Multi-omics signatures of alcohol use disorder in the dorsal and ventral striatum

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Alcohol Use Disorder (AUD) is a major contributor to global mortality and morbidity. Postmortem human brain tissue enables the investigation of molecular mechanisms of AUD in the neurocircuitry of addiction. We aimed to identify differentially expressed (DE) genes in the ventral and dorsal striatum between individuals with AUD and controls, and to integrate the results with findings from genome- and epigenome-wide association studies (GWAS/EWAS) to identify functionally relevant molecular mechanisms of AUD. DNA-methylation and gene expression (RNA-seq) data was generated from postmortem brain samples of 48 individuals with AUD and 51 controls from the ventral striatum (VS) and the dorsal striatal regions caudate nucleus (CN) and putamen (PUT). We identified DE genes using DESeq2, performed gene-set enrichment analysis (GSEA), and tested enrichment of DE genes in results of GWASs using MAGMA. Weighted correlation network analysis (WGCNA) was performed for DNA-methylation and gene expression data and gene overlap was tested. Differential gene expression was observed in the dorsal (FDR < 0.05), but not the ventral striatum of AUD cases. In the VS, DE genes at FDR < 0.25 were overrepresented in a recent GWAS of problematic alcohol use. The ARHGEF15 gene was upregulated in all three brain regions. GSEA in CN and VS pointed towards cell-structure associated GO-terms and in PUT towards immune pathways. The WGCNA modules most strongly associated with AUD showed strong enrichment for immune response and inflammation pathways. Our integrated analysis of multi-omics data sets provides further evidence for the importance of immune- and inflammation-related processes in AUD.

INTRODUCTION

Alcohol Use Disorder (AUD) is a major contributor to the global disease burden, with a prevalence of ~17% among 12-month alcohol users in the US [1, 2] and an estimated heritability of 49% [3]. Knowledge about the molecular mechanisms can foster understanding of causes and promote prevention. Recent genome-wide association studies (GWASs) have identified 29 genetic loci associated with Problematic Alcohol Use (PAU), a proxy of AUD [4]. While GWASs identify increasing numbers of disease-associated loci, the functional interpretation of many of these findings remains inconclusive. Analyzing the transcriptome can extend the understanding of the molecular mechanisms underlying AUD, by identifying associated gene expression patterns. Findings can in turn be integrated with results from GWASs and epigenome-wide association studies (EWASs) to identify the pathomechanisms underlying disease.

Processes in the central nervous system are considered to play a major role in the etiology of addiction, and the transition from chronic alcohol consumption to AUD [5]. Therefore, it is of particular interest to examine molecular changes associated with addiction in brain tissue. So far, only few studies have been conducted in postmortem human brain tissue to identify transcriptional changes associated with AUD [6–8]. These studies mainly focused on the prefrontal cortex (PFC) one important part of the neurocircuitry of addiction [9, 10]. The first transcriptome-wide study in the PFC found DE genes implicated in neuronal processes, such as myelination, neurogenesis, and neural diseases, as well as cellular processes, such as cell adhesion and apoptosis [11]. In Brodmann Area 9 down-regulation of calcium signaling pathways has been observed in individuals with AUD compared to controls [12]. In the same study, a weighted gene co-expression analysis (WGCNA) pointed towards modules associated with AUD case/control status, which were enriched for nicotine and opioid signaling, as well as immune processes. Another study in the PFC (Brodmann Area 8) showed that co-expression networks associated with lifetime alcohol consumption were enriched for GWAS signals of alcohol dependence [6].

Despite the importance of striatal regions in addiction processes, genome-wide human omics studies of these brain regions are still missing. The striatum is divided into the ventral striatum (VS), consisting of the nucleus accumbens and olfactory tubercle; and the dorsal striatum, comprising the caudate nucleus...
Table 1. Descriptive statistics of demographic data.

| Characteristic          | Cases | Controls | p    |
|------------------------|-------|----------|------|
| N                      | 48    | 51       | 0.51 |
| Age, years             | 55.58 (10.62)  | 57 (10.64) | 0.51 |
| Sex (M/F)              | 31/17 | 37/14    |      |
| pH-value               | 6.63 (0.26) | 6.65 (0.25) | 0.026* |
| PMI (hours)            | 37.07 (15.79) | 30.7 (15.57) | 0.047* |
| Blood alcohol level (N)| 7     | 0        |      |
| Blood alcohol level (g/100 ml)| 0.21 (0.21) |        |      |
| Smoking (yes/%)        | 32 (66.7%) | 12 (23.5%) | <0.001* |

Samples per brain region

| Caudate nucleus        | 36    | 37       |      |
| Putamen                | 35    | 42       |      |
| Ventral striatum       | 31    | 32       |      |

Data are presented as count (n/c; n (%) or mean (±SD).

PMI post-mortem interval, pH pH-value of the brain, p p-value of t-Test/Chi-squared test comparing cases and controls.

*Significant difference between cases and controls.

(CN) and putamen (PUT) [13]. The nucleus accumbens is involved in mediating motivational processes such as aversion and reward, which play a significant role in the development and maintenance of substance use disorders (SUD) [13]. In addition to regulating motor function, the CN and PUT are involved in cognitive processes relevant for addiction, such as executive functioning and cognitive control, reinforcement learning and habit formation [14]. Analyses of omics data from striatal regions could complement the knowledge on global molecular changes in the neurocircuitry of addiction in AUD.

In a recent EWAS of AUD in postmortem brain tissue, we identified differentially methylated CpG-sites and regions in the ventral and dorsal striatum [15]. Previous studies have shown the utility of integrating epigenetic and transcriptomic data in postmortem brain tissue of SUDs using weighted correlation network analysis (WGCNA) [16]. WGCNA clusters genes or CpG-sites into co-expressed or co-methylated modules based on correlation matrices. By relating modules to each other, WGCNA can be used for data integration, providing more insights than descriptive overlap. For example, whereas a descriptive comparison can be used for data integration, providing more insights than regulatory overlap of histone H3 lysine 4 trimethylation (H3K4me3) and mRNA expression in individuals with AUD and cocaine use disorder revealed no consistent overlap between H3K4me3 trimethylation and gene expression [17], a network analysis identified overlapping modules pointing towards co-expressed genes associated with H3K4me3 trimethylation [6]. Modules associated with AUD were enriched for CNS functions, such as synaptic transmission and regulation of neurogenesis [6]. WGCNA has also been used for integrating epigenetic and transcriptomic data and investigating their association with opioid use disorder (OUD) in postmortem human brain, identifying immune-related transcriptional regulation to be enriched in co-expressed and co-methylated modules [18]. The aim of the present study was to investigate differential gene expression associated with AUD status in the ventral and dorsal striatum, relate these to GWAS findings, and to integrate the findings with DNA-methylation data using a network approach (WGCNA) in order to identify functionally relevant molecular mechanisms in AUD.

Materials and Methods

Samples

Postmortem human brain tissue from CN, PUT and VS of a total of 48 individuals with AUD and 51 control individuals (68% male) was obtained from the New South Wales Tissue Resource Centre at the University of Sydney. The Ethics Committee II of the University of Heidelberg approved the study (reference number 2021–681). After quality control (QC), the total sample sizes for each brain region were NCN = 71, NPUT = 77 and NVS = 63. Phenotypic information was assessed by next-of-kin interviews. Inclusion criteria for this study were: age > 18 years, Western European Ancestry, no history of severe psychiatric or neurodevelopmental disorders, or SUDs other than AUD and nicotine use disorder or smoking. AUD was defined as meeting DSM-IV criteria for alcohol dependence and consuming 80 g of alcohol a day or more (control group: <20 g/day). Descriptive information can be found in Table 1 and Supplementary Table S1.

RNA extraction and -sequencing

RNA was extracted from frozen tissue according to the manufacturer’s protocol using the Qiagen RNeasy microKit (Qiagen, Hilden, Germany). The RNA Integrity Number (RIN) of all samples was determined using a TapeStation 4200 (Agilent, Santa Clara, CA); RIN values of 273 samples were larger than 5.5, for which libraries were prepared using the TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA). RNA sequencing was performed on the NovaSeq 6000 (Illumina) at the Life & Brain Center in Bonn, Germany with read lengths of 2 x 100 bp and a sequencing depth of 62.5 M read pairs per sample on average. Technical replicates were sequenced for all but four samples.

DNA extraction and methylation profiling

DNA extraction, methylation profiling, and QC was performed as described in Zillich et al. [15]. In brief, DNA was extracted using the DNeasy extraction kit (Qiagen, Hilden, Germany); the Illumina HumanMethylation EPIC BeadChip and the Illumina HiScan array scanning system (Illumina, San Diego, CA) were used to determine DNA-methylation levels. We used an updated and customized version of the CPACOR pipeline to extract beta values from raw intensities [19]. Criteria for the removal of samples and probes can be found in Zillich et al. [15]. In the present analyses, DNA methylation data were included from all subjects from whom gene expression data were available after QC.

Statistical analyses

All analyses apart from QC and read mapping were performed using R version 3.6.1 [20]. An overview of the analysis workflow can be found in Fig. 1. The Benjamini–Hochberg (FDR) [21] procedure was used to correct for multiple testing. Differentially expressed genes were considered statistically significant at FDR < 0.05. All downstream analyses were performed using genes significantly differentially expressed at FDR < 0.25.

Mapping and quantification

Sequencing quality was determined using FastQC [22] and 24 samples (11 cases and 13 controls) were excluded due to insufficient sequencing quality (e.g., strong overrepresentation of sequences, GC distribution). Raw reads were mapped to the human genome (hg38) using HISAT2 (v.2.1.0) [23]. Quantiﬁcation was performed with the featureCounts function of the Rsubread package (v.2.0.1) [24], with hg38 annotation.

Differential gene expression analysis

Differential gene expression was determined using DESeq2 (v.1.26.0) [25]. Minimal pre-filtering was applied, removing genes with normalized counts <10 for more than two samples. Technical replicates were merged prior to differential expression analysis using the collapseReplicates function as implemented in DESeq2. For the differential gene expression analysis, we included age, sex, RIN, pH-value of the brain, and postmortem interval (PMI) as covariates, because of their known influence on gene expression [26–28]. To assess residual bias after adjustment for covariates, we generated Q-Q plots and calculated genomic inﬂation factors (Supplementary Fig. 1). We further conducted a variance partition analysis using the variancePartition() function of the corresponding R package [29], which conﬁrmed the covariates. Results of this analysis can be found in Supplementary Fig. 2. Results were filtered for differentially expressed (DE) genes with an absolute log2 fold change larger than 0.02. Volcano plots displaying up- and downregulation of genes for each brain region are shown in Supplementary Fig. 3.

Gene-set enrichment analysis

Gene-set enrichment analysis was performed using the R package gsea (v.1.12.0) [30], for which DE genes were ranked according to p-value.
Enrichment analysis was performed for Gene-Ontology (GO) terms [31] and Hallmark gene sets [32] and the results were adjusted using FDR correction.

Cell-type enrichment analysis
To identify cell-type-specific expression signatures, we performed cell-type enrichment analysis using DE genes (FDR < 0.25) from the three brain regions. As a reference gene set for brain cell types, we used the "top ranked cell-type-enriched genes based on human data" as provided by McKenzie et al. [33]. These contain the 1000 most enriched genes in a cell type and cover astrocytes, endothelial cells, microglia, neurons, and oligodendrocytes. Using the R package GeneOverlap (v.1.22.0) [34], we assessed the overlap of AUD-status associated DE genes with markers from the different cell populations. Results were adjusted for multiple testing using the Benjamini–Hochberg method as implemented in GeneOverlap.

Differential methylation analysis
Effect sizes and p values for CpG sites were used from the EWAS results as presented in the original publication [15]. In brief, the EWAS model was based on methylation M-values as the dependent variable and AUD status as the predictor. As covariates, sex, age, postmortem interval (PMI), pH-value, estimated smoking, standardized neuronal cell count, and the first ten principal components of the EPIC array internal control probes were included.

WGCNA
Weighted correlation network analyses (WGCNA, v.1.70-3) [16] were performed to identify modules of co-expressed genes and co-methylated CpG-sites. We assessed the relationship of these modules with AUD case/control status and tested the overlap between associated modules. WGCNA clusters the input matrix according to a dynamic tree-cutting algorithm, using a soft power threshold that approximates the criterion of scale-free topology (R² > 0.80). Resulting soft power thresholds for expression networks were 6 for CN, 5 for PUT, and 14 for VS; for methylation networks, all power thresholds were 2.

To identify methylation networks associated with gene expression, beta values from normalized intensities of all samples from which gene expression data were available were filtered for promoter-associated CpG-sites based on the manufacturer's manifest (Illumina, San Diego, CA). The resulting 105,796 CpG-sites were used as input.

For the RNA-seq data, count matrices were normalized using the DESeq2 function normalizeCounts and variance stability transformation was applied. Networks were constructed using following settings: minimum module size = 30, mergeCutHeight = 0.25, maxBlockSize = 36,000. In WGCNA, modules are labeled using colors. In the "Results" section modules are labeled according to type of data, brain region, and color assigned in the analysis, e.g., "e-VS-pink" for module "pink" from the WGCNA analysis of gene expression data in the ventral striatum. For each module, its eigengene was calculated and correlated with AUD status. Association of
modules with AUD status and covariates is shown in Supplementary Fig. 4. For modules associated with AUD status, we performed enrichment analysis using the GEnrichmentAnalysis function implemented in the WGCNA package for expression data and the R package missMethyl (v.1.20.4) [35] for methylation modules. Further, we extracted hub genes of AUD-associated WGCNA expression modules by calculating the product of the module membership and gene significance for each gene of a module. Based on this score, the 10% of highest-ranking genes were defined as module hub genes. To investigate the biological relevance of hub genes, protein-protein interaction networks were generated using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, v.11.5) [36]. Graphical representation of gene networks was restricted to high confidence interactions (interaction score threshold 0.7).

**Expression and methylation data integration**

To identify genes both DE and differentially methylated, we analyzed the overlap of DE genes (FDR < 0.25) with the results of an EWAS (p < 0.001) in the same sample [15]. We prioritized CpG-sites based on their functional relevance in gene expression regulation. Thus, promotor-associated CpG-sites were used in the analysis.

At the module level, gene-set overlap tests were performed using the R package GeneOverlap (v.1.22.0) [34]. Here, Fisher’s exact test is used to identify significant overlap. For each brain region, the overlap of the AUD-associated co-expression and co-methylation modules was tested.

**GWSA enrichment analysis**

We analyzed enrichment of DE genes with an FDR < 0.25, and genes in AUD-associated WGCNA modules in GWAS summary statistics using Multi-marker Analysis of GenoMic Annotation (MAGMA, v.1.08b) [37]. We performed enrichment of DE genes with an FDR < 0.25, and genes in AUD-associated WGCNA expression modules by calculating the product of the module membership and gene significance for each gene of a module. Based on this score, the 10% of highest-ranking genes were defined as module hub genes. To investigate the biological relevance of hub genes, protein-protein interaction networks were generated using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, v.11.5) [36]. Graphical representation of gene networks was restricted to high confidence interactions (interaction score threshold 0.7).

**RESULTS**

**Differential gene expression**

Gene expression analysis of postmortem brain tissue from AUD cases and controls revealed DE genes at FDR < 0.05 in both dorsal striatal regions. In the caudate nucleus, 49 DE genes were identified at FDR < 0.05 (39 up- and 10 downregulated). Tubulin Tyrosine Ligase Like 4 (TTL4, log2FC = 0.11, p = 2.3 × 10^{-8}) and GATA Binding Protein 2 (GATA2, log2FC = −0.27, p = 8.6 × 10^{-7}) were the most significantly upregulated and downregulated genes, respectively. Top up- and downregulated genes in the putamen were found to be Transcription Elongation Factor A Like 2 (TCEAL2, log2FC = 0.09, p = 5.8 × 10^{-4}) and Desmin (DES, log2FC = −0.86, p = 2.6 × 10^{-5}), the latter being the only significant gene after correction for multiple testing. Nine genes were downregulated in both dorsal striatal regions, with HLA-DOB having the highest log2FC in both regions. In the ventral striatum, no DE genes were detected at FDR < 0.05. The most significant differential gene expression in the ventral striatum was observed for Ankyrin Repeat And Ubiquitin Domain Containing 1 (ANKUB1) which was upregulated in AUD cases (log2FC = 1.35, p = 5.8 × 10^{-5}). In the VS of AUD cases, Caseinolytic Mitochondrial Matrix Peptidase Chaperone Subunit B (CLPB, log2FC = −0.11, p = 5.2 × 10^{-6}) was the most significantly downregulated gene.

None of the DE genes at FDR < 0.05 overlapped between multiple brain regions. Therefore, the less conservative significance threshold of FDR < 0.25, which was also used for downstream analyses, was applied to compare the overlap of DE genes. At FDR < 0.25 the cardiomyopathy associated 5 (CMI5) gene showed an upregulation in both caudate nucleus and putamen. ARHGEF15 (Rho Guanine Nucleotide Exchange Factor 15) was upregulated in all three brain regions at FDR < 0.25. The Top 5 DE genes from each brain region are listed in Table 2; complete summary statistics are listed in Supplementary Table S2 (CN), S3 (PUT), and S4 (VS). Overlap between DE genes in the different brain regions is shown in Fig. 2A.

**Gene-set enrichment analysis**

Pathway analysis using a pre-ranked enrichment analysis revealed significant enrichment of dorsal striatum DE genes for several GO terms and Hallmark gene-sets. Genes in the CN were found to be related to cilia- and microtubule-associated GO-terms, while none of the Hallmark gene-sets was significantly enriched. GO-term and Hallmark gene-set analysis in PUT samples showed enrichment for immune processes, such as “acute inflammatory response to antigenic stimuli” (pFDR = 0.006) and “adaptive immune response” (pFDR = 0.006). In the VS the most significantly enriched GO-terms

| Table 2. | Top 5 differentially expressed genes in caudate nucleus, putamen, and ventral striatum. |
|----------|-------------------------------------------------------------------------------------|
| **Entrez Gene ID** | **Gene name** | **baseMean** | **log2(FC)** | **IfcSE** | **Stat** | **P-Value** | **FDR** |
| Caudate nucleus | | | | | | | |
| 9654 | TTL4 | 1125.89 | 0.11 | 0.02 | 5.59 | 2.33 × 10^{-8} | 0.0005 |
| 2624 | GATA2 | 51.17 | −0.27 | 0.05 | −4.92 | 8.58 × 10^{-7} | 0.0091 |
| 25904 | CNOT10 | 695.68 | 0.06 | 0.01 | 4.84 | 1.27 × 10^{-6} | 0.0091 |
| 222256 | CDHR3 | 1483.68 | 0.19 | 0.04 | 4.75 | 1.99 × 10^{-6} | 0.0106 |
| 375611 | SLC26A5 | 63.80 | 0.28 | 0.06 | 4.62 | 3.81 × 10^{-6} | 0.0163 |
| Putamen | | | | | | | |
| 1674 | DES | 22.06 | −0.86 | 0.18 | −4.70 | 2.64 × 10^{-6} | 0.0486 |
| 2050 | EPHB4 | 118.04 | −0.19 | 0.05 | −4.19 | 2.78 × 10^{-5} | 0.0939 |
| 9144 | SYN2 | 499.84 | −0.20 | 0.05 | −4.19 | 2.76 × 10^{-5} | 0.0939 |
| 55741 | EDEM2 | 348.56 | −0.07 | 0.02 | −4.23 | 2.30 × 10^{-5} | 0.0939 |
| 84245 | MRR1 | 662.19 | −0.13 | 0.03 | −4.13 | 3.57 × 10^{-5} | 0.0939 |
| Ventral striatum | | | | | | | |
| 81570 | CLPB | 1188.85 | −0.11 | 0.02 | −4.56 | 5.16 × 10^{-8} | 0.0653 |
| 22899 | ARHGEF15 | 54.23 | −0.26 | 0.06 | −4.49 | 7.04 × 10^{-6} | 0.0653 |
| 55584 | CHRNA9 | 5.58 | −1.17 | 0.27 | −4.44 | 9.14 × 10^{-6} | 0.0653 |
| 100463488 | MTRNR2L10 | 4.91 | −2.07 | 0.50 | −4.16 | 3.23 × 10^{-5} | 0.1730 |
| 389161 | ANKUB1 | 30.26 | 1.35 | 0.34 | 4.02 | 5.79 × 10^{-5} | 0.2480 |
were also related to cilia and microtubules, as well as antigen processing. All GO-terms and Hallmark gene-set with FDR < 0.10 are listed in Supplementary Tables S5 (CN), S6 (PUT), and S7 (VS).

Cell-type enrichment analysis
In the CN, upregulated DE genes were significantly enriched for astrocytic markers (p_{FDR} = 7 \times 10^{-6}), whereas an enrichment for endothelial cell marker genes was detected among downregulated genes (p_{FDR} = 2 \times 10^{-7}). No significant cell-type enrichment of DE genes was found in the putamen and the ventral striatum. GeneOverlap heatmap visualizations for the three brain regions are displayed in Supplementary Fig. 5.

WGCNA
Expression. In the CN, 21 modules with a median size of 352 genes (range: 64–7259) were identified. Module “e-CN-magenta”, consisting of 328 genes, showed the strongest positive association with AUD status (r = 0.42, p = 2.89 \times 10^{-4}). In the PUT, of the 25 modules (median size 249 genes, range: 33–5381) identified, module “e-PUT-black” was most strongly correlated with AUD with a positive direction of effect (r = 0.41, p = 2.31 \times 10^{-4}). For expression data from the ventral striatum, 16 modules with a median size of 429 genes (range: 35–9708) were identified; module “e-VS-pink” had the strongest positive association with AUD (r = 0.41, p = 0.009). Interestingly, in a GO-term analysis the three AUD-associated modules were all enriched for immune processes, such as “defense response” and “inflammation response”. Gene network representation of hub genes in modules “e-CN-magenta”, “e-VS-pink”, and “e-PUT-black” revealed the signal transducer and activator of transcription 3 (STAT3) gene as a conserved hub node in all three brain regions (Fig. 2D-F). There was also a wide overlap of the genes in the three modules: 174 (22.54%) were partially shared between all three modules corresponding to the three brain regions, while another 21.76% were shared between at least two modules (Fig. 2B). A gene network analysis of the 174 shared genes between regions identified STAT3, TP53, ICAM1, MYC, and NFκBIA as the top 5 hub nodes of the network. A visualization of the network is depicted in Fig. 3.

Methylation. In the CN, WGCNA resulted in 36 modules with a median size of 346 CpG-sites (range: 66–41,423). Module “m-CN-red”, consisting of 2117 CpG-sites, showed the strongest association with AUD case/control status (r = −0.27, p = 0.021). This module was most highly enriched for the biological processes “cell activation” (p = 1.52 \times 10^{-5}) and “leukocyte activation” (p = 2.09 \times 10^{-5}). In PUT 177 modules were identified (median size = 57 CpG-sites, range: 30–42,248). Module “m-PUT-plum” consisted of 70 CpG-sites and was significantly associated with AUD case/control status (r = −0.29, p = 0.023) and enriched for the biological processes “positive regulation of IκB kinase/NF-κB signaling” (p = 0.002) and “regulation of IκB kinase/NF-κB signaling” (p = 0.005). WGCNA in the VS methylation data resulted in 85 modules (median size = 178 CpG-sites, range: 35–30,370). The module with the strongest association with AUD was “m-VS-lavender” (r = −0.29, p = 0.023), which consisted of 117 CpG-sites and was enriched for the molecular function “natural killer cell lectin-like receptor binding” (p = 3.43 \times 10^{-4}) and the biological process “susceptibility to natural killer cell mediated cytotoxicity” (p = 3.65 \times 10^{-4}). The top 10 enriched GO-terms for all AUD-associated modules can be found in Supplementary Tables S8–S10.

Expression and methylation data integration. In the CN, 12 genes showed both differential methylation and differential gene expression. DE statistics, EWAS summary statistics and functional
annotation for these genes are provided in Supplementary Table S11. No overlap was observed in the VS and PUT. At the module-level, co-expression module “e-CN-magenta” showed significant overlap with the methylation modules “m-CN-red” ($p = 0.003$) and “m-CN-midnightblue” ($p = 0.014$) (Fig. 2C), while expression module “e-CN-purple” did not show significant overlap with the methylation modules in CN. Of the 3 AUD-associated expression modules in the VS, only “e-VS-salmon” showed significant overlap with the methylation module “m-VS-turquoise” ($p = 0.003$), but not “m-VS-lavender”. No overlap was observed for gene expression and DNA-methylation in PUT.

GWAS enrichment analysis of DE genes and WGCNA modules. In the VS, but not in the dorsal striatum, we observed enrichment of DE genes in the GWAS signal of PAU ($p = 0.045$). In the putamen DE genes were enriched for GWAS signal from a study comparing individuals with OUD to unexposed controls ($p = 0.025$). None of the DE genes in any of the brain regions showed enrichment for signals from a GWAS of Cannabis Use Disorder or AUD.

From the WGCNA modules showing the strongest association with AUD, only module e-VS-pink showed significant enrichment for GWAS signals of CUD ($p = 0.043$). None of the findings remained statistically significant after multiple testing correction. Results from the respective analyses are depicted in Supplementary Fig. 6 and enrichment p values as well as the number of overlapping genes are displayed in Supplementary Table S12.

DISCUSSION

In the present study, we identified DE genes, co-expression networks, and pathways associated with AUD in the dorsal and ventral striatum. The results were integrated with DNA-methylation data and results from GWASs of SUDs.

We discovered that one gene ($ARHGEF15$) was consistently upregulated in all investigated brain regions of AUD cases compared to controls. $ARHGEF15$ encodes a specific guanine nucleotide exchange factor for the activation of Ras homolog family member A (RhoA), a GTPase, which has been linked to higher blood pressure and hypertension over the Rho/ROCK signaling cascade [40]. It is postulated that the Rho Guanine Nucleotide Exchange Factor 15 negatively regulates excitatory synapse development by suppressing the synapse-promoting activity of EPHB2 [41]. EPHB2 deficiency has been linked to depression-like behaviors and memory impairments in animal studies [42]. In line with this, genetic variation within $ARHGEF15$ has been associated with hematocrit, red blood cell count, and hemoglobin concentration [43], but also with psychiatric traits,

Fig. 3 Network Plot of Co-Expressed Genes in AUD-associated Modules. Network plot of genes, co-expressed between the WGCNA modules e-CN-magenta, e-PUT-black and e-VS-pink.
such as neuroticism and worries [44] as well as bipolar disorder [45].

Among the genes that were downregulated in both dorsal striatal regions, HLA-DOB displayed the highest fold change. HLAs of the Major Histocompatibility Class II are an essential part of the acquired immune system presenting antigens to T-lymphocytes (for review: Howell, Carter [46]). The most significantly downregulated gene in the VS is CLPB, a mitochondrial chaperone, which has been associated with progressive brain atrophy [47] and with the cellular response to alcohol-induced stress [48]. In a recent GWAS, CLPB was associated with the amount of alcohol consumed on a typical day ($p = 9.67 	imes 10^{-5}, N = 116,163$) [49].

DE genes in the ventral striatum were enriched for GWAS signals of PAU, but not AUD. This could be a result of the larger sample size of the PAU GWAS, but also point towards differences in genetic variation as responsible for differential expression.

Our results from the pathway and network analyses further underline immune-related effects of chronic alcohol exposure; the pathway and network modules most strongly associated with AUD case-control status were also enriched for immune system and inflammation processes. This was observed for all three brain regions, and both in expression and methylation data, providing further evidence for the important role of immune processes in AUD.

Gene networks derived from WGCNA hub genes similarly revealed genes related to inflammatory processes as strongly connected network nodes. Here, STAT3 represents a conserved network hub node in all three brain regions. STAT3 is a member of the JAK/STAT pathway and acts as a transcription factor upon activation by cytokines, hormones and growth factors [30]. Interestingly, a recent study assessing expression signatures of alcohol withdrawal in rats discovered a very similar gene network in the hippocampus with STAT3 as a hub node surrounded by a network of downstream targets [51]. The authors also discovered increased levels of STAT3 and its neuroinflammation-related target genes in postmortem brain tissue of subjects with AUD. Activation of the STAT3 gene network was found to be primarily restricted to astrocytes. This supports the results of the cell type enrichment analyses, where enrichment of astrocytic expression signatures was detected for upregulated DE genes in the CN.

These results strongly reflect the well-described effect of chronic alcohol exposure on different aspects of the innate and acquired immune systems [52]. Chronic alcohol exposure accelerates the inflammatory response and reduces anti-inflammatory cytokines [52]. An activated immune response in response to chronic alcohol exposure has been shown on the cell level [53], as well as on the transcription [53], and protein levels [54, 55]. In a previous EWAS, we found strong enrichment of immune processes in differentially methylated CpG-sites associated with alcohol withdrawal [56].

Neuroinflammation has been repeatedly associated with AUD and both the glutamate excitotoxicity and the production of acetaldehyde, key processes in AUD metabolism, have been suggested to produce an inflammatory response in the brain [57]. On a phenotypic level, there is also widespread overlap between symptoms of inflammation and of SUDs, such as anhedonia, depression, and decreased cognitive functioning [58]. In addition, in candidate gene studies in postmortem human PFC, hippocampus, and orbitofrontal cortex, increased mRNA levels of HMGB1, which encodes a proinflammatory cytokine and toll-like receptor genes have been associated with alcohol consumption in AUD cases, providing evidence for chronic neuroinflammation in response to alcohol [59–61]. Notably, there is an overlap of findings not only on the single-gene level but also on the level of pathways and networks/modules. This overlap underlines that alcohol consumption has common biological effects in different brain regions, i.e., most prominently, effects on immune and inflammation processes.

Several limitations apply to our study. First, we cannot distinguish between effects being a consequence of chronic alcohol consumption or addiction. Second, although we corrected for PML, which can influence tissue quality as a confounding factor, it cannot be ruled out that other characteristics not easily accounted for, such as cause of death, or blood alcohol level for which the majority of individuals have missing data, influenced gene expression. Third, although the sample size is comparatively large for postmortem brain studies in the addiction field, the small number of differentially expressed genes is likely attributable to limited power. Lastly, analyzing bulk tissue does not adequately reflect the diversity of cell types across different brain regions and future studies on the single-cell level are needed to investigate cell-type-specific transcriptional changes associated with AUD.

It has to be noted that besides DNA methylation, epigenetic mechanisms such as histone and chromatin modifications, or microRNA expression profiles can influence gene expression and are especially important in addiction research [62]. Future studies should therefore expand the epigenetic profiling of AUD to include these mechanisms.

In summary, the present study provides further evidence from multi-omics data sets for the importance of immune- and inflammation-related processes in AUD. Notably, drugs that reduce neuroinflammation to reduce drinking, such as phosphodiesterases, may be promising approaches for novel treatment options for AUD. Recently published randomized controlled trials suggest that a phosphodiesterase inhibitor reduces heavy drinking whereas an antibiotic compound was not effective [63, 64]. A deeper understanding of the underlying mechanisms will enhance the discovery of drug targets and drive forward the development of precision medicine within this field.

**DATA AVAILABILITY**
Raw data are available from the corresponding author upon reasonable request.

**CODE AVAILABILITY**
Analysis codes are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS
LZ, SHW, MR, RS, MMN, GB, and ACH planned the investigation. MMF and ACH performed the DNA and RNA extraction and SHH, PH, FD, AH, and MMN were responsible for generating genome-wide methylation and gene expression data. LZ, EP, JF, JCF, and FS developed the analysis plan. LZ and EP performed all statistical analyses. LZ, EP, MMF, JCF, LS, MR, FS, and SHW reviewed the literature for the paper. LZ, EP, and SHW drafted the manuscript. All authors contributed, revised, and edited the final manuscript critically. All authors agreed to the publication of the final version of the manuscript.

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COMPETING INTERESTS
The authors declare no competing interests.