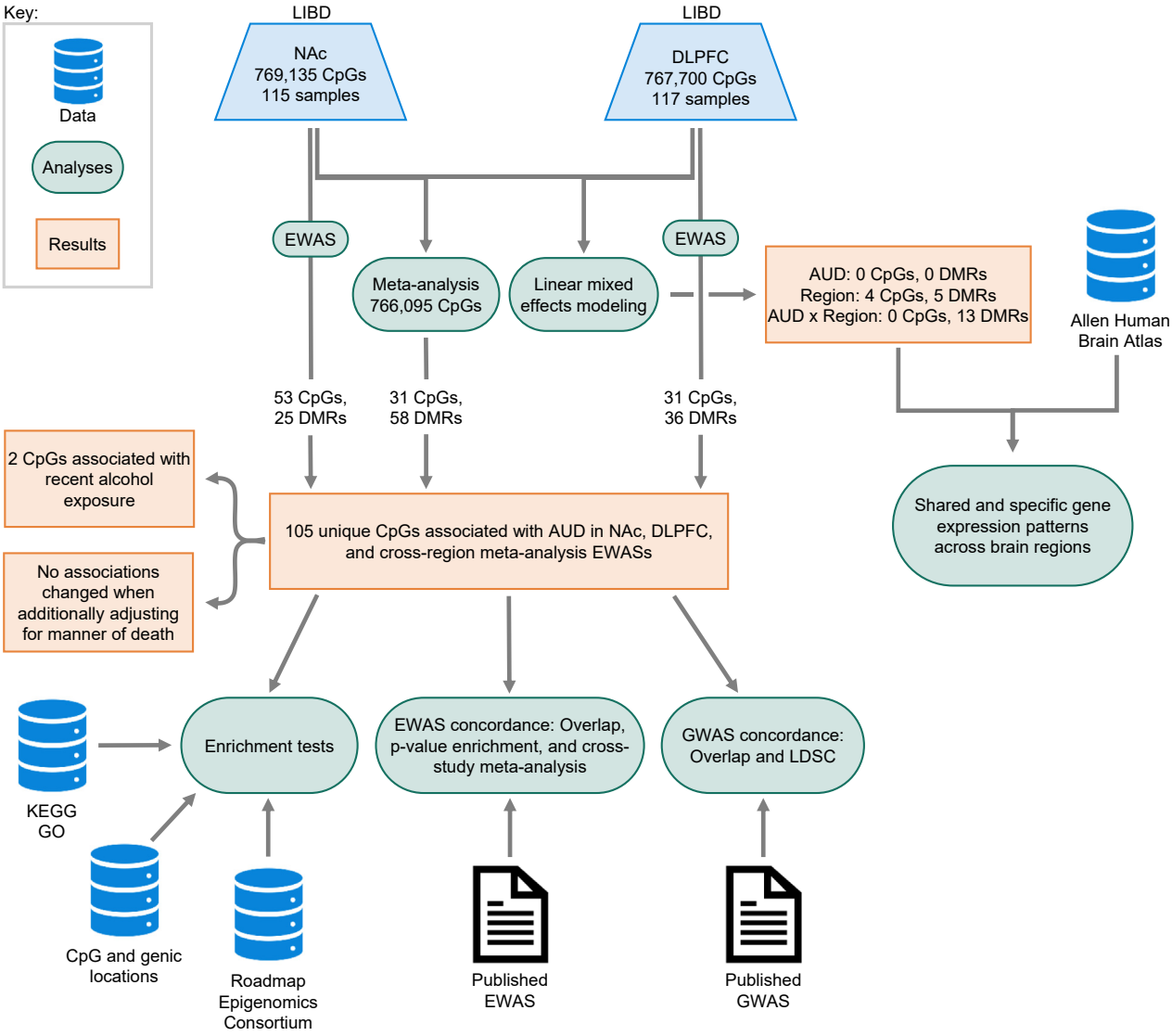

Figure 1. Overview of all data, analyses, and results. AUD, alcohol use disorder; DLPFC, dorsolateral prefrontal cortex; DMR, differentially methylated region; EWAS, epigenome-wide association study; GO, Gene Ontology; GWAS, genome-wide association study; KEGG, Kyoto Encyclopedia of Genes and Genomes; LDSC, linkage disequilibrium score regression; LIBD, Lieber Institute for Brain Development; NAc, nucleus accumbens.

and was among the top 20 sites with AUD-associated hydroxymethylation in BA 10 from Clark *et al.* (11). At the gene level, 3 genes annotated to significant probes in our study overlapped with genes annotated to significant probes from Hagerty *et al.* (*CAPS2*, *PTPRN2*, and *SLIT3*) (Figure 4) (10).

Comparing full summary statistics with Clark *et al.* (11) and Zillich *et al.* (12), no significant enrichment was found with Clark *et al.* (Table S9 in the Supplement). However, enrichment was identified between the top 1% of our NAc results and the top 1% of Zillich's putamen and VS results (Table S9 in the Supplement). The cross-study meta-analysis with Zillich *et al.*'s results identified 10 FDR-significant CpGs for the combination of the NAc and VS and 5 CpGs for the combination of the DLPFC and BA 9 (Table S10).

Concordance With GWAS Results

Stratified linkage disequilibrium score regression results did not indicate significant heritability enrichment of alcohol-associated genetic loci in genomic windows around significant CpGs from our primary analyses (Table S11). However, 23 genes annotated to significant CpGs from our study were previously associated with GWAS results for substance use phenotypes, some of which were also reported in previous EWASs of the same phenotypes (Table 2).

Shared Versus Brain Region–Specific Effects

CpGs significant in the NAc and DLPFC analyses had low similarity in effect sizes (Figure S4A in the Supplement) and high I^2 heterogeneity values. In contrast, CpGs significant

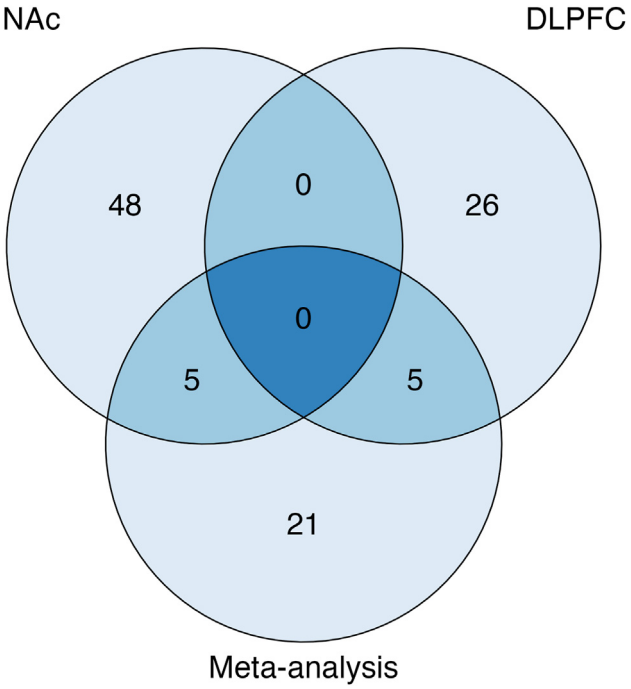

Figure 2. Overlap of significant probes from the NAc and DLPFC epigenome-wide association study analyses and the corresponding meta-analysis across brain regions. DLPFC, dorsolateral prefrontal cortex; NAc, nucleus accumbens.

in the cross-region meta-analysis had greater similarity of effect sizes (Figure S4B in the Supplement) and lower I^2 heterogeneity values (Table S12 and Figure S4C in the Supplement). Thus, as expected, the cross-region meta-analysis identified CpGs with consistent effects, while significant CpGs from the within-brain region EWAS were more dissimilar.

To explicitly test for brain region–specific or brain region–shared associations with AUD, we used linear mixed-effects modeling to analyze differential CpG methylation by brain region, AUD, and a brain region × AUD interaction. Four CpGs had an FDR ≤ .05 for the region term, suggesting different methylation profiles across brain regions (Table S13). No CpGs reached genome-wide significance for the AUD or AUD × region terms.

When testing DMRs, we identified 5 significantly associated with the brain region term and 13 with the interaction term (Table S14). To test whether genes annotated to these DMRs also had brain region–specific or brain region–shared expression profiles, we used microarray expression data from the AHBA MFG and Acb brain regions, corresponding to the DLPFC and NAc, respectively. In total, 17 unique genes were annotated to significant DMRs, with 15 (or their synonyms) being present in the AHBA data. Three of the 4 brain region–annotated genes (75%) and 2 of the 11 interaction-annotated genes (18%) had expression probes with median paired *t* test *p* values ≤ .05, indicating different expression profiles between MFG and Acb brain regions (Figure S5 in the Supplement; Table S14).

DISCUSSION

We report the largest EWAS of AUD in postmortem human brain to date. In within-brain region EWASs, we identified 53 AUD-associated CpGs (65 genes) in the NAc and 31 AUD-associated CpGs (36 genes) in the DLPFC. A meta-analysis across the 2 brain regions resulted in 31 CpGs significantly associated with AUD, 10 of which were already identified in the DLPFC or NAc EWAS. Only 2 of the 105 unique CpGs from these analyses showed association with ethanol toxicology, and controlling for manner of death did not change the associations with AUD, indicating robustness to recency of drinking and manner of death.

We identified enriched overlap between significant CpGs from our NAc and cross-region meta-analysis results and H3K27ac, H3K9ac, and H3K4me3 histone marks. H3K27ac is a classic marker of active enhancers and promoters, and H3K4me3 marks are commonly associated with transcription activation in nearby genes (50). Given that we also identified enrichment in H3K9ac marks, which are typically associated with active promoters, but not in H3K4me1 marks, which are typically associated with gene enhancers, our results suggest promoter-specific regulation of nearby genes (38,50). This is corroborated by our findings of significant enrichment for significant CpGs from the NAc and cross-region meta-analysis in islands, gene promoters, 5′ UTR, and exon regions.

EWASs of alcohol phenotypes in postmortem brain have not identified any genome-wide significant sites that overlap across published studies, which suggests limited robustness of the AUD-DNAm associations to date. When we compared our results to previous EWAS findings of alcohol use phenotypes in blood, we identified 3 CpGs that reached look-up FDR significance in one of our primary results. Concordance was higher at the gene level, with 7 genes implicated in our study and 2 previous blood-based EWASs of alcohol consumption. The CpGs and genes with AUD-associated differential DNAm across brain and blood may represent systemic gene regulatory responses or predisposing risk factors for AUD. When narrowing down to comparisons within the brain, we identified 3 genes annotated to significant CpGs in a previous brain EWAS and those observed in our study. Some concordance was also evident in the enrichment of top CpGs from our NAc results in results from EWASs of alcohol dependence in the putamen and VS. The VS contains the NAc and is proximal to the putamen, suggesting greater concordance for nearby brain regions with similar functions. However, we did not identify enrichment between our DLPFC results and previous EWASs of BA 9 (12), which correspond anatomically, or BA 10 (11), which is nearby. Cross-study meta-analyses with Zillich *et al.*'s results identified 10 FDR-significant CpGs for the NAc and VS combination and another 5 FDR-significant CpGs for the DLPFC and BA 9 combination. These 15 CpGs are promising candidates for robust AUD-associated methylation in the brain and include CpGs annotated to *PTPRN2*, *PTPRJ*, *MYO18A*, *KCNB2*, and *GRIN2A*. These genes are involved in neurotransmitter release, cell signaling, intracellular transport, neuronal excitability, and synaptic plasticity, respectively, implying significant roles in the mechanisms that underlie AUD. As with all comparisons that rely on summary statistics and technologically variable assays, overlap between our results

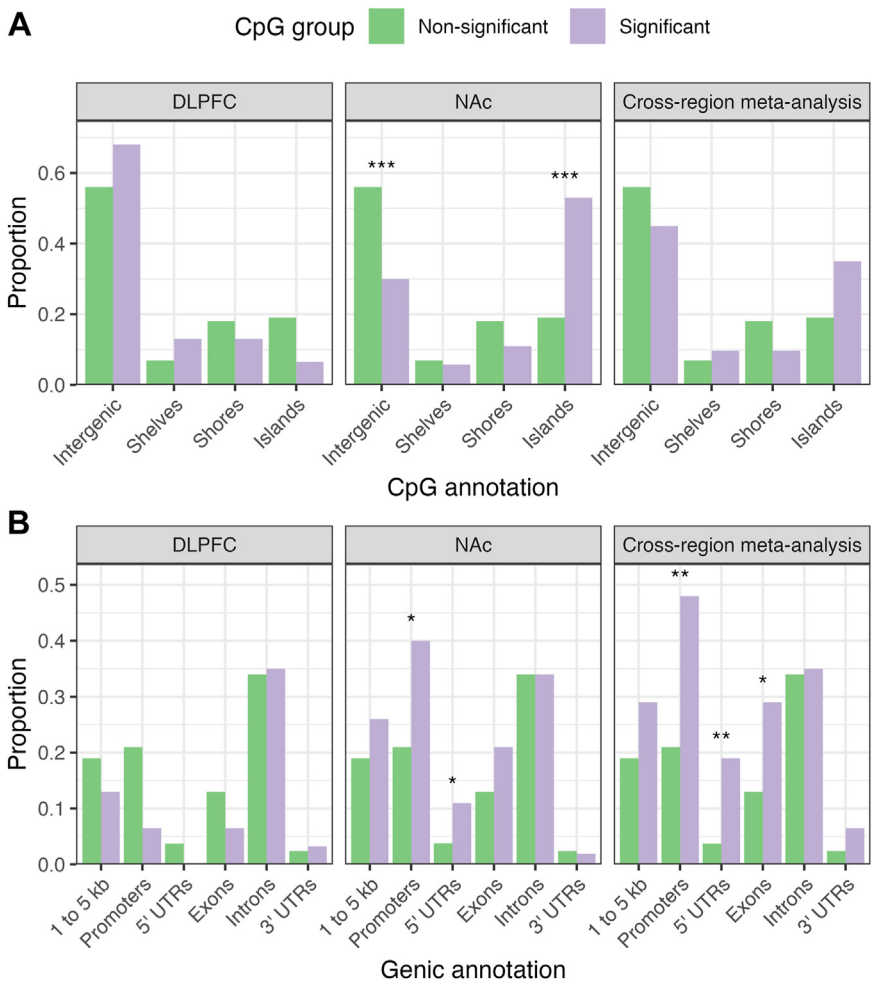

Figure 3. Enrichment of genomic features. Proportions of nonsignificant vs. significant CpGs were compared based on **(A)** CpG contexts and **(B)** gene-centric contexts. Stars represent significance of a two-sided Fisher's exact test for count data, based on false discovery rate–corrected *p* values. ***$p < .001$, **$p < .01$, *$p < .05$. DLPFC, dorsolateral prefrontal cortex; NAc, nucleus accumbens; UTR, untranslated region.

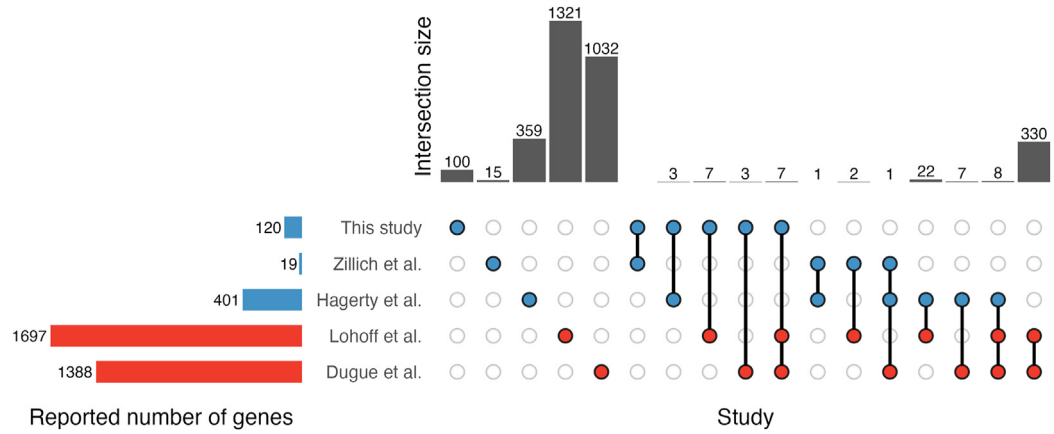

Figure 4. Gene-level concordance for statistically significant CpGs among previously published epigenome-wide association studies of alcohol use disorder and alcohol consumption. This UpSet plot shows intersections between genes annotated to significant CpGs from our study (nucleus accumbens, dorsolateral prefrontal cortex, or cross-region meta-analysis) and previously published epigenome-wide association studies. Studies of blood DNA methylation are colored red, while studies of brain methylation are colored blue. For genes annotated to CpGs passing each publication's significance threshold, the bar plot on the left illustrates the total number of unique genes from each publication, and the bar plot on top shows the number of unique genes annotated to significant CpGs in each intersection set. Clark *et al.*'s results (11) were not included because no brain methylation or hydroxymethylation sites passed that study's significance threshold. The comparison between our study and Zillich *et al.* (12) revealed no overlapping annotated genes.

Table 2. CpGs and Annotated Genes That Were Significant in Our Analyses and Associated With Alcohol or Other Substance Use Behaviors in Published GWASs and EWASs

Probe	Gene	GWAS Associations	EWAS Associations
Significant in Our NAc Analysis			
cg16163981	*KCNF1*	Smoking initiation (44)	
cg03751356	*ZNF789*	Smoking initiation (44,54,55) Lifetime smoking index (capturing smoking duration, heaviness, and cessation) (56)	Current smoking (57)
cg23088510	*FAM53B*	Smoking initiation (44) Cocaine dependence (58)	Current smoking (57,59)
cg05114676	*DPF3*	Smoking initiation (44)	
cg24612305	*TOM1L2*	Drinks per week (44,54) Smoking initiation (44,54) Smoking cessation (44)	Current smoking (57)
cg21028171	*LARGE1*	Smoking initiation (44)	
Significant in NAc and Cross-Region Meta-Analysis			
cg03119639	*GPR85*	Smoking initiation (44)	Prenatal cigarette exposure (57,60)
cg25077654	*PSMG1*	Smoking initiation (44)	
Significant in Our DLPFC Analysis			
cg01879507	*NFIA*	Drinks per week (44) Externalizing behavior (61) Smoking initiation (44)	Alcohol consumption (40)
cg22217235	*MYO1B*	Smoking initiation (44,54)	
cg03349057	*ITIH4*	Smoking initiation (62)	
cg25368989	*LSAMP*	Smoking initiation (44) Age of smoking initiation (44)	
cg21343292	*STAG1*	Cigarettes smoked per day (44,54) Smoking cessation (44)	
cg15112081	*EEF1AKMT4-ECE2*	Drinks per week (44)	
cg23352885	*PTPRN2*	Externalizing behavior (61) Smoking initiation (44) Smoking status (63,64)	Current smoking (57,65,66) Alcohol dependence (10)
cg25683478	*CDH23*	Externalizing behavior (61)	Alcohol consumption (39,40,67)
cg07945177	*CAPS2-AS1*	Smoking initiation (44)	
cg02911569	*NUBPL*	Smoking initiation (44)	
cg13804024	*ANKRD11*	Drinks per week (44)	Alcohol consumption (39,40,67) Current smoking (57,59,65,66,68,69) Prenatal cigarette exposure (60)
Significant in DLPFC and Cross-Region Meta-Analysis			
cg10315231	*EPHA3*	Drinks per week (44) Cigarettes smoked per day (44)	
cg04933990	*GRIN2A*	Externalizing behavior (61) Smoking initiation (44,54) Age of smoking initiation (44,54)	Alcohol consumption (40)
Significant in Our Cross-Region Meta-Analysis			
cg02675896	*MACIR*	Smoking initiation (44)	
cg21156771	*HIKESHI*	Smoking initiation (44)	

DLPFC, dorsolateral prefrontal cortex; EWAS, epigenome-wide association study; GWAS, genome-wide association study; NAc, nucleus accumbens.

and those from previous studies are impacted by different sampling and analytic strategies, although our larger sample sizes and approach have enabled the identification and confirmation of some robust signals at both the CpG and gene level.

Differing methylation levels across brain regions, regardless of disease status (51–53), could help explain different AUD associations across brain regions. We formally tested this hypothesis to distinguish between brain region–shared versus brain region–specific associations with AUD and identified 5 DMRs associated with brain region and 13 DMRs with brain region–specific AUD associations. Importantly, the brain region–associated CpGs/DMRs from this exploratory analysis are likely a subset of the true number of CpGs/DMRs with different methylation patterns across brain regions because some of the main effect of brain region is captured by the interaction term. We followed up this analysis by testing for expression differences between the MFG, which contains the

DLPFC, and the Acb (NAc) in the AHBA. As expected, only 2 of the 11 genes annotated to brain region–specific AUD DMRs showed evidence of different expression profiles across the MFG and Acb, while 3 of 4 genes annotated to the brain region–associated DMRs had evidence of different expression patterns across brain regions. These analyses suggest that while harmonized analytic strategies and increased statistical power may increase discovery and overlap of results among studies, human brain–derived alcohol-related epigenetic associations should be considered in as specific locations as possible, because associations found in one brain region may not translate to another.

We did not identify enrichment for GWAS variants of alcohol behaviors in genomic regions surrounding our significant CpGs, which suggests that these DNAm sites were largely not genetically driven and predisposing. However, 23 genes were previously associated with addiction-related traits, many with previous addiction-related EWAS associations. These converging associations could indicate that variants around these genes impact methylation in the NAc and DLPFC, thereby potentially altering key functions of these brain regions and contributing to alcohol use.

Although this EWAS is the largest reported to date for AUD in postmortem human brain, our statistical power for genome-wide analyses remains limited, and the sample composition of White decedents limits generalizability. Future studies are critically needed to increase the sample sizes available, especially for non-White individuals. Including decedents with a lifetime history of AUD as opposed to an active AUD diagnosis at death could also reduce power because some differentially methylated CpGs might revert to control levels if drinking stopped well before death. Because we analyzed DNAm from bulk tissue, cell type–specific patterns for AUD-associated methylation were not captured, although future single-cell sequencing efforts will be informative for this question.

Despite limitations, our study design allowed us to integrate and compare AUD associations across brain regions important to the addiction cycle. Our results, which show concordance with previous EWASs, suggest that the strongest AUD-associated signals are brain region specific, helping to illuminate potential relevant gene regulatory mechanisms. Many associations were annotated to genes that have been implicated in substance use GWASs, particularly for cigarette smoking. This convergence may indicate some genetic variants at these genes alter methylation and predispose an individual to general addiction liability. Identified associations that did not overlap with previous GWASs could reflect consequences of excessive alcohol intake, potentially explaining some neurological changes in response to AUD. Larger sample sizes and integrative efforts will help clarify these relationships, promote further understanding of the molecular mechanisms that underlie AUD, and identify therapeutic options.

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Illumina EPIC methylation data generated from this study has been deposited on GEO (GSE252501), and summary statistics for all association analyses are provided as supplemental tables on Figshare at https://doi.org/10.6084/m9.figshare.24871662.v3.

JEK is a member of a drug monitoring committee for an antipsychotic drug trial for Merck. LBJ is listed as an inventor on U.S. Patent 8,080,371, "Markers for Addiction," covering the use of certain single nucleotide polymorphisms in determining the diagnosis, prognosis, and treatment of addiction. All other authors report no biomedical financial interests or potential conflicts of interest.

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