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A systematic review and meta-analysis on the transcriptomic signatures in alcohol use disorder

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Currently available clinical treatments on alcohol use disorder (AUD) exhibit limited efficacy and new druggable targets are required. One promising approach to discover new molecular treatment targets involves the transcriptomic profiling of brain regions within the addiction neurocircuitry, utilizing animal models and postmortem brain tissue from deceased patients with AUD. Unfortunately, such studies suffer from large heterogeneity and small sample sizes. To address these limitations, we conducted a cross-species meta-analysis on transcriptome-wide data obtained from brain tissue of patients with AUD and animal models. We integrated 36 cross-species transcriptome-wide RNA-expression datasets with an alcohol-dependent phenotype vs. controls, following the PRISMA guidelines. In total, we meta-analyzed 964 samples – 502 samples from the prefrontal cortex (PFC), 282 nucleus accumbens (NAc) samples, and 180 from amygdala (AMY). The PFC had the highest number of differentially expressed genes (DEGs) across rodents, monkeys, and humans. Commonly dysregulated DEGs suggest conserved cross-species mechanisms for chronic alcohol consumption/AUD comprising MAPKs as well as STAT, IRF7, and TNF. Furthermore, we identified numerous unique gene sets that might contribute individually to these conserved mechanisms and also suggest novel molecular aspects of AUD. Validation of the transcriptomic alterations on the protein level revealed interesting targets for further investigation. Finally, we identified a combination of DEGs that are commonly regulated across different brain tissues as potential biomarkers for AUD. In summary, we provide a compendium of genes that are assessable via a shiny app, and describe signaling pathways, and physiological and cellular processes that are altered in AUD that require future studies for functional validation.

Molecular Psychiatry; https://doi.org/10.1038/s41380-024-02719-x

INTRODUCTION

Alcohol is one of the most widely consumed psychoactive drugs with approximately 2.3 billion adults drinking globally [1]. Alcohol consumption is associated with many harms. Especially when used chronically, alcohol use disorder (AUD) may develop, which represents a complex, multifaceted human disease that poses a significant public health challenge with an extensive economic burden [2]. AUD is associated with numerous comorbidities, including major depressive disorder, antisocial and borderline personality disorders and other psychiatric conditions, liver disease and multiple other organ injuries, as well as numerous cancer types [3]. Although alcohol has a systemic effect on a variety of organs, AUD is primarily considered a brain disease [4, 5].

A key goal of AUD research is to unravel the underlying molecular alterations that contribute to disease progression, maintenance, and relapse after abstinence and to pinpoint essential molecular targets for the development of new medications [6–8].

Preclinical transcriptome-wide sequencing studies in different model organisms such as mice, rats and monkeys can provide valuable information on molecular changes associated with alcohol dependence but these findings may not always translate to humans. Another major limitation of transcriptome-wide approaches both in animal models and humans is the often inadequate sample size, leading to insufficient statistical power and unreliable findings [9]. Additionally, inter-study variability due to e.g., breeding facility, laboratory conditions, different strains and experimental protocols, leads to diverse outcomes among published studies. These limitations challenge the reproducibility and furthermore, hinder the translatability of preclinical research and underscore the need for optimizations to yield meaningful conclusions [10].

Likewise postmortem brain studies from deceased patients with AUD suffer from large variations in tissue quality and the large heterogeneity of AUD. Furthermore, different genetic

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Received: 19 February 2024 Revised: 20 August 2024 Accepted: 22 August 2024
Published online: 06 September 2024

backgrounds and personal life histories such as early life adversity, age, sex, infectious history, tobacco and poly-drug use, and many other factors contribute to the large heterogeneity the investigated cohorts. Hence, large cohorts are required to achieve sufficient statistical power and to allow the identification of generalizable and reproducible transcriptome-wide signatures.

Here, we integrated multiple publicly available and also some unpublished datasets into a cross-species analysis with the idea of overcoming some of the aforementioned limitations and to identify alterations in gene expression profiles and regulatory pathways that could have previously been missed due to low statistical power and high heterogeneity of the experimental and pathological condition. Therefore, we meta-analyzed data from different transcriptome-wide sequencing platforms comprising data from brain areas of the addiction neurocircuitry from patients with AUD, rats, and mice that underwent the chronic intermittent ethanol exposure paradigm (CIE) [11] as well as monkeys with alcohol-naïve controls, respectively, and further validated them by human proteomics data. Based on the number of studies identified through our initial systematic literature screening, the meta-analyses of this study are concentrated on three critically important areas of the addiction neurocircuitry [12, 13], namely the prefrontal cortex (PFC), nucleus accumbens (NAc) and amygdala (AMY). Other brain sites could not be considered for meta-analyses due to low study and sample numbers.

The convergent cross-species findings of differentially expressed genes (DEGs) and pathways provide a foundation for further functional research into understanding the mechanisms of AUD in the brain and eventually, development of new therapeutic targets. The finding of common mechanisms across species also gives credence to the clinical relevance of AUD models.

MATERIALS AND METHODS

Systematic literature screening

The systematic literature screening was performed according to the PRISMA Guidelines [14]. The protocol for this review has been registered at PROSPERO and Open Science Framework (Open Science Framework: https://doi.org/10.17605/OSF.IO/TF8R4; PROSPERO: CRD42020192453). Briefly, two researchers independently screened the literature on PubMed and EMBASE with the predefined keywords that were determined to specify four main categories of interest: alcohol/ethanol AND species-specific & model-defining terms AND RNA-specific terms AND brain (Fig. 1A, for detailed keyword information see Suppl. Table 4).

The literature screening was conducted using the SysRev screening tool, which was developed by the CAMARADES research group at the University of Edinburgh (www.syrf.org.uk). Following each screening step (title/abstract and full text), the researchers compared the respective resulting studies. In case of disagreement, a third, independent expert in the field chose whether the study was included or excluded for the next screening step. The general structure of the predefined keywords, as well as the complete PRISMA flow diagrams with the resulting number of studies for each screening step for the rodent, monkey and human study screening is depicted in Fig. 1. The screening and data extraction yielded a substantial number of studies for the PFC (n_{human}= 8; n_{rodent} = 8; n_{monkey} = 4), NAc (n_{human}= 3; n_{rodent} = 5) and AMY (n_{human}= 2; n_{rodent} = 6), enabling us to conduct a comprehensive meta-analysis.

Inclusion and exclusion criteria for human, rodent, and monkey studies

Only studies providing transcriptome-wide RNA-expression datasets, such as microarray or RNA-Seq experiments, were considered for the meta-analysis.

For the rodent meta-analysis, we focused our attention on the CIE paradigm, where rodents undergo several cycles of exposure to vaporized ethanol resulting in blood alcohol concentrations of 180–300 mg/dl [11]. This animal model mimics the development of AUD in humans characterized by several intermittent drinking episodes interspersed with withdrawal periods [11]. Importantly, CIE results in robust and stable behavioral symptoms of alcohol dependence that persist during prolonged abstinence and is characterized by long-lasting molecular neuroadaptations that mediate such symptoms [11].

The inclusion criteria for rodent studies were the following: rodents (mice/rats), alcohol exposure (≥ 2 weeks), post-dependence (≥ 3 days abstinence prior to death), transcriptome-wide RNA-expression data (microarray/RNA-Seq) from brain tissue, and controls without alcohol intake. Studies were excluded in case of: adolescence, prenatal alcohol exposure, too short exposure/abstinence times, or additional treatments. Before the start of the screening process, no specific strain restrictions were set to allow an unbiased investigation. However, after data extraction, all rat data turned out to be conducted from the Wistar strain and all mouse data retrieved were from the C57BL/6 strain. The time point of abstinence of at least three days was chosen, because this study aimed to find transcriptomic signatures without the effect of acute ethanol intoxication or early withdrawal states.

For human postmortem studies, the inclusion criteria were: human postmortem tissue, diagnosed AUD (DSM-IV), transcriptome-wide RNA-expression data (microarray/RNA-Seq) from brain tissue, and non-AUD controls. Studies were excluded in case of other psychiatric comorbidities or brain injury.

For screening of monkey studies, the following inclusion criteria were applied: non-human primate, transcriptome-wide RNA-expression data (microarray/RNA-Seq) from brain tissue, alcohol-naïve controls. Studies were excluded if monkeys received any additional treatments or were subject to prenatal alcohol exposure. The summary statistics, which included the comprehensive profiles of all detected genes from the sequencing experiments, were either obtained by downloading from the supplementary materials of the original publications or by reaching out to the corresponding authors to request the respective datasets. Details of the included studies are listed in Suppl. Tab. 1–3.

As is customary in systematic reviews and meta-analyses, we aimed to assess the risk of bias (RoB) for the original studies. Unfortunately, inadequate reporting of the conducted experiments hindered our ability to access the complete information required for a proper RoB assessment.

Meta-analysis approach for the three species

The aim of this project was to provide a highly comprehensive input dataset that consists of as many original datasets as possible matching our criteria. Due to data availability constraints, the analysis was performed using summary statistics. Accessing the original data was not feasible, as many of the studies had been conducted some years ago. Despite attempts to contact most of the corresponding authors, the raw data could no longer be retrieved. To maximize the number of studies to combine, as the data was derived from different platforms and some lacked standard error distribution information, a *p*-value combination approach was chosen. This type of meta-analysis has been previously used for transcriptome-wide approaches [15–17], especially when data from different platforms are combined, demonstrating its suitability for our study. Standard *p*-value combination meta-analyses fail to account for contradictory expression patterns, while Stouffer's *p*-value combination approach uniquely accounts for contradictory expression patterns by using one-tailed *p*-values as input [18]. The meta-analyses were performed by transforming the two-tailed *p*-values from the original publications into one-tailed *p*-values according to the observed effect direction in the respective studies as previously described [15, 19, 20].

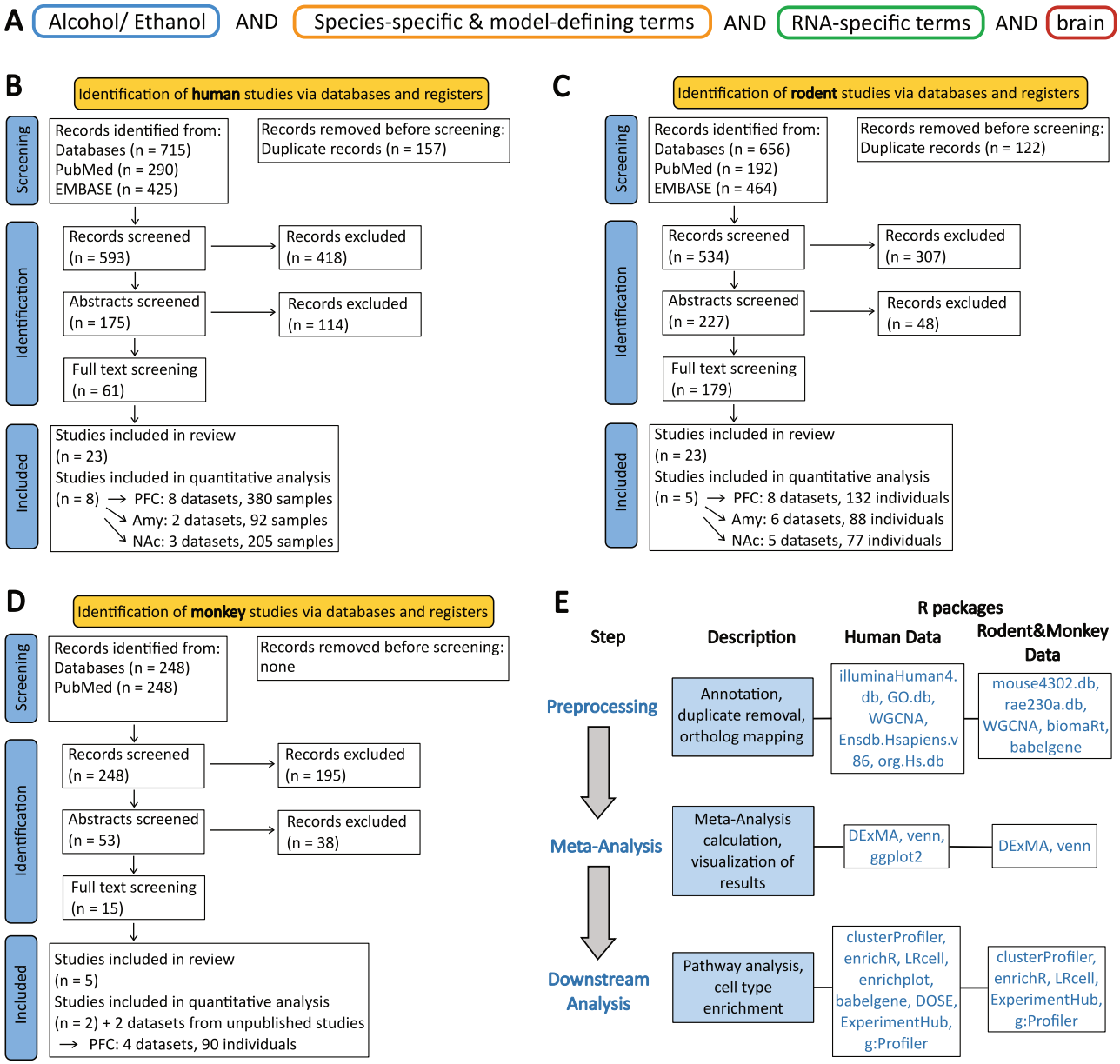


Fig. 1 Systematic literature screening and meta-analysis. Systematic literature screening of PubMed and EMBASE to retrieve transcriptome-wide expression datasets derived from (**B**) postmortem brain tissue of deceased AUD patients and controls, **C** from CIE rodents, and **D** from monkeys that had long-term intermittent voluntary alcohol consumption. **A** General structure for the keyword design for systematic literature research (**B**) PRISMA workflow for the screening of human studies. Before the screening procedure, duplicates due to overrepresentation in the two databases screened were removed by using EndNote. The template for overview of the screening procedure and the resulting studies was taken from www.prisma-statement.org. Eventually, we identified ten human postmortem studies matching our criteria [22, 120–128] (**C**) PRISMA workflow for the screening of rodent studies resulted in five studies to include [129–133] (**D**) PRISMA workflow for the screening of monkey studies identified two studies to include in the meta-analysis [134, 135]. In addition, unpublished data were kindly provided by Dr. Kathleen A. Grant and Dr. Suzanne Fei. **E** General workflow of the meta-analysis pipeline with the respective packages used for the statistical software R.

Subsequently, two separate meta-analyses were conducted per species using the R package DExMA (https://www.mdpi.com/2227-7390/10/18/3376), one on left-sided *p*-values testing for downregulation and one on right-sided *p*-values testing for upregulation for each gene. Both results were then combined by excluding the larger *p*-value for each gene and a false discovery rate was calculated. Stouffer's method additionally provides the possibility to include weights, which is considered as the square root of the respective sample size for each original study, previously shown to improve power substantially [21]. To avoid significant results due to these transcripts being measured in only a small number of studies, we set a threshold for the proportion of studies in which the gene must be measured to be included in the analysis. This threshold was at least three datasets or 50% of the datasets. While this approach did not provide a pooled estimator for effect size, the median fold change across studies for each gene was calculated to indicate the direction of the observed effect. For the analysis, we excluded non-protein coding genes. For cross-species comparison analyses, the rodent and monkey gene lists were converted into their human orthologs using the babelgene package in R with the respective genome settings.

Assessment of robustness and heterogeneity

To assess the robustness of meta-analysis results, we conducted leave-one-out-meta-analyses (LOO-MA) on the human PFC datasets. In this analysis, we systematically excluded the study with the highest sample size and the largest number of reported DEGs, which is Kapoor et al. [22].

As an indicator of robustness, we examined the overlap among the results obtained from the main meta-analysis (list of DEGs with FDR < 0.1), the LOO-MA results, and the reported DEGs from Kapoor et al. [22]. To assess the heterogeneity of DEGs across the studies included in our analysis and their respective meta-analyses, we created Venn diagrams illustrating the intersection of the identified DEGs (FDR < 0.1).

The majority of rodent studies as revealed by the systematic literature screening, predominantly consisted of mouse studies – depending on the brain region the range was 75% to 83% of the total. Therefore, to understand the species-specific impact on the meta-analysis outcomes for CIE rodents and to assess heterogeneity across species, we conducted subgroup analyses by categorizing the original studies based on the species involved, specifically mice and rats. Subsequently, we performed separate meta-analyses within the subgroups and compared their results with the DEGs obtained from the combined meta-analysis that considered both rodent species together in Venn diagrams.

Gene set enrichment analysis

To identify potential pathways and biological functions associated with the DEGs identified in the meta-analyses, we carried out gene set enrichment analysis (GSEA) utilizing GO-terms, Reactome-pathways and KEGG-pathways. This analysis was performed using the R packages ClusterProfiler [23] and g:Profiler [24]. As input for ClusterProfiler, we ranked DEGs based on the formula -log10(*p*-value) multiplied by the median fold-change. Additionally, we performed cell-type enrichment for the PFC in the human and rodent datasets using the R package LRcell [25]. To explore protein network associations, we employed STRING to estimate the level of connectivity of the identified DEGs on the protein level.

Ingenuity pathway analysis

We performed Ingenuity Pathway Analysis (IPA) with the IPA software (Qiagen, Hilden, Germany) to compare species-specific pathways and upstream regulators statistically and identify potential common and distinct regulatory patterns in a cross-species setting. As input for the core analyses, we filtered the meta-analysis results by $p < 0.01$. The threshold was set as rather non-conservative, since the software requires a decent number of genes to obtain meaningful results. We first performed core analyses per species and brain region separately and combined these afterward in a comparison analysis.

Rank-rank hypergeometric overlap (RRHO) of human transcriptomic findings with human proteomics data

To evaluate the importance of the identified transcriptomic alterations from the meta-analysis at the protein level, we performed rank-rank hypergeometric overlap (RRHO) analysis [26] with a recently published human AUD brain proteomics atlas [27]. We performed the analysis for the human meta-analysis results of PFC, NAc, and AMY separately. For this analysis only genes with a respective protein in the included dataset were considered, which resulted in a number of 3359- 4685 transcripts per brain region, respectively. Input genes and proteins were ranked based on -log10(*p*-value) multiplied by the median fold-change and the RRHO was performed using the RRHO2 package in R [28].

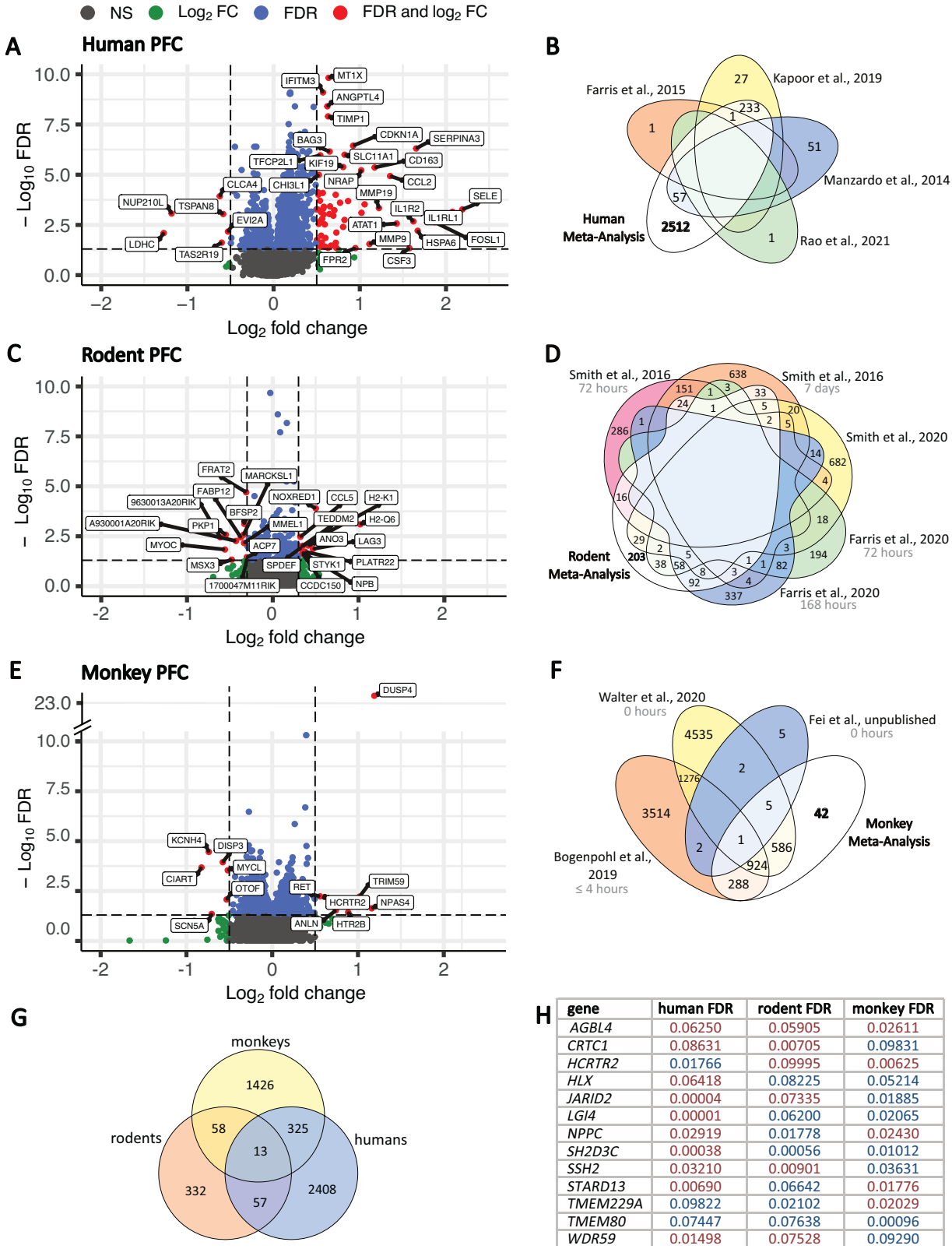
The analysis codes to all above-mentioned analyses can be available on request.

RESULTS

Meta-analysis of the human PFC results in DEGs involved in neuroimmune functions and newly identified gene families

For the human postmortem PFC, eight datasets comprising 380 samples were included (Fig. 1, Suppl. Tab. 2). The meta-analysis resulted in a total number of 1945 DEGs with FDR < 0.05 (2803 with FDR > 0.1), whereby 78% (n = 1525 DEGs) of the transcripts were up- and 22% (n = 421 DEGs) were down-regulated (Fig. 2A, B; **Suppl 1, sheet 1**). The top 10 DEGs are mainly involved in neuroimmune functions e.g., *SELE*, *IL1R2*, *CSF3*, and cell cycle regulation e.g., *FOSL1*, *MMP19* and *HSPA6*. STRING analysis was performed to identify the most inter-connected genes among the significant DEGs of the meta-analysis. For the human PFC, the genes with the highest number of connective nodes were *TP53* (n(nodes)=243), *ACTB* (n(nodes)=242), *MYC* (n(nodes)=193), *JUN* (n(nodes)=155), and *MAPK3* (n(nodes)=150) (**Suppl 1, sheet 9**). Further assessment of the enrichment of the determined DEGs in specific pathways by GSEA showed the dysregulation of neuroimmune signaling pathways, cell cycle, and cancer-associated pathways, such as interleukins e.g., IL-1, 4, 10, 13, and 17, TNF signaling components, as well as MAPK and ERBB signaling pathway. Cell type enrichment analysis resulted in significant enrichment of the identified DEGs in endothelial cells (FDR<9e-12) and astrocytes (FDR<3e-06) (**Suppl 1, sheet 8**). Since Kapoor et al. [22] contributed the by far highest number of samples to this analysis (n = 138 individuals), we performed leave-one-out analysis (LOO) to observe any potential bias introduced by this study (Suppl. Fig. 2). LOO analysis identified 1909 DEGs (FDR < 0.1) with 1788 DEGs (93.7%) overlapping with the meta-analysis results, suggesting a rather small bias towards this study.

In comparison with the original datasets included in the analysis, 2512 unique DEGs (FDR < 0.1) were found that were not identified as significantly dysregulated by any of the original studies (Fig. 2B). Among those genes are well-known AUD-related transcripts, such as calcium signaling components e.g., *CACNA1* and *CACNA2*, metallothioneins, alcohol dehydrogenases, and glutamate transporters. However, other transcriptional classes that have not yet been considered in AUD research were identified. For example, ankyrins (e.g., ANKRD54 (FDR = 0.005), ANKRD31 (FDR = 0.007)) that work closely in concert with cell adhesion molecules to secure cytoskeleton integrity represent one of those gene families among the uniquely identified transcripts resulting from the human PFC meta-analysis. Even though this gene family has so far not been identified in the context of AUD in particular, a recent study identified ankyrin-G among the top altered proteins in a single-dose ethanol intoxication experiment in mice [29]. In addition, gene families previously associated with tumor development, such as the Kelch-like gene family (KLHs) (e.g., *KLHL36* (FDR = 0.023), *KLHL5* (FDR = 0.046)) as well as lymphotoxin beta receptors (LTBRs) (LTB4R (FDR = 0.002), LTBR (FDR = 0.01)) have been uniquely found in our dataset. Moreover, six members of adenylyl cyclases (ADCs) (e.g., *ADCY3* (FDR = 0.003), *ADCY7* (FDR = 0.009)) as well as the ADC activator *ADCYAP1* (FDR = 0.003) and its respective receptor *ADCYAP1R1* (FDR = 0.008) have been identified. Previous studies reported that this enzyme class is of particular importance in neuroprotective astrocyte reactivity as well as in opioid and alcohol addiction [30–33]. Another astrocyte-specific mechanism identified by the unique DEGs is NIMA-related kinases (NEKs) (e.g., *NEK2* (FDR = 0.047), *NEK6* (FDR = 0.027)) that are responsible for regulating reactive astrogliosis [34, 35]. Additionally, Nudix hydrolases (NUDs) (e.g., *NUDT11* (FDR = 0.009), *NUDT12* (FDR = 0.047)) have been uniquely identified as impaired in AUD. These enzymes are suggested to regulate mitochondrial dynamics in concert with poly-(ADP-ribose) polymerases, which were significant in our dataset as well and, therefore, might promote the development of neurodegenerative diseases as co-morbid occurrences in AUD [36].



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gene	human FDR	rodent FDR	monkey FDR
AGBL4	0.06250	0.05905	0.02611
CRTC1	0.08631	0.00705	0.09831
HCRTR2	0.01766	0.09995	0.00625
HLX	0.06418	0.08225	0.05214
JARID2	0.00004	0.07335	0.01885
LGI4	0.00001	0.06200	0.02065
NPPC	0.02919	0.01778	0.02430
SH2D3C	0.00038	0.00056	0.01012
SSH2	0.03210	0.00901	0.03631
STARD13	0.00690	0.06642	0.01776
TMEM229A	0.09822	0.02102	0.02029
TMEM80	0.07447	0.07638	0.00096
WDR59	0.01498	0.07528	0.09290

Pathway analysis considering the unique DEGs pointed towards enrichment in immune pathways comprising cytokine and interleukin signaling pathways, phagocytosis, adaptive immune response, and TNF signaling (**Suppl 1, sheets 17-20**). Furthermore, apoptosis and cell death-related pathways, as well as cell signaling and cell stability processes were enriched. Cell type enrichment analysis of the unique DEGs showed significant enrichment in endothelial cells (FDR = 0.0005) and astrocytes (FDR = 0.046). Among the top regulatory elements, TNF, TGFB1, IL4, IL1B, CREB1, and immunoglobulin were identified (**Suppl 4,**

Fig. 2 Transcriptome-wide meta-analysis of PFC data. Meta-Analyses of the transcriptome-wide gene expression data derived from the PFC of humans (**A**, **B**), rodents (**C**, **D**) and monkeys (**E**, **F**) identified by species-specific Stouffer's *p*-value combination with FDR < 0.05 as threshold for significantly altered transcripts (DEGs). **A** Volcano plot depicting all genes analyzed in the human PFC meta-analysis. Transcripts with FDR < 0.05 and log2 fold-change (FC) > 0.5 are highlighted in red, DEGs with FDR < 0.05 and FC < 0.25 are highlighted in blue, DEGs with FDR > 0.05 and FC > 0.25 are highlighted in green. DEGs with FDR > 0.05 and FC < 0.5 are highlighted in gray (**B**) Venn diagram comparing DEGs with FDR < 0.1 of the human PFC studies and the meta-analysis identified 2,512 unique genes being significant in the meta-analysis, that have not reached significance in the original studies. No significant DEG has been identified across all studies commonly. Only datasets representing significant DEGs are shown. **C** Volcano plot depicting all genes analyzed in the rodent PFC meta-analysis. Transcripts with FDR < 0.05 and log2 fold-change (FC) > 0.3 are highlighted in red, DEGs with FDR < 0.05 and FC < 0.25 are highlighted in blue, DEGs with FDR > 0.05 and FC > 0.25 are highlighted in green. DEGs with FDR > 0.05 and FC < 0.3 are highlighted in gray (**D**) Venn diagram comparing DEGs with FDR < 0.1 of the rodent PFC studies and the meta-analysis identified 203 unique genes being significant in the meta-analysis, that have not reached significance in the original studies. No significant DEG has been identified across all studies commonly. Only datasets representing significant DEGs are shown. Studies include the time span of last alcohol experience and time point of death as indicated in light gray below the respective study. **E** Volcano plot depicting all genes analyzed in the monkey PFC meta-analysis. Transcripts with FDR < 0.05 and log2 fold-change (FC) > 0.5 are highlighted in red, DEGs with FDR < 0.05 and FC < 0.25 are highlighted in blue, DEGs with FDR > 0.05 and FC > 0.25 are highlighted in green. DEGs with FDR > 0.05 and FC < 0.5 are highlighted in gray (**F**) Venn diagram comparing DEGs with FDR < 0.1 of the monkey PFC studies and the meta-analysis identified 42 unique genes being significant in the meta-analysis, that have not reached significance in the original studies. One significant DEG has been identified across all studies commonly. Only datasets representing significant DEGs are shown. **G** Venn diagram depicting the cross-species comparison considering DEGs with FDR < 0.1 across the human, rodent and monkey meta-analysis results. **H** Thirteen DEGs were detected as significantly altered in the PFC across all three species with two transcripts being dysregulated in the same direction - AGBLA4 and TMEM80. FDR values depicted in blue represent down-regulated transcripts, while FDR values in red stand for up-regulated DEGs.

sheet 5), which adds additional emphasis on neuroimmune and cell proliferation regulation as well as MAPK-related mechanisms.

Taken together, our analysis identified multiple genes that were not detected in the original studies that will contribute to our knowledge of molecular alterations in AUD.

Meta-analysis of the rodent PFC results in DEGs with multiple functions

Systematic literature screening and data extraction of the rodent studies led to 8 datasets including 132 subjects for the meta-analysis of PFC data (Fig. 1, Suppl. Tab. 1). The meta-analysis resulted in 299 DEGs with FDR < 0.05 (520 DEGs with FDR < 0.1) and a distribution of 22% (n = 65 DEGs) up- and 78% (n = 234 DEGs) down-regulated transcripts (Fig. 2C, D; **Suppl 2, sheets 1 & 2**). When observing the DEGs resulting from the meta-analysis of the rodent datasets, the most significantly altered genes are involved in neuronal signaling e.g., *Ano3*, *Prepl*, *Marcksl1*, and *Btbd8*, immune-regulation e.g., *Lag3*, *Pkp1*, and *H2-q6*, and cancer-related pathways e.g., *Atl1*, *Styk1*, *Spdef*, and *Frat2*. Additionally, the aspect of neuro-immune alterations is further supported by the dysregulation of three histocompatibility complex II genes (H2s), namely *H2-d1* (FDR = 0.004), *H2-k1* (FDR = 0.009), and *H2-q6* (FDR = 0.0008) as well as correlated direct interactors, such as *B2m* (FDR = 0.01). When observing the highest inter-connected transcripts via STRING, *Src* (n(nodes)=39), *Jun* (n(nodes)=26), *Ubxn7* (n(nodes)=25), *Hspa8* (n(nodes)=21), and *Yes1* (n(nodes)=21) had the highest number of nodes.

The comparison of the meta-analysis and the published datasets included in the analysis identified 203 unique DEGs (FDR < 0.1, Fig. 2D**; Suppl 2, sheet 6**). Among those are previously well-characterized alcohol-sensitive genes, such as *Fkbp5*, *Hcn2*, and *Hcrtr2*. The unique DEGs of the rodent meta-analysis did not result in as large a number of new gene classes as in the human analysis but rather complemented the compendium of previous transcripts associated with the phenotype that were not fully represented in the original data sets. However, we identified three classes of genes that have not been found in AUD research before, namely diacylglycerol kinases (DGKs) (*Dgkb* (FDR = 0.08), *Dgkl* (FDR = 0.08)), dedicators of cytokinesis (DOCKs) (*Dock2* (FDR = 0.22), *Dock4* (FDR = 0.03)), and PHD finger proteins (PHFs; e.g., *Phf13* (FDR = 0.004), *Phf24* (FDR = 0.06)). DGKs have previously been associated with cortical aging [37] and directly interact with MAP kinases. DOCKs and PHFs are involved in neurodegenerative diseases and cognitive decline [38–40].

GSEA of the unique DEGs did not lead to significant enrichment in specific pathways or cell types. Potential upstream regulators were found to be mir-34 ($p = 9.75e-04$), and TP53 ($p = 6.13e-03$). TP53, encoding for p53, is the most frequently altered transcript reported in cancer studies. Furthermore, it directly interferes with mir-34, which was found to be a fundamental mechanism in the development of myeloma [41].

Meta-analysis of the monkey PFC results in DEGs that are enriched in cell signaling and MAPK cascade-related processes

For the monkey PFC, systematic literature screening identified two datasets. In addition, we retrieved unpublished data kindly provided by Suzanne Fei, Robert Hitzemann, and Kathleen Grant, which resulted in a final number of four studies comprising 90 animals (Fig. 1, Suppl Tab. 3). Meta-analysis resulted in 1173 DEGs at FDR < 0.05 (1846 DEGs at FDR < 0.1) with a distribution of 61% (n = 719 DEGs) up-regulated and 39% (n = 454 DEGs) down-regulated genes (Fig. 2E, F; **Suppl 3**). The top 10 dysregulated transcripts are mainly involved in the regulation of the innate immune system e.g., *TRIM59*, and *ANLN*, cell differentiation and survival e.g., *RET*, *DISP3*, and *MYCL*, and the circadian rhythm e.g., *HCRTR2*, *CIART*, and *NPAS4*. Also, among these highest significantly dysregulated genes, the MAP kinase phosphatases *DUSP4* and *DUSP6* were both significantly up-regulated in the alcohol drinking monkeys compared to alcohol-naive controls, which links the findings in monkeys to the MAPK-related outcomes identified in humans and rodents. GSEA resulted in a particular enrichment in cell signaling processes, such as "signal transduction", "cell communication", "cellular integrity", "NCAM signaling for neurite out-growth", and "response to stimulus". Furthermore, MAPK-cascade regulatory processes, such as "MAPK signaling pathway", "MAPK targets/ Nuclear events mediated by MAP kinases", "ERK/ MAPK targets", and "AGE-RAGE signaling pathway in diabetic complication", were highly over-represented, which further strengthens the importance of this pathway in monkeys with history of chronic alcohol consumption (**Suppl3**). Cell type enrichment analysis did not show any significant results. Upstream analysis revealed potential activation of *ESR1*, *CREB1*, *XBP1*, and *KRAS* and inhibition of *miR-16-5p*, *GABA*, and *GRIN3A* (**Suppl 4, sheet 7**). STRING analysis did not yield in any significantly interacting networks or genes on the protein level.

The number of unique genes that were identified as significant in the meta-analysis but not in the included datasets was remarkably less than in the human and rodent analyses (FDR < 0.1, n = 42; Fig. 2F) and did not lead to new gene classes. Six unique DEGs were overlapping with the human unique DEGs, namely *AP2B1*, *CA14*, *DUSP9*, *TRIM21*, and *TUBA8*. Interestingly,

all these genes have been previously associated with neurodegeneration in the context of Alzheimer's disease. None of the DEGs overlapped with the rodent's unique DEGs.

Cross-species comparison of the PFC results in DEGs that converge to an enrichment in neuroimmune system-related pathways, BBB regulation and MAPK-signaling

Since we were able to retrieve a sufficient number of DEGs for the human, rodent, and monkey PFC, we were next interested in a direct comparison of the gene expression patterns across these species. Because we were expecting a higher degree of heterogeneity when comparing cross-species specific transcriptomic profiles, we set the threshold for DEGs to FDR < 0.1. Considering a direct comparison of two species, we found 70 common DEGs in humans and rodents. Among the top common DEGs, we identified main involvement in neuronal signaling (e.g., *Clstn2*, *Rapgef2*, *Tnr*, *Ap2a1*) and neuroimmune pathways (e.g., *Fkbp5*, *Zc3hav1*, *Card19*) (**Suppl 5, sheet 1**). In humans and monkeys, 338 common DEGs were found. Represented gene families are the NF-κB regulating COMMD genes (COMMD8, COMMD10), the MAPK-interacting DUSP family (DUSP5, DUSP9, DUSP14), and the potassium channel family KCTD (KCTD2, KCTD6, KCTD16) (**Suppl 5, sheet 2**). Rodent and monkey comparison yielded 71 overlapping genes (**Suppl 5, sheet 3**). The top common DEGs are involved in cell matrix stability (e.g., *Slf2*, *Pnpla8*), innate immune system (e.g., *Sh2d3c*, *Myo18a*), DNA repair (e.g., *Pms1*, *Oma1*), and neuronal signaling (e.g., *Ap3b2*, *Slf2*).

In the overall cross-species comparison, 13 common DEGs were identified (Fig. 2G). Among these, two genes were dysregulated in the same direction across all species, *AGBL4* and *TMEM80*. The remaining 11 transcripts were not dysregulated in the same direction across all three species (Fig. 2G, H). Furthermore, when comparing the direction of these 13 DEGs across the species in a pairwise manner, seven DEGs are dysregulated for humans and rodents, six for rodents and monkeys and four for humans and monkeys. The number of overlapping DEGs across the three species indicates that a higher number of DEGs in the human PFC are overlapping with the DEGs of the monkey PFC than with the rodent PFC (Fig. 2G). Cross-species specific STRING analyses identified *JUN* as a highly interconnected gene in humans and rodents. This gene has already been listed among the dysregulated DEGs with low FDR values in both of the species. However, in humans *JUN* is up-regulated (FDR(hum)=0.0004), while in rodent PFC, it is down-regulated (FDR(rod)=0.0002). In monkeys, *JUN* is down-regulated but does not reach significance (FDR(mon)=0.94). GSEA identified commonly dysregulated pathways in neuroimmune functions, vascular system regulation and BBB, cell cycle regulation, apoptosis and cancer-related pathways. The neuroimmune system- as well as the MAPK-related pathways were especially impaired across humans, rodents, and monkeys. While in humans and monkeys, the majority of MAPK members appeared in the immune regulatory MAP3 kinase family, in rodents predominantly stress-related MAPKs were found. The rodent GSEA mainly identified STAT-regulating mechanisms related to cell cycle-regulatory pathways, which are present in the human downstream analysis results, as well. When further comparing the species-specific GSEA outcomes in both humans as well as monkeys, mechanisms of BBB and regulation of vascular development and angiogenesis were significantly altered in the alcohol-dependent phenotype.

Meta approach further supports activation of neuroimmune pathways in a cross-species manner

To understand the biological relevance of the transcripts detected in this study in greater detail, we performed IPA analysis in a comparative setting (**Suppl 4, sheets 1 & 2**). The observation of conserved canonical pathways (z-score ≥ 2) across humans, rodents, and monkeys revealed two significant pathways, which

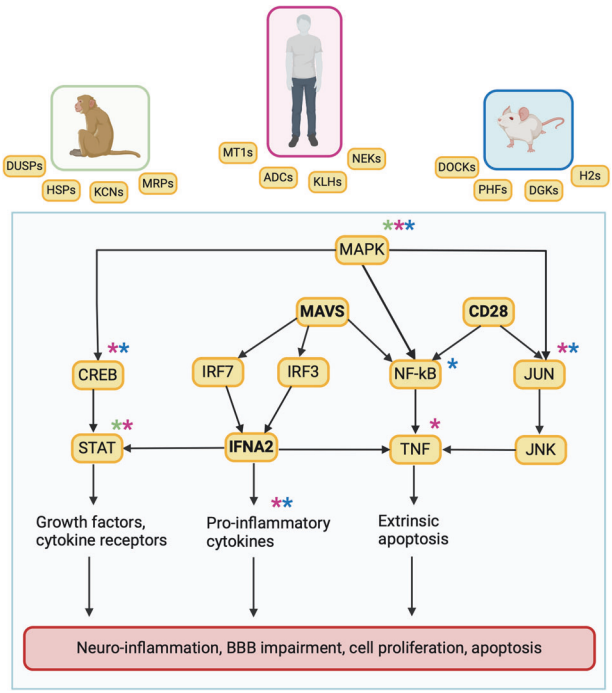


Fig. 3 Proposed conserved cross-species mechanism for chronic alcohol consumption/AUD. In the gray box, the conserved mechanism includes the upstream regulators MAVS, CD28, and IFNA2 (highlighted in bold) and underlying pathways are highlighted. These pathways work together to cause neuroinflammatory responses as well as impairment in BBB integrity, cell proliferation and apoptosis regulation. Above the gray box, the three analyzed species monkey, human, and rodent are shown with their top distinct findings and how those interact with the conserved mechanism as indicated by asterisks in the respective colors (green for monkey, pink for human, blue for rodent). This figure was created with Biorender. DUSP dual specificity phosphatases, HSPs heat shock proteins, KCNs potassium channels, MRPs multidrug resistance-associated proteins, MT1s metallothionines 1, ADCs adenylyl cyclases, KLHs Kelch-like genes, NEKs NIMA related kinases, DOCKs dedicators of cytokines, PHFs PHD finger proteins, DGKs diacylglycerol kinases, H2s histocompatibility complex II genes.

are up-regulated serotonin receptor signaling and the KEAP1-NFE2L2 pathway (**Suppl 4, sheet 2**). The latter functions as a protection mechanism against homeostatic responses comprising oxidative stress, inflammation, and proteotoxic effects [42]. Three upstream regulators have been identified as consistently enriched, which are *IFNA2*, *CD28*, and *MAVS* (Fig. 3**; Suppl4, sheet 1**). These genes are involved in proinflammatory processes comprising activated *IRF3*, *IRF7*, and *NF-κB*.

Since three out of four of the monkey datasets included animals that were sacrificed immediately after the last drinking session, these data combine the effects of long-term alcohol consumption and acute intoxication. Hence, the picture of dysregulated pathways and upstream regulators becomes more consistently enriched when considering rodents and humans solely. By doing so, inflammatory processes such as interferon signaling, CREB signaling in neurons, and the Rho GTPase cycle appeared among the most commonly upregulated canonical pathways (**Suppl 4, sheet 4**). In addition, serotonin receptor and glutamatergic receptor signaling as well as cell proliferation pathways comprising the RAF/MAP cascade and ALK signaling, were positively enriched. Common upstream regulators are nearly exclusively represented by interferons and respective receptors e.g., *IFNAR1*, *IFNL1*, *IFNA2*, *IFNAR*. Down-regulated regulators were *CITED2*, *RPSA*, *IKZF1*, *APOE*, and *IRGM1* (**Suppl 4, sheet 3**). Therefore, the down-regulated neuroimmune modulators *APOE*, *IRGM1*, and *IKZF1*,

seem to further enhance the inflammation induced by the proinflammatory interferons.

Meta-analysis of datasets derived from the NAc across human and rodent data results in a limited to absent number of DEGs

Systematic literature screening of the human postmortem brain studies resulted in three datasets derived from NAc tissue (n = 205 samples), while for the rodent species, five datasets (n = 77 samples) were identified and retrieved. Meta-analysis in the human NAc resulted in 17 DEGs at FDR < 0.05 (44 DEGs at FDR < 0.1), which were all up-regulated in AUD patients compared to healthy control individuals (Fig. 4A, B**; Suppl 1, sheet 2**). The DEGs with the lowest FDR value were *ENPEP* (FDR = 0.00035), *SERPINA3* (FDR = 0.0042), *SLC7A2* (FDR = 0.014), and *EDN1* (FDR = 0.014). Comparison with the included original studies resulted in 44 unique DEGs (FDR < 0.1; Fig. 4B**; Suppl 1, sheet 23**). Among the highest dysregulated genes are *ENPEP* (FDR = 0.0004), *SERPINA3* (FDR = 0.004), *ANXA1* (FDR = 0.014), and *EDN1* (FDR = 0.014). GSEA pointed towards enrichment in cell composition and signaling e.g., extracellular region (FDR = 0.022), cell-cell signaling (FDR = 0.036), and cytoplasmic vesicle (FDR = 0.065) and as well as immune response e.g., granulocyte chemotaxis and migration (FDR = 0.036), myeloid leukocyte migration (FDR = 0.036), and defense response (FDR = 0.065) (**Suppl 1, sheet 24**).

STRING analysis of the full meta-analysis outcome did not yield significantly inter-connected genes. GSEA resulted in dysregulated pathways of the neuroimmune system (e.g., "inflammatory response", "response to cytokine", "granulocyte migration"), blood vessel system ("leukocyte migration & chemotaxis", "Fluid shear stress and atherosclerosis", "blood vessel development", "blood vessel morphogenesis"), cell integrity and signaling ("TNF signaling pathway", "JAK-STAT signaling pathway", "vesicle lumen") (**Suppl 1, sheet 11-13)**.

In the rodent analysis, no DEGs at FDR < 0.05 as well as FDR < 0.1 appeared to be significant (Fig. 4G) and GSEA did not lead to any significant finding. When considering the mouse datasets separately, neither significant DEGs nor enriched pathways could be identified (**Suppl 2, sheets 11& 12)**.

Meta-analysis in the human AMY yielded DEGs that are mainly enriched in immune regulatory and BBB-related pathways, while in the rodent AMY no significant DEGs were detected

The meta-analysis of the AMY samples comprised six datasets from humans (n = 92 samples) and two from rodents (n = 88 samples). In the human meta-analysis, 171 DEGs with FDR < 0.05 (339 DEGs with FDR < 0.1) were identified, with 100% of transcripts being up-regulated in AUD patients compared to healthy control individuals (Fig. 4C, D). Among these DEGs, 123 genes were uniquely identified as significantly dysregulated (FDR < 0.1) in comparison to the included datasets (Fig. 4D**; Suppl 1, sheet 21**). This set of unique genes does not point towards newly identified gene classes. However, it is worth mentioning that among the unique genes, members of collagens (e.g., *COL7A1*(FDR = 0.01), *COL4A1* (FDR = 0.03)), heat shock proteins (*HSPA2* (FDR = 0.03), *HSPB1* (FDR = 0.06), as well as solute carrier families (e.g., *SLC39A1* (FDR = 0.04), *SLC7A2* (FDR = 0.29)) and calcium-binding proteins (*S100A10* (FDR = 0.07), *S100A8* (FDR = 0.08)). GSEA of these unique DEGs did not yield significant enrichment in specific pathways or cell types (**Suppl 1, sheet 22**).

Subsequent GSEA of all DEGs resulted in enrichment in dysregulated pathways in immune regulation (e.g., "inflammatory response", "PPAR signaling pathway", "IL-4 and IL-13 signaling", "Cytokine signaling in immune system"), and factors of BBB integrity, such as "angiogenesis", "hemostasis", "blood vessels morphogenesis", "regulation of vasculature development", and "vasculature development", and "JAK-STAT signaling pathway". Pathways suggesting a direct influence on the protein level were also significantly enriched, such as "negative regulation of hydrolase activity", "regulation of endopeptidase activity", and "post-translational protein phosphorylation" (**Suppl 1, sheets 14-16)**.

The rodent meta-analysis of the AMY included six datasets with a total of 88 animals. The gene expression analysis did not yield any significant DEGs at FDR < 0.05 and only one DEG at FDR < 0.1, namely *Cxcr6* (FDR = 0.078) (Fig. 4H). GSEA did not result in any significant outcome for the rodent AMY. According to the sample descriptive that we extracted from the original studies, half of the datasets did not report the exact sub-region of the AMY. Since the AMY is known to be a very heterogeneous brain region that consists of multiple nuclei, we additionally analyzed the datasets that specifically stated the central AMY (CeA) (n(datasets)=3; n(animals)=43). These datasets were focusing on mice as experimental animals and were conducted from the same research laboratory and the same first author (Suppl. Tab. 1). However, this analysis did not yield any significantly altered transcripts at FDR < 0.05. At the threshold of FDR < 0.1, one gene appeared as significant, which is *Cxcr6* (FDR = 0.074) that already occurred in the rodent AMY meta-analysis after applying the same threshold for significance (**Suppl 2, sheet 14**).

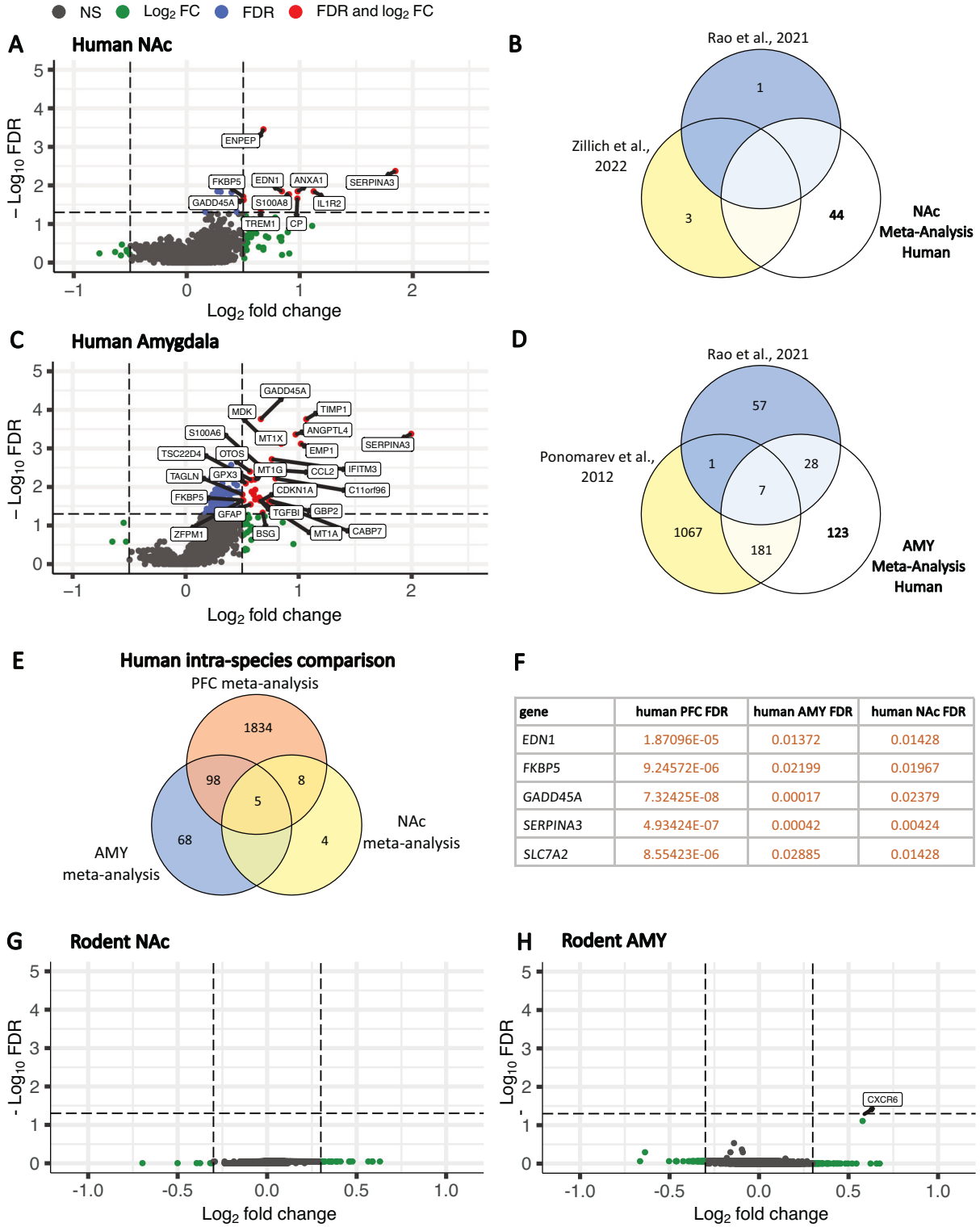
Human cross-region analysis points towards new biomarkers for AUD diagnosis

As described in the sections above, humans were the only species that resulted in significantly altered transcripts within all the three brain regions analyzed in this study. Therefore, we wanted to determine whether there are transcripts that are dysregulated across these brain regions within the human species. As indicated in Fig. 4E, five DEGs –*EDN1*, *FKBP5*, *GADD45A5*, *SERPINA3*, and *SLC7A2*– overlap across PFC, NAc and AMY with FDR < 0.05. These five transcripts are consistently up-regulated (Fig. 4F**; Suppl 1, sheet 4**). We hypothesize that the common DEGs across PFC, NAc, and AMY, might be potential biomarkers for AUD, as they show a common pattern of dysregulation due to the alcohol-dependent phenotype, irrespective of the brain region.

On the level of enriched pathways, common signatures were observed across the PFC, NAc and AMY that point towards a general impairment of certain biological mechanisms. As already pointed out in the cross-species comparison of the PFC, also in the intra-species comparison the four main categories neuroimmunity, vascular system and BBB regulation, cell cycle regulation, and apoptosis-related pathways such as MAPK signaling were dysregulated due to the AUD phenotype in all three brain regions with the majority of these terms being positively enriched. In addition, glia cell-specific terms, such as "regulation of glial cell apoptotic process", "glia cell differentiation", "gliogenesis" and "glial cell development" were enriched across the three brain regions.

Integrative analysis of AUD transcriptomics and proteomics data identified substantial overlap of the DEGs and their respective protein homolog

To identify the impact of the DEGs identified by the human meta-analysis on the protein level, we performed RRHO of these DEGs with a recently published proteomics dataset of human AUD postmortem brain [27]. As input for the PFC, 4685 transcripts and their respective protein homologs were included. Among those, 1701 transcripts were identified as concordantly dysregulated towards the same direction as their respective protein, which makes up 36.3% of the input transcripts (representation factor:1.5, $p < 1.34e\text{-}117$; Fig. 5**; Suppl 1, sheet 25**). Among the top up-regulated overlapping transcripts, we identified *SERPINA3*, *CHI3L1*, *S100A6*, and *MAOB*. *PVALB*, *NPY*, *HOMER3*, and *ALDH1A1* were detected among the highest down-regulated overlapping transcripts/proteins. GSEA of the overlapping transcripts/proteins pointed towards enrichment in cell stability and signaling (Fig. 5C**; Suppl 1, sheet 26**). Cell type enrichment analysis suggested astrocytes as the only enriched cell type (OR = 3.46, FDR = 0.0008).



gene	human PFC FDR	human AMY FDR	human NAc FDR
EDN1	1.87096E-05	0.01372	0.01428
FKBP5	9.24572E-06	0.02199	0.01967
GADD45A	7.32425E-08	0.00017	0.02379
SERPINA3	4.93424E-07	0.00042	0.00424
SLC7A2	8.55423E-06	0.02885	0.01428

Fig. 4 Transcriptome-wide meta-analysis of NAc and AMY data. Meta-Analysis of the transcriptome-wide gene expression data from NAc and AMY from humans (**A**–**F**) and rodents (**G**, **H**) identified by Stouffer's *p*-value combination with FDR < 0.05 as threshold for significant DEGs. Transcripts with FDR < 0.05 and log2 fold-change (FC) > 0.5 are highlighted in red; DEGs with FDR < 0.05 and FC < 0.25 are highlighted in blue; DEGs with FDR > 0.05 and FC > 0.25 are highlighted in green; DEGs with FDR > 0.05 and FC < 0.5 for humans and FC < 0.3 are highlighted in gray. **A** Gene expression pattern of the transcriptome-wide meta-analysis in human NAc. **B** Overlapping transcripts derived from human NAc original studied and the meta-analysis (FDR < 0.1). **C** Gene expression pattern of the transcriptome-wide meta-analysis in human AMY. **D** Overlapping transcripts derived from human AMY original studied and the meta-analysis (FDR < 0.1). **E** Venn diagram depicting the interspecies comparison considering the DEGs with FDR < 0.05 across the human PFC, NAc and AMY meta-analysis results. **F** Five DEGs have been detected to be consistently up-regulated across these brain regions: *EDN*, *FKBP5*, *GADD45A*, *SERPINA3*, and *SLC7A2*. **G** Gene expression pattern of the transcriptome-wide meta-analysis in rodent NAc. **H** Gene expression pattern of the transcriptome-wide meta-analysis in rodent AMY. Considering a threshold of FDR < 0.05, no DEGs were detected for both rodent NAc and AMY meta-analysis.

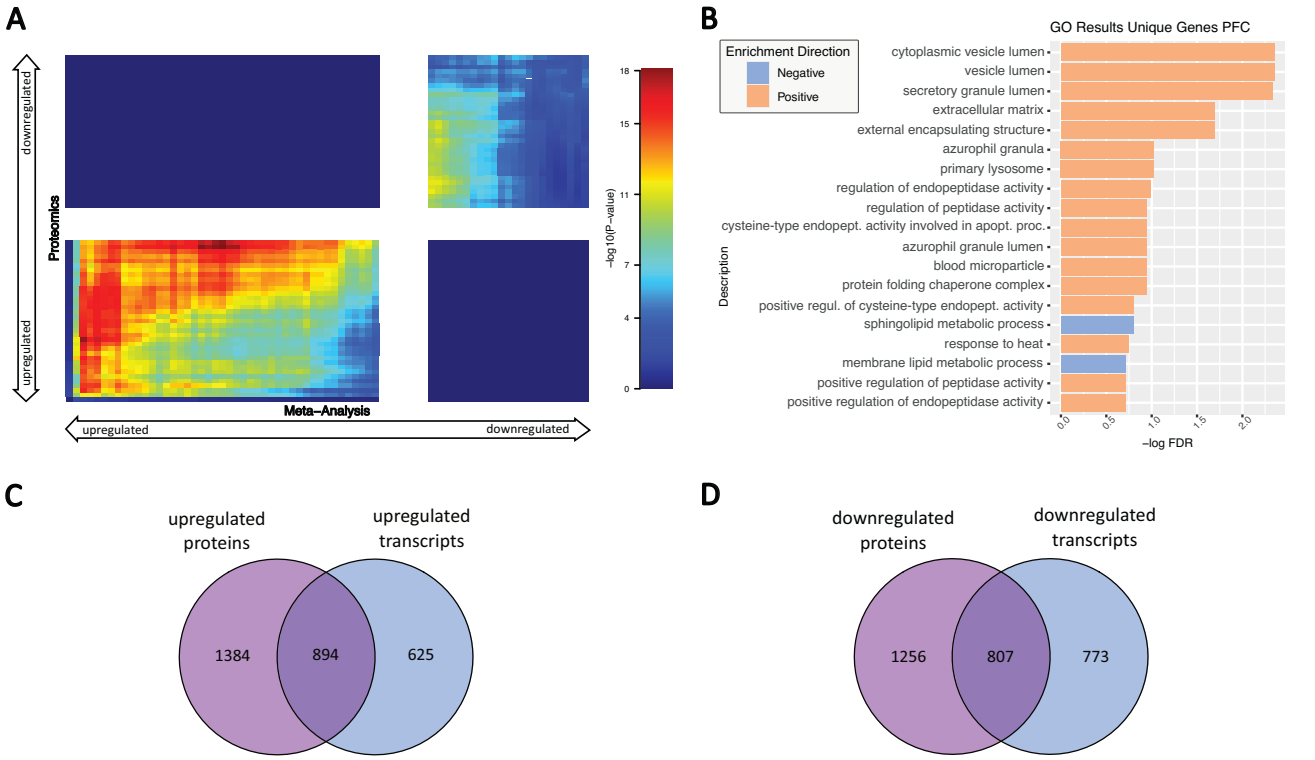


Fig. 5 Integrative analysis of AUD transcriptomics and proteomics data to obtain direct effects of the mRNA alterations identified in the meta-analysis on the protein level. Since RRHO only allows inclusion of identically named targets, this analysis comprised 4,685 transcripts and their respective protein homolog. **A** Enrichment plot of stratified RRHO considering all four possibilities of concordant and discordant overlaps. Top left quadrant represents up-regulated gene expression and down-regulated protein expression, top right quadrant represents concordant down-regulation of gene and protein expression, bottom left quadrant represent concordant up-regulation of gene and protein expression and bottom right quadrant represents down-regulated gene expression and up-regulated protein expression. 1,701 transcripts (36.3%) were dysregulated into the same direction as their respective protein (representation factor:1.5, p < 1.34e-117). **B** GSEA of the overlapping transcripts. **C** Venn diagram depicting transcript-protein overlaps that are concordantly up-regulated. **D** Venn diagram depicting transcript-protein overlaps that are concordantly down-regulated.

Since this extensive dataset from Teng et al. enabled also the proteomic validation of the NAc and AMY data, we pursued these analyses as well. For NAc, 3,384 transcripts/proteins were included in the analysis, which resulted in an overlap of 393 genes (11.6%, representation factor: 0.7, $p < 1.64e-21$) with 16 concordantly up-regulated and 377 down-regulated transcripts (**Suppl 1, sheets 29&30;** Suppl. Fig. 4). 3,359 transcripts/proteins identified in the AMY were included for RRHO with an overlap of 441 genes (13.1%, representation factor: 0.8, $p < 9.91e-11$). Among those transcripts, 19 were concordantly up-regulated and 422 were down-regulated (**Suppl 1, sheets 27&28;** Suppl. Fig. 5). Neither the overlapping transcripts in NAc nor in AMY were significantly enriched in any pathways or cell types.

DISCUSSION

The aim of this study was to conduct a comparative meta-analysis of transcriptome-wide data obtained from brain tissue samples collected from both animal models of alcohol dependence and individuals diagnosed with AUD and healthy controls. Following a rigorous process of systematic literature screening and data collection, we acquired a sufficient number of datasets to perform meta-analyses for three specific brain regions: the PFC, the NAc, and the AMY. Key findings from this meta-analyzed 36 cross-species (mice, rats, monkeys, humans) transcriptome-wide RNA expression datasets with an alcohol-dependent phenotype, comprising a total of 964 samples, include: (i) the transcriptomic profiles of the PFC exhibit the most pronounced alterations across all three species and point out 13 common DEGs. (ii) The transcriptomic profile in the NAc appears to be less susceptible to long-term alcohol consumption followed by extended abstinence. (iii) Human intra-species comparison between the three brain areas suggests five potential biomarkers for AUD (e.g., SERPINA3). (iv) The most consistent finding across all meta-analyses performed here is the dysregulation of numerous genes encoding inflammatory processes, BBB integrity, and cell proliferation; in conjunction with the cell type-specific accumulation of these DEGs in astrocytes and endothelial cells, this finding is consistent with the proposed critical role of inflammatory mechanisms in AUD [43–46]. (v) The cross-species meta-analyses also revealed numerous gene clusters that have so far not been considered in AUD research, and that allowed us to propose a conserved mechanism for chronic alcohol consumption/AUD incorporating IFNA2, MAVS, and CD28 as common regulatory components (Fig. 3). Together with MAPKs, which are abundantly dysregulated across the datasets as well, these factors regulate cell proliferative and apoptotic processes.

For validation, we used RRHO analysis to compare our human transcriptomic findings with human proteomics data. We found a substantial overlap (>36%) of the identified DEGs and their respective protein homologs. This not only confirms a large number of genes at different system levels but also supports the validity of our cross-species meta-analytic approach.

Meta-analysis of the human and rodent PFC led to the majority of DEGs that are enriched in inflammatory processes

In the human PFC, 1945 DEGs (FDR < 0.05) resulted from the meta-analysis combining eight datasets with 360 samples. The volcano plot depicting the outcome DEGs of this analysis shows that the

majority of DEGs are up-regulated. As a consequence, the majority of enriched pathways were positively enriched. The main finding is that most of the DEGs are enriched in inflammatory processes. Previous research has repeatedly shown that especially inflammation-related genes are up-regulated in alcohol dependence [43–45]. And furthermore, that neuroinflammatory processes indeed promote AUD development and progression [46, 47]. Consequently, the role of anti-inflammatory approaches is suggested to be particularly promising for AUD treatment [48]. Moreover, the cell type-specific enrichment in glia cells, such as astrocytes underlines the overrepresentation of inflammatory mechanisms in the PFC of AUD individuals. This cell type specificity is further supported by our RRHO approach, where cell type enrichment analysis reveals astrocytes as the only enriched cell type. This finding is in line with one of the key hypotheses in the field, namely that glial cells, such as astrocytes and microglia, are considered new cellular targets for treating alcohol-induced inflammatory and behavioral responses [43–45, 48].

The human PFC meta-analysis results are in line with the findings in rodents. In addition, rodent GSEA also pointed towards a major impairment of immune system-related pathways, such as leukocyte functioning, cytokine production, and especially T cell regulatory mechanisms. Indeed, T cell functioning seems to be altered by chronic alcohol consumption in both AUD patients and rodent models thereof. Thus, a recent methylation study on CD3 + T cells of AUD patients and matched controls identified numerous methylation sites to be altered in the AUD condition [49], while epigenetic (chromatin immunoprecipitation [ChIP]-seq) analysis in the PFC of CIE rats showed significant enrichment in the interleukin signaling pathway resulting in a diminished anti-inflammatory IL-6 response [50, 51].

In summary, our cross-species meta-analytic data on a set of dysregulated genes is in line with the hypothesis that neuroimmune signaling is a key component of alcohol abuse [9]. Among the key players of this neuroimmune response are toll-like receptors, such as TLR3 and TLR7, and their direct interferon regulatory factors 3 and 7 (IRF3 and IRF7) [12, 13]. Furthermore, a recent meta-analysis on neuroimaging, cerebral spinal fluid, and post-mortem studies [14] reported neuroimmune markers for AUD, including IL-1β, HMGB1, IL-1, RAGE, and NF-κB, which are closely working together in interferon signaling activating inflammatory responses [15].

DEGs in the human PFC that encode for the metallothionein (MT) family may explain zinc deficiency in AUD patients

The DEG of the human PFC meta-analysis with the lowest FDR (FDR = 1.5E-10), *MT1X*, is a gene that encodes for metallothionein 1X. Additional members of the metallothionein (MT) family, such as *MT1A*, *MT1M*, *MT1E*, *MT1G*, *MT2A*, and *MT3*, reached significance in our study, as well, and all of these were up-regulated in the AUD patient samples. MT genes are involved in zinc ion binding activity, and zinc, in turn, is a key element for the activation and binding of certain transcription factors through its participation in the zinc finger region of the protein. MT genes have been associated with AUD in previous studies. MT genes were found to be elevated in the hippocampus of AUD patients [52]. Another study that focused on mRNA and miRNA transcription patterns in the NAc and PFC of AUD patients and matched controls, identified similar MT gene candidates (*MT1E*, *MT1F*, *MT1G*, *MT1H*, *MT1HL1*, *MT1X*, *MT2A*, and *MT3*) being up-regulated in both brain regions investigated [53]. The upregulation of MT genes explains the well-known phenomenon of zinc deficiency in AUD, a phenomenon that may contribute to disease maintenance [54, 55]. Because of zinc deficiency, a number of zinc finger proteins are known to be impaired by AUD, which can be observed in our data, as well. In sum, it is concluded that not only daily oral zinc supplementation but also regulation of zinc homeostasis through MTs might be helpful for treating AUD patients.

In silico validation of the altered transcripts on the proteome level in the human PFC points towards promising astrocyte-specific targets for functional validation

Investigation of the impact of the identified DEGs in humans on the proteome identified a significant RRHO of transcripts with their respective proteins and furthermore, most of these transcripts were dysregulated in the same direction on the proteome level. Among the top-ranking overlaps were previously alcohol-associated transcripts, such as *MAOB*, *ICAM*, *CHI3L1*, *NPY* as well as *ALDH1A1* [56–59] which were found among the highest overlapping transcripts. Furthermore, *SERPINA3* and *FKBP5* were among the top up-regulated overlaps, which is once more suggesting the importance of these genes in AUD. Another interesting finding is the occurrence of genes that have been found to play a substantial role in neurodegenerative diseases, such as *CLU*, *CHI3L1*, *MAOB*, and *PVALB* [60–63]. GSEA of the overlapping transcripts pointed towards dysregulation in neuronal signaling and a cell type-specific enrichment in astrocytes. Despite their function in neuroimmune-regulatory mechanisms, astrocytes are directly interacting with neurons to mediate their signaling processes in a bidirectional communicative way [64]. Furthermore, transcriptional alterations in astrocytes have been suggested to mediate alcohol-dependent behavior [65].

However, this significant overlap was only observed in the PFC, while for NAc, and AMY, the overlap was less than expected by chance. Since in NAc, both our study as well as the reference study did not show a high number of significantly altered molecules, the reduced number of overlaps was not surprising. However, for the AMY, both studies identified a high number of alterations, which suggested that this brain region represents a particular vulnerability for the observed AUD phenotype. One potential explanation for the lack of significant overlaps might be that while in our study most of the tissue was derived from CeA, the Teng et al. study did not specify from which of the various and distinct nuclei of the AMY the tissue was collected and therefore, we assume a non-distinct mix of these nuclei, which might result in deviant outcomes on the expression patterns. Furthermore, all individuals included in the proteomics study had high blood alcohol levels at time of death, suggesting that the AMY may be particularly sensitive to acute alcohol intoxication, even after long-term alcohol consumption, whereas the PFC may not be.

In conclusion, the RRHO validated of the transcriptional alterations on the proteome level. Especially, transcripts being concordantly dysregulated suggest interesting targets for further functional investigation.

Meta-analysis of the monkey PFC results in alteration of MAPK/ERK-signaling mainly by *DUSP4*

The meta-analysis of the monkey PFC datasets resulted in 1173 DEGs (FDR < 0.05). The DEG of the monkey PFC meta-analysis with the lowest FDR (FDR = 6.1E-24 and FC 1.3), *DUSP4*, is a gene that encodes for the Dual-Specificity Protein Phosphatase 4, which is a major inhibitor of the MAPK pathway and is also involved in circadian rhythmicity [66–69]. So far, in alcohol-related research, it was reported that *DUSP4* is involved in ethanol-induced liver injury, where an increase of *Dusp4* expression was suggested to cause autophagy via miR-26a [70]. In the monkey PFC results, *DUSP4* was not the only family member of DUSPs that were significantly altered in the chronic alcohol-drinking monkeys. In total, seven DUSPs were identified as dysregulated with five up-regulated (*DUSP1*, *DUSP14*, *DUSP4*, *DUSP5*, *DUSP6*) transcripts. As one of the major interaction partners of MAPK/ERK pathway-related components, it does not come as a surprise that MAPK- and ERK-signaling is the most frequently represented enriched pathway in the GSEA. Additionally, five MAPK genes were dysregulated in the monkey PFC, further strengthing the impairment of this signaling pathway. *DUSP4* regulates several components of the MAPK pathways and thereby also impacts

circadian control [66]. In this respect, it is of note that circadian rhythmicity is severely altered in AUD patients, and in animal models of alcohol dependence [71] and *DUSP4* should thus be considered as a new target to normalize circadian rhythmicity in AUD patients. Furthermore, a recent study focusing on monkeys that underwent repeated alcohol abstinence phases identified *DUSP1*, *4*, *5*, *6*, and *18* as significantly up-regulated and correlated these findings with a negative regulation of MAPK activity [72].

In conclusion, the monkey meta-analyzed data are in line with numerous publications showing the importance of the MAPK/ERK pathway in mediating acute and long-term effects of alcohol [73–76]. However, this is not an alcohol-specific effect since reinforcing effects of other drugs of abuse, such as cocaine [77, 78] and nicotine [79, 80] are also mediated by this pathway. Therefore, clinically relevant, systemically administered drugs that target ERK signaling in the brain could be used to treat AUD and other substance use disorders in concordance [77].

Convergent transcriptomic signature in the PFC across AUD patients and alcohol-dependent rodents and monkeys

When observing the two-species overlaps, humans and monkeys have a remarkably higher number of common DEGs but with a low consistency in fold-change direction. This effect might be explained by the fact that monkey data was retrieved from animals that were sacrificed immediately after their last drinking session; the effects measured in the transcriptomic profile thus combine signatures of long-term alcohol consumption and effects of acute intoxication. It is known that gene regulation exhibits a bidirectional pattern during intoxication and phases of abstinence [81, 82]. Given that the human AUD signatures are retrieved from patients who have been abstinent for a prolonged time before the time of death, bi-directionality of gene regulation during intoxication in monkeys and prolonged periods of abstinence in humans may well explain the opposing findings in fold-change direction.

The inter-species comparison resulted in 13 DEGs that were significant across all three species, with two genes - *AGBL4* and *TMEM80* - being dysregulated in the same direction. ATP/GTP binding protein-like 4 (*AGBL4*) was found to be associated with alcohol, heroin, and methamphetamine addiction [83] as well as smoking [84] in recent GWAS studies. The function and relevance of transmembrane protein 80 (*TMEM80*) in health and disease is currently not known. However, another DEG that encodes for transmembrane protein *TMEM229A* also emerged in all three species, and a particular gene variant of *TMEM229A* is associated with alcohol consumption in a GWAS [85]. Another DEG with convergent transcriptomic signature for all three species is *HCRTR2*, which encodes for the orexin receptor 2. The involvement of orexin receptor 2 signaling in alcohol consumption [86] and relapse-like behavior in alcohol-dependent rats [87–90] supports our convergent cross-species finding.

However, the focus on single gene findings in a disease with high heterogeneity, such as AUD might not be the best way for medication development. Hence, the investigation of dysregulated pathways and physiological processes may lead to more promising targets for intervention in AUD patients. In this respect, the dysregulation of the integrity and function of the BBB stands out in our convergent analysis. Since the BBB is regulating the in- and efflux of components derived from the periphery, it protects the brain from inflammatory factors derived from the periphery. Cell type enrichment analysis resulted in endothelial cells being the strongest enriched cell type in the PFC. Since endothelial cells are one of the principal components making up the BBB, DEGs within endothelial cells of the BBB may contribute to a physiological dysfunction of BBB integrity and will thereby contribute to the aforementioned inflammatory processes in the PFC and other brain sites. The impairment of the BBB due to endothelial cell dysfunction and subsequent inflammatory brain responses have been reported previously and re-establishing the BBB integrity has been suggested as a promising therapeutic tool in abstinence and withdrawal [91–94].

Consistent with the result of the convergent analysis in the PFC, we also found numerous DEGs in the meta-analysis of the human AMY that were mainly enriched in inflammatory processes and BBB integrity, supporting the idea of a general pathological mechanism. The chain of pathological processes can include the toxic effect of alcohol, which leads to peripheral inflammatory processes and injuries to various organs. Thus, the toxic effect of alcohol may cause blood-brain barrier leakage due to damage to the endothelial cells, allowing mediators of inflammation to enter the brain. Some brain areas, particularly the PFC, may be more sensitive to these processes and then suffer cell death and loss of function. Furthermore, especially the uniquely identified transcripts link AUD to neurodegenerative diseases, such as Alzheimer's disease. This potential link could be explained by B12 deficiency introduced by overall malnutrition and the alcohol-specific effects on the overall metabolism [95].

Transcriptome-wide meta-analysis identified new molecular targets of AUD

As shown by the comparisons of the DEGs identified by the included datasets and the meta-approach (Fig. 2B, D, F and Fig. 4B, D), we identified unique genes and even gene classes that were not identified in any of the original studies. These unique DEGs point towards particular importance in neuroimmune and cell proliferation processes. Furthermore, the most significant upstream regulators, *CREB1*, *TNF*, *IL1B1*, *IL4*, and *TGFB1*, are mainly involved in immune regulatory and cell proliferation processes. Interestingly, *IL4*, *IL1B* and *TGFB* have been recently demonstrated to interfere with cell differentiation in cancer [96]. Furthermore, *TGFB1* and *CREB1* were found to work in concert to stimulate MAPK expression and activation [97], which is furthermore pointing towards MAPK-related pathways as one of the major targets in AUD [98]. Upstream regulators *mir-34*, and *TP53* suggest a network correlated to neurodegeneration [99, 100].

Eventually, we propose a conserved mechanism for chronic alcohol consumption/AUD incorporating *IFNA2*, *MAVS*, and *CD28* as common regulatory components (Fig. 3). Together with MAPKs, which are abundantly dysregulated across the datasets, these factors regulate cell proliferative and apoptotic mechanisms indicated by CREB and STAT as well as cytokine storm and neuroinflammation mediated by IRF7 and NF-κB. Neuroinflammation is a well-known phenomenon observed in AUD and therefore, a potentially conserved neuroinflammatory mechanism across species enables translational investigation to a bigger extent. Especially, the activation of TLR7 and its directly related transcription factors IRF7 and NF-κB, as well as the resulting activation of pro-inflammatory cytokines, has been repeatedly found in different rodent models of alcohol addiction [101] and TLR7 antagonists have been suggested as new therapeutic treatments [102]. Furthermore, this mechanism suggests the interplay of these components to induce impairment in BBB integrity, cell proliferation and apoptosis, besides neuroinflammation per se. These pathways are known to interfere with each other, which leads to a complex spiral-down reaction causing further damage by the progression of the dysregulation of each component. Despite microglia, astrocytes have been shown to be strongly involved in neuroinflammatory and BBB regulatory processes. For example, previous research demonstrated that inducing neuroinflammation by reactive astrocytes mediated by STAT3 and TNF causes BBB dysfunction [103]. Our data clearly show that, the transcriptomic alterations were particularly enriched in astrocytes. Therefore, our findings do not only emphasize the importance of specific neuroimmune pathways in chronic alcohol consumption models but also link these mechanisms to glia cells and particularly astrocytes.

The transcriptomic profile of the NAc seems to be less vulnerable to long-term alcohol consumption followed by protracted abstinence

The NAc, also known as the center of reward processing, has an essential role in the development of AUD, mainly where alcohol is experienced as pleasurable and rewarding [104–106]. Our meta-analysis of human and rodent NAc transcriptomic profiles showed limited to no significant findings in alcohol-dependent subjects compared to non-dependent controls. Since this study is focused on more advanced stages of AUD, it could well be that the NAc transcriptome is less involved during the maintenance of alcohol dependence.

Previous research describes that the NAc may mostly be involved in the initiation of AUD characterized by the binge and acquisition phase [107, 108]. The data we included in our analyses also underline these findings, as no DEGs were reported in the rodent studies and very little in the human postmortem studies. A recent single nucleus RNA sequencing (snRNA-Seq) on the NAc of human postmortem brain [109] reported 26 DEGs that were mainly attributed to D1- and D2 medium spiny neurons, oligodendrocytes, and microglia. In comparison, another snRNA-Seq study that was performed on human postmortem PFC identified 10-fold more DEGs (253 DEGs at FDR < 0.05) [110]. Therefore, our study supports the notion that the NAc has little relevance in maintaining AUD. Alternatively, the lack of transcriptomic response could reflect a form of alcohol-induced anaplasticity. Such an inability to adaptively respond to specific challenges or stimuli has been observed in some animal models of addiction [111] as well as certain tumor cells [112].

Human intra-species comparison suggests five potential biomarkers for AUD

Since significant DEGs for all three brain regions were only observed in AUD patients, an intra-species comparison was performed to identify potential commonly dysregulated genes in these brain regions. Identified genes or their combinations may represent potential biomarkers for AUD that could lead to the much-needed improvement in the diagnosis of AUD. Five DEGs were overlapping across PFC, NAc, and AMY – namely *EDN1*, *FKBP5*, *GADD45A*, *SLC7A2*, and *SERPINA3* – and all of these genes were consistently up-regulated. For example, *SERPINA3* might be a promising target for biomarker identification, as previous research on the hippocampus and peripheral blood of AUD patients reported up-regulated *SERPINA3* gene expression, as well [52, 113]. Furthermore, we found SERPINA3 among the top hits of our RRHO analysis, further supporting the role of this gene at the protein level.

However, upregulation of *SERPINA3* also occurs in the aging brain and in neurodegenerative diseases [114] and is therefore not only specific to AUD disease. Same holds true for *FKBP5*, a gene that has not only been shown to be affected in AUD but also other psychiatric conditions such as bipolar disorder, major depressive disorder, and schizophrenia. Furthermore, regulation of cortisol levels via *FKBP5* has been addressed as therapeutic target for these disorders [115]. Also, GADD45A was shown to be altered in psychiatric conditions such as depression [116]. *EDN1* and *SLC7A2* have not been found in psychiatric research to our knowledge. However, *EDN1* has been found to be associated with BBB leakage in Alzheimer's disease [117] and also SLC7A2 is involved in neuroimmune regulatory processes that are associated with a more rapid progression of Huntington's disease [118]. Therefore, the here identified set of potential biomarkers might rather be a broad set to diagnose psychiatric disorders and might link AUD to its most common neurodegenerative comorbidities.

Limitations

Even though this study comes with strengths such as high statistical power due to sample sizes that can usually not be realized by molecular studies in this field, as well as the cross-species aspect that leads to targets that are potentially interesting to follow up in a translational aspect, it also faces some limitations. The data included in this study was conducted within a wide time span considering method-specific sensitivity differences. Since RNA-Seq and microarray experiments are usually less sensitive to lowly expressed transcripts such as neuropeptides, neurohormones, and their respective receptors that have been shown to be sensitive to the addictive phenotype [119], these transcripts are underrepresented in this study. Since sex-specific aspects in AUD have just started to be explored within the most recent years, we were not able to include sex-specific analyses. Postmortem studies in general come with the limitation that it is impossible to distinguish between signatures that were driving the development of the disorder and signatures that were caused by AUD, respectively. In addition, it is highly challenging to distinguish the origin of neurotoxic effects observed in the data since they can be caused by either the toxicology induced by chronic high-level alcohol consumption or the multiple phenotypes seen in addictive phenotypes. However, by setting our exclusion criteria to the presence of pathological conditions, long agonal state, neurodegenerative disorders, and brain injury as case of death we aimed to minimize the neurotoxic effect that could have been induced by other factors than AUD. Nevertheless, we believe that this study contributes important new findings to the field of AUD research, especially emphasizing pathways that can be studied in a translational setting.

CONCLUSION

This study demonstrates that enhanced statistical power induced by increased sample size leads to numerous genes that previously could not have been detected as significantly altered in the alcohol-dependent phenotype. The meta-analyzed, transcriptome-wide, cross-species data provided here represents a compendium of genes, signaling pathways, and altered physiological and cellular processes in AUD. The processed data can be accessed and visualized using our Shiny app: https://wcaar.shinyapps.io/friske_MA/. Future studies should focus on functional validation of the DEGs reported here, particularly those that converge across all three species studied. The pathomechanisms described here, which include damage to BBB integrity by endothelial cell-enriched DEGs and subsequent inflammatory processes in vulnerable brain regions such as PFC and AMY, warrant further research, and drugs that counteract these pathomechanisms may indeed be useful for future treatments of AUD. In particular, DEGs that are similarly dysregulated on the protein level, as revealed by our RRHO analysis are interesting targets for further functional validation of an alcohol-dependent phenotype.

Furthermore, our results once again underline the notion that the NAc is not of great interest for studies on addictive processes, while there is no doubt that the NAc plays a crucial role in the initiation and acquisition of alcohol drinking behavior. Finally, the combination of DEGs proposed here, which are commonly upregulated in brain tissue and probably also in the periphery, should be further investigated in the context of the biological diagnosis of AUD. For future translational research, this compendium of transcriptomic alterations across rodents, monkeys, and humans will deliver a solid basis for designing studies in a cross-species setting to pave the way for identifying promising treatments.

DATA AVAILABILITY

The meta-analysis in rodents (Open Science Framework: https://doi.org/10.17605/OSF.IO/TF8R4) as well as in human postmortem brain tissue (Prospero: CRD42020192453) has been pre-registered in advance. As mentioned in the methods section, the analysis strategy was deviating from the pre-registered methods, since

the obtained data were not enabling the initially planned random effect size combination. Datasets as well as analysis pipelines will be publicly available from the day of publication onwards. The processed data can be accessed and visualized using the Shiny app: https://wcaar.shinyapps.io/friske_MA/.

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ACKNOWLEDGEMENTS

The authors thank the CAMARADES research group Edinburgh for their very helpful support on the study design and the systematic literature screening and data extraction process. We want to thank Michael Keist for generating the shiny app. In addition, we are very grateful to all the corresponding authors of the original studies that made it possible to include these datasets in our analyses. Without this involvement, it would not have been possible to conduct the study to this extent. The authors declare no biomedical financial interests or potential conflict of interest.

AUTHOR CONTRIBUTIONS

MMF, AMB, FG and RS designed the study. MMF, AMB and FG designed the keywords for systematic literature screening. MMF, ECT, AMB and FG performed systematic literature screening and data extraction. MMF and WHS contacted the corresponding authors in case datasets were not publicly available. MMF, ECT, MJWH and RS designed the analysis plan. MMF, ECT and MJWH performed data analysis. ACH, MAP, WHS, SF and RH provided unpublished data. MAP, JS, WHS, RDM, and RS provided scientific support. MMF and RS wrote the manuscript. All authors have read and approved the manuscript.

FUNDING

Financial support for this work was provided by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) with the TRR 265 (A05; Spanagel et al. [136]) to RS and support by the German Federal Ministry of Education and Research (BMBF), "A systems-medicine approach towards district and shared resilience and pathological mechanisms of substance use disorders" (01ZX01909) to RS, and "Inflamed Brain Response in Alcohol Addiction (IBRAA): Understanding the role of neuroinflammation, immunotypes, and gut-brain reactivity for heterogeneity in disease course and therapy response (01EW2402 (to WHS), and the Ministry for Science, Research and Art of Baden-Wuerttemberg (MWK) for the 3R-Center Rhein-Neckar to RS. Several datasets were provided by the Oregon National Primate Research Center Bioinformatics & Biostatistics Core, which is funded by NIH OD P51OD011092 and NIAAA P60AA010760.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41380-024-02719-x.

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