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Transcriptomic Signatures in Alcohol Use Disorder – A Translational Approach

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“The art and science of

asking questions

is the source of

all knowledge”

-Thomas Berger

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Summary

To date, there are only limited pharmacological treatments to cure AUD and the success rate is very low. Therefore, new therapeutic routes are warranted. This thesis aims to contribute to this need by identifying molecular mechanisms that are altered in AUD, considering the patient aspect through the use of human post-mortem brain samples, and compare the findings to a frequently used animal model of alcohol dependence. By combining multiple cutting-edge techniques, this study provides high evidence for further follow-up studies, where it will be possible to validate the findings in pre-clinical approaches and predict the relevance for the patient more precisely than previous studies. In addition, biomarkers for AUD, that are defined by this study, contribute substantially to the diagnosis of AUD on a molecular basis.

The meta-analysis of three brain regions (study 2) –prefrontal cortex (PFC), nucleus accumbens (NAc) and amygdala (AMY)– identified common signatures comparing rodent, monkey, and human post-mortem brain tissue. In the PFC, we found commonly enriched pathways for cancerogenesis, pro-inflammatory processes, and oxidative stress. The analysis of the NAc resulted in no differentially expressed genes (DEGs) in the rodent model and less than 10 DEGs in humans, suggesting that this brain region is not significantly vulnerable to long-term alcohol abuse with prolonged abstinence. Further evidence was also found in the bulk sequencing approach (Study 1), where both methylome- and transcriptome-wide data of prefrontal and striatal regions were conducted, and the ventral striatum (VS) showed a limited number of DEGs and no overlapping genes in the data integration of methylation and transcription data. However, on the level of differentially methylated positions and regions, the VS was one of most affected regions observed. When comparing the results of all three brain regions in humans, SERPINA3 appears to be up-regulated independently of the region, suggesting this gene as a new biomarker for AUD.

First preliminary snRNA-Seq data from the dorsomedial striatum of PD rats (Study 3) – the brain region that was also found in the human post-mortem brain to be most significantly altered on the multiomics level (Study 1) – suggests a pronounced relevance for oligodendrocytes and microglia in regard to altered transcripts that persist after long-term abstinence. These cell types have been previously found to be relevant in AUD patients and the post-dependent rats on several brain regions.

As indicated by studies 1-3, the immune system is strongly dysregulated in AUD. Therefore, in study 4, chronic alcohol consumption and alcohol dependence as potential risk factors for COVID-19 infection and severity was observed across multiple rat models of alcohol intake. Especially, the consistent up-regulation of ACE2 in lung tissue detected in all models as well as the reduction of Mas expression in the olfactory bulb led to the conclusion that alcohol intake – especially in a sub-chronic to chronic manner – might increase the propensity to develop a SARS-CoV2 infection and potentially, suffer from severe long-term consequences, such as anosmia.

Zusammenfassung

Bis heute sind nur wenige pharmakologische Präparatee für die Bewältigung von Alkoholkonsumstörung (AUD) verfügbar und deren Erfolgsrate ist stark limitiert. Deshalb sind neue therapeutische Wege unerlässlich. Diese Dissertation hat zum Ziel dieser Notwendigkeit nachzukommen, indem molekulare Mechanismen identifiziert werden, die in AUD verändert sind. Hierzu wird der Patientenaspekt durch Verwendung von humanem post-mortem Hirngewebe in Betracht gezogen und die Ergebnisse mit einen frequent eingesetzten Tiermodel von Alkoholabhängigkeit verglichen. Durch die Kombination von zahlreichen cutting-edge Techniken liefert diese Arbeit hohe Evidenz für weitere aufbauende Studien, die es ermöglichen werden die Ergebnisse in präklinischen Ansätzen zu validieren und deren Relevanz für den Patienten genauer vorherzusagen als vorherige Studien. Zusätzlich können Biomarker für AUD, die in dieser Studie identifiziert werden, substanziell zur AUD-Diagnose auf molekularer Basis beitragen.

Die Meta-Analysis von drei Hirnregionen (Studie 2) –präfrontaler Kortex (PFC), nucleus accumbens (NAc) und Amygdala (AMY) – hat gemeinsame Signaturen identifiziert, indem Nager, Affen und humanes post-mortem Hirngewebe verglichen wurden. Im PFC wurden Kanzerogenese, pro-inflammatorische und Metabolismen von oxidativem Stress identifizieret. Die Analyse im NAc zeigte keine signifikanten unterschiedlich exprimierten Gene (DEGs) im Nager-Modell und weniger als zehn DEGs in Menschen, was vermuten lässt, dass diese Hirnregion wenig vulnerabel für Langzeit-Alkoholkonsum mit anhaltender Abstinenzphase ist. Weitere Evidenz konnte ebenfalls aus bulk Sequnzierungsanwendungen (Studie 1) entnommen werden, in denen Methylom- und Transkriptom-weite Daten aus präfrontalen und striatalen Regionen erhoben wurden und das ventrale Striatum (VS) eine geringe Anzahl an DEGs und keine überlappenden Gene in der Datenintegration von Methylierungs- und Transkriptionsdaten aufwies. Jedoch auf der Ebene von differenziell methylierten Positionen und Regionen, wurde das VS als eine der am stärksten beeinflussten Regionen beobachtet. In einem Vergleich durch alle drei Hirnregionen in der humanen Spezies ist SERPINA3 als konsistent hoch-reguliertes Gen aufgefallen, was unabhängig von der Hirnregion beobachtet werden konnte und dieses Gen als neuen Biomarker für AUD in Betracht zieht.

Erste vorläufige Daten aus einem snRNA-Seq-Experiment (Studie 3) aus dem dorsomedialen Striatum –die Hirnregion, die in humanem post-mortem Gewebe die stärksten Veränderungen auf dem multiomics Level zeigte (Studie 1)– von Alkohol abhängigen Ratten zeigen eine potenziell verstärkte Relevanz für Oligodendrozyten und Mikroglia in Bezug auf veränderte Transkripte, die Langzeit-Abstinenz überdauern. Diese Zelltypen haben bereits in vorangehende Studien an AUD-Patienten Relevanz gezeigt.

Wie in den Studien 1-3 gezeigt, ist das Immunsystem in AUD stark beeinträchtigt. Deshalb wurde in Studie 4 chronischer Alkoholkonsum und Alkoholabhängigkeit als potenzielle Risikofaktoren für COVID-19-Infektion und Komplikationen durch multiple Rattenmodelle von Alkoholkonsum betrachtet. Besonders die konstante Hochregulation von Ace2 im Lungengewebe aller Modelle, sowie die Reduktion von Mas-Expression im olfaktorischen Bulbus führten zur Schlussfolgerung, dass Alkoholkonsum –insbesondere in einer sub-chronischen und chronischen Weise– die Wahrscheinlichkeit mit SARS-CoV2 infiziert zu werden, sowie potenziell unter schweren Langzeitkonsequenzen, wie beispielsweise Anosmie, zu leiden, erhöhen könnte.

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Symbols and abbreviations

AcbC	Nucleus accumbens core
AcbS	Nucleus accumbens shell
ACC	Anterior cingulate cortex
ARH(1)	Heterogeneous autoregressive
AUD	Alcohol Use Disorder
BAC	Blood alcohol concentration
BLA	Basolateral amygdala
BMA	Basomedial amygdala
BNST	Bed nucleus of stria terminalis
CA1, 3, 4	Cornu ammonis area 1,2 and 4
CeA	Central amygdala
Cg	Cingulate cortex
CI	Consistency index
CIE	Chronic-intermittent ethanol exposure
CN	Caudate nucleus
COVID-19	Coronavirus disease 2019
CPu	Caudate putamen
CRF	Corticotrophin-releasing factor
DEG	Differentially expressed gene
DG	Dentate gyrus
DLS	Dorsolateral striatum
DMP	Differentially methylated positions
DMR	Differentially methylated region
DMS	Dorsomedial striatum
FC	Fold-change
GEM	Gel bead in emulsion
GEx	Gene expression
GO	Gene-Ontology
GSEA	GSEA
IL	Infralimbic cortex

IP	Intraperitoneal
LMM	Linear mixed model
LOO	Leave-one-out
MA	Meta-analysis
MeA	Medial amygdala
ML	Maximum Likelihood
MSN	Medium spiny neurons
NAc	Nucleus accumbens
NGS	Next generation sequencing
NSWBTRC	New South Wales Brain Tissue Resource Center
NTS	Nucleus tractus solitarii
OF	Orbitofrontal cortex
PCA	Principal component analysis
PD	Post-dependent
PFC	Prefrontal cortex
PrL	Prelimbic cortex
PUT	Putamen
PVN	Paraventricular nucleus
QC	Quality control
RIN	RNA integrity number
RoB	Risk of bias
SARS-CoV2	Severe acute respiratory syndrome coronavirus 2
scRNA-Seq	Single cell RNA-Sequencing
snRNA-Seq	Single nucleus RNA-Sequencing
VS	Ventral striatum
VTA	Ventral tegmental area
WGCA	Weighted gene network co-expression analysis
ZT	Zeitgeber-Time

List of publications

Thesis related publications

Friske MM, Giannone F, Senger M, Seitz R, Hansson AC, Spanagel R (2023). Chronic alcohol intake regulated expression of SARS-CoV2 infection-relevant genes in an organ specific manner. *Alcohol Clin Exp Res.* 47(1):76-86. (Study 4 in this thesis)

Friske MM, Spanagel R (2023). Chronic alcohol consumption and COVID-19 infection risk: a narrative review. *Alcohol Clin Exp Res.* 47(4):629-639. (Study 4 in this thesis)

Friske MM, Torrico EC, Borruto AM, …, Spanagel R (2022). A Systematic Review and Meta-analysis on the Transcriptomic Signatures in Alcohol Use Disorder – a Translational Approach. *bioRxiv* 2022.12.19.521027. (Study 2 in this thesis)

Zillich L, Poisel E, Frank J, Foo JC, **Friske MM**, …, Rietschel M, Spanagel R, Witt SH (2022). Multi-omics signatures of alcohol use disorder in the dorsal and ventral striatum. *Transl Psychiatry*. 12(1):190. (Study 1 in this thesis)

Zillich L, Frank J, Streit F, **Friske MM**, …, Rietschel M, Spanagel R, Witt SH (2022). Epigenome-wide association study of alcohol use disorder in five brain regions. *Neuropsychopharmacology*. 47(4):832-839. (Study 1 in this thesis)

Thesis unrelated publications

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Zillich L, Poisel E, Streit F, Frank J, Fries GR, Foo JC, **Friske MM**, …, Spanagel R, Rietschel M (2022). Epigenetic Signatures of Smoking in Five Brain Regions*. J Pers Med* 12(4):566.

Poisel E, Zillich L, Streit F, Frank J, **Friske MM**, …, Spanagel R, Rietschel M (2023). DNA methylation in cocaine use disorder– An epigenome-wide approach in the human prefrontal cortex. *Front Psychiatry.* 14:1075250.

Friske MM, Uhrig S, Giannone F, Vengeliene V, Hyytiä P, Cicciocioppo R, Bakalkin G, Sommer WH, Spanagel R, Hansson A. Cacna1c and Cacna1d mRNA expression in human post-mortem brain tissue and link to genetic and functional rat models of alcohol dependence. (In preparation)

1. Introduction

1.1 Alcohol use disorder – a common mental health problem

Alcohol is one of the few drugs that is consumed legally in the majority of countries worldwide. This is potentially encouraging an elevated consumption rate of this substance, where, e.g., in the US 86% of all people aged 18 year or older drank alcohol at least once in their lifetime and 70% of people of the same age reported drinking over the last year, according to a report of the National Institute on Alcohol Abuse and Alcoholism (NIAAA) (SAMHSA, 2019). Furthermore, alcohol-related emergencies and deaths account for about 20% of all cases reported in the US, making alcohol the third-leading preventable cause of death in this country (Jones et al., 2014; Mokdad et al., 2004). Globally, 5.3% of all deaths are caused by alcohol consumption (World Health Organization, 2018), making alcohol abuse the highest risk factor for premature disability and death for people aged between 15 and 49 years (Collaborators, 2018). Therefore, it might not be surprising, that alcohol was classified as the most harmful drug, in comparison to other licit and illicitly commonly consumed drugs (Nutt et al., 2010). Furthermore, Alcohol Use Disorder (AUD) is the most prevalent substance use disorder worldwide (Collaborators, 2018). Despite cultural and religious aspects, alcoholic beverages can be found in TV advertisements, public bars, and restaurants and are freely available even on public transportation, such as planes and trains. This omnipresence of alcohol is not just engaging consumption in general, but also represents a high number of trigger points and cues for patients suffering from AUD, which enhances their relapse potential. 10-15% of the global population suffers from AUD, whereby the number of undiagnosed AUD patients is potentially even higher (Egervari et al., 2021). One of the most challenging aspects of AUD is withdrawal, and relapse. More than 50 % of the patients do not manage to break through the steady cycles of abstinence, withdrawal and relapse (Sliedrecht et al., 2019). Furthermore, less than 10% of all AUD patients receive treatment (Fairbanks et al., 2020), and pharmacotherapeutic options are comparably low for these patients, since, to date, there are only three commonly approved therapeutic drugs available (Egervari et al., 2021; Littleton et al., 2004). Disulfiram, FDA-approved since 1951, interacts with aldehyde dehydrogenase to block alcohol catabolism, which leads to the accumulation of acetaldehyde. If the patient is consuming alcohol during this medication, strong negative side effects such as nausea, vomiting, and heart palpitations occur, which is supposed to establish a negative

association with alcohol and therefore initiate an aversive behavior in the patient, eventually (Vallari & Pietruszko, 1982). Additionally, disulfiram was suggested to interact with the dopamine system, thereby inducing the metabolization of dopamine to noradrenaline, leading to reduced withdrawal symptoms (Bagdy & Arato, 1987). However, the efficacy of disulfiram is still questioned. While a meta-analysis from 2014 showed, that disulfiram treatment seems to be solely affective in open-label, but not in blinded trials (Skinner et al., 2014), it was suggested that just awareness of the direct negative physiological consequences of alcohol consumption might improve treatment outcome (Mutschler et al., 2016). To date, disulfiram is mainly recommended for abstinence maintaining, but not for a slight reduction of drinking behavior (Kranzler & Soyka, 2018). The second FDA-approved pharmacotherapeutic drug is naltrexone, which was approved in 1994 for treating AUD, and was initially used for opioid use disorders (OUD). Naltrexone interacts with the μ-opioid receptor (MOR) as an antagonist, which results in a reduced rewarding effect of alcohol, caused by impairment of the dopamine system (Nestler, 2005). Previous studies reported a significant reduction of drinking days, relapse episodes, and the general rewarding effect of alcohol (O'Malley et al., 1992; Ray & Hutchison, 2007; Volpicelli et al., 1992). Nevertheless, for naltrexone, mixed outcomes have been observed. Additional clinical trials reported no significant positive outcome to placebo, which, considering the aversive side effects of this medication, points towards a limited utility (Kranzler et al., 2000). In general, it has to be taken into account that naltrexone might enhance OUD withdrawal signs, and therefore AUD patients should be screened for additional OUD as a comorbidity before initiating the therapy (Kranzler & Soyka, 2018). Additionally, patients with alcoholic liver disease are suggested to abstain from naltrexone treatment, since acute hepatitis and liver failure are contraindications (McDonough, 2015). The third pharmacotherapy, acamprosate, was approved in 2004. Even though the mechanism of action is yet not fully understood, it is suggested to act on *N*-methyl-d-aspartic acid (NMDA) receptors as a partial co-agonist to reduce the hyperexcitability of neuronal cells and therefore, reducing the effects of acute and protracted withdrawal (Mason & Heyser, 2010). Furthermore, it has previously been shown that a hyper-glutamatergic state, induced by a mutation in the circadian clock gene Per2 gene, which leads to the accumulation of glutamate, induces enhanced alcohol consumption and that this effect can be reversed by acamprosate (Spanagel et al., 2005). In addition, the efficacy of acamprosate was dedicated to calcium, as it has been found that that replacing

the calcium ligand in the acamprosate compound with sodium was leading to no positive effects on alcohol preference, and craving. Also, increased levels of calcium in the plasma of AUD patients were positively correlated with acamprosate treatment outcomes. (Spanagel et al., 2014)

Also, for acamprosate, several studies showed only little to no effect over placebo (Anton et al., 2006; Chick et al., 2000; Kampman et al., 2009; Paille et al., 1995; Richardson et al., 2008; Shabunin, 1991). However, confirmatory studies were also published, stating an increase in abstinence duration with acamprosate treatment and these additionally recommended applying it for abstinence maintenance (Mann et al., 2004; Rosner et al., 2010; Rosner et al., 2008).

This short introduction to AUD pharmacological treatments shows the necessity for further investigations to reduce the burden caused by long-term withdrawal, relapses and repeated abstinence from high-level drinking cycles. Since AUD and the associated relapse behavior strongly affect the brain (Cui et al., 2015; Koob & Volkow, 2016; Uhl et al., 2019), research is mostly focusing on the neurocircuits that are altered in this disease phenotype. Therefore, pre-clinical studies on post-mortem human brain tissue as well as on animal models of alcohol dependence are used to study the molecular processes underlying the variety of aspects that define AUD to develop new successful therapeutic approaches. Additionally, the more scientific communication and education to the public takes place, the higher the likelihood will be that AUD becomes less stigmatized and eventually, more patients become diagnosed and receive treatment.

1.2 Dysregulated neurocircuits in AUD

At the time alcohol abuse and addiction arose as a topic in society, it was mainly considered a social issue, and as a consequence, it was concluded that the justice system had to take care of eliminating this public problem (Leshner, 1997). Over time, with increased general understanding of mental disorders, AUD has been added to diagnostic handbooks in psychiatry and has been officially characterized as a brain disease (Heilig et al., 2021; Leshner, 1997). Furthermore, the phenomenon of the “dark side of addiction” was established, describing the impact of negative emotions on the negative reinforcement for drug intake (Koob, 2017; Koob & Le Moal, 2005, 2008). As the term indicates, this aspect focuses on the

counterpart of the positive reinforcement factors that come with the pleasurable, rewarding effects of the drug. To this date, this terminus serves as a basis for neurobiological observations and interpretation in addiction research.

By now, various neurobiological studies have observed which brain components might be dysregulated by alcohol consumption, dependence, withdrawal, and relapse. Hence, most of these studies reported that when developing an AUD, the brain activity patterns are substantially altered compared to those of social drinkers. Besides the activity patterns the brain volume is also shrinking in AUD patients. As a neurotoxic agent, alcohol causes a reduction in grey matter volume through damage and induced autophagy in neurons. Even though this effect was observed globally throughout the brain, the major affected regions were reported to be the prefrontal cortex (PFC), cingulate cortex, insula, and striatum (Nutt et al., 2021) and these effects were found to have a negative effect of biological aging with an increase of up to 11.7 years with the strongest effect in older AUD patients (Guggenmos et al., 2017). Also, the white matter is impaired by long-term alcohol abuse, as microstructural changes have been observed that even progress in early abstinence in a translational approach focusing on alcohol preferring rats as well as AUD patients (De Santis et al., 2019).

The pleasurable, stimulating effect of alcohol, when occasionally consumed, has been attributed to the striatum (Weafer et al., 2018). In AUD, the rewarding properties of alcohol are experienced less, and the pleasure of alternative natural rewards is declining until it is completely absent. This impairment in reward processing is mediated by a reduced reward-driven motivational function from the ventral striatum and projections to the extended amygdala (Koob & Le Moal, 2008). As part of the driving forces in reward processing, the endocannabinoid system as well as endogenous opioids interact directly with the dopamine system in a positively enhancing manner, but also stimulates glutamate release, mainly in the nucleus accumbens (NAc) (Spanagel, 2009, 2020). When comparing the severity of AUD and the level of impairment of the driving neurocircuits, a stronger impairment of these circuits was reported in heavy drinking AUD patients, compared to moderate AUD patients (Fede et al., 2022).

In addition, so-called antireward circuits are activated, which drive aversive aspects, such as dysphoria, emotional pain, and loss of motivation for natural rewards via an imbalance in neurotransmitters through a down-regulation of dopamine and opioids and an up-regulation of corticotrophin-releasing factor (CRF) in the HPA axis and extended amygdala (Koob & Le

Moal, 2008). Also, an inner-striatal shift takes place, inducing a shift in activity from ventral to dorsal striatum, when developing AUD (Vollstädt-Klein et al., 2010). The aspect of craving, including compulsivity and loss of control over alcohol intake despite the awareness of negative consequences, is mainly driven by alterations in the PFC (de Paiva Lima et al., 2017; Frascella et al., 2010; Koob & Volkow, 2010). Reduced dopamine receptor expression was assumed to lead to decreased activity of the PFC, which might explain impaired self-control leading to compulsive drinking behavior in AUD patients (Volkow et al., 2017). The aspect of craving was additionally related to an increase in MORs in the ventral striatum of short-term abstinent AUD patients (Heinz et al., 2005). However, in non-abstinent patients and human post-mortem brain tissue, reduced levels of MOR were measured, and even further, the decreased availability of MOR was interpreted as a worse treatment prognosis with naltrexone, and MORs were suggested as molecular markers for AUD relapse prediction (Hermann et al., 2017).

When presenting an alcoholic cue, AUD patients show activation of several brain regions, e.g., nucleus accumbens (NAc), anterior cingulate cortex (ACC), insula, and ventral tegmental aera (VTA), while social drinking individuals only show minor activation of the cingulate gyrus (Myrick et al., 2004). However, AUD is a complex disorder with a broad spectrum of characteristics that are directed by different brain regions and their interactions with each other (Figure 1). The amygdala is impaired in AUD by, e.g., dysregulation of neuropeptides, such as endocannabinoids, substance P, and CRF, which all are stress-relevant molecules (Egervari et al., 2021; Koob, 2009). Inhibition of CRF neurons in the amygdala projecting to the bed nucleus of the stria terminalis (BNST) initiates escalated drinking and physiologic withdrawal symptoms (Centanni et al., 2019). In terms of connective projections between brain regions, the interaction between the orbitofrontal cortex (OF) and the dorsomedial striatum (DMS) is impaired by chronic alcohol consumption, leading to reduced excitation of outputs from the OF to the DMS (Nimitvilai et al., 2017a; Nimitvilai et al., 2017b). Additionally, projections from the prefrontal cortex (PFC) to striatal regions are bidirectionally controlling alcohol consumption in a long-lasting manner (Ma et al., 2018). Incentive salience is one of the key features of AUD. It describes the desire to consume the drug and goes along with the loss of alternative rewards. Therefore, the main brain regions involved refer to the mesocorticolimbic system, which includes the midbrain to forebrain projections as well as the striatum and is mainly driven by dopamine (Berridge & Robinson, 2016). Figure 1 depicts a

schematic overview of the brain regions that are involved in AUD and their major neuronal projections.

Based on these neuroscientific studies, new hypotheses were established, focusing on, e.g., neuroinflammation as the main alcohol-sensitive process (Grantham et al., 2023; Mayfield & Harris, 2017; Warden et al., 2016). The stress hypothesis explains stress as a major driving force for alcohol acquisition and relapse (Zhou & Kreek, 2014; Zorrilla et al., 2013). Another hypothesis focuses on anxiety that comes along with acute withdrawal, but also as an initial factor for alcohol consumption, since alcohol has high anxiolytic properties (Silberman & Winder, 2015; Tolic & Soyka, 2018). On the cellular level, e.g., the glial dysfunction theory has been described that suggests glia cells as the main cell type that is impaired by chronic alcohol consumption and AUD (Erickson et al., 2019; Kim et al., 2015; Miguel-Hidalgo & Rajkowska, 2003; Orellana et al., 2017). These hypotheses aim to describe major factors that drive the development and maintenance of the disorder, with the goal of modulating them to induce long-term abstinence and eventually, overcome craving and relapse. The number of hypotheses point out not only the complexity of this disorder, but also the variety of ways scientists try to intervene to develop new targets for treatment.

In conclusion, this section aimed to give a short overview of dysregulated brain regions and neurotransmitters that have previously been identified in AUD research. However, even though this paragraph does not represent the full content of this research field, it might still be sufficient to point out that in AUD patients, neuroadaptation takes place in diverse brain regions and connectivities between the regions are dysregulated, which eventually leads to addictive behavior. However, the full picture of these neurocircuitries is not yet clear, since especially the sex-specific differences and the involvement of cell type-specific components are not well understood so far (Borrego et al., 2022). The aspects discussed in this section underline that AUD is a multidimensional disease, that still requires further investigation into a variety of molecules, interactions, and regions to fully understand its extent on the neurophysiological level.

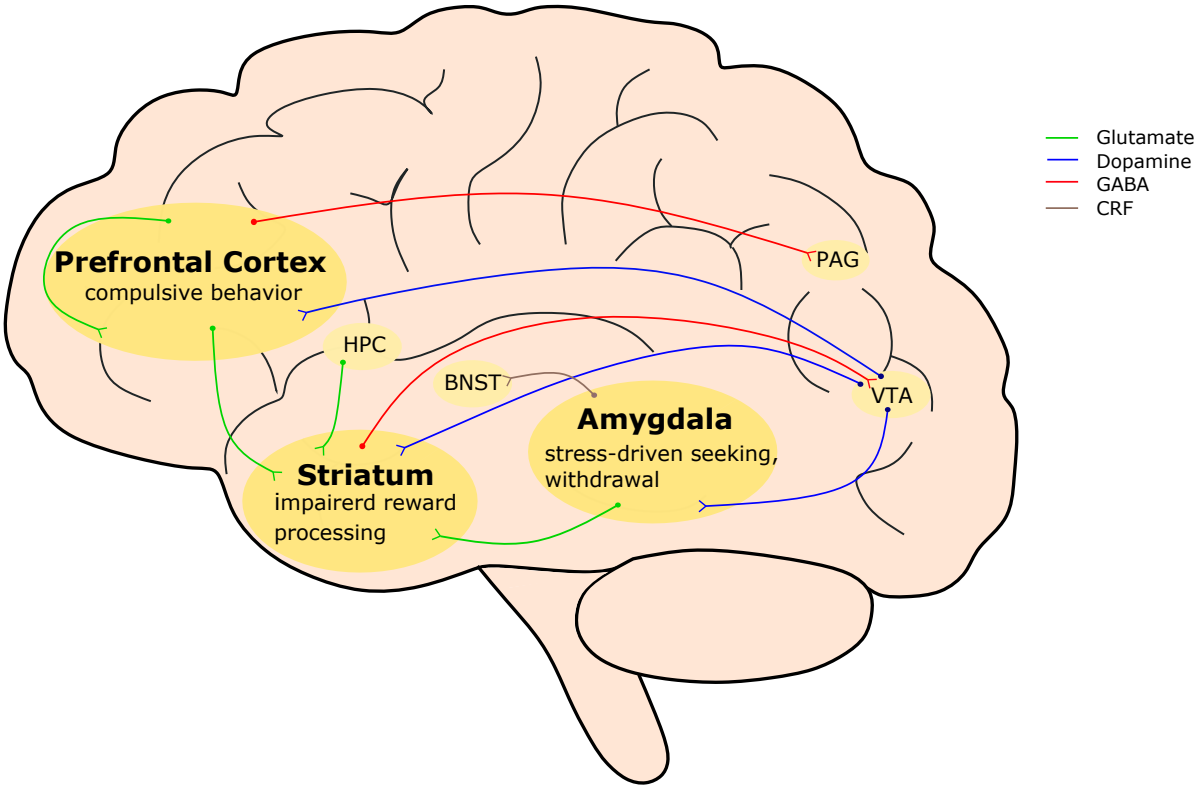

Figure 1: Neurocircuits of AUD.

Schematic overview of the most abundantly studied brain regions, their function in AUD and their major neuronal projections (based on Egervari et al. (2021)). As central regions the prefrontal cortex, the striatum and the amygdala are highlighted, since they play key roles in AUD. BNST= Bed nucleus of stria terminalis, HPC= Hippocampus, PAG= periaqueductal grey, VTA= ventral tegmental area.

1.3 Pre-clinical models of alcohol dependence – the post-dependent (PD) model

The observation of affected neurocircuits and the outcome of their modulation on addictive behavior in patients is very limited. Therefore, animal models are valuable tools to observe alterations underlying the development of dependent behavior.

The modulation of dysregulated molecules and circuits can be applied on the behavioral level to predict their efficacy as therapeutic targets. Over the past decades, several studies have shown the successful induction of alcohol-dependent behavior in rodent models (Gilpin et al., 2008; Rodd et al., 2004; Spanagel, 2003). The use of rodent species to study the molecular effects of alcohol dependence comes with several advantages, since the environmental factors can be controlled to have harmonized conditions across the individuals and the genome can be manipulated with a variety of tools adequate for numerous different research questions. In addition, over the years, a variety of animal models for studying different aspects of AUD have been established, which has enabled the observation of the factors of AUD in diverse settings. To date, there is no “all-rounder model” that can mimic all the aspects

of AUD combined in animals, but it is possible to study the criteria, such as neurocircuits (on the genetic, epigenetic, transcription, and network level), and their behavioral consequences (reinforcing factors, craving, and dysphoria), separately (Koob & Le Moal, 1997). Additionally, the stages of developing dependence, withdrawal, and craving can be studied longitudinally. Due to common diagnostic tools, such as the Diagnostic and Statistical Manual of Mental Disorders 5th Edition (DSM-V), AUD is classified by loss of control over alcohol consumption, alcohol seeking and craving behavior, consumption despite negative consequences, enhanced risk-taking behavior, increasing tolerance to alcohol, and withdrawal symptoms during abstinence periods (American Psychiatric Association, 2013). The post-dependent (PD) animal model, which is induced by chronic intermittent ethanol (CIE) exposure, is frequently used in pre-clinical studies, since it is known to fulfill a high proportion of these criteria, such as withdrawal, tolerance to alcohol over time, excessive alcohol consumption, increased motivation for alcohol seeking, and evidence for loss of control over alcohol intake (Meinhardt & Sommer, 2015). In addition, due to the intense treatment characterized by numerous repeated cycles of ethanol exposure and abstinence, the animals show strong and persistent changes in neurocircuits on several molecular levels (Hansson et al., 2008; Heilig et al., 2017; Kisby et al., 2021; Meinhardt et al., 2013; Meinhardt et al., 2015; Olsen & Liang, 2017; Rimondini et al., 2002).

The PD phenotype emerges from a chronic intermittent exposure (CIE) treatment that usually consists of ethanol vapor exposure. In our laboratory, rodents usually undergo ethanol vapor cycles of 16 h followed by 8 h of abstinence per day for seven weeks in total. Hereby, the measurement of the blood alcohol concentration (BAC) ensures that the animals stay consistently in a high intoxication state ranging from 150 to 300 mg/dl (1.5-3.0‰) throughout the whole treatment period. After one to three weeks of abstinence, persistent alterations can be observed in alcohol dependence-related behavioral experiments, such as ethanol reinstatement behavior, to measure the relapse potential of the animals, as well as the persistent alterations on the molecular level (Carpio et al., 2022; Eisenhardt et al., 2015; Hansson et al., 2018; Uhrig et al., 2017). The treatment paradigm can vary across laboratories and research questions, but the general principle of high intoxication due to repeated circles of ethanol exposure and recurring abstinence periods to induce dependence is the same. Recent research has also included the model in translational approaches as a comparison to human findings or to elucidate behavioral relevance of targets observed in AUD patients

(Griffin, 2014). Functional magnetic resonance imaging (fMRI) studies have shown significant overlap between the brain activity patterns in CIE animals and AUD patients (Fritz et al., 2022). When measuring the dynamic changes of dopamine receptor expression during abstinence, the observed expression pattern in the human post-mortem brain was matched to a longitudinal observation of the expression changes in CIE rats at different time points of protracted abstinence (Hirth et al., 2016). Eventually, the consequence of this hyperdopaminergic state found in long-term abstinent rats as well as in human tissue, was suggested as an increased relapse vulnerability in additional behavioral data from the animal model.

For testing treatment drug candidates, the CIE model has demonstrated that pregnenolone derivates can reduce alcohol intake in dependent individuals (Macedo et al., 2023). The model has also been used to investigate potential optogenetic treatment interventions during withdrawal (Alberto et al., 2023). In a study comparing CIE animals with human post-mortem brains as well as alive AUD patients, oxytocin was described as an anti-craving component. Since the findings in the preclinical experiments, were directly investigated in the clinics, this study led to a highly comprehensive and robust finding (Hansson et al., 2018).

In summary, the use of animal models in preclinical research of AUD is essential. Therefore, the PD model plays a central role in examining the behavioral and molecular processes underlying this disorder to deliver target molecules for clinical drug testing. The combination with human post-mortem brain tissue and the establishment of translational approaches provides a promising basis for the understanding of AUD and the development of psychopharmacotherapy.

1.4 Human post-mortem brain tissue

Human post-mortem brain tissue is a valuable, but sparse resource for pre-clinical research on AUD to observe molecular changes in patients and establish connections between the observations in patients and animal models of alcohol addiction. There are only a limited number of brain banks worldwide that provide brain tissue from deceased AUD patients and healthy controls under high standards of archiving. Probably, the most frequently approached brain bank is the New South Wales Brain Tissue Resource Center (NSWBTRC) at the University of Sydney, Australia, which has been providing high quality tissue for decades (Dedova et al.,

2009; Sutherland et al., 2016). The NSWBTRC is collaborating with the National Institute of Alcohol Abuse and Alcoholism (NIAAA) in the US as their major founder and is a certified biobank that aims to provide post-mortem brain tissue from patients with AUD and other mental disorders and healthy individuals. Since 2002, the brain bank has established a donor program called “Using our Brains” that aims to collect brain tissue from individuals before they die, and focuses on both, healthy and mentally disordered people (Sheedy et al., 2008). The requested tissue can be provided as either fresh-frozen or formalin-fixed, depending on the research purposes. In addition, each sample comes with a health record of the individual, including ethnic origin, psychiatric history, family history, cause of death, age at time of death, post-mortem interval and substance use, which provides a highly informative set of meta-data for the molecular analyses.

Especially with the development of high-throughput sequencing methods, human post-mortem brain tissue was applied for DNA, RNA and protein expression profiling, which identified new risk alleles, biomarkers, and drug targets of AUD (Sutherland et al., 2014; Warden & Mayfield, 2017). Specifically, in transcriptomic observations, it has to be stated that even though the number of significantly altered transcripts is high, the effect size is comparably small. Therefore, the previous studies point towards the understanding of network interactions, by using, e.g., weighted gene network co-expression analysis (WGCNA), instead of the observation of single genes (Warden & Mayfield, 2017). On the network level, it has been suggested that impaired homeostatic regulation of gene networks might be the cause for neurotoxicity of high level ethanol consumption in AUD. In addition, dysregulation of numerous transcription factors as well as signalling cascades, such as the MAPK cascade, were identified across several alcohol-related studies (Farris & Miles, 2012). On the basis of epigenetic regulation, both non-coding RNAs (ncRNA) as well as micro RNAs (miRNA) are potentially influenced by alcohol and might have an important role in gene expression regulation (Farris & Mayfield, 2014). Furthermore, the combination of human post-mortem brains with blood samples from AUD patients in the clinics is a usual approach to find biomarkers that are commonly altered in brain tissue as well as peripheral organs, and also in finding brain-specific effects. Therefore, the $GABA_B$-receptor promotor GABBR1 was suggested as a biomarker for neuronal dysregulation in the brain with a sex-specific outcome, showing effects only in male AUD subjects (Meyer-Bockenkamp et al., 2023). Another study using the same approach claimed that these alterations in the methylation pattern of the

DRD2 gene observed in several brain regions, could not be predicted by blood samples solely (Arfmann et al., 2023).

Even though the previous findings in human post-mortem studies were highly promising, they were limited in sample size and lacked generalizability. The disease history of AUD patients is diverse, since every patient has a different onset of drinking, duration of drinking, number of relapses, and additional influencing factors, such as comorbidities, mainly additional psychiatric disorders and smoking, but also physiological diseases. All these factors describe the high heterogeneity of this mental health disorder, and it has been reported that these factors have influence on the molecular patterns of AUD (Bohnsack et al., 2019; Lohoff, 2020; Zahr et al., 2016). It is well known that also the environment has a high impact not only on the behavioral, but also on the molecular outcome (Gorwood et al., 2007; Sher et al., 2010). Therefore, according to statistics, heterogeneous cohorts that represent a set of numerous variables in addition to the main outcome measure, require a decent sample size to enable clear-cut statements of molecular processes underlying a certain disease (B-Rao, 2001; Jung, 2010; Lai et al., 2003; Lau et al., 2023). To date, we are facing these limitations, as the approaches with the highest gain of information also require the highest amount of funding. In addition, even though the human post-mortem samples are expanding, the sample size is still limited. Especially, the observation of sex-specific differences in AUD, as well as ethnicity-specific outcomes, is strongly underrepresented. There are several reasons for this. As mentioned above, the number of diagnosed AUD patients does not by far represent the total number of patients. In the past, women were significantly less diagnosed than men, which is why the number of potentially available study samples for sex-specific observations is restricted. However, recent data from the US indicate that the number of female AUD patients is increasing (Grant et al., 2017). It is known that female AUD patients differ from male AUD patients in their drinking patterns and general vulnerability to AUD (Stanesby et al., 2018). Women seem to be less likely to develop an AUD, but if they do, women sustain more severe physiological consequences than men (Erol & Karpyak, 2015). One potential explanation for these sex-specific effects might be the differences in density of neurotransmitter receptors in women, such as dopamine, GABA, and glutamate (Ceylan-Isik et al., 2010). However, the number of studies investigating the sex-specific effects of AUD, including risk factors, treatment needs, and molecular patterns, is highly limited so far. But the currently existing points towards the need for further exploration of female AUD patients

and their comparison to men. Most of the individuals who have donated post-mortem brain tissue so far are of Caucasian origin. This might be explained by the high percentage of white people in industrial countries, which is the area where the majority of patients are diagnosed, and samples are donated. However, it has been shown that ethnicity is an important factor to consider when investigating potential risk factors for developing AUD (Evans et al., 2017; Wall et al., 2016).

Bottom line, human post-mortem brain tissue provides the unique chance to study the molecular changes at the genomic, transcriptomic, and proteomic level within the patient. Therefore, numerous studies investigated this valuable resource and contributed substantially to the understanding of the disorder. However, since it is only possible to study these phenomena in deceased individuals, it cannot be determined whether the observed patterns are caused by AUD or if they are initiating factors leading to the development of the disease that persist over lifetime.

1.5 Bulk sequencing and the era of transcriptome-wide approaches

Since the end of the last century, interest in capturing more than just a limited set of target genes has increased, and therefore the development of sequencing (seq) methods for transcriptome- and genome-wide observations has been initiated and developed further. Over time, the sensitivity for captured molecules has increased dramatically, leading to reliable observations of lowly expressed transcripts. One of the first techniques for transcriptome-wide analysis were microarrays. With these arrays, several thousand genes could be detected at the same time by hybridization of the pre-labeled sample cDNA to custom-made adaptor sequences on the microarray chip (Celis et al., 2000). The samples were distributed on the microarray chip, and the cDNA strands hybridized to the probes on the chip were detected. Afterwards, the sample cDNA could be washed away to re-use the chip, which made this technique cost-effective and approachable for financially limited studies, as well. Since the adaptor sequences on those chips were custom-made, a broader spectrum of gensets and networks of interest could be detected than before. This was the first time, thousands of genes could be detected at the same time, which enhanced the number of investigated genes dramatically in comparison to Northern Blots, qPCR and any other target-molecule approach. Nevertheless, capturing the full transcriptome was first possible with the

development of the Next Generation Sequencing (NGS) approaches at the beginning of the second decade of the 21st century. Hence, the number of detected genes had significantly increased by enabling the capture of the whole transcriptome, and a reliable observation of lowly abundant transcripts was granted. Not only was the resolution enhanced, but due to automated sample processing and high-throughput sequencing methods, the hands-on working time was strongly reduced (Ambardar et al., 2016). In many research fields, numerous key publications have identified target genes and pathways that are strongly dysregulated in a variety of diseases, thereby providing the basis for follow-up studies of treatment target identifications. Also, in the field of personalized medicine, bulk sequencing approaches contributed extensively to establishing cost-effective solutions to optimize diagnosis procedures in the clinics (Rabbani et al., 2016). As mentioned in Section 1.4, in pre-clinical research on AUD, important findings were conducted applying these seq techniques and are still ongoing, which is contributing extensively to better understanding of the underlying molecular mechanisms and interactions of the disorder. However, even though bulk seq approaches have pioneered the field of molecular analyses at the DNA, RNA and protein level, one crucial limitation of these methods is that bulk seq measures a merged average of all cell types within one sample. Through the homogenization process applied in the RNA isolation procedure, all cell types appear in a merged interphase in the sequencing. Therefore, findings in bulk analyses represent the average of transcripts from a specific tissue type, but cell type-specific signatures cannot be observed accurately. By now, it is known that cell types show individual epigenetic and transcriptomic expression profiles, which leads to the expectation that the effects observed in bulk analyses might not represent the full picture of important aspects of a transcriptome-wide or epigenome-wide profiling.

1.6 Single cell sequencing approaches deliver new insights to understanding the cell-type specific signatures in AUD

Since the first study published results from single cell RNA- Seq (scRNA-Seq) in 2011 (Islam et al., 2011), single-cell seq (scSeq) techniques have been developed, and contributed fundamentally to investigations of several diseases, and to date are even part of diagnostic tools. E.g., in the cancer field, scSeq techniques are used to more precisely identify the appropriate target therapy for the patient since the previous bulk analyses led to a high

number of patients not being treated with their respective optimal medications (Baslan & Hicks, 2017). Therefore, scSeq approaches are the pioneering methods for understanding diseases in greater detail on a cell type-specific level, which is enabling the development of new, promising therapeutic diagnostics and approaches. Also, in the context of general organ development, which is also linked to physiological malfunctioning, longitudinal scSeq studies have enlightened the interaction of the individual cell types at different time points throughout embryonic development and further (Herring et al., 2022). In the AUD field and in addiction research in general, so far, only a very few studies have applied this approach. Brenner et al. (2020) performed single nuclei RNA (snRNA) seq in human post-mortem brain samples of the prefrontal cortex (PFC) from deceased AUD patients and observed the highest number of differentially expressed genes (DEGs) in astrocytes, oligodendrocytes, and microglia. Furthermore, a snRNA-Seq study published this year analyzed NAc tissue derived from human post-mortem brains of AUD patients (van den Oord et al., 2023). Here, the cell types with the strongest effect were identified as medium spiny neurons (MSN), both dopaminergic subtypes D1 and D2, microglia, and oligodendrocytes. The authors integrated their dataset with the already existing one from Brenner et al. (2020) of the PFC and found replicating findings. The main finding, that CD53 is differentially expressed in microglia, was also observed in the previous PFC study in post-mortem AUD brains. In the central amygdala of vapor exposed rats with acute withdrawal, the most affected cell types were found to be astrocytes and GABAergic neurons (Dilly et al., 2022). When looking further into subclasses of GABAergic neurons, the protein kinase C delta expressing GABAergic neurons were identified as the most affected subclass, which was also validated using fluorescent in-situ staining and quantification methods. This study has identified a new cellular subpopulation that might play an important role in alcohol- and withdrawal-related transcriptomic changes. In addition, a recent study focusing on an animal model of cocaine intake identified dopamine-receptor positive MSN and immediate early genes expressed in this cell type as key driving aspects of cocaine intake (Savell et al., 2020). Taken together, these limited number of single-cell resolution studies have pointed towards the high importance of medium spiny neurons as well as glia cells being affected by AUD on the transcriptomic level. Without doubt, in the near future, numerous additional snSeq studies will add further understanding of the cell types mainly involved in AUD, which has great potential for better understanding the disease to a greater extent. However, even though the number of snSeq studies is by now very limited in

the addiction field, past studies have focused on the impact of specific cell types on alcohol drinking behaviors using, e.g., cell type enrichment analysis as a downstream analysis in bulk sequencing approaches (Mavromatis et al., 2022; Ponomarev et al., 2012) and targeted cell type-specific approaches (O'Sullivan et al., 2021; Warden et al., 2021), which are adding further evidence to snSeq findings.

To understand the molecular alterations of AUD and other addiction-related disorders in greater detail, additional single cell studies need to be conducted on different molecular layers. One of the aims of this thesis is to contribute to these previous findings and add further information on genes and molecular pathways that might be involved in AUD and represent potential future targets for pharmacotherapeutic treatment.

1.7 Alcohol and the COVID-19 pandemic

At the end of 2019, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) and the resulting coronavirus disease (COVID-19) were a rapidly and long-lastingly evolving global health problem, resulting in a pandemic. Millions of people required severe hospitalization, treatments, or died due to COVID-19, which represented a tremendous burden for the health system in nearly all countries worldwide. Therefore, it has been intensively discussed how to protect the global population most effectively, reduce the amount of severely suffering patients, and end this pandemic as soon as possible. In this context, the identification of risk groups for severe COVID-19 was of great importance. Hence, the elderly, obesity, smoking, gender, respiratory dysfunction, and pre-existing autoimmune diseases were some of these identified risk groups that were found to be more often infected by SARS-CoV2, but also showed an increased number of severe and fatal cases. Since alcohol is known to cause impairment on a broad spectrum of physiological health conditions, it might well be that this could contribute to enhanced conditions for the coronavirus to infect and cause severe symptoms. However, even though it has been recorded that throughout the pandemic, the global alcohol consumption increased, the impact of alcohol on the pandemic as a potential risk factor was only sporadically discussed. This is surprising, considering that more than two billion people worldwide regularly drink and hundreds of millions of people are diagnosed with AUD.

Epidemiological data from AUD patients and SARS-CoV2 infection, hospitalization, and mortality rates provide no basis for a clear-cut conclusion about whether AUD, or above average drinking, can be considered a risk factor for COVID-19. While most studies examined increased risk for complications in AUD patients (Bailey et al., 2022; Bhalla et al., 2021; Pavarin et al., 2022; Ramakrishnan et al., 2022; Varela-Rey et al., 2013), there were also observational studies that reported increased general COVID-19 infection without increased risk for complications (Kianersi et al., 2022; Saurabh et al., 2021). Moreover, some studies reported no significant increase in COVID-19 infection or disease severity compared to individuals with no comorbidity (Dai et al., 2022; S. Rao et al., 2021; Zhong et al., 2020). Interestingly, an early onset study by Hamer et al. (2020) even reported data suggesting over average drinking as a potential protective factor (Hamer et al., 2020).

On the molecular level, it is known that the virus is infecting the cells via the cell-surface protein ACE2, that is, in collaboration with the transmembrane protease TMPRSS2, facilitating virus entry (Hoffmann et al., 2020). Therefore, an increased expression of ACE2 has been correlated with a higher risk of COVID-19 infection in general, but also with an increased potential to develop complications. In terms of ACE2 expression in AUD patients, most studies reported up-regulation of this gene and protein in several tissues, such as the brain and liver, before and after the infection (Muhammad et al., 2021; Nuovo et al., 2022; Testino et al., 2022).

It has been reported that throughout the pandemic, AUD patients consumed significantly more alcohol than before, and the number of alcohol-related organ damage cases in AUD patients has increased since 2020 (Hutchison et al., 2022; Kothadia et al., 2022; Marano et al., 2022; Schecke et al., 2022; Tikaria et al., 2022). Therefore, it is of great importance to further investigate the consequences for AUD patients of the COVID-19 pandemic. In addition, the stressful effects of the pandemic, such as social isolation and life uncertainties, led several healthy individuals to drink alcohol more often alcohol to cope with these suffocating worries. This has an impact on the global health system even after the pandemic. Hence, further investigations into the potential risk of AUD patients to COVID-19 infections are warranted to eventually convey strong evidence for the government to protect this group of the population with, e.g., a higher priority for vaccination and other preventive measures.

1.8 Study aims

The aim of this thesis is the observation of molecular patterns in AUD, with the main focus on transcriptomic changes in human post-mortem brain tissue of AUD patients as well as in the brain of a rodent animal model of alcohol dependence across different brain regions. Therefore, bulk as well as scSeq approaches were applied in a comprehensive manner to find commonly altered mechanisms in the patient samples and the animal model in four studies: Study 1 focuses on bulk DNA- and RNA-sequencing on human post-mortem brain tissue from multiple brain regions and the separate and combined analysis of these two omics layers. Study 2 represents a meta-analysis of transcriptome-wide sequencing approaches in three brain regions of human post-mortem and animal brain samples in a translational setting. In Study 3, single nuclei RNA-sequencing is applied to identify cell-type specific patterns in the dorsomedial striatum of alcohol dependent rats. Finally, as mentioned in the introduction, AUD has been previously associated with impairment in neuroimmune processes and since this thesis was predominantly conducted during the COVID-19 pandemic, Study 4 investigated the potential of chronic alcohol consumption on COVID-19 severity and disease progression by measuring Ace2 gene expression across several animal models of alcohol intake and dependence using RT-qPCR.

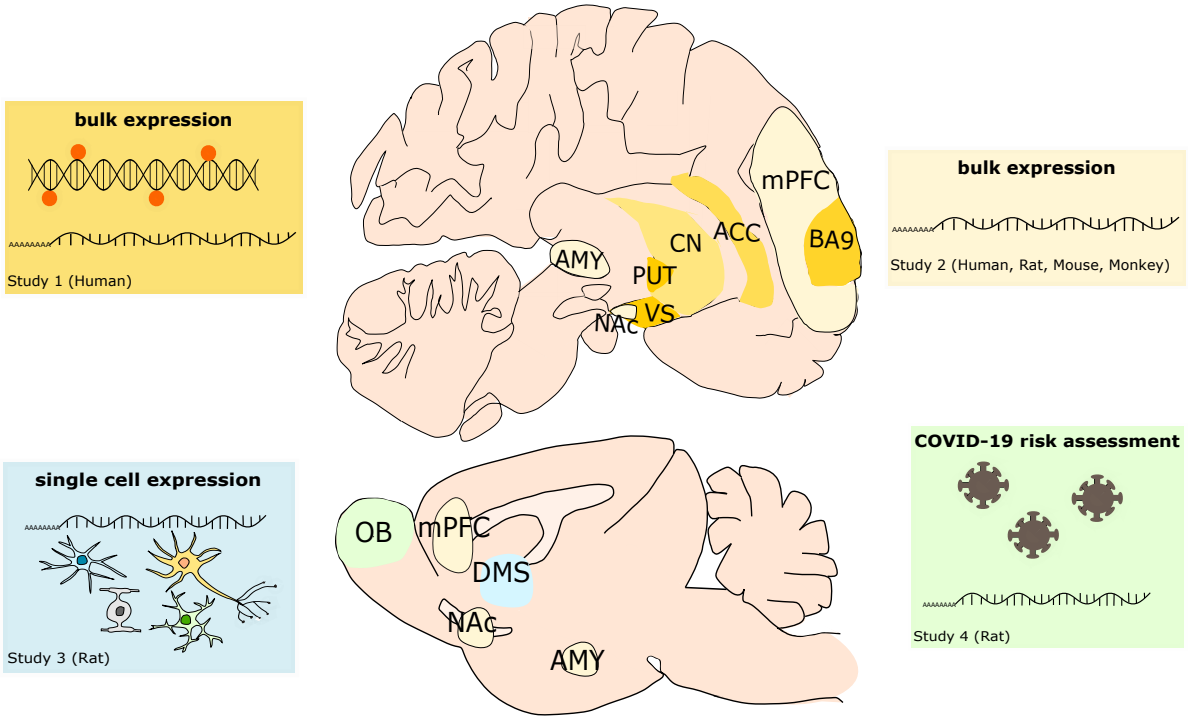

Figure 2: Schematic overview of the studies in this thesis and how they contribute to the observation of neurocircuits in AUD.

To distinguish the brain regions included in study 1 and study 2, the brain regions of study 2 (mPFC, AMY and NAc) are highlighted in light yellow and additionally surrounded by a black stroke. Human post-mortem brain tissue was included in study 1 (dark yellow) and 2 (light yellow), while study 3 (blue) and 4 (green) focused on rat brain tissue. PFC= medial prefrontal cortex; BA9= Brodmann Area 9; ACC= anterior cingulate cortex; CN= caudate nucleus; PUT= putamen; VS= ventral striatum; NAc= nucleus accumbens; AMY= amygdala; OB= olfactory bulb; DMS= dorsomedial striatum.

As highlighted in Figure 2, these studies all contribute to the main study aim of the translational comparison of neurocircuits impaired by AUD in different species and at diverse levels of molecular investigations. We believe that the targets identified in these studies will provide profound evidence for further exploration of their functional importance using behavioral experiments in animal models of alcohol dependence and will eventually lead to new therapeutic approaches to overcome the high relapse risk in AUD patients, which is still a tremendous challenge in the treatment of this psychiatric disorder.

2. Materials and Methods

2.1 Study 1: Bulk sequencing in human post-mortem brain tissue

As extensively demonstrated in the introductory part of this thesis, AUD is a brain disease. Therefore, the study of this organ to identify the brain regions, that are showing the strongest impairment by AUD, is highly relevant to understanding the disorder. On the molecular level, it is only possible to observe these changes within the patient through the use of post-mortem samples. As described in 1.4, human post-mortem brain tissue is a sparse and valuable source in the AUD research field, and even though there is pre-existing data derived from this kind of sample, the heterogeneity of the patients is too high to determine the generalized effects of AUD on the patient's brain. In addition, the observation of several brain regions in the same individual has only been partially possible to date. Therefore, this study examines molecular changes in DNA methylation as well as the RNA expression level by observing five and three brain regions, respectively, with high individual overlap across the brain regions. This approach aims to identify altered genes and pathways on two omics layers, both separately and combined, which adds to the already existing findings of previous studies. The findings of this study are published as Zillich et al. (2022a) and Zillich et al. (2022b). While I performed sample preparation, Lea Zillich (Department of Genetic Epidemiology in Psychiatry, ZI Mannheim) analyzed the data.

2.1.1 DNA sequencing for epigenome-wide analyses

DNA extraction in human post-mortem brain tissue of deceased AUD patients and matched controls was performed to analyze DNA methylation patterns in the following brain regions: PFC/BA9, anterior cingulate cortex (ACC), caudate nucleus (CN), ventral striatum (VS), and putamen (PUT). All samples were derived from the NSWBTRC at the University of Sydney. Characterization of the subjects based on next-of-kin interviews revealed DSM-IV criteria of alcohol dependence for the case individuals and additionally, an estimated daily ethanol consumption of >80 g per day and <20 g per day for control individuals. In addition, the study subjects were older than 18 years, had no diagnosis of an additional severe psychological disorder, no neurodevelopmental impairments or brain injuries, no additional substance use disorder (despite having nicotine use disorder), and were of Caucasian ethnicity. Each

individual contributed between one and five brain region-specific samples. Further details on the samples can be obtained from Table 1.

Table 1: Demographic description of all human post-mortem brain samples included for DNA methylation analysis.

Control and case individuals were matched by age and sex. PMI = post-mortem interval; BAL = blood alcohol level. The number of detected CpG sites varies between 657 000 and 690 000 sites per brain region. Table adapted from Zillich et al. (2022a).

Characteristic	AUD Cases	Controls	Difference (p value)
n	53	58	
Age (years)	56.72 ± 10.81	56.69 ± 10.29	0.989
Sex	34M/19F	40M/18F	0.737
pH value	6.5 ± 0.28	6.57 ± 0.32	0.189
PMI (hours)	35.46 ± 16.1	28.17 ± 15.29	0.038*
Smoking status	0.72 ± 0.26	0.51 ± 0.31	>0.001*
BAL >0 at time of death	8	0	
Number of brain regions coming from same patient			
5	19 (35.8%)	19 (32.8%)	
4	9 (17.0%)	8 (13.8%)	
3	18 (34.0%)	21 (36.2%)	
2	0 (0%)	3 (5.1%)	
1	7 (13.2%)	7 (12.1%)	
Number of samples per brain region			
ACC	28	26	
BA9	25	21	
CN	45	49	
PUT	44	50	
VS	46	47	

DNA extraction was performed using the Qiagen DNeasy extraction kit (Qiagen, Hilden, Germany). Afterward, the samples were placed randomly on processing plates, for each brain region separately. The DNA methylation levels were measured using the Illumina HumanMethylationEPIC Beadchip and the Illumina HiScan array scanning system under the leadership of Dr. André Heimbach at the Life & Brain GmbH at the University Bonn.

2.1.2 RNA sequencing for transcriptome-wide analyses

RNA was extracted from human post-mortem brain tissue derived from the VS, CN, and PUT. The sample criteria were similar to the ones mentioned in Section 2.1.1 with only the inclusion

of an RNA integrity number (RIN) of greater than 5.5 of the extracted RNA. Tissue was also obtained from NSWTRC at University of Sydney, Australia. A detailed sample description can also be extracted from Table 2.

Table 2: Demographic description of included samples for RNA sequencing.

Demographic description of the included samples for RNA sequencing. PMI = post-mortem interval; BAL = blood alcohol level. The significance of difference is calculated as t-test/ chi²-test comparing cases and controls. Content adapted from Zillich et al. (2022b).

Characteristic	AUD Cases	Controls	Significance of Difference (p value)
n	48	51	
Age (years)	55.58±10.62	57±10.64	0.51
Sex	31M/17F	37M/14F	
pH value	6.53±0.26	6.65±0.25	0.026*
PMI (hours)	37.07±15.79	30.7±15.57	0.047*
Smoking status	0.67	0.24	<0.001*
BAL >0 at TOD	7	0	
Number of samples per brain region			
CN	36	37	
PUT	35	42	
VS	31	32	

RNA extraction was performed using the RNeasy micro kit from Qiagen (Qiagen, Hilden, Germany), and RIN value measurement was performed using an Agilent Bioanalyzer and TapeStation 4200 (Agilent, Stanta Clara, CA). Samples with a RIN threshold of <5 were excluded for sequencing. Afterward, sequencing libraries were prepared using the TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA), and sample sequencing was performed in technical replicates via Illumina NovaSeq 6000 with a read length of 2 x 100 bp and a sequencing depth of 62.5 M read pairs per sample. The library preparation as well as the sequencing was performed at the Life & Brain GmbH at the University Bonn under the leadership of Dr. André Heimbach.

2.1.3 Data analysis

Data analysis of bulk DNA- and RNA-Seq was performed by the Department of Genetic Epidemiology in Psychiatry at the Central Institute of Mental Health Mannheim. Detailed information can be extracted from Zillich et al. (2022a) and Zillich et al. (2022b). Since the

data analysis is not the main scope of this thesis, the respective methods will be described briefly in the following sections.

2.1.3.1 Data analysis of DNA methylation in human post-mortem brain tissue

The CPACOR-pipeline (Lehne et al., 2015) was used for the first processing of the raw intensity data into methylation data and following Quality Control (QC). Removal criteria for samples were either a low quality of the extracted DNA or methylation-based, and phenotypic sex was not matching. Exclusion criteria for probes were call-rate of <0.95, allele frequency for SNPs of >0.1, or probe allocation to sex-specific chromosomes (X or Y chromosome). After filtering, 657.593- 694.791 methylation sites remained for further analysis, depending on the brain region. Afterwards, association analyses were used with the log2-transformed methylation values as dependent variables. Principal component analysis (PCA) was used to correct for batch effects, and the resulting first ten PCs were used in further association testing as covariates. Since more than 10% of all patient samples did not include information about the smoking status, smoking was estimated by the methylation pattern using a validated set of methylation sites as reference (Maas et al., 2019). This smoking estimate for each sample was included in the analysis as a continuous variable.

Epigenome-wide analysis was performed to compare AUD with control samples, using a linear model including sex, age, PMI, pH, smoking estimate, standardized neuronal cell count, and the first ten PCs as covariates. The brain regions were analyzed separately. Afterward, CN and VS were combined using a meta-analysis approach and the METAL package for R (Willer et al., 2010). P value correction was performed with the Benjamini-Hochberg (FDR) method, and CpG sites were annotated via the Illumina's manifest files (http://webdata.illumina.com.s3-website-us-east-1.amazonaws.com/downloads/product-files/methylationEPIC/infinium methylationepic-v-1-0-b4-manifest-file-csv.zip; downloaded on 10th of August 2018). Differentially methylated regions (DMRs) were calculated by the com-p-algorithm (Pedersen et al., 2012), where autocorrelation of methylation sites in a given window are calculated. The settings were set to Seed-p value <0.01, minimum 2 probes per window, and a sliding window of 500 bp. Multiple testing correction was performed with the Šidák method.

For validation of the most significant finding, pyrosequencing was applied in the DMR of DDAH2 as well as TaqMan Assay on the respective mRNA transcript.

In addition, GO-term analysis as well as GWAS-enrichment and weighted gene correlation network analysis (WGCNA) were performed in the downstream analysis. Detailed information about the analysis methods of this study can be extracted from Zillich et al. (2022a).

2.1.3.2 Data analysis of RNA expression in human post-mortem brain tissue

Despite QC, the analyses were performed with the statistical software R version 3.6.1. For the QC of the fastq files, HISAT2 was used for mapping the raw reads and Rsubread for quantification. After QC, 11 AUD and 13 control samples had to be excluded due to low quality, defined by e.g., significant overrepresentation of specific sequences. The resulting input data was loaded in the DESeq2 (v.1.26.0) package to estimate significant DEGs, which were defined as log2 fold change >0.02 and a Benjamini-Hochberg corrected FDR value of $p<0.05$. Covariates such as smoking, sex, age, pH-value, RIN ad PMI were included in the model to correct for these known influencing cofactors (Durrenberger et al., 2010; Trabzuni et al., 2011). Gene-set enrichment analysis was performed with the R package fgsea (v.1.12.0) using Gene-Ontology (GO) terms and Hallmark gene sets. DEGs with an FDR <0.25 were included for cell-type specific enrichment using GeneOverlap (v.1.22.0).

As a follow-up downstream analysis, WGCNA using the WGCNA package in R (v.1.70.3) was performed to detect co-expression modules, including co-expressed genes and co-methylated CpG sites. Additionally, an overlap of DEGs with FDR<0.25 and significant CpG-sites ($p<0.001$) from the methylation analysis within the same patient sample was estimated using the GeneOverlap package (v.1.22.0) with Fisher's exact test for each brain region separately.

2.2 Study 2: Meta-analysis of transcriptome-wide sequencing approaches across four species

Especially, in context with the low degree of translation into the clinics, that resulted in multiple failed therapeutic approaches, identified by preclinical research, animal experiments in preclinical research become questioned regarding their usefulness and applicability. In the AUD research field, this phenomenon can be observed, as well, since the lack of translation represents one of the major explanations for the limited offer of psychotherapeutic compounds for AUD. We are facing a reproducibility crisis that is driven by multiple examples

where researchers failed to succeed in observing the same effects than the originally published study (Llovera & Liesz, 2016). Eventually, this encouraged the pharmaceutical industry sector to no longer rely on preclinical findings derived from basic research but to rather invest in drug discovery themselves in a more extensive manner (Hyman, 2014). Recently, an ongoing debate in the scientific society about the reproducibility crisis and its potential initiated conglomerates of researchers that aim to establish guidance for high quality pre-clinical research, including experimental planning, pre-registration, and comprehensive reporting (Kilkenny et al., 2010; Spanagel, 2022). In line with these, the 3R principles –reduce, replace, and refine– (Russell & Burch, 1959), which were established at the end of the 50's, got critically revised (Tannenbaum & Bennett, 2015), and their current expansion into the 6Rs –including robustness, registration, and reporting– was introduced (Strech & Dirnagl, 2019). These principles also consider meta-approaches to combine previous studies into one analysis, which increases the sample size, the statistical power, and eventually the meaningfulness of the outcome. The number of individuals included in animal research is usually restricted by the number of scientists and experimental conditions. Even though, experimental animals are usually bred in controlled settings, which ensures that they are genetically more similar than humans, the animal experiments are most of the time statistically underpowered.

To overcome these limitations and to consider certain heterogeneity, that comes with the inclusion of different institutions, individuals, and treatment paradigms, we aimed in this study to perform a meta-analysis on transcriptome-wide datasets derived from four species: mouse, rat, monkey, and human, in a comparative setting. By combining datasets of numerous independent experiments, which increases the sample size to a meaningful extent, this study is meant to provide statistically solid results, that can be used as a basis for follow-up studies on the investigation of the involvement of these identified genes on additional molecular levels as well as on alcohol-related behavior. In addition, by comparing the most frequently used animal species to model alcohol dependence in comparison to the human post-mortem brain, potential species-specific and eventually alcohol-specific features can be observed. A preliminary version of this study can be found at bioRxiv (Friske et al., 2022). In addition, the human meta-analysis was conducted together with Maximilian Haas –who I supervised throughout his Bachelor thesis– and is published as his Bachelor thesis. The meta-analysis in rodents was pre-registered in Open Science Framework

(https://doi.org/10.17605/OSF.IO/TF8R4) and the human post-mortem brain project was pre-registered in Prospero (ID: CRD42020192453).

2.2.1 Systematic Literature Screening

One of the biggest restrictions of transcriptome-wide sequencing approaches is the limited sample size. Therefore, meta-approaches including datasets conducted from various studies are warranted to enable the observation of transcriptomic signatures, considering a larger sample size and higher level of heterogeneity to determine distinct patterns for the alcohol dependent phenotype. Therefore, in this study, we included datasets derived from microarray and RNA-Seq approaches in four species –rats, mice, monkeys, and humans –and three brain regions –the PFC, nucleus accumbens (NAc), and amygdala (AMY)– to determine transcriptomic signatures in a highly comprehensive manner.

The initial step of meta-analytical approaches consists of a systematic literature screening. In this study, the databases PubMed and EMBASE were screened using pre-defined keywords, optimally suited to the respective databases (Figure 3A). For rodent studies on the PD phenotype, the following keywords were used: (alcohol OR ethanol) AND (rats OR mice OR rat OR mouse) AND (ribonucleic acid OR RNA OR RNA-seq OR mRNA OR ncRNA OR miRNA OR siRNA OR piRNA OR snRNA OR microRNA OR expression OR gene OR cDNA OR transcription OR transcript OR transcripts OR transcriptional OR transcriptomic OR translation OR transcriptome OR transcriptomics OR translation) AND (PD OR "post dependent" OR postdependent OR cie OR "chronic intermittent"). For monkey studies, we applied the following keywords: (alcohol OR ethanol) AND ("Primates"[Mesh] OR "monkey" OR "monkeys" OR "ape" OR "apes" OR "macaques") AND (ribonucleic acid OR RNA OR RNA-seq OR mRNA OR ncRNA OR miRNA OR siRNA OR piRNA OR snRNA OR microRNA OR expression OR gene OR cDNA OR transcription OR transcript OR transcripts OR transcriptional OR transcriptomic OR translation OR transcriptome OR transcriptomics OR translation) AND brain. For the screening of the human post-mortem studies, we used the keywords: (“post-mortem” OR “post-mortem” OR “human” OR “patients”) AND (“brain”) AND (“alcohol” OR “alcoholic” OR “alcohol dependence” OR “alcoholism” OR “AUD” OR “alcohol use disorder” OR “alcohol addiction”) AND (“transcriptome” OR “transcriptomic” OR “transcriptomics” OR “gene-expression” OR "gene expression" OR “mRNA” OR “messenger RNA”). Before the start of the screening procedure, inclusion and exclusion criteria were defined to ensure a

consistent and reproducible screening outcome (Figure 3). Since two databases were screened, the literature managing software EndNote was used to deduplicate potential over-represented studies before initiation of the first screening step. The full data screening and extraction procedure was performed based on the well-established PRISMA Guidelines (Page et al., 2021). Hence, two researchers independently screened the literature at each screening step, title, abstract, full text, and data extraction. After each step, the resulting studies were compared, and in terms of discrepancies, a third independent party decided on the inclusion or exclusion of the study for the next screening step. The SysRev screening tool, developed by the CAMARADES research group at the University of Edinburgh (www.syrf.org.uk), was applied to minimize bias and increase traceability and reproducibility. In Figure 3B-C, the total number of resulting studies per screening step is visualized for each species separately, including the eventually resulting number of individuals.

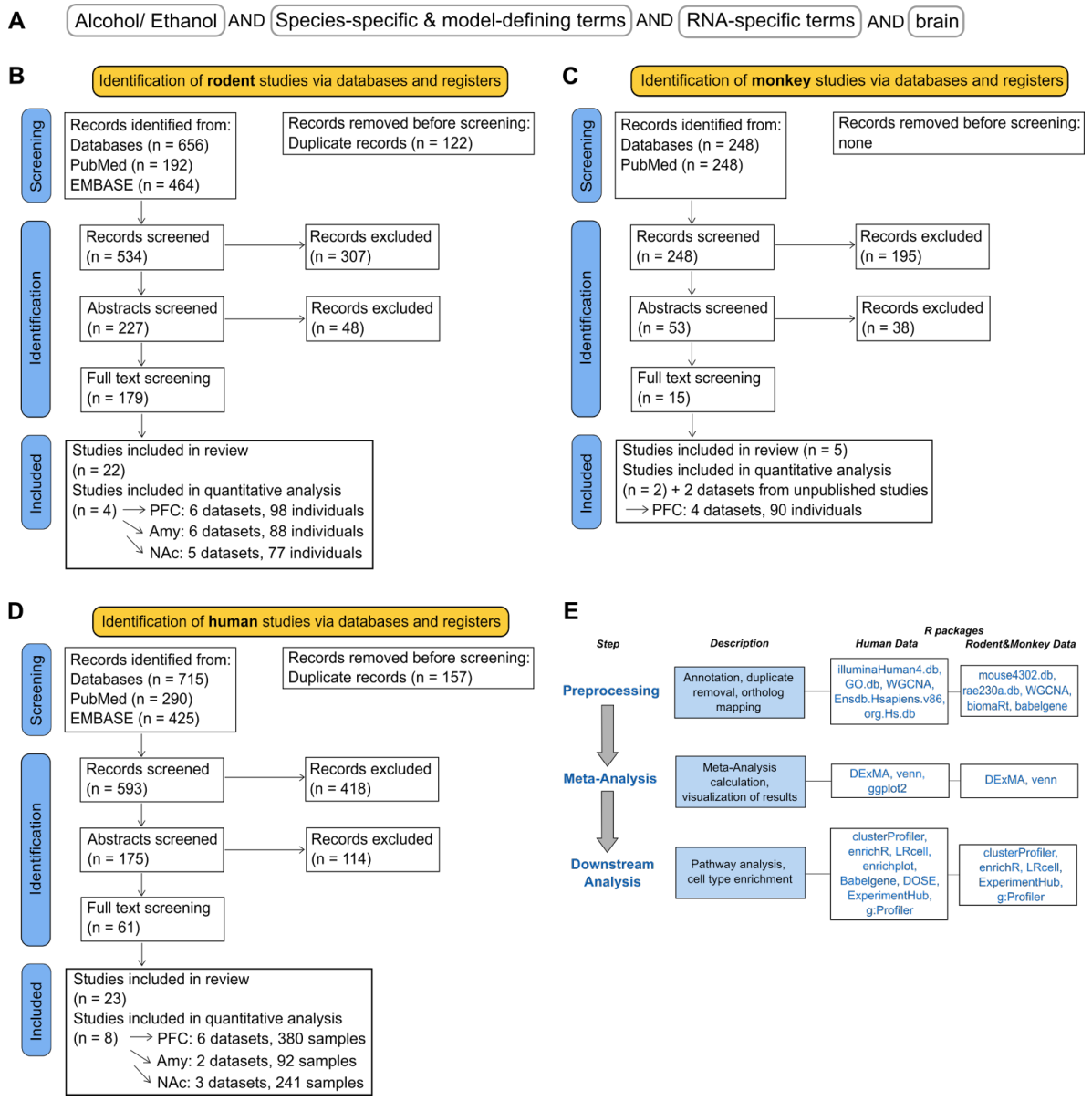

Figure 3: General outline of the keywords designed for systematic literature research in PubMed and EMBASE.

*(**A**) The general design of the search keywords; (**B**) the resulting number of studies for rodent studies; (**C**) monkey studies; (**D**) as well as human post-mortem brain studies. (**E**) Overview of the analysis steps of the meta-analysis using Stauffer's p value combination, including all approached packages for the statistical software R. Before the first screening step (title/abstract screening), duplicates were removed using EndNote. Inclusion criteria rodents: species (rats/mice), alcohol exposure ≥ 2 weeks, post-dependence defined as abstinence prior to death ≥ 3 days, transcriptome-wide RNA expression data from post-mortem brain tissue, high quality RNA samples with RIN ≥ 5, EtOH-naïve controls. Exclusion criteria rodents: adolescence, prenatal alcohol exposure, too short exposure or abstinence period, additional interventions, ethanol-preferring lines/selective breeds for high ethanol intake, non-accessible datasets. Inclusion criteria monkeys: ethanol experience in a chronic treatment, ethanol naïve control animals, transcriptome-wide RNA expression data from post-mortem brain tissue, high quality RNA samples with RIN ≥ 5. Exclusion criteria monkeys: prenatal exposure, no ethanol naïve controls, target gene studies, non-accessible datasets. Inclusion criteria humans: human post-mortem brain, diagnosed AUD based on DSM-IV or similar diagnostics, transcriptome-wide RNA expression data, high quality RNA samples with RIN ≥ 5, control post-mortem samples from healthy individuals. Exclusion criteria humans: comorbid psychiatric disorders, co-abuse of additional substances (despite nicotine), brain injury as cause of death, age < 18 years, non-accessible datasets. Among the studies that matched the inclusion criteria, two rodent studies each included two datasets fulfilling our criteria, which explains the higher number of datasets compared to studies. The template for the overview of the resulting studies was extracted from www.prisma-statement.org.*

Table 3: Details of the included rodent studies for transcriptome-wide meta-analysis.

As indicated in Figure 3, two studies delivered two datasets per study. Details about the screening criteria and keywords can be extracted from Figures 3 A and B.

Study	n	Strain	Treat-ment	Exposure	Abstinence	Brain region	Method
Meinhardt et al., 2013	18(PFC), 16(AMY), 14(NAc)	Wistar rats	CIE Vapor	7 wk	3 wk	mPFC, AMY, NAc	Microarray
Meinhardt et al., 2013	16(PFC), 13(AMY)	Wistar rats	CIE Vapor	4 wk	3 wk	mPFC, AMY	Microarray
Osterndorff-Kahanek et al., 2015	16(PFC), 16(AMY), 16(NAc)	C57BL/6 mice	CIE Vapor	8 wk	120 h	mPFC, AMY, NAc	Microarray
Smith et al., 2016	12(PFC), 12(AMY), 11(NAc)	C57BL/6 mice	CIE Vapor	6 wk	72 h	mPFC, AMY, NAc	Microarray
Smith et al., 2016	12(PFC), 12(AMY), 11(NAc)	C57BL/6 mice	CIE Vapor	6 wk	7 d	mPFC, AMY, NAc	Microarray
Smith et al., 2020	24(PFC), 19(AMY), 24(NAc)	C57BL/6 mice	CIE Vapor (non-drinker)	6 wk	7 d	mPFC, AMY, NAc	Microarray

Table 4: Details of the included human studies for transcriptome-wide meta-analysis.

Details about the screening criteria and keywords can be extracted from Figures 3A and D. Studies including women are represented by the split number of individuals in f= female and m = male. All studies except Hade et al. (2021) obtained brain tissue from the NSWBTRC at the University of Sydney, Australia. The tissue analyzed in Hade et al. (2021) was conducted at the Estonian Forensic Science Institute.

Study	*n (AUD / Ctr)*	*Diagnosis*	*PMI (±SD) (hrs)*	*Brain Region*	*Method*
Liu et al., 2006	13m, 1f/ 10m, 3f	≥80g alcohol per day	28(±14)	Superior FC	Microarray
Ponomarev et al., 2012	17/ 15	DSM-IV	not reported	Superior FC, BLA	Microarray
Manzardo et al., 2013	6m, 3f/ 6m, 3f	DSM-IV	26.3 (±9.6)	PFC	Microarray
Wang et al., 2013	16m, 7f/ 16m, 7f	DSM-IV	not reported	BA9	Microarray
Farris et al., 2015	16/ 16	≥7 yrs drinking, DSM-IV	≤48	BA8	RNA-Seq

Kapoor et al., 2019	51m, 14f/ 60m, 13f	DSM-IV	33.66(±15.59)/ 26.63(±13.25)	BA8	RNA-Seq
Drake et al., 2020	34m, 7f/34m 7f	-	31.3(±12.1)/ 27.4(±12.5)	NAc	RNA-Seq
Rao et al., 2021	30/ 30	DSM-IV	≤48	Superior FC, BLA, NAc	RNA-Seq
Hade et al., 2021	14/13	alcohol-use-disorder-related diagnosis	31.92(±12.88)	PFC	Microarray
Zillich et al., 2022b	48/51	DSM-IV	37.07(±15.79)/ 30.7 (±15.57)	NAc	RNA-Seq

Table 5: Details of the included monkey studies for transcriptome-wide meta-analysis.

Details about the screening criteria and keywords can be extracted from Figures 3A and C.

Study	n	Strain	Treat-ment	Drinking	Absti-nence	Brain region	Method
Bogenpohl et al., 2019	43	Macaca mulatta	Drinking ad libitum	1 yr	Up to 4 h	mPFC (BA24, 15, 32)	Microarray
Walter et al., 2020	23	Macaca fascicularis	Drinking ad libitum	6 m	None	PFC (BA46)	RNA-Seq
Fei et al. (unpublished)	28	Macaca mulatta	Drinking ad libitum	646 d (avg.)	4 wk	PFC (BA46)	RNA-Seq
Fei et al. (unpublished)	24	Macaca mulatta	Drinking ad libitum	226 d (avg.)	None	PFC (BA46)	RNA-Seq

As indicated by the inclusion and exclusion criteria, only studies focusing on transcriptome-wide approaches, such as microarray and RNA-Seq experiments, were considered for the analyses. If provided in the supplementary material, the summary statistics of the eventually resulting studies were downloaded from the original publication. Otherwise, the corresponding authors were contacted to request the respective datasets. Details of the included studies are listed in Tables 3-5. Eventually, the number of datasets to be included in the brain region-specific meta-analyses of rodents was six for the PFC, five for the NAc and five for the AMY. Monkey datasets were mainly retrieved from the PFC (n=5), while for AMY and NAc not sufficient studies were identified to perform a meaningful analysis. For the human meta-analyses, six datasets for PFC, three datasets for NAc, and two datasets for AMY were included (Figure 3B-D). As an indication of the potential bias of the included original studies, systematic reviews and meta-analyses report an assessment of the risk of bias (RoB)

to add input about the value of each study contributing to the final analysis results (Sterne et al., 2019). Unfortunately, in regard to the included studies for these analyses, inadequate reporting was impeding the access to the full information necessary for the RoB assessment. Therefore, I want to strongly point out the warranty of full and comprehensive experimental reporting in publications, not only for analytical approaches like this study, but also for traceability to enable other researchers to fully understand the experiments and interpret the respective outcomes properly.

2.2.2 Meta-Analysis

At the end of the 20th century, microarray-based cDNA analysis was invented (Brewster et al., 2004). The development of this method enabled, for the first time, the simultaneous capturing of thousands of transcripts. The basic principle of microarray consists of pre-existing probes presented on an Affymetrix chip. As depicted in Figure 4, once the RNA is extracted, the molecules are labeled, before reverse transcription into cDNA. Then, the complementary cDNA strand binds to the respective probe on the chip, and after washing away the non-hybridized unspecific strands, the fluorescent signal of the remaining cDNA molecules is detected. The measured differences in intensity of the fluorescent signal correspond to the concentration of the respective transcript. The final measurement outcome is based on relative quantification, whereby the intensity of a treatment is compared to the intensity of a control condition.

In the early 2010's, the RNA-Seq method, as part of the next generation sequencing (NGS) methods, was invented, which enabled a high-throughput method with increased sensitivity. Instead of hybridization to whole sequences of interest and detection of the fluorescent signal of the successfully hybridized strands, the RNA-Seq detection workflow is different (Figure 4). Here, the cDNA first hybridizes to an adapter primer on a flow cell. Then, the remaining cDNA that has not bound to any adapter is washed away, and the complementary strand of the cDNA sequence gets elongated by adding one nucleotide at a time to the adapter primer by performing a so-called bridge amplification. Whenever a nucleotide is successfully hybridized to the cDNA template, the corresponding signal is detected (Ambardar et al., 2016). This sequencing-by-synthesis method generates read counts as a measurement outcome, representing the number of base pairs and their respective nucleotide code per gene sequence. The sample processing, as well as the analysis tools and pipelines for RNA-Seq, are

more harmonized from the beginning of its development on, which is supposed to enable a higher comparability across individual experiments. However, previous studies have shown that microarray and RNA-Seq measure outcomes are highly overlapping but with a higher sensitivity for lowly abundant transcripts in RNA-Seq (Giorgi et al., 2013; Kogenaru et al., 2012; Mooney et al., 2013). Since this study aimed at obtaining the broadest possible picture of pre-existing transcriptome-wide data in the field of AUD, across several species and brain regions and the number of microarray experiments performed in this field is still predominant, I integrated both types into the meta-analytical approach.

Over the years, a variety of different detection and especially, analysis platforms have been developed, which results in a high heterogeneity across the datasets, mainly for the microarrays (Kontou et al., 2018). To overcome this methodological challenge, when aiming to combine these datasets, we integrated the summary statistics of each measured gene per dataset. The retrieved summary statistics consisted of the log2 fold-change, that represents the expression difference between the treatment/disease group and the respective control group. In addition, the respective p value and the corrected p value of each log2 fold-change were reported. By including the probe IDs that were used to identify each measured gene, we were able to map the gene sequences to the latest version of the genome. Since the time frame in which the studies were conducted is comparably large and the precision of the respective genomes has tremendously increased within this time span, it was of great importance to obtain the probe ID for each detected gene per dataset. Additionally, by including the full summary statistics of each detected gene in the experiment and not exclusively focusing on the genes that have been reported as significantly altered within each study, we were able to retrieve the full spectrum of the transcriptome. All analysis steps, including the respective R packages used to perform the analysis, can be retrieved from Figure 3E).

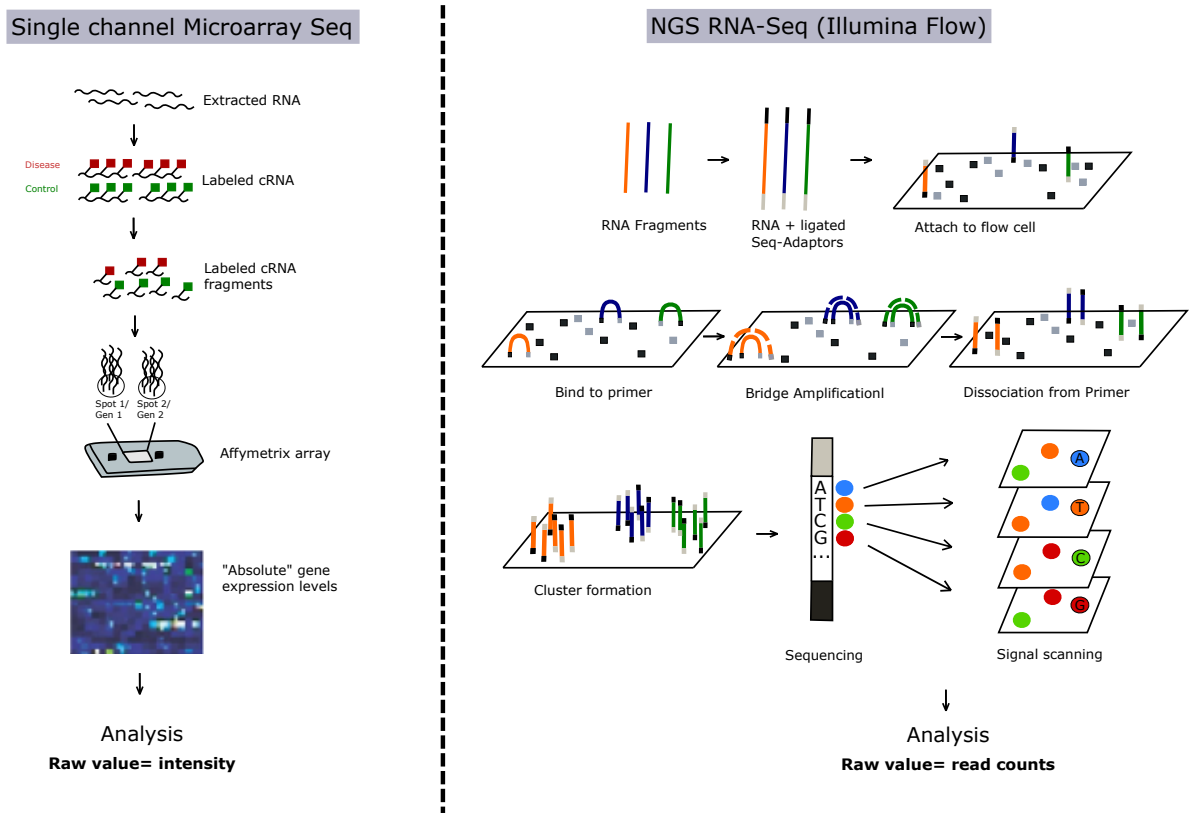

Figure 4: Comprehensive overview of the basic principles of microarray and RNA-Seq.

The visualization of the single-channel microarray workflow was modified based on Staal et al. (2003). The general workflow of Illumina RNA-Seq was modified based on Lu et al. (2016).

Since the effect size, which is represented by the log2 fold-change, was estimated by various methods in the original studies, and additionally, most of the obtained summary statistics did not include a standard error measurement, which is required for estimating effect sizes in an effect size combinatorial meta-analysis approach, we decided to perform a weighted Stouffer's p value combination instead, using the R package DExMA (Villatoro-García et al., 2022). When considering the two methos of meta-analysis despite effect size combination, rank- and p value- based approaches remain (Toro-Dominguez et al., 2021). For rank combination approaches, usually the fold-change is used as basis for the ranking procedure. However, this method is highly sensitive to variance and could therefore lead to false positive results. P value-based meta-analysis methods are suited for data derived from different platforms and heterogeneous analyses. Furthermore, the Stouffer's method enables the usage of weights, which are regularly estimated by including the sample size per study as the root square of the total sample size per datasets (Marot et al., 2009; Whitlock, 2005; Zaykin, 2011) (Equation 1.2). Stouffer's p value combination, which is also referred to as the "inverse normal" method, consists of the transformation of the p value per gene into a Z-score by

including the inverse standard normal cumulative distribution. Afterward, the Z-score is combined with the study weight, which is defined by the square root of the total sample size for the respective study. The resulting p value of the respective final Z-score can then be determined by applying a standard normal distribution table (Stouffer et al., 1949) (Equation 1.1). The mathematical equations of the Stouffer's p value combination method can be extracted from Equation 1.

$$Z_i = \phi^{-1}(1 - p_i) \quad with\ \phi^{-1} = inverse\ standard\ normal\ cumulative\ distribution \quad (1)$$

$$\frac{\sum_{i=1}^{k} \omega i Z i}{\sqrt{\sum_{i=1}^{k} \omega i^2}} \quad with\ \omega = \sqrt{n}\ for\ study\ i \quad (2)$$

Equation 1: Stouffer's p value combination method.

Description of the calculation of the Z-score (1) and the respective test-statistic (2). Weights are introduced by ω, which is calculated as the square root of the sample size per study.

Since the p value combination approach does not include information on the fold-change and whether a gene is up- or down-regulated, per gene the median of the fold-changes derived from the original datasets is calculated as final fold-change indication of the meta-analysis outcome. P values per se depend on the consistent direction of the measured effect. Therefore, to add a value of trustworthiness of the results coming from this meta-analysis, a consistency index (CI) was established, that considers the direction of the fold-changes across the studies and compares them with the final outcome fold-change from the meta-analysis. As described in Equation 2, the CI is calculated by the number of datasets that detect the fold-change in the same direction as the median divided by the total number of included datasets. For the interpretation of the meta-analysis results as well as for the downstream analysis, such as gene set and pathway enrichment analysis, a CI of >0.7 was set as threshold for meta-analyses focusing on one species. For the rodent meta-analysis outcome, which consists of rat and mouse datasets, a less conservative CI of ≥0.5 was determined.

$$CI = \frac{n(fold\text{-}change\ in\ same\ direction\ as\ median\ from\ meta\text{-}analysis)}{n\ (studies\ in\ which\ gene\ is\ measured)}$$

Equation 2: Formular for the calculation of the consistency index (CI) to add value to the outcome of the meta-analyses.

To estimate a level of consistency for the direction of dysregulation of each gene, the direction of the gene in the meta-analysis result, measured as an average across all datasets that detected this gene, was compared to the direction of the same gene within each dataset. The number of datasets that measured the same direction as the meta-analysis fold-change is divided by the total number of studies that detected the gene. In this formula, only the direction of the fold-change (up- or down-regulated) is considered and not the value of the fold-change itself, since an error measure for the fold-change was not provided by most of the datasets.

To minimize potential bias by extreme outliers measured by only a few datasets, the criteria for considering a gene in the meta-analysis were set to a threshold of 60% presence across all datasets per species. As stated above, each dataset was annotated to the latest version of the respective genome to ensure comparability across the datasets. Therefore, the most current databases mouse4302.db, rae230a.db, rheMac10, illuminaHuman4.db, org.Hs.db, and EnsDb.Hsapiens.v86 were used for annotation of the probe IDs into the respective gene encoded by the detection sequence (Carlson, 2021a, 2021b, 2022; Dunning et al., 2015; Rainer, 2017; TBD, 2019). Since some genes were detected by multiple probes, the collapseRows function derived from the R package WGCNA (Langfelder & Horvath, 2008) was applied to estimate an average across these multiple entries per gene. For the combined analysis of rodent datasets, the R package Biomart (Durinck et al., 2009) converted rat genes into their respective mouse orthologs to correct for potentially deviating nomenclatures of genes across these species. Meta-analysis was performed separately for the rodent datasets as well as monkey and human datasets. In addition, each brain region was considered separately within each species. In the visualization of the overlapping DEGs of the included datasets among each other and with the meta-analysis, a Venn Diagram was used with a threshold for defining significant DEGs of FDR<0.1. Subgroup analysis within the rodent analyses was performed to estimate the contribution of rat and mouse datasets to the final outcome of the meta-analysis. The complete workflow of the meta-analysis pipeline and all applied packages is shown in Figure 3E. Each analysis step was performed with the statistical program R Version 4.1.2.

2.2.3 Subgroup analysis rodent datasets

For the datasets derived from the rodent species, both, a combined and a separate analysis was performed to observe common and diverse genes that are dysregulated in the PD rodent models. As mentioned above, the rodent meta-analysis included a less stringent CI threshold because of the heterogeneity of the cohort, while in the other meta-analyses of human and monkey datasets, the same species was considered and therefore, a more conservative CI threshold was applied. To observe how the two species, mouse and rat, contribute to the overall rodent meta-analysis results, the subgroup analysis was performed by considering the species separately. Since for the rat species, two datasets were available, the threshold for inclusion of a gene was 100%, and a CI of 1.0 was set. In the mouse species, the inclusion

threshold of a gene was 70%, and the threshold for CI was 0.7. The results were then compared across the species-specific analyses and additionally considering the rodent meta-analysis by Venn Diagram.

2.2.4 Downstream analysis

To further investigate the meaning of the resulting DEGs of the meta-analyses, biological information was conducted by downstream analyses. Therefore, genes with a CI of at least 0.7 were filtered for analysis of a potential enrichment in gene ontology (GO)-terms, KEGG-pathways, and reactome components. Gene set enrichment analysis (GSEA) was performed using the R package ClusterProfiler (Wu et al., 2021). Network analysis on the protein level was performed with the STRING database (Szklarczyk et al., 2021). In addition, the level of interaction per gene was estimated based on the STRING results, where the number of nodes per gene and the score per node were considered and the corresponding ranking was estimated. The scoring system in STRING is based on various characteristics, such as neighborhood on chromosome, gene fusion, phylogenetic occurrence, homology, co-expression, experimentally determined interaction, database annotated, and automated text mining, which are eventually combined into a final score for each node (Szklarczyk et al., 2023). To test for a potential enrichment of the DEGs in specific cell types, the R package LRcell (Ma, 2021) was applied. The commonly used approach of WGCNA could not be performed in this study since this analysis required the input of raw data, which does not go in line with the general approach of combination of Summary Statistics.

2.2.5 Overlap between human and animal DEGs

One of the main aims of this study is the comparison of transcriptome-wide sequencing results across species in a translational context to identify commonly dysregulated genes for the alcohol-dependent phenotype. Therefore, the investigation into overlapping genes across species, defined as FDR<0.1 and consistency index≥0.7 for each species, is of great importance. The genes derived from rodent, mouse, and monkey meta-analyses were converted into the human orthologs by the Babelgene package for R (Dolgalev, 2022). Visualization of the overlapping DEGs (FDR<0.1) across species was performed using Venn Diagrams.

2.2.6 Molecular validation of the top finding identified in the PFC

Since in the meta-analysis approach –as a p value combinatorial method– not the effect size, but the p value was in the center of the analysis, molecular validation of the most promising finding was performed to ensure the trustworthiness of this finding. Therefore, we performed RT-qPCR on human post-mortem dlPFC/BA9 samples of AUD patients and matched controls derived from the NSWBTRC at the University of Sydney, Australia. The sequences of the designed primers can be extracted from Table 6.

Table 6: Sequences of the primer pairs designed for RT-qPCR to validate the finding of one of the top hits of the meta-analysis.

Gene	Sequence Forward	Sequence Reverse	Product length
Hsd11b1	5'-GGCTTATCATCTGGCGAAGA-3'	5'-CATGGGCAAGGCAGCTACAGT-3'	322
Gapdh	5'-CATGAGAAGTATGACAACAGCCT-3'	5'-AGTCCTTCCACGATACCAAAGT-3'	113
AluSx	5'-GAGGCTGAGGCAGGAGAATCG-3'	5'-GTCGCGCAGGCTGGAGTG-3'	110

A first analysis regarding an observation of the overall effect was performed using analysis of variants (ANOVA) considering condition (AUD, control) and sex as factors. In addition, a follow-up analysis with analysis of covariates (ANCOVA) was performed, considering additional factors such as smoking status, PMI, BAL at timepoint of death, and RIN value of the samples. All statistical analyses were performed using the statistical software R Version 4.1.2. Table 7 represents the detailed sample information of the patients coming from these two cohorts.

Table 7: Demographic information of the cohort that served for the validation experiment of RT-qPCR on Hsd11b1.

Samples from deceased AUD patients and controls are derived from the NSWBTRC at the University of Sydney, Australia. The total number of samples included into this experiment is 153. PMI = post-mortem interval; RIN= RNA integrity number; BAC= blood alcohol concentration (at timepoint of death); Ex= Ex-smokers.

Criterion	AUD (± SD)	Control (± SD)	p value
N	73	80	
Age	55± 11.43	56± 11.26	0.78
Brain pH	6.55± 0.33	6.67± 0.30	0.018*
PMI	30.5± 15.33	27± 15.53	0.20
BAL	47 %	6.4 %	8.895e-06*
Smoking			
Yes	75 %	33 %	8.14e-07*

No **Ex**	16 % 8.3 %	42 % 24 %	0.0013* 0.01*
RIN	7.9± 0.69	7.6± 1.30	0.14
%Women	35.2	31.1	0.75

2.3 Study 3: Single nuclei RNA sequencing in the DMS of PD rats

As evaluated in the introduction, a variety of brain regions and neuronal circuits are affected by alcohol and are supposed to drive the development and maintenance of AUD. One hypothesis focuses on the striatum and states that during the development of AUD, there is a shift in neuronal activity within the striatum from the ventral (VS) to the dorsomedial (DMS; caudate nucleus in primates) and eventually to the dorsolateral (DLS; putamen in primates) part (Chen et al., 2011; Vollstädt-Klein et al., 2010). The VS is involved in cue- conditioning, the DLS is associated with habitual behavior, and the DMS is activated in goal-directed behavior (Balleine & O'Doherty, 2010). Therefore, the working hypothesis of this study is, that during the development of alcohol dependence in the PD rat model, there is a shift from goal-directed to habitual behavior, which is mirrored in the transcriptomic signatures of both, the DMS and the DLS. Besides the molecular examination using cutting-edge techniques of single cell seq, which is part of this thesis, operant experiments observing the self-administration behavior of CIE rats were performed together with Francesco Giannone (Institute of Psychopharmacology, ZI Mannheim).

To date, the number of transcriptome-wide approaches in striatal regions, especially in the DMS and DLS are limited. Furthermore, scSeq approaches are arising in the AUD research field and cell type-specific alterations are highly necessary to understand the disease phenotype and eventually, develop future therapeutic interventions. Therefore, the data derived from this project will add molecular information to the behavioral changes observed in PD rats in an operant setting and in addition, will contribute to understand molecular signatures of AUD in the striatum on a single-cell level.

2.3.1 The PD animal model

As described extensively in paragraph 1.3, the PD phenotype in rats is a commonly used animal model to research alcohol dependence and is induced by chronic intermittent ethanol

exposure (CIE). In our laboratory (approval license by regional council Karlsruhe, Germany: AZ 35-9185.81/G-10/16), male RccHan:Wistar rats at the age of 6 wk derived from Envigo (Pforzheim, Germany) were randomly distributed into treatment and control group and were acclimatized to the housing room and the scientists for one week before the beginning of the experiment. The vapor exposure treatment was performed as reported previously (Gilpin et al., 2008; Meinhardt et al., 2013; Vendruscolo & Roberts, 2014). At the beginning of the experiment, the weight difference between the treatment groups was not significant (PD=184.08±2 g; Ctr=190.67±8 g, n=16/group). Throughout the complete experiment, both groups received ad libitum access to food and water. In the CIE treatment, which endured seven weeks in total, animals were exposed to ethanol (VWR, Darmstadt, Germany, purity: 96%) vaporized air for 16 h per day. For the remaining 8 hours, the animals were exposed to pure air to recover from the treatment. Control animals were housed under equal conditions, but did not receive ethanol-vaporized air at any timepoint. To maintain the optimum ethanol concentration in the vapor chambers, blood alcohol concentrations (BAC) were determined twice per week, directly after the end of the exposure cycle. Therefore, approximately 10 µl blood was taken from the tail tips of the animals and afterwards the samples were centrifuged and the plasma was inserted in the Analox system (Analox, Stokesley, UK) to estimate the BAC of the exposed animals. According to these measurements, the ethanol vapor treatment was adjusted to maintain the animals' BAC between 150 and 300 mg/dl throughout the whole exposure time. Seven hours after the end of the last vapor exposure cycle, the withdrawal scores of both, ethanol-vapor-exposed and control animals were determined by a previously reported method (Macey et al., 1996). Therefore, in a blinded examination process, the aspects of abnormal gait, vocalization, ventral limb retraction and tremors were measured. A value of 0 indicates no noticeable behavior, while a value of 2 indicates highly noticeable behavior. Each criterion was evaluated separately, and the animals were grouped based on the overall withdrawal score, which represents the sum of the four measures. Afterward, both groups were left without any treatment for three consecutive weeks, before they were sacrificed using isoflurane as short-term anesthesia and a guillotine for effective brain removal. At timepoint of death, the PD group weighted 395.09±5 g and control group 466.52±8 g. All rats included in this study were sacrificed at the same timepoint (2 h before the end of the active cycle; light on: 3 am, lights off: 3 pm; ZT22), and the procedure of sacrifice was performed with alternating exposed and control individuals. Directly after

decapitation and consecutive brain removal, the brains were frozen in isopentane and stored at -80°C immediately. The rats with the highest withdrawal score in the PD group ($score_{PD}$=3-6; 1x6, 2x4, 1x3) and the lowest withdrawal score in the Ctr group ($score_{Ctr}$= 0) were chosen for sample preparation for the snRNA-Seq experiment. Afterward, the brain regions DMS and DLS were punched bilaterally and collected, respectively, in Eppendorf tubes until further use. A brief overview of the experimental design can be extracted from Figure 5A.

2.3.2 Tissue preparation for single nuclei RNA sequencing

This project was focused on observing cell type-specific alterations in the DMS. Therefore, this region was punched out using a micro-punching tool on 10 µm brain slices. Since the brains were fresh frozen in -80°C directly after sacrifice of the animals, single nuclei RNA-Seq (snRNA-Seq) was performed instead of single cell RNA-Seq (scRNA-Seq). Hence, the first step was isolating the nuclei from the frozen brain tissue. This step turned out to be challenging, since the isolation of nuclei requires delicate sample handling and specific adjustments due to the instability of nuclei when isolated from the initial cell. Therefore, it was necessary to test a variety of previously well-established protocols to isolate cells for their suitability on nuclei. In the end, we decided to use the 10X Genomics Chromium Nuclei Isolation Kit for single cell 3' and 5' gene expression assays (10X Genomics, Pleasanton, CA, USA), as one of the main advantages is the standardized processing across batches. Briefly, the frozen tissue was first shortly acclimatized to the crushed ice temperature and then homogenized in pre-cooled lysis buffer using one pestle per sample. After centrifugation to pellet the isolated nuclei, debris removal, followed by several washing steps, was performed until a nuclei suspension of good-quality nuclei with a low number of debris was obtained. Since I aimed for a recovery of 10,000 nuclei per sample for the sequencing, I loaded 20,000 nuclei per sample to the 10X Chromium controller for GEM generation (Figure 5) as the first step of the snRNA-Seq library preparation protocol.

2.3.3 snRNA-Seq library preparation and sequencing

Library preparation of the RNA from isolated nuclei for snRNA-Seq was performed according to the 10X Genomics Chromium Next GEM Single Cell 3' Kit v3.1 user protocol. Briefly, the library preparation procedure consists of the generation of so-called gel beads in emulsion

(GEMs) due to the droplet-based methodology (Figure 5B), which consist of the fusion of one 10X gel bead to one nucleus and contain the important barcodes for capturing the nuclei and also the individual molecule to enable correction of inconsistent amplification in the follow-up PCR steps. Once the beads have fused with the nuclei using the 10X Controller (10X Genomics, Pleasanton, CA, USA) to form the GEMs, and the reverse transcription has taken place, the GEMs are broken up to release the constructed cDNA, including the barcodes coming from the GEMs. Afterward, adaptor ligation, fractionation and amplification are performed until the library is complete for sequencing. Due to previous experience with RNA-Seq experiments in the alcohol field, we were expecting a high number of lowly abundant genes in the snRNA-Seq experiment. Hence, we set the sequencing depth of the Illumina NovaSeq 6000 on a S2 100 flow cell to 50,000 reads per nucleus, for 10,000 nuclei per sample. Sequencing was performed at the Life & Brain GmbH at the University of Bonn under the leadership of Dr. Stefanie Heilmann-Heimbach. Figure 5 depicts the experimental design and droplet-based methodology of the 10X Genomics platform for 3' snRNA-Seq.

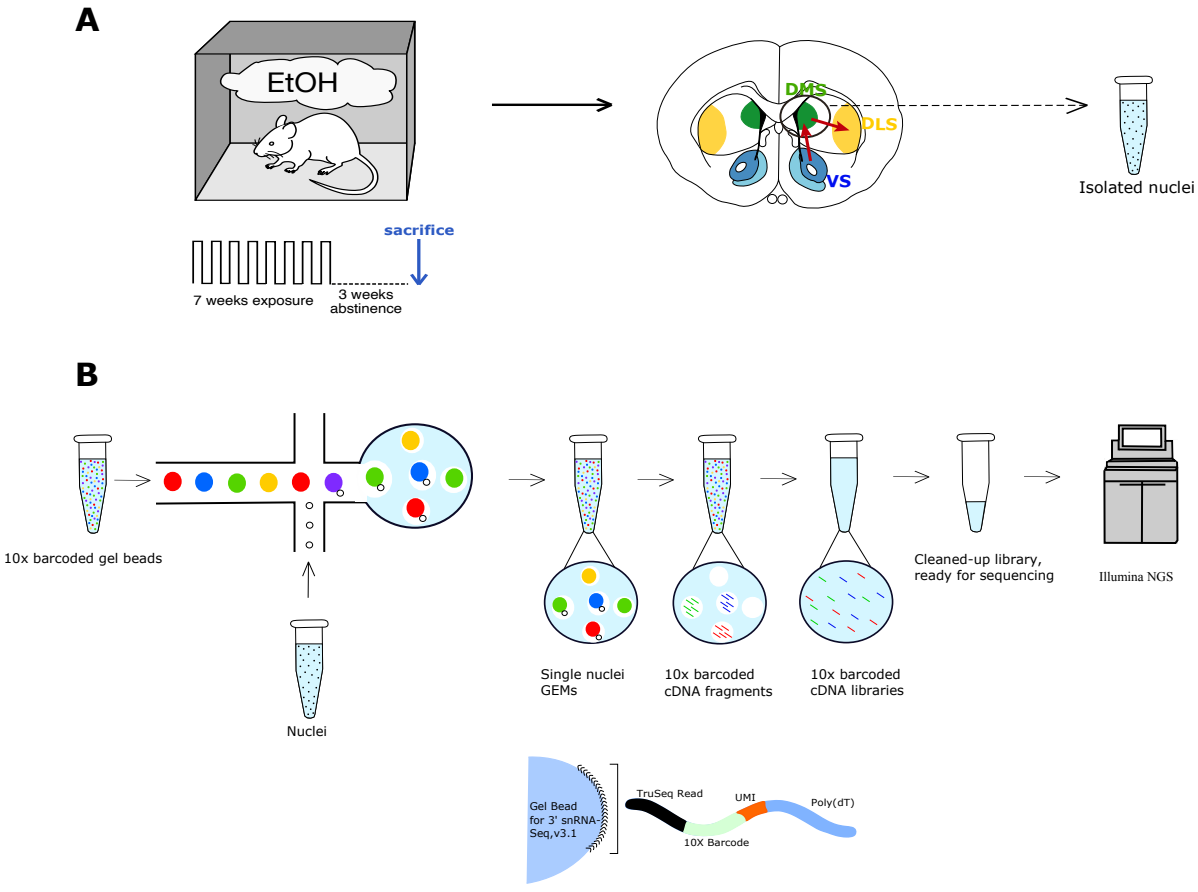

Figure 5: Workflow of the snRNA-Seq experiment on PD rat tissue of the DMS.

*Parts adapted from www.10Xgenomics.org. **A)** Rats receive vaporized ethanol for 7 weeks daily in a chronic intermittent exposure pattern, while control rats are exposed to air. After 3 weeks of abstinence the animals are sacrificed and tissue from the dorsomedial striatum (DMS) is extracted by micro punctures and nuclei are extracted for snRNA-Seq. **B)** Workflow of snRNA-Seq by 10X Genomics. The extracted nuclei are brought together with 10x barcoded gel beads to form gel beads in emulsion (GEMs), which contain one nucleus fused with one gel bead in a lipid droplet. The number of gel beads is a multiple of the number of extracted nuclei that are entered in this fusion process, to prevent doublet formation, which would consist of more than one nucleus fused with the same gel bead. Within the GEMs reverse transcription of the nucleic RNA into cDNA takes place by which the 10X barcode as well as the unique molecular identifier (UMI) are attached to each cDNA molecule. 10x barcodes are individual for each gel bead, which is enabling to obtain information from each captured nucleus in the analysis. The UMI is unique for each molecule and is used in the analysis to correct for amplification bias during the PCR amplification steps of the library generation.*

2.3.4 Data analysis

Data analysis was performed using the 10X Genomics Cell Ranger pipeline to pre-process the raw data. Then, a first observation of the reads mapped to the genome was performed with the 10X Loupe Browser software, and eventually the analysis was performed using the statistical software R with the respective packages for snRNA-Seq analysis.

Since the raw data obtained from the sequencing center in Bonn was already in the required fastq-format, the first step of the data pre-processing consisted of aligning the raw reads to the rat genome. To do so, a custom-made reference genome was developed using the mkref pipeline and the Rnor 6.0 genome for Rattus norvegicus. This step was required because the default genome set provided by 10X Genomics exclusively contains the human and the mouse genome. Subsequently, the sequence reads of the samples were mapped to the genome by the 10X cellranger count command, which additionally performed filtering, barcode counting, and UMI counting. This pipeline generates feature-barcode matrices, determines cell-clusters, and performs gene expression analysis by applying the 10X barcodes (depicted in Figure 5). The output web summary files were examined to receive feedback on the efficacy of the genome mapping procedure and overall sequencing performance. Finally, the cellranger aggr command is used to have one combined file including every sample in one genome-mapped output file by normalizing the runs to the same sequencing depth and then recomputing the feature-barcode matrices and gene expression analysis across all samples. This file was then uploaded into the Loupe Browser software for a first examination of the outcome data.

For the more advanced follow-up analysis, the statistical analysis software R and the packages Seurat v4 (Hao et al., 2021), tidyverse v.2.0, and magrittr v.2.0.3 were applied to perform QC, sample integration, batch correction, normalization and filtering, principal component

analysis (PCA), data visualization, clustering, cluster calling/cell-type identification, and downstream analysis including GSEA.

During the QC, nuclei with more than 5% of mitochondrial reads as well as nuclei with less than 200 UMIs were filtered out. As input, the separate Cell Ranger files, with one file representing one sample and animal, respectively, was used. Before filtering, 1,006-4,110 nuclei per sample (n_{PD}=2,965-3,190 nuclei/sample, median= 3,100 nuclei/sample, total= 12,403 nuclei; n_{Ctr}= 1,006-4,110 nuclei/sample, median= 2,744 nuclei/sample, total= 10,979 nuclei) were identified with 152,591-201,713 reads per nucleus (n_{PD}=152,591-166,880 reads/sample, median= 157,006 reads/sample; n_{Ctr}= 156,123-201,173 reads/sample, median= 273,344 reads/sample) and 935-2,880 median genes captured per nucleus (n_{PD}= 935-1,978 genes/nucleus, median= 1,290 genes/nucleus; n_{Ctr}= 1,988-2,880 genes/nucleus, median= 2,449 genes/nucleus). The final number of reads per nucleus was three times higher than it was initially set up, as we aimed for 50,000 reads per nucleus. Since the set up was estimated for a total number of 10,000 nuclei per sample, whereby eventually, I only obtained one third of this aimed number of nuclei, the sequencing depth per nucleus increased accordingly. After QC, in total 11,819 nuclei remained, which consisted of 6,404 nuclei from control and 5,415 nuclei from PD rats. Subsequently, the Seurat Objects of each sample were integrated into one combined object with the Seurat scTransform function. For dimensionality reduction, the first 40 principal components were included in the uniform manifold approximation and projection (UMAP). For cluster calling, the clusters were tested for cell type- markers, based on the literature, describing cell type-specific experiments in rats, mice, and humans. Once the clusters were identified, DEGs were estimated using the Seurat function FindMarkers per cell type/cluster using the Wilcox test to estimate the significant difference in GEx between the two treatment groups. GSEA was performed using the singleseqgset package v.0.1.1.9000.

2.4 Study 4: Gene expression of COVID-19-related genes across different ethanol treatment paradigms

As stated in the introductory part, the COVID-19 pandemic had a high impact on the global population on both a physical and mental health level. However, the effect of chronic alcohol consumption on the overall propensity to become infected with SARS-CoV2 and to develop

severe disease outcomes, has not been extensively studied. Therefore, we generated target gene expression data from three cohorts with different treatments of ethanol intake: the sub-chronic, the abstinent, and the non-abstinent alcohol paradigm. These treatments were diverse in route of application and duration to mimic diverse ethanol intake behaviors. Since the abstinent and non-abstinent groups underwent ethanol-vapor treatment as described detailed in Section 2.3.1, this procedure will be briefly described in this section. The data of this project is published in Alcohol: Clinical and Experimental Research (Friske et al., 2023), and a comprehensive review targeting the potential influencing factors of chronic alcohol consumption and AUD on the COVID-19 infection propensity and disease severity has been published by us, in addition (Friske & Spanagel, 2023).

2.4.1 Sub-chronic ethanol IP treatment

Male RccHan:Wistar rats (Envigo, Ettlingen, Germany; n=12) at the age of seven weeks were randomly distributed in two categories, with three animals per cage, each and were acclimatized to the housing conditions and the scientists for one week. Throughout the whole treatment, both groups had ad libitum access to food and water. At the beginning of the intra-peritoneal (IP) treatment, T and C animals weighed 242.78 ± 5 g and 235.62 ± 4 g (mean ± SE), respectively. The ethanol treatment group received ethanol injections twice-daily (1.5 g/kg; intraperitoneal (IP); Ethanol supplied from VWR, Darmstadt, Germany, purity: 96%) for two weeks consecutively. At the same time, control animals received IP injections containing corresponding volumes of saline. The injections were given 2 h before the beginning of the active cycle and 6 h after the first injection. On the last day of treatment, 6 h after the second IP injection (ZT22), the animals were euthanized with isoflurane (CP-Pharma, Burgdorf, Germany; purity: 1ml/ml) and sacrificed by decapitation (weight T: 296.43 ± 8 g, C: 322.33 ± 12 g). Immediately after, lung, liver, ileum, kidney, and heart were dissected, flash-frozen in isopentane and stored at -80°C until further use. The time of sacrifice was chosen based on the circadian regulation of the Ace2 gene expression, which peaks at ZT22. In addition, it was of great importance to ensure that the ethanol from the last injection was completely metabolized. A detailed overview of the whole experimental procedure is depicted in Figure 6. The included experimental procedures were approved by the regional council Karlsruhe, Germany (license: AZ 35-9185.81/G-208/16) and performed according to the European and German guidelines.

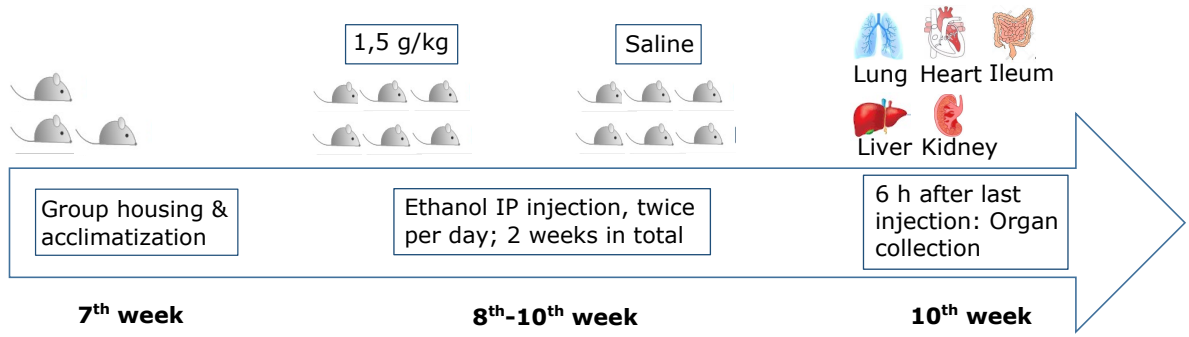

Figure 6: Illustrated description of the sub-chronic ethanol IP treatment in male Wistar rats.

According to the timetable, the animals arrived in the laboratory at the age of 7 weeks. The two weeks ethanol IP treatment started after one week of acclimatization to the housing and handling conditions. For molecular analysis, lung, heart, ileum, liver, and kidney were dissected 6 h after the last injection and afterwards stored at -80°C until RNA extraction was accomplished. The figure is based on Friske et al. (2023).

2.4.2 Chronic intermittent ethanol vapor exposure

The procedure to generate the PD phenotype was followed, as described in Section 2.3.1. In addition, the non-abstinence group was included, whereby the animals underwent the same schedule of vapor exposure but were sacrificed during the last day of vapor treatment and consequently, did not undergo any abstinence phase after the vapor treatment. Since these animals were sacrificed two hours before the end of the last vapor exposure, withdrawal scores of all groups were measured one day before the last exposure cycle, according to section 2.3.1. All groups had ad libitum access to water and food throughout the whole procedure. In the following, the PD phenotype will be called “abstinence group” to appropriately point out the two different treatment strategies of the vapor experiment. The animals of each group were sacrificed at the same time point of the circadian cycle, which is also similar to the time point of sacrifice in the sub-chronic IP treatment (ZT22). Throughout the vapor treatment, one animal died, resulting in a total sample size of 5 + 6 animals in the non-abstinence group and 6 + 6 animals in the abstinence group. Figure 7 is depicting a detailed overview of the experimental procedure.

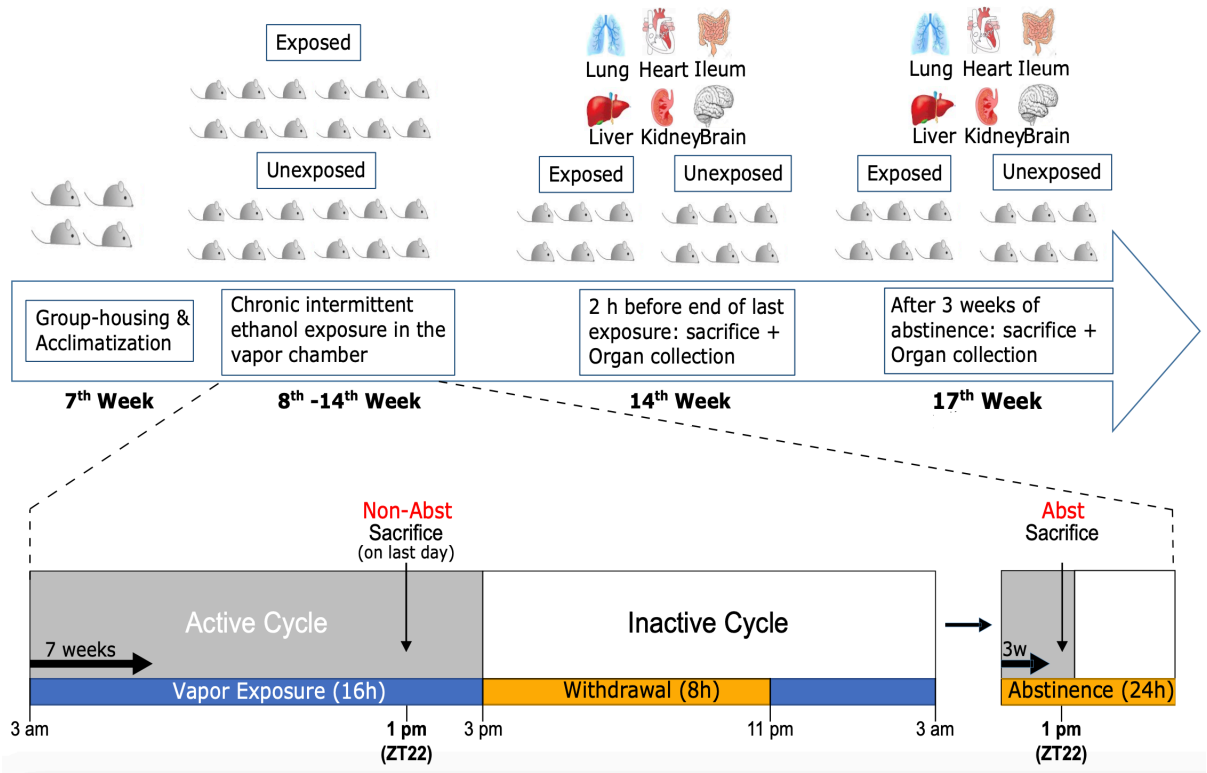

Figure 7: Graphical description of the two vapor treatment groups and their respective control groups.

***Top**: Experimental procedure of vapor exposure, sacrifice and organ collection. After one week of acclimatization to housing conditions and the scientists, the animals were exposed to ethanol vapor for seven weeks consecutively. The non-abstinence group was sacrificed throughout the last exposure cycle, whereas the non-abstinence group was sacrificed after three weeks of abstinence at the same circadian timepoint. **Bottom**: Detailed description of the ethanol vapor treatment. The animals were exposed for 16 h daily for seven weeks. One exposure cycle ended with the end of the active cycle. Animals were sacrificed two hours before end of the active cycle both (ZT22). This timepoint was chosen to match the sub-chronic group from experiment 2.4.1. The dissected organs were flash-frozen in isopentane and stored at -80°C until further use. The figure is based on Friske et al. (2023).*

2.4.3 RNA extraction and RT-qPCR

The fresh frozen organs kidney, lung, heart, liver, ileum, and brain of the three treatment groups and their respective controls were used for RNA extraction using Trizol (Thermo Fisher, Waltham, MA, USA) and the RNeasy MicroElute RNA extraction kit (Qiagen, Hilden, Germany). Reverse transcription was performed using the High- Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Waltham, MA, USA), before the samples were randomly distributed on RT-qPCR plates to measure RNA expression levels of Ace2, Tmprss2, Mas and Gapdh as housekeeping gene (primer sequences are listed in Table 8). RT-qPCR was performed with three technical replicates, and outliers were defined as deviation of greater than 5% from the mean of the triplicates.

Table 8: Primer pair constitution of the three COVID-19 target genes Ace2, Tmprss2 and Mas and the housekeeping gene Gapdh for normalization of the target gene expressions.

Each gene primer consists of one forward and one reverse sequence

Gene	Sequence forward	Sequence reverse	Product length
Ace2	5'-TGCGTATGAATGGACCGA-3'	5'-CAGGATAACAATGCCAACCAC-3'	388 bp
Tmprss2	5'-GGTTTGGGCACATAGG-3'	5'-CACAGGCAATGGGTAGTGTTC-3'	251 bp
Mas	5'-CCCAAGCACCAGTCGGCATTC-3'	5'-AGGCCCATGTGTTCTTCCGTA-3'	229 bp
Gapdh	5'-CATGAGAAGTATGACAACAGCCT-3'	5'-AGTCCTTCCACGATACCAAAGT-3	113 bp

2.4.4 In-situ hybridization in rat brain tissue

To measure the gene expression across a variety of brain regions, in situ hybridization on 10 µm thick coronal sections was performed as previously demonstrated (Uhrig et al., 2017). The 18 brain regions of interest, covered the following regions: cingulate cortex (Cg), infralimbic cortex (IL), prelimbic cortex (PrL), orbitofrontal cortex (OF), caudate putamen (CPu), nucleus accumbens core (AcbC) and shell (AcbSh), paraventricular nucleus (PVN), cornu ammonis area 1 (CA1), cornu ammonis area 3 (CA3), cornu ammonis area 4 (CA4), dentate gyrus (DG), basolateral amygdala (BLA), central amygdala (CeA), basomedial amygdala (BMA), medial amygdala (MeA), ventral tegmental area (VTA), nucleus tractus solitarii (NTS). The slices, representing the respective areas of interest, were collected using the rat brain atlas of Paxinos and Wilkins (Paxinos & Watson, 1998). For appropriate analysis of the expression patterns, the Allen Brain Atlas was used (https://mouse.brain-map.org/gene/show/30018).

2.4.5 Statistical analysis

Data analysis for RT-qPCR was performed for each target gene separately, considering the individual organs as factor. When observing the Ct values of the housekeeping genes, we discovered that ß-Actin showed organ- and treatment-specific expression changes. Therefore, this gene did not serve as a housekeeping gene in this study and was excluded from the analysis. Hence, we solely included Gapdh to normalize the gene expression analysis of the target genes. dCt values, defined by the difference between the gene of interest and the housekeeping gene, were calculated, normal distribution was observed, and extreme outliers were excluded, defined by greater than ±3x interquartile range. Linear Mixed Model (LMM) was utilized to test differences in dCt expression values in an organ-wise expression

comparison of the treatment groups. LMM was chosen over repeated measure ANOVA, since for LMM, covariance structures, that best fit the dataset, can be modeled, which is ensuring appropriate analysis of longitudinal data (Gueorguieva & Krystal, 2004). The categorical factors were represented by groups, whereas the organ was chosen for the repeated factors. Maximum Likelihood (ML) estimation was applied, since random effects were excluded in the analysis model (Feld, 2013). Due to the lowest BIC value, first-order autoregressive (AR(1)) covariance or heterogeneous autoregressive ARH(1) covariance was applied (Wolfinger, 1996). In addition to the peripheral organs, data derived from the PFC and the olfactory bulb were analyzed, using LMM, with treatment group as categorical factors and brain region as repeated factors. Follow-up Fisher LSD post-hoc test was conducted, regardless of the significance of the interaction of a significant main effect, to maintain the exploratory aspect of this study and eventually, to obtain as much information about the data as possible. Additionally, effect sizes represented by Cohen's d were reported for every organ and brain region, that showed significant treatment-specific outcome after the post-hoc test.

For the RNA in-situ hybridization experiment, data analysis was conducted using multiple t-tests. Input values were obtained in Nanocurie per gram (nCi/g), via conversion of the final mean gray values, using the software MCID Analysis 7.1. Extreme biological outliers, defined by greater than $\pm$3x interquartile range, were excluded. Technical outliers were defined by greater than $\pm$2x standard deviation. Further information can be obtained from Table 8.

The sample sizes for each treatment were estimated in advance by the power analysis method G*Power. The corresponding Cohen's d was calculated for two-tailed unpaired t-test with $\alpha = 0.05$ and power = 0.8 in order to obtain an effect size of Cohen's d of 2.0. Statistical analysis as well as graphical visualization of the data were conducted with IBM SPSS Statistics 27 and GraphPad Prism 8.4.3.

3. Results

3.1 Study 1: Epigenetic and transcriptomic signatures of human post-mortem brain tissue using bulk seq approaches

3.1.1 Epigenetic signatures of human post-mortem brain tissue result in the highest number of altered methylation sites and regions in the VS and CN

In this study, we performed epigenome-wide association analysis on five brain regions derived from human post-mortem brain tissue of AUD patients and matched control individuals: ACC, Brodmann Area 9 (BA9), as part of the dorsolateral PFC, CN, VS, and the PUT. The results of this project have already been published (Zillich et al., 2022a). While I performed sample preparation, Lea Zillich (Department of Genetic Epidemiology in Psychiatry, ZI Mannheim) analyzed the data. The identification of epigenome-wide significant methylation sites (DMS, Table 9) resulted in 18 DMS in the VS, and two CpG sites in the CN. The remaining three regions, BA9, ACC, and PUT, did not show any epigenome-wide significant DMS. Two significant CpG-sites located in *PIEZO2* and *GLANT9*, respectively, were additionally found to be differentially methylated in the CN with a nominal p value of ≤0.023. When comparing the results with a previously published epigenome-wide association study (EWAS) of peripheral blood (Witt et al., 2020), three epigenome-wide significant CpG-sites from the VS were significantly overlapping with the findings in blood: cg27512762 (*PCAT29* gene), cg06427508 (*KLHL6* gene), and cg02849689 (non-coding position).

Table 9: Overview of the significant DMS in CN and VS.

ES= Effect size; Std Err= Standard Error; FDR= FDR-corrected p value; n.c.= non-coding position. Since the methylation detection was performed in two batches, the direction of the respective methylation sites is represented with two arrows.

Chr	Position	CpG-Site	Gene symbol	ES	Std Err	P value	FDR	Region
15	78729669	cg04214706	*IREB2*	-0.39	0.07	7.6e-08	0.03	CN
5	74633012	cg26685658	*HMGCR*	-5.92	1.11	8.5e-08	0.03	CN
8	117961971	cg17163967	*SLC30A8*	0.50	0.09	1.1e-08	0.007	VS
1	178998656	cg23933289	*FAM20B*	0.27	0.05	2.4e-08	0.008	VS
15	69908472	cg27512762	*PCAT29*	0.17	0.03	6.8e-08	0.016	VS
7	1008720	cg02028351	*COX19*	0.18	0.03	1.3e-07	0.017	VS
16	68563886	cg02941431	n.c.	-0.25	0.05	1.3e-07	0.017	VS
3	183274235	cg06427508	*KLHL6*	0.38	0.07	1.4e-07	0.017	VS
12	132882652	cg16767842	*GALNT9*	0.24	0.05	1.7e-07	0.017	VS

16	4901809	cg02741291	*UBN1*	0.58	0.11	2.6e-07	0.023	VS
16	1946176	cg10824492	n.c.	-0.15	0.03	3.4e-07	0.026	VS
19	35168316	cg18564234	*SCH1B2P*; *ZNF302*	-0.78	0.15	4.1e-07	0.029	VS
13	73687406	cg06630619	n.c.	-0.43	0.09	4.8e-07	0.03	VS
11	1215457	cg23618269	*MUC5AC*	-0.43	0.09	5.4e-07	0.03	VS
14	96177134	cg13545750	*TCL1A*	-0.23	0.05	7.2e-07	0.039	VS
5	79331052	cg04360099	*THBS4*	0.30	0.06	1.0e-06	0.048	VS
6	29400397	cg26754552	n.c.	0.28	0.06	9.9e-06	0.048	VS
11	59390857	cg02849689	n.c.	-0.29	0.06	1.2e-06	0.048	VS
18	11147785	cg12049992	*PIEZO2*	-0.28	0.06	1.2e-06	0.048	VS
17	18210650	cg16021181	*TOP3A*	-0.31	0.06	1.1e-06	0.048	VS

The determination of differentially methylated regions (DMRs) resulted in ten DMRs for the CN, where the three hits with the lowest p values were annotated to *DDAH2*, *CCDC152*, and *CAMSAP1* (Table 10). The VS showed six DMRs, with the genes *TMEM232*, *FANCD20S*, and *HM13* representing the regions with the lowest p values. One DMR was found in the ACC, which is associated with the *HCG9* gene, while no DMR was detected in PUT or BA9. For the purpose of validation, the DMR in the *DDAH2* gene in the CN was replicated using pyrosequencing (cg04074004: t(76.77)=2.39, p=0.019). Additionally, the gene expression of this gene was measured using RT-qPCR to explore the effect of this DMR on the transcriptomic level. However, there was no significant differential expression of *DDAH2* in the CN observed.

Table 10: Overview of the significant DMRs in the CN, VS, and ACC.

Chr= Chromosome; Start= Starting sequence for sequencing; End= End sequence for sequencing; N= number of probes; Gene= Gene Symbol describing the gene represented by the respective sequence reads.

Chr	Start	End	N	CpG-Site	Gene	Z_p	Sidak p	Region
6	31696063	31696520	17	cg08550588	*DDAH2*	1.9e-20	2.8e-17	CN
5	42756786	42757024	6	cg18472410	*CCDC152*	3.1e-11	9.1e-08	CN
9	138800324	138800391	3	cg00022024	*CAMSAP1*	2.8e-10	2.9e-06	CN
6	55039232	55039383	4	cg10725720	*HCRTR2*	1.1e-08	5.3e-05	CN
3	10149974	10150225	7	cg00166722	*FANCD2OS*	1.6e-07	0.0004	CN
3	49459909	49460058	5	cg21926782	*AMT; NICN1*	1.6e-07	0.0007	CN
3	170136766	170136921	4	cg02241055	*CLDN11*	4.4e-07	0.002	CN
2	175190675	175190822	4	cg02216981	*LINC01305*	5.1e-07	0.002	CN

1	15272238	15272384	5	cg11648522	*KAZN*	5.9e-07	0.003	CN
14	67940414	67940426	2	cg06215536	*TMEM229B*	5.7e-08	0.003	CN
16	68270252	68270388	6	cg04513006	*ESRP2*	7.7e-07	0.004	CN
5	110062343	110062730	11	cg23279021	*TMEM232*	4.0e-11	7.3e-08	VS
3	10149963	10150225	8	cg10729496	*FANCD2OS*	8.4e-10	2.2e-06	VS
20	30135144	30135292	5	cg15815607	*HM13*	3.6e-08	0.0002	VS
15	22833149	22833309	6	cg07959104	*TUBGCP5*	5.2e-07	0.002	VS
17	18210638	18210651	2	cg03540694	*TOP3A*	6.2e-07	0.03	VS
7	99765805	99765819	2	cg18810646	*GAL3ST4*	6.8e-07	0.03	VS
6	29943401	29943426	4	cg23244913	*HCG9*	1.2e-10	3.3e-06	ACC

GO-Analysis in CN revealed association with “homophilic cell adhesion via plasma-membrane adhesion molecules” (p=5.37e-6, q=0.12) and “cell-cell adhesion via plasma-membrane adhesion molecules” (p=1.68e-5, q=0.19) and in the VS “Lsm 1-7 Pat2 complex” (p=6.49e-5, q≈1). However, none of these pathways remained significant after correction for multiple testing.

Since the initial methylation analyses showed significant results only in the CN and VS, WGCNA of these two regions exclusively will be described here. WGCNA identified 15 modules for CN (median size= 965 CpG sites, range: 49- 10330 CpG sites per module), with the module “black” having the strongest association with AUD. The CpG sites of this module were additionally enriched for the GO term “PML body” (p=0.001) and “G-rich strand telomeric DNA binding” (p=0.001). In the VS, 14 modules were identified with a median size of 611 CpG sites (range: 38-12721 CpG sites per module), where the “purple” module had the strongest association with AUD. In addition, the CpG sites of this module were enriched for “regulation of T-cell proliferation” (p=4.32e-6), “regulation of leukocyte cell-cell adhesion” (p=6.83e-6) and other immune system-related pathways.

3.1.2 Transcriptomic signatures of human post-mortem brain tissue

We observed the gene expression (GEx) in human post-mortem brain tissue derived from three striatal regions: VS, CN, and PUT. The outcome of this Multiomics study, including the findings from Section 3.1.1, is published in Translational Psychiatry (Zillich et al., 2022b). While I performed sample preparation, Lea Zillich (Department of Genetic Epidemiology in

Psychiatry, ZI Mannheim) analyzed the data. In the CN, 49 genes resulted as significantly dysregulated (FDR<0.05), while in the PUT, one gene was found significantly altered, and in the VS, none were below the threshold of FDR<0.05. Interestingly, in the CN ¾ of the significant DEGs were up-regulated, with the lowest FDR for *TTLL4* (FDR= 0.0005). Among the significantly down-regulated genes in the CN *GATA2* (FDR= 0.0091) represented the lowest FDR corrected value. In the PUT, the only gene with an FDR corrected p value <0.05 was *DES* (FDR=0.0486), which was found to be down-regulated. Even though in the VS, no gene reached FDR<0.05, three genes were close to significance. *CLPB*, *ARHGEF15*, and *CHRNA9* reached an FDR-corrected p value of 0.065, and all three genes were down-regulated in the AUD samples. Since the resulted genes had on average high p values, a less conservative FDR-corrected p value cut-off of 0.25 was chosen for the downstream analysis and the comparison across the striatal brain regions. Therefore, *ARHGEF15* has been found to be the only common DEG across the three regions and was consistently up-regulated.

Table 11: Significant differentially expressed genes (DEGs) resulting from the RNA-Seq experiment in the striatal regions VS, CN, and PUT with a significance threshold of FDR<0.05.

For CN 49 DEGs were identified, for PUT one. Due to space reasons, for CN only the top 10 DEGs are listed in this table. Data is also reported in Zillich et al., 2022b.

Gene Symbol	Log2FC	P value	FDR	Brain Region
TTLL4	0.11	2.3e-08	0.0005	CN
GATA2	-0.27	8.6e-07	0.009	CN
CNOT10	0.06	1.3e-06	0.009	CN
CDHR3	0.19	1.9e-06	0.011	CN
SLC26A5	0.28	3.8e-06	0.016	CN
ASTL	1.01	6.8e-06	0.024	CN
HSPA6	0.98	8.9e-06	0.027	CN
LGR6	0.64	1.2e-05	0.031	CN
EGFL7	-0.22	2.0e-05	0.037	CN
RIPPLY2	-0.15	2.1e-05	0.037	CN
DES	-0.86	2.6e-06	0.0486	PUT

For downstream analysis, GSEA was performed with all DEGs having an FDR<0.25. In this analysis, only the PUT showed significant enrichment in GO-terms and Hallmark gene-sets with FDR<0.05, which resulted in significant enrichment for “adaptive immune response” (FDR=0.006) and “acute inflammatory response to antigenic stimuli” (FDR=0.006). Cell type

enrichment analysis resulted in significant results in the CN exclusively, where the up-regulated DEGs were enriched for astrocytes (FDR=7e-6) and the down-regulated DEGs were enriched for endothelial cells (FDR=2e-7). WGCNA analysis identified the highest number of modules for the PUT, with 25 modules representing a median size of 249 genes with a range between 33 and 5381 per module. The strongest correlation with AUD status was found in the "e-PUT-black" module (r=0.41, p=2.31e-4). In the CN, 21 modules were identified with median size of 352 genes per module (range from 64 to 7259 genes), where the strongest association with AUD was shown in the "e-CN-magenta module", represented by 328 genes (r=0.42, p=2.89e-4). 16 modules were identified in VS (median size 429 genes, range: 35-9708 genes per module) and the "e-VS-pink module" was associated with AUD (r=0.41, p=0.009). The gene STAT3 was represented within all the three modules "e- PUT-black", "e-CN-magenta", and "e-VS-pink", which led to the identification as conserved hub node across the three striatal regions. Gene network analysis, considering the in total 174 shared genes across the modules of the three brain regions, *STAT3*, *TP53*, *ICAM1*, *MYC*, and *NFKBIA* were the most significant hub nodes of the network representing conserved nodes across the observed striatal regions.

Table 12: Genes commonly significantly dysregulated in both omics layer methylation and GEx with a significance threshold of FDR<0.25 in the gene expression analysis.

Significant findings were detected in the CN solely. Values are represented as ES meth. (=effect size of methylation) for methylation or log2FC (log2 fold change) for gene expression with the respective p value and FDR corrected p value.

Gene Symbol	ES meth.	P value	FDR	Log2FC	P value	FDR
ERMAP	0.3	8.2e-05	0.273	0.07	1.6e-02	0.243
IP6K2	0.2	4.0e-04	0.436	-0.05	1.7e-02	0.247
BRD2	-7.69	2.9e-04	0.409	0.06	8.3e-03	0.188
FAM299B	-0.34	5.5e-04	0.462	0.09	1.4e-02	0.225
DYNLT1	0.61	2.8e-04	0.405	0.10	8.5e-03	0.189
KLHL7	5.26	4.7e-04	0.449	-0.05	6.3e-04	0.078
C9orf34	-0.29	9.3e-04	0.535	0.22	4.2e-03	0.143
PFKFB3	3.03	9.7e-04	0.544	0.13	2.6e-03	0.120
HIPK3	0.39	8.9e-04	0.531	0.05	1.1e-02	0.205
SAC3D1	-0.13	7.6e-04	0.509	-0.12	3.2e-03	0.129
TMEM10	-0.14	4.8e-04	0.449	-0.18	2.5e-03	0.119
ZNF295-AS1	0.19	1.3e-04	0.319	0.33	1.4e-04	0.0511

When considering the genes that were commonly significantly dysregulated in GEx as well as methylation analyses, the CN was the only brain region that showed overlapping genes (n= 12 genes). WGCNA considering both the methylation and GEx analysis outcome, resulted in a significant overlap between GEx module “e-CN-magenta” and the methylation modules “m-CN-red” (p=0.003) and “m-CN-midnightblue” (p=0.014). When considering the WGCNA modules of GEx and methylation in the VS, the module “e-VS-salmon” had significant overlap with the methylation module “m-VS-turquoise” (p=0.003). In the PUT none of the GEx and methylation patterns showed significant overlap.

3.2 Study 2: Meta-analysis of transcriptome-wide sequencing approaches across four species

This study aimed to perform a meta-analysis of transcriptome-wide datasets derived from previously published human post-mortem brain as well as animal models of alcohol dependence. Therefore, the brain regions PFC, NAc and AMY were investigated, since the number of studies identified by systematic literature screening led to the scope of the study (Figure 3). In addition, previous research has pointed out, that these brain regions have high relevance in AUD (Figure 1). For the PFC, the species human, mouse, rat, and monkey were analyzed, while for NAc and AMY, there were no sufficient numbers of monkey datasets identified. A preliminary version of this study can be found at bioRxiv (Friske et al., 2022). In addition, the human meta-analysis was conducted together with Maximilian Haas –who I supervised throughout his Bachelor thesis– and is published as his Bachelor thesis.

3.2.1 Meta-Analysis in the PFC of four species

Comparison of DEGs derived from the original datasets result in high heterogeneity across studies.

After systematic literature screening of the rodent studies, six datasets derived from four studies were conducted (Meinhardt et al., 2013; Osterndorff-Kahanek et al., 2015; Smith et al., 2016; M. L. Smith et al., 2020), which resulted in a total of 106 individuals (Figure 3A, Table 3). When observing the reported significant DEGs with a significance threshold of FDR <0.1, three original datasets detected DEGs (Smith et al., 2016, 72h abstinence; Smith et al, 2016, 7d abstinence; Smith et al., 2020) (Figure 8A). In comparison to the rodent meta-analysis,

seven genes were commonly significant across these three original datasets and the meta-analysis, namely *4921534E14RIK* (FDR_{MA}=5.82e-05), *9430092D12RIK* (FDR_{MA}=1.71e-07), *Fgf16* (FDR_{MA}=0.049), *H4c9* (FDR_{MA}=5.59e-07), *Nid2* (FDR_{MA}=0.0001), *Slc30a5* (FDR_{MA}=0.042), and *Spata48* (FDR_{MA}=0.0007). In addition, between 16-51 % of the identified DEGs from the original datasets overlapped with at least one other dataset, which describes a high heterogeneity across the individual datasets; especially when considering, that only half of the datasets in total identified any significantly altered transcripts. Hereby, it has to be addressed, that the two datasets derived from Smith et al., 2016 have a notably higher amount of shared DEGs (n=184 DEGs) than the dataset from Smith et al., 2020 (n= 11-32 DEGs). The two datasets derived from Smith et al., 2016 had both the same ethanol exposure duration, but differing abstinence periods, since this study was investigating a time-course analysis, considering diverse abstinence periods after a common exposure length (Smith et al., 2016). The three original datasets that reported significant DEGs were derived from mice, which leads to the hypothesis that the result of the rodent meta-analysis might be primarily driven by the mouse species. Therefore, a subgroup analysis was performed by considering the datasets from the rat and mouse species separately and then comparing these results with the rodent meta-analysis.

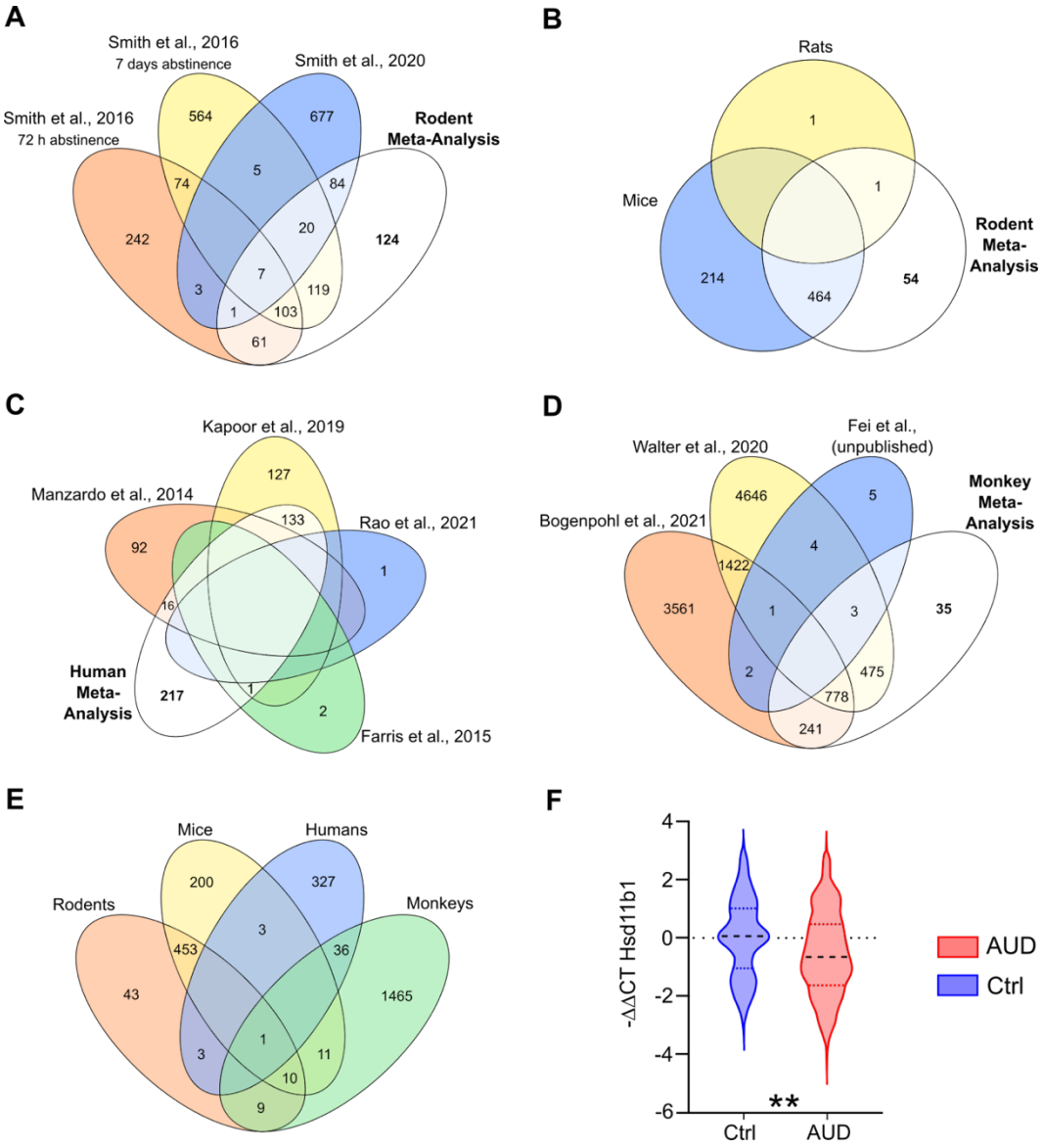

Figure 8: Venn Diagrams comparing significant DEGs (FDR<0.1) found in the PFC from original studies and different species-specific meta-analyses.

***A) Rodent meta-analysis**. Comparison of DEGs from original rodent studies with the rodent meta-analysis. The original studies that identified significant DEGs were all derived from mouse brain tissue. **B) Sub-group analysis** considering rat and mouse datasets separately. According to the overall number of DEGs derived from this species-specific meta-analysis and the respective overlap with the rodent meta-analysis, it can be concluded that the mouse datasets (n=4) contribute to a higher extend to the overall outcome of the rodent meta-analysis than the rat datasets (n=2). **C) Human meta-analysis**. DEGs of original studies from human post-mortem brain tissue and the results of the human meta-analysis. No gene was found to be commonly dysregulated across in all studies. 217 genes were uniquely identified as DEGs by the meta-analysis, which have not been found to be significantly dysregulated in any of the original datasets. **D) Monkey meta-analysis**. Three out of the four identified studies reported significantly altered transcripts, whereby most of these genes do not overlap with any of the other studies. The meta-analysis resulted in 1532 DEGs, whereby 35 DEGs were uniquely identified. **E) Cross-species comparison**. Comparison of the DEGs derived from the species-specific meta-analyses in rodents, mice, humans and monkeys. One DEG is conserved throughout all species, namely Hsd11b1. This gene is down-regulated in human, rodent and mouse meta-analysis and up-regulated in monkey meta-analysis. **F) Molecular validation of top finding Hsd11b1**. Validation of Hsd11b1 down-regulation in an independent human brain sample cohort by performing RT-qPCR of Hsd11b1 mRNA in human brain tissue from AUD (n=73) and control (n=80) samples derived from both the NSWBTRC Sydney. The significant down-regulation of this gene, as identified in the meta-analysis could be successfully replicated by this sample set (FC= 0.76, p= 0.0002). Overall, only studies that reported significant DEGs (FDR<0.1) appear in the venn diagrams, whereas for the meta-analysis, all datasets were included, regardless of the presence of significant results. Further information about the top 10 DEGs as well as the enriched pathways can be drawn from Table 13-15 and Table 16-18, respectively.*

Table 13: Top 10 DEGs identified by rodent meta-analysis in the PFC, sorted by FDR-corrected p value.

MedianFC= median fold-change; FDR= FDR-corrected p value; PropData=Proportion Data, describes the proportion of datasets that detected the gene in the experiment; CI= consistency index.

Gene	MedianFC	p value	FDR	PropData	CI
9430092d12Rik	-0.33	7.9e-12	1.7e-07	0.5	1.0
492153e14Rik	-0.48	5.4e-09	5.8e-05	0.5	1.0
D5ertd163e	-0.28	9.4e-09	6.8e-05	0.5	1.0
Sox14	0.53	2.4e-08	0.00013	0.5	0.67
Fam243	-0.25	4.8e-08	0.00017	0.5	1.0
Nid2	0.14	4.7e-08	0.00017	0.83	1.0
Pkp1	-0.46	1.72e-07	0.0005	0.5	1.0
C78704	-0.23	2.2e-07	0.0006	0.5	1.0
D730005E14Rik	0.21	3.12e-07	0.0007	0.5	0.67
Spata48	-0.25	3.27e-07	0.0007	0.5	0.67

Table 14: Top 10 DEGs identified by human meta-analysis in the PFC, sorted by FDR-corrected p value.

MedianFC= median fold-change; FDR= FDR-corrected p value; PropData=Proportion Data, describes the proportion of datasets that detected the gene in the experiment; CI= consistency index.

Gene	MedianFC	p value	FDR	PropData	CI
KCNK10	0.19	2.49e-10	2.17e-06	0.625	1.0
MT1X	0.59	8.42e-11	2.17e-06	0.75	0.83
MGEA5	0.20	1.94e-10	2.17e-06	0.625	1.0
ODC1	0.18	1.94e-09	1.26e-05	0.75	0.83
IFITM3	0.36	2.68e-09	1.40e-05	0.875	0.71
GPR85	-0.22	7.96e-09	3.47e-05	0.625	0.8
KCNJ16	-0.25	9.49e-09	3.55e-05	0.875	1.0
RERGL	-0.45	2.24e-08	7.31e-05	0.625	0.8
ANGPTL4	0.37	3.97e-08	9.43e-05	0.875	0.86
RASD1	0.27	5.92e-08	0.0001	1.0	0.75

Table 15: Top 10 DEGs identified by monkey meta-analysis in the PFC, sorted by FDR-corrected p value.

MedianFC= median fold-change; FDR= FDR-corrected p value; PropData=Proportion Data, describes the proportion of datasets that detected the gene in the experiment; CI= consistency index.

Gene	MedianFC	p value	FDR	PropData	CI
DUSP4	1.18	1.05e-34	3.68e-30	0.75	1.0

SPRY2	0.17	2.9e-14	5.08e-10	0.75	1.0
DUSP6	0.39	1.53e-10	6.61e-07	1.0	1.0
SPRY4	0.42	1.53e-10	6.61e-07	1.0	0.75
TRIM63	-0.27	1.7e-10	6.61e-07	0.75	1.0
CHORDC1	0.26	2.74e-09	7.36e-06	1.0	1.0
SPRYD3	-0.15	3.61e-08	5.74e-05	1.0	0.75
FLNA	-0.25	4.18e-08	6.09e-05	1.0	1.0
BHLHE40	0.39	5.09e-08	6.85e-05	1.0	1.0
CTDSPL	-0.26	8.89e-08	9.72e-05	0.75	1.0

Table 16: Pathway enrichment analysis of the rodent PFC meta-analysis results using the Reactome database with a p value cut-off of FDR<0.2.

ES= Enrichment score; NES= normalized enrichment score.

Pathway	ES	NES	p value	FDR
Formation of the cornified envelope	-0.741	-1.798	8.1e-05	0.07
Keratinization	-0.706	-1.796	0.0001	0.07
Deposits of new CENPA-containing nucleosomes at the centromere	-0.798	-1.749	0.0006	0.123
Nucleosome assembly	-0.749	-1.749	0.0007	0.123
Chromatin modifying enzymes	-0.639	-1.639	0.0005	0.123
Chromatin organization	-0.615	-1.639	0.0005	0.123
Nonhomologous End-Joining (NHEJ)	-0.799	-1.775	0.0008	0.124
RMTs methylate histone arginines	-0.802	-1.721	0.001	0.124
HDR through homologous recombination (HRR) or single strand annealing (SSA)	-0.662	-1.684	0.001	0.124
Homology directed repair	-0.659	-1.678	0.0009	0.124

Table 17: Pathway enrichment analysis of the human PFC meta-analysis results using the Reactome database with a p value cut-off of FDR<0.2.

ES= Enrichment score; NES= normalized enrichment score.

Pathway	ES	NES	p value	FDR
Innate immune system	0.642	1.547	4e-06	0.004
Neutrophil degranulation	0.683	1.618	5e-05	0.017
Cytokine signaling in immune system	0.652	1.566	4e-05	0.017
Antimicrobial peptides	0.96	1.657	0.0001	0.028
Cellular responses to stimuli	0.612	1.471	0.0002	0.037
Platelet degranulation	0.803	1.736	0.0004	0.057

ER-Phagosome pathway	0.827	1.695	0.0005	0.057
Interleukin-10 signaling	0.881	1.683	0.0005	0.057
Hemostasis	0.633	1.518	0.0004	0.057
Response to elevated platelet cytosolic Ca2+	0.781	1.701	0.0009	0.081

Table 18: Top 10 findings of the pathway enrichment analysis of the monkey PFC meta-analysis results using the Reactome database with a p value cut-off of FDR<0.2.

Since the reactome database does not contain information about the monkey species, I first converted the DEGs derived from the meta-analysis in their human orthologs and run the reactome approach afterwards. ES= Enrichment score.

Pathway	ES	NES	p value	FDR
Extracellular matrix organization	-0.6	-1.946	2.5e-05	0.022
Collagen chain trimerization	-0.829	-1.985	5.5e-05	0.025
NCAM1 interactions	-0.79	1.968	0.0001	0.025
Muscle contraction	-0.613	-1.875	9.6e-05	0.025
ECM proteoglycans	-0.716	-1.983	0.0002	0.046
NCAM signaling for neurite out-growth	-0.726	-1.941	0.0003	0.046
Smooth muscle contraction	-0.793	-1.909	0.0003	0.046
Interleukin-17 signaling	0.923	2.238	0.0007	0.06
Integrin cell surface interactions	-0.699	-1.923	0.0006	0.06
Collagen biosynthesis and modifying enzymes	-0.761	-1.912	0.0006	0.06

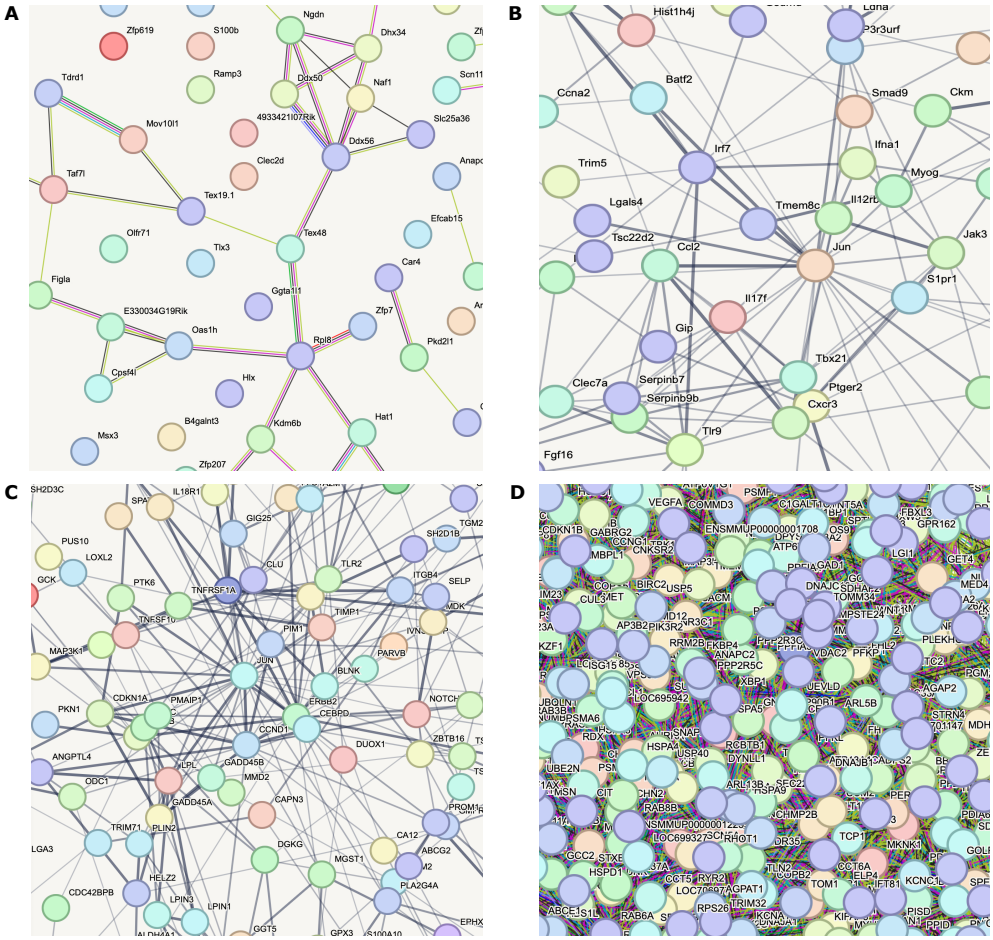

Figure 9: STRING network analysis.

*Due to comprehensibility, only a representative cutout of networks representing nodes with the highest degree of connections are presented per species. As input served the interaction of the significant DEGs (FDR<0.1) identified by rodent (**A**), mouse (**B**), human (**C**), and monkey (**D**) meta-analysis. **A**) Representative cutout of the **rodent** analysis, including top 10 proteins with the highest degree of connections, such as DDX56 (n=6), RPL8 (n=5), NGDN (n=5) and DHX34 (n=4). **B**) Representative cutout of the **mouse** analysis including top 10 proteins with the highest degree of connections, such as JUN (n=26), TBX21 (n=13), CCL2 (n=12) and TLR9 (n=12). **C**) Representative cutout of the **human** analysis including top 10 proteins with the highest degree of connections, such as JUN (n=30), ERBB2 (n=27), CCND1 (n=21) and TLR2 (n= 14). **D**) Representative cutout of the **monkey** analysis. As depicted in the figure, the density of nodes is too high to identify strongly interacting proteins by eye. The proteins with the highest degree of connections are AKT1 (n=109), POLR2A (n=91), CUL1 (n=78) and ISG15 (n=77).*

Rodent meta-analysis identified 520 significant DEGs, while the outcome seems to be mainly driven by the datasets derived from the mouse species.

The rodent meta-analysis identified an overall number of 520 genes that were significantly dysregulated in the PD phenotype, when considering an FDR threshold of <0.1. Furthermore, 124 DEGs were solely identified by combining the datasets, which corresponds to 24% of all significant genes identified by the meta-analysis. When considering the original datasets with significant findings, the highest overlap with the meta-analysis results was detected for the Smith et al., 2020 datasets (72h: n=172 DEGs, 33% of DEGs of MA; 7d: n=249, 48% of DEGs of

MA), while the least overlap was found for Smith et al., 2020 (n=112, 22% of DEGs of MA). GSEA pointed towards dysregulation of chromatin organization and maintenance, as well as apoptotic processes. STRING protein-network analysis was conducted to identify potentially high interconnected genes on the protein level, considering the identified DEGs of the meta-analysis with an FDR threshold <0.1. *Ddx56* was identified as the highest interconnected DEG for the rodent outcome (Figure 9A). However, the node degree, which is calculated by the number of connections to other nodes, to describe the interaction of the respective protein with the other proteins of the dataset, is comparably low in respect to the mouse, human and monkey STRING analysis result (Figure 9 B-D).

As mentioned above, the observation of DEGs in the original datasets led to the hypothesis that the mice data might generally have a higher contribution to the outcome of the rodent meta-analysis results. Therefore, I performed a subgroup-analysis considering mouse and rat data, separately (Figure 8B). Mouse meta-analysis resulted in 678 DEGs with an overlap of 68% with the rodent meta-analysis results. Rat-specific meta-analysis led to 2 DEGs with an 50% overlap with the rodent meta-analysis and 0% overlap with the mouse-specific meta-analysis (Figure 8B). The high number of common DEGs between the mouse-specific meta-analysis and the rodent meta-analysis, while rat-specific meta-analysis is leading to a limited number of results, strengthens the hypothesis, that the results of the rodent meta-analysis are strongly driven by the mouse datasets. However, it has to be recognized that the number of rat datasets (n=2) is comparably low. Since mouse-specific meta-analysis resulted in an additional interesting outcome, we included these results in the cross-species comparison approach, that will be described further in the following paragraphs.

In the human PFC, 370 DEGs were identified from which 217 DEGs were not identified by the original studies.

For the human meta-analysis of the PFC, eight datasets (Farris et al., 2015; Hade et al., 2021; Kapoor et al., 2019; Liu et al., 2006; Manzardo et al., 2013; Ponomarev et al., 2012; X. Rao et al., 2021; Wang et al., 2013) resulting in 380 samples were included (Figure 3D, Table 4), whereby four of these studies reported significantly altered genes (Figure 8C). Notably, no common DEG was identified across these four datasets, and only one gene was identified as significant in two datasets –Kapoor et al. (2019) and Farris et al. (2015)– and was significant in the meta-analysis, as well. Any other overlap was solely identified between one original

dataset and the meta-analysis. The portion of DEGs identified in the original studies that were overlapping with the meta-analysis outcome ranged from 0-52 %, whereby Kapoor et al. (2019) showed the highest overlap, while the remaining studies had 0-15 % overlap (Figure 8C).

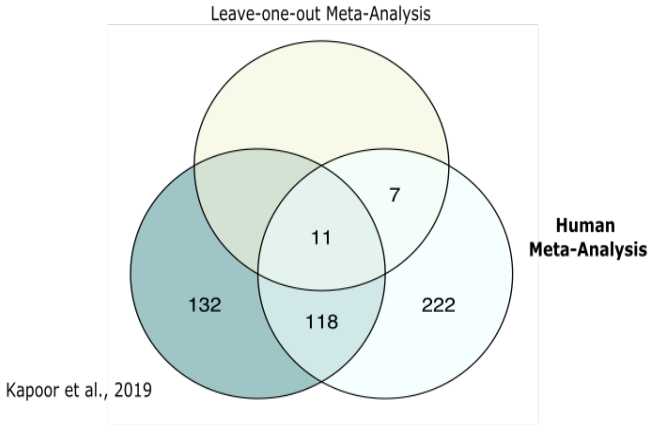

Figure 10: Leave-one-out (LOO) meta-analysis.

Kapoor et al. (2019) showed the highest overlap of DEGs with the meta-analysis outcome (Figure 8C) and also the biggest sample size. Therefore, a LOO meta-analysis was performed to estimate the potential bias introduced by this study. It was assumed that, if the LOO meta-analysis represents a subset of the meta-analysis outcome, Kapoor et al. (2019) does not introduce a significant bias to the overall findings, but does contribute to additional findings that are pronounced by the increased sample size.

Since Kapoor et al. (2019) had the highest overlap with the meta-analysis results and also the biggest sample size (Figure 8C, Table 4), which is more than double the sample size of the other datasets included, it was hypothesized that this study might contribute significantly stronger to the outcome of the human meta-analysis outcome than the remaining datasets. Therefore, a leave-one-out (LOO) meta-analysis was performed by excluding the Kapoor et al. (2019) dataset in a separate meta-analysis and comparing the outcome with the human meta-analysis result (Figure 10). It was assumed, that, if the LOO meta-analysis represents a subset of the total human meta-analysis, the dataset from Kapoor et al. (2019) does not introduce a pronounced bias to the overall human meta-analysis outcome. As depicted in Figure 10, the outcome of the LOO meta-analysis does represent a subset of the human meta-analysis with a 100% overlap, which leads to the conclusion, that the inclusion of the dataset from Kapoor et al. (2019) does not lead to a warped result, but does enable the detection of additional DEGs that would not have been detected without increasing the sample size to this extent. However, the number of DEGs identified by the LOO meta-analysis are only 5% of the DEGs identified by the human meta-analysis ($DEGs_{MA}$=358, $DEGs_{LOO}$=18). Next, the potential enrichment of the 370 significant DEGs from the meta-analysis in biological pathways was

investigated. Hereby, basic cell membrane functions, such as ether lipid metabolism and linoleic acid metabolism, immune system-related components, mineral absorption as well as ErbB signaling, and COVID-19-relevant processes, were enriched (Table 17). STRING analysis pointed towards a high cross-interaction of *Jun*, *Erbb2*, and *Ccnd1*, with a number of connections per node between 21 and 30 (Figure 9C). The human meta-analysis results were the only output with a significant enrichment of the DEGs in specific cell types, which resulted in astrocytes (FDR=7.57e-07), endothelial cells (FDR=1.76e-05), and inhibitory neurons (FDR=0.0013).

Meta-analysis of the monkey PFC resulted in 1532 DEGs with 35 genes uniquely identified by this approach.

Systematic literature screening identified two datasets from monkey PFC tissue (Bogenpohl et al., 2019; Walter et al., 2020). In addition, Prof. Robert Hitzemann and Prof. Suzanne Fei from the OHSU Oregon (USA) provided us with two unpublished datasets, which increased the sample size to 90 individuals in total (Figure 3C). By observing the original datasets of the monkey PFC (Figure 8D), it is apparent, that the overall number of DEGs is higher than in the datasets derived from rodents and humans. While rodent and human datasets identified between 1-892 DEGs, the monkey studies reported a maximum number of 7,329 DEGs. Therefore, it is not surprising, that the meta-analysis of the monkey datasets resulted in 1,532 DEGs. Additionally, the overlap with at least one original dataset is comparably higher than in the other species with only 35 genes (=2% of the overall meta-analysis outcome) uniquely identified by the monkey meta-analysis. The tremendously increased number of DEGs for both, the original datasets and the monkey meta-analysis, might be explained by the timepoint of tissue collection. For most of the included datasets, the monkeys were still intoxicated when they were sacrificed (Table 5), and it is known, that the alcohol intoxication state causes an acute alteration of multiple transcripts, which do not sustain after extended abstinence (Smith et al., 2016). GSEA resulted in cell adhesion, immune signaling, and muscle contraction being significantly enriched in the PFC of the alcohol-dependent monkeys (Table 18). STRING protein network analysis showed the highest interactions for *AKT1*, *POLR2A*, and *CUL1*.

Cross-species comparison in the PFC across four species suggests that *HSD11B1* dysregulation might be conserved for the alcohol dependent phenotype.

Since the subgroup analysis of the rodent data pointed towards a high number of DEGs derived from the mouse species (Figure 8B), the mouse-specific meta-analysis was added as a fourth dataset in the cross-species comparative approach (Figure 8E). When observing the Venn Diagram, it is notable, that a higher number of DEGs from the human meta-analysis overlap with the monkey meta-analysis (n=37, 10 %) than with the rodent (n=4, 1 %) or mouse (n=4, 1 %) meta-analysis. The overlap of human and rodent meta-analysis results in four common DEGs: one commonly down-regulated, *HSD11B1* ($FDR_{rodents}$= 0.001, FDR_{humans}= 0.001), two commonly up-regulated genes, *ITM2C* ($FDR_{rodents}$= 0.09, FDR_{humans}= 0.053) and *RNF122* ($FDR_{rodents}$= 0.16, FDR_{humans}= 0.07), and one inconsistently regulated gene, which was down-regulated in rodents and up-regulated in humans, *RAB26* ($FDR_{rodents}$= 0.03, FDR_{humans}= 0.09). Three additional genes were commonly significant in the mouse and human meta-analysis, namely *TSPAN12* (FDR_{mice}= 0.058, FDR_{humans}= 0.029), *EVA1C* (FDR_{mice}= 0.098, FDR_{humans}= 0.02), and *JUN* (FDR_{mice}= 0.03, FDR_{humans}= 0.079). Interestingly, *JUN* was also found to be the highest interacting gene in the STRING analysis for both, mice and humans (Figures 9B&C). When considering all species, one gene was significant among all of them, which is *HSD11B1* ($FDR_{rodents}$= 0.001, FDR_{mice}= 0.027, FDR_{humans}= 0.001, $FDR_{monkeys}$= 0.059). The fold-change direction of this gene was negative in rodent (medianFC= -0.02), mouse (medianFC= -0.32) and human (medianFC= -0.19) meta-analysis and positive in the monkey meta-analysis (medianFC= 0.79). The opposite direction of *HSD11B1* in the monkey species might be explained by the lack of abstinence in most of the included individuals, which leads to the hypothesis, that the direction of the dysregulation of *HSD11B1* might be depending on the state of intoxication. However, these data suggest that *HSD11B1* might be relevant for the alcohol dependent phenotype, regardless of species and treatment paradigm. Since the chosen meta- analytical approach was Stouffer's p value combination, an effect size estimation is not included, which points out the importance to validate the findings of the analysis. Therefore, I investigated the validation of the down-regulated *HSD11B1* in our in-house sample of human post-mortem brain tissue of the PFC in a total sample size of n=153 (Figure 8F). The significant down-regulation of *HSD11B1* in the PFC was replicated (FC= 0.76, FDR= 0.0002) and in addition, the previously reported up-regulation in brain tissue depending on age, which has been associated with cognitive decline (Bini et al., 2020; Gregory et al.,

2020), was also found in this sample. However, when considering age as a cofactor, *HSD11B1* remains significantly down-regulated, irrespective of the age of the individuals.

When comparing the downstream analyses across the species, it is apparent that each species shows its own pronounced emphasis of dysregulated pathways (Table 16-18). However, some common phenomena can be observed, such as immune-regulatory pathways that appear to be significantly impaired in both, the human and monkey species (Table 17&18).

3.2.2 Meta-Analysis in the NAc and the AMY of ethanol exposed rodent and human AUD patient samples

The brain regions NAc and AMY were meta-analyzed, since systematic literature screening was resulting in a sufficient number of studies for these brain regions in rodents and humans. Including these brain regions was considered to add value to the analyses in a brain region-specific, but also in a brain-wide manner, leading to region-specific as well as brain-wide signatures.

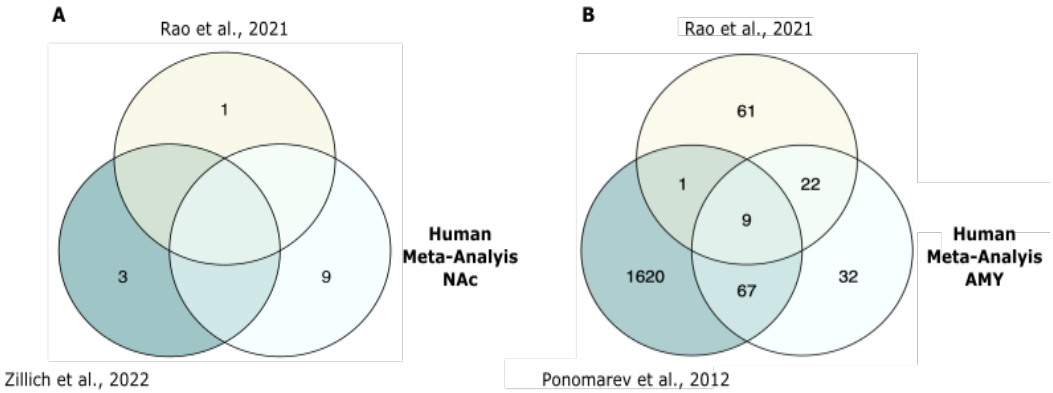

Figure 11: Venn Diagrams comparing significant DEGs (FDR<0.1) found in the NAc and AMY from the original studies and the meta-analyses.

In both, the NAc as well as the AMY, I did not identify any DEGs in rodents in both, the original data as well as the meta-analysis outcomes. Also, subgroup-analysis considering datasets derived from mice and rats separately, did not result in any significant DEGs. ***A) Human meta-analyses of the NAc*** *found nine DEGs (FDR<0.1) with no overlapping gene between the meta-analysis and the included studies.* ***B) Human meta-analysis of the AMY*** *identified 130 DEGs with nine DEGs commonly appearing in Rao et al. (2021), Ponomarev et al. (2012) and the meta-analysis.*

Table 19: Top 9 DEGs identified by human meta-analysis of the NAc, sorted by FDR-corrected p value.

MedianFC= median fold-change; FDR= FDR-corrected p value; PropData= Proportion Data, describes the proportion of datasets that detected the gene in the experiment; CI= consistency index.

Gene	MedianFC	p value	FDR	PropData	CI
ENPEP	0.68	1.12e-07	0.003	1.0	1.0
SERPINA3	0.68	7.79e-06	0.045	1.0	1.0
SLC7A2	0.27	2.77e-05	0.09	1.0	1.0
EDN1	0.84	4.64e-05	0.09	0.84	1.0
IL1R2	1.12	3.16e-05	0.09	1.0	1.0
ANXA1	0.98	3.49e-05	0.09	1.0	1.0
MCL1	0.3	4.06e-05	0.09	1.0	1.0
S100A8	0.91	6.11e-05	0.09	1.0	1.0
CEBPD	0.39	6.03e-05	0.09	1.0	1.0

Table 20: Top 10 DEGs identified by human meta-analysis of the AMY, sorted by FDR-corrected p value.

MedianFC= median fold-change; FDR= FDR-corrected p value; PropData=Proportion Data, describes the proportion of datasets that detected the gene in the experiment; CI= consistency index.

Gene	MedianFC	p value	FDR	PropData	CI
GADD45A	0.67	3.71e-08	0.0004	1.0	1.0
TIMP1	1.07	7.26e-08	0.0004	1.0	1.0
SERPINA3	1.99	3.65e-07	0.0009	1.0	1.0
ANGPTL4	0.97	6.95e-07	0.001	1.0	1.0
MDK	0.65	6.3e-07	0.001	1.0	1.0
MT1X	0.85	1.81e-06	0.003	1.0	1.0
EMP1	1.02	3.13e-06	0.003	1.0	1.0
SERPING1	0.39	3.84e-06	0.004	1.0	1.0
HMGB2	0.35	5.99e-06	0.005	1.0	1.0
IFITM3	0.76	7.06e-06	0.006	1.0	1.0

Table 21: Pathway enrichment analysis of the human NAc meta-analysis results using the Reactome database with an input p value cut-off of FDR<0.2.

ES= Enrichment score NES= normalized enrichment score.

Pathway	ES	NES	p value	FDR
Neutrophil degranulation	0.813	1.65	1.57e-05	0.004
Immune system	0.698	1.44	1.06e-05	0.004
Signaling by Interleukins	0.806	1.63	0.0001	0.019
Cytokine Signaling in Immune system	0.76	1.55	0.0001	0.019

Translation	-0.603	-1.79	0.0002	0.028
Hemostasis	0.789	1.59	0.0004	0.039
Innate immune system	0.729	1.49	0.0005	0.042
Ion channel transport	-0.658	-1.75	0.004	0.2
Platelet degranulation	0.87	1.57	0.003	0.2
Response to elevated platelet cytosolic Ca^{2+}	0.87	1.57	0.003	0.2

Table 22: Pathway enrichment analysis of the human AMY meta-analysis results using the Reactome database with a p value cut-off of FDR<0.2.

ES= Enrichment score; NES= normalized enrichment score.

Pathway	ES	NES	p value	FDR
Interleukin-4 and Interleukin-13 signaling	0.88	1.67	9.49e-05	0.04
Signaling by Interleukins	0.74	1.52	0.0001	0.04
Cytokine Signaling in Immune system	0.71	1.47	7.6e-05	0.04
Hemostasis	0.69	1.43	0.0002	0.06
Cellular responses to stimuli	0.65	1.34	0.0004	0.08
Platelet degranulation	0.82	1.59	0.0006	0.11
Resolution of D-Loop Structures	-0.88	-1.88	0.001	0.11
Resolution of D-loop Structures through Holliday Junction Intermediates	-0.88	-1.88	0.001	0.11
Response to elevated platelet cytosolic Ca^{2+}	0.81	1.59	0.001	0.11
Apoptotic cleavage of cellular proteins	0.91	1.58	0.001	0.11

Rodent and human Meta-analysis in NAc suggests a less important meaning of this brain region for long-term alcohol dependence.

Rodent PD samples were conducted from five datasets coming from four studies (Meinhardt et al., 2013; Osterndorff-Kahanek et al., 2015; Smith et al., 2016; M. L. Smith et al., 2020), which results in 77 individuals in total, whereby all of these studies were already included in the meta-analysis of the PFC (Figure 3B, Table 3). When observing DEGs across the original datasets, it is notable, that none of the datasets reported significant DEGs and combining these datasets in the meta-analysis, still no gene appeared to be significantly altered. In human post-mortem brain tissue of the NAc, three datasets with a total number of 241 samples were included in the analysis (Drake et al., 2020; X. Rao et al., 2021; Zillich et al., 2022b) (Figure 3D, Table 4). Among these, the RNA-Seq dataset conducted in Study 1, which is Zillich et al. (2022b), was included as well. In the human studies, it was of notice that the

number of significant DEGs in the original datasets was low with 3 to 12 DEGs. In the meta-analysis, nine DEGs appeared significant with no overlapping gene being commonly dysregulated with one of the studies as well as there were no commonly dysregulated transcripts between Rao et al. (2021) and Zillich et al. (2022b) (Figure 11A). The observation of transcriptomic signatures in rodent and human post-mortem brain tissue of the NAc results in little to none dysregulated transcripts, which suggests that the mRNA expression in the NAc seems to be less vulnerable to long-term alcohol consumption followed by prolonged abstinence. However, GSEA of the human meta-analysis outcome pointed towards significantly dysregulated pathways with a major focus on immune-regulatory pathways, such as cytokine signaling, interleukin signaling and the innate immune system (Table 21).

In the AMY, human meta-analysis showed strong alterations, while in the PD rodent studies, the data suggests limited relevance of this region on the transcriptome-wide level. The number of identified datasets targeting transcriptome-wide sequencing in the AMY was six for rodents (Meinhardt et al., 2013; Osterndorff-Kahanek et al., 2015; Smith et al., 2016; M. L. Smith et al., 2020) with a sample size of 88 individuals and two for humans (Ponomarev et al., 2012; X. Rao et al., 2021) with 92 samples included. The rodent datasets were derived from the studies already identified for PFC and NAc. As reported for the NAc analysis, also in the AMY, the original datasets from rodents did not report any significantly altered transcripts. However, the human studies identified 95 (Rao et al., 2021) and 1133 (Ponomarev et al., 2012) DEGs, respectively (FDR< 0.1). The meta-analysis of the rodent datasets did not result in any significant DEGs, considering a significance threshold of FDR< 0.1, while the meta-analysis of human AMY brought up 130 DEGs (FDR< 0.1) with 32 unique DEGs, 67 DEGs overlapping with Ponomarev et al. (2012), and 25 DEGs overlapping with Rao et al. (2021) (Figure 11B). In addition, nine DEGs appeared significantly in both, the original datasets and the meta-analysis, namely *ANGPTL4* (medianFC_{MA}= 0.97, FDR_{MA}= 0.001), *GADD45A* (medianFC_{MA}= 0.67, FDR_{MA}= 0.0004), *IFITM3* (medianFC_{MA}= 0.76, FDR_{MA}= 0.0056), *LMNA* (medianFC_{MA}= 0.38, FDR_{MA}= 0.021), *MAL* (medianFC_{MA}= 0.31, FDR_{MA}= 0.092), *MT1X* (medianFC_{MA}= 0.85, FDR_{MA}= 0.0025), *S100A6* (medianFC_{MA}= 0.65, FDR_{MA}= 0.0086), SERPINA3 (medianFC_{MA}= 1.99, FDR_{MA}= 0.0009), and *TIMP1* (medianFC_{MA}= 1.07, FDR_{MA}= 0.0004). GSEA pointed towards dysregulation of immune signaling pathways, mainly containing interleukin signaling processes (Table 22).

Comparing the meta-analyses from the rodent and human AMY suggests that the PD animal model is leaking translatability to the patient situation in this brain region. Since all included original datasets in the rodent AMY reported no significantly altered transcripts and the meta-analytic approach could not identify significant DEGs either, this strengthens the finding on two levels of statistical power. However, the observation of the human post-mortem AMY samples indicates that the AMY is a brain region that might be sensitive to AUD on the transcriptomic level, even when the patient has been abstinent for years before the timepoint of death.

Intra-species comparison in humans across three brain regions suggests *SERPINA3* as a potential new biomarker for AUD.

Since the meta-analysis of the post-mortem brain samples from human AUD patients resulted in significant DEGs in all three brain regions observed, an intra-species comparison was performed to estimate potentially commonly dysregulated genes across these regions, which might suggest a new biomarker for AUD diagnosis. Indeed, one gene appears consistently up-regulated in the PFC, NAc and AMY, which is *SERPINA3* (FDR_{PFC}= 0.0001, FDR_{NAc}= 0.045, FDR_{AMY}= 0.0009). Further support comes from previous human studies, that found this gene to be up-regulated in the hippocampus and blood from AUD patients (McClintick et al., 2013; B. Zhang et al., 2021), which provides further evidence, that *SERPINA3* might be a new potential biomarker for AUD. However, Zhang et al. (2021) additionally indicated, that *SERPINA3* might not imply information regarding the relapse potential of the individual patients.

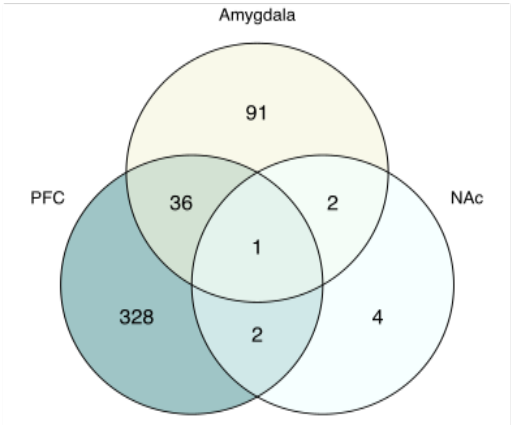

Figure 12: Human intra-species comparison considering the meta-analysis outcomes from PFC, NAc and AMY.

Across all three brain regions, one DEGs was commonly up-regulated, which is Serpina3. Additional overlaps between brain regions: NAc-AMY: EDN1 (FDR_{AMY}=0.048, FDR_{NAc}=0.098), SLC7A2 (FDR_{AMY}=0.093, FDR_{NAc}=0.098); PFC-NAc: CEBPD (FDR_{PFC}=0.049, FDR_{NAc}=0.098); PFC-AMY: ALDF1L1 (FDR_{PFC}=0.0004, FDR_{AMY}=0.042), ANGPtl4 (FDR_{PFC}=9.43e-05, FDR_{AMY}=0.001), ARRDC3 (FDR_{PFC}=0.0001, FDR_{AMY}=0.044), C1ORF54 (FDR_{PFC}=0.0006, FDR_{AMY}=0.042), CDKN1A (FDR_{PFC}=0.0027, FDR_{AMY}=0.074), CHST3 (FDR_{PFC}=0.0019, FDR_{AMY}=0.017), CRISPLD2 (FDR_{PFC}=0.023, FDR_{AMY}=0.099), DCXR (FDR_{PFC}=0.0019, FDR_{AMY}=0.048), FKBP5 (FDR_{PFC}=0.012, FDR_{AMY}=0.066), GADD45A (FDR_{PFC}=0.00029, FDR_{AMY}=0.0004), GADD45B (FDR_{PFC}=0.022, FDR_{AMY}=0.042), GPX3 (FDR_{PFC}=0.056, FDR_{AMY}=0.026), HMGB2 (FDR_{PFC}=0.0006, FDR_{AMY}=0.005), HMGN2 (FDR_{PFC}=0.0006, FDR_{AMY}=0.005), IFITM3 (FDR_{PFC}=1.4e-05, FDR_{AMY}=0.0056), ITGB4 (FDR_{PFC}=0.0002, FDR_{AMY}=0.042), JARID2 (FDR_{PFC}=0.022, FDR_{AMY}=0.086), KCNK10 (FDR_{PFC}=2.17e-06, FDR_{AMY}=0.051), MDK (FDR_{PFC}=0.0023, FDR_{AMY}=0.0014), MT1X (FDR_{PFC}=2.17e-06, FDR_{AMY}=0.0025), ODC1 (FDR_{PFC}=1.27e-05, FDR_{AMY}=0.019), PDPR (FDR_{PFC}=0.056, FDR_{AMY}=0.065), POLR38 (FDR_{PFC}=0.026, FDR_{AMY}=0.079), RASSF4 (FDR_{PFC}=0.013, FDR_{AMY}=0.065), ROBO3 (FDR_{PFC}=0.065, FDR_{AMY}=0.019), S100A6 (FDR_{PFC}=0.0018, FDR_{AMY}=0.0087), SDSL (FDR_{PFC}=0.082, FDR_{AMY}=0.087), SERPING1 (FDR_{PFC}=0.0002, FDR_{AMY}=0.0041), SHISA5 (FDR_{PFC}=0.012, FDR_{AMY}=0.087), SLC6A8 (FDR_{PFC}=0.011, FDR_{AMY}=0.012), TIMP1 (FDR_{PFC}=0.0005, FDR_{AMY}=0.0004), ZBTB16 (FDR_{PFC}=0.059, FDR_{AMY}=0.038), ZNF395 (FDR_{PFC}=0.0059, FDR_{AMY}=0.079), ZNF559 (FDR_{PFC}=0.067, FDR_{AMY}=0.048).

3.3 Study 3: Single nuclei RNA sequencing in the DMS of PD rats

The habit formation theory suggests that the striatum, mainly the DMS and DLS, is a central brain region involved in the development of addictive behavior (Balleine & O'Doherty, 2010; Chen et al., 2011; Lipton et al., 2019). In addition, the outcome of Study 1 strongly pointed towards the importance of the CN particularly, which is reflected by the DMS in rats, for the alcohol dependent phenotype. Therefore, as a first step to understanding the cell interaction processes that might underlie these alterations seen in the CN of the AUD patients, I observed the transcriptomic changes in the DMS of PD rats and matched alcohol naïve-control rats using snRNA-Seq. In combination with the behavioral data conducted by Francesco Giannone (Institute of Psychopharmacology, CIMH Mannheim), we aimed to identify cell types that might explain the behavioral outcome of the animals. In this thesis, however, exclusively the preliminary results derived from the first snRNA-Seq trial cohort of the DMS will be presented and discussed.

snRNA-Seq in the DMS of PD rats resulted in 16 distinct clusters.

When it comes to snSeq experiments, the nuclei isolation technique is probably the most delicate step in the sample processing procedure, which is reflected by the number of nuclei isolation protocols and further adaptations of these that have been published so far (Alvarez et al., 2023; Krishnaswami et al., 2016; Matson et al., 2018; Nadelmann et al., 2021). With the development of the nuclei isolation kit from 10X Genomics, which was released in the first half of 2022, it was possible to harmonize the nuclei isolation procedure even across several batches, which yielded to a high quality across samples due to the standardized procedure and chemicals (https://www.10xgenomics.com/products/nuclei-isolation). Therefore, after several trials of using FACS sorting as well as manual nuclei isolation protocols, I eventually applied the nuclei isolation kit for this experiment. Especially with respect to further experiments that are planned for this project to expand the sample size and for intra-species comparisons, including additional brain regions, the involvement of a standardized procedure reduced potential batch effects that might occur due to differing sample preparation time points. However, once isolated from the tissue and the cells, nuclei and their respective RNAs are quite unstable since they tend to lyse rapidly after isolation (Hancock & Hadj-Sahraoui, 2009). This effect is something I also encountered during the analysis of these data. While I included 20,000 nuclei per sample for the GEM generation to end up with a recovery of 10,000 nuclei for the sequencing, only around 2,000 nuclei per sample remained after QC of the Seq data. When observing the cell clustering depending on the treatment (Figure 13A), there seem to be no striking differences in the general clustering between PD and control rats. However, when observing the cell type-specific outcomes and the percentage reflecting the contribution of the treatment groups to each cell type (Figure 13B, Table 23), it is apparent that there might be treatment-specific differences and furthermore, these differences might lead to a treatment-specific vulnerability to cell degradation. Since for both treatment groups, oligodendrocytes made up the cell population with the highest proportion of nuclei ($\%_{Ctr}$= 24.8, $\%_{PD}$= 17.8; Table 23), already the cell population with the second-highest appearance rate is differing (Ctr: D2-MSN, $\%_{Ctr}$=18.3; PD: Pyramidal cells, $\%_{PD}$=16.7, Table 23). When observing additional cell types identified in this dataset, it is apparent that the distribution of nuclei varies between the PD and Ctr group. This effect is pronounced the strongest in Neurons_1 and Neurons_2, where the difference in proportion of nuclei per cell type between the treatment groups is 2-4x with a higher proportion in the PD group. After the nuclei

isolation procedure, degradation processes were initiated due to unexpected delays during the cell counting. Therefore, these data on the differing distribution of propensity across the cell types might suggest a treatment-specific vulnerability of certain cell types to degradation processes.

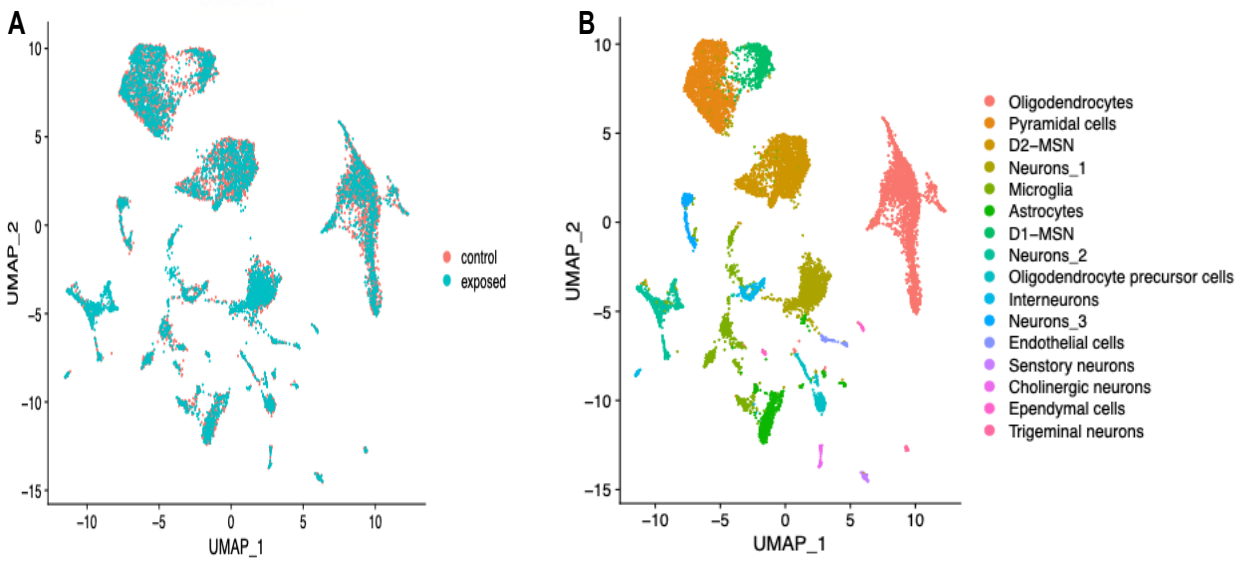

Figure 13: snRNA-Seq of the DMS of PD rats.

***A**) Clustering respective the treatment condition. PD rats (exposed) are highlighted in turquoise and control rats (control) in red. The general clustering seems to be common across the treatment groups. **B**) Cluster calling resulted in 16 distinct cell type clusters. In general, around 1,800 nuclei per sample passed the QC of the analysis of the Seq raw data. Neurons_1-_3 reflect clusters that were positive for general neuronal marker, but no further specification could be made. Further description of the treatment-specific distribution of these cell types can be extracted from Table 23.*

According to the literature, MSN are the leading neuronal cell population of the striatum, with more than 90% of all neurons in this brain region (Gokce et al., 2016). In general, around 70% of the nuclei in my sample are made up by neurons. However, MSN only made up 35% of this population (Figure 13B, Table 23). In addition, the neuronal population with the highest appearance rate across the dataset is pyramidal cells, which is also the most abundant neuronal population in the PD group, while in the Ctr group, D2-MSN are the neuronal cell type with the highest proportion of nuclei. Oligodendrocytes –the glia cells with the generally highest abundance in the rodent brain (Valerio-Gomes et al., 2018)– are the cell type with the highest proportion in both PD and Ctr rats.

Table 23: Distribution of nuclei per cell type.

The number of nuclei is represented as the total sum of nuclei per cell type (Sum), the percentage in respect to the total number of nuclei (%) and their distribution in the control and PD group with the respective proportion ($\%_{Ctr}$, $\%_{PD}$). Already during sample processing, one sample of the PD group resulted in a drastically reduced number of nuclei, which might explain the difference in the overall number of nuclei in the PD group in respect to the Ctr group. Neurons_1-_3 reflect clusters that were positive for general neuronal marker, but no further specification could be made.

Cluster	Sum	%	SumCtr	$\%_{Ctr}$	SumPD	$\%_{PD}$	n(DEGs)
Oligodendrocytes	2550	19	1587	24.8	963	17.8	36
Pyramidal cells	2025	17	1120	17.5	905	16.7	11
D2_MSN	1984	16.7	1172	18.3	812	14.9	11
Neurons_1	1349	11.4	463	7.2	886	16.4	13
Microglia	901	7.6	420	6.6	481	7.7	333
Astrocytes	693	5.8	383	5.9	310	5.7	14
D1-MSN	661	5.6	389	6.1	272	5.0	9
Neurons_2	612	5.2	160	2.5	452	8.3	187
Oligodendrocyte precursor cells	360	3.0	186	2.9	174	3.2	2
Interneurons	313	2.6	183	2.9	130	2.4	1
Neurons_3	305	2.6	169	2.6	136	2.5	0
Endothelial cells	100	<1	37	<1	63	1.2	0
Sensory neurons	99	<1	53	<1	46	<1	0
Cholinergic neurons	67	<1	36	<1	31	<1	0
Ependymal cells	58	<1	21	<1	37	<1	0
Trigeminal neurons	42	<1	25	<1	17	<1	0
Total	**11.819**		**6.404**		**5.415**		

GEx analysis considering the transcripts per cell type, resulted in microglia, neurons_2, and oligodendrocytes as the cell types with remarkable alterations in mRNA expression considering the comparison between PD and Ctr rats (Figure 14, Table 23-26) with 333 DEGs in microglia, 187 DEGs in neurons_2 and 36 DEGs in oligodendrocytes. Among the Top 10 DEGs for microglia (Table 24, Figure 14A), most of the genes are associated with synaptic transmission and signaling. In neurons_2 (Table 25, Figure 14B), the most dysregulated transcripts are involved in neuroimmune pathways and basic cellular regulatory mechanisms. The Top 10 DEGs identified in oligodendrocytes (Table 26, Figure 14C) pointed towards a potential alteration in neuronal migration.

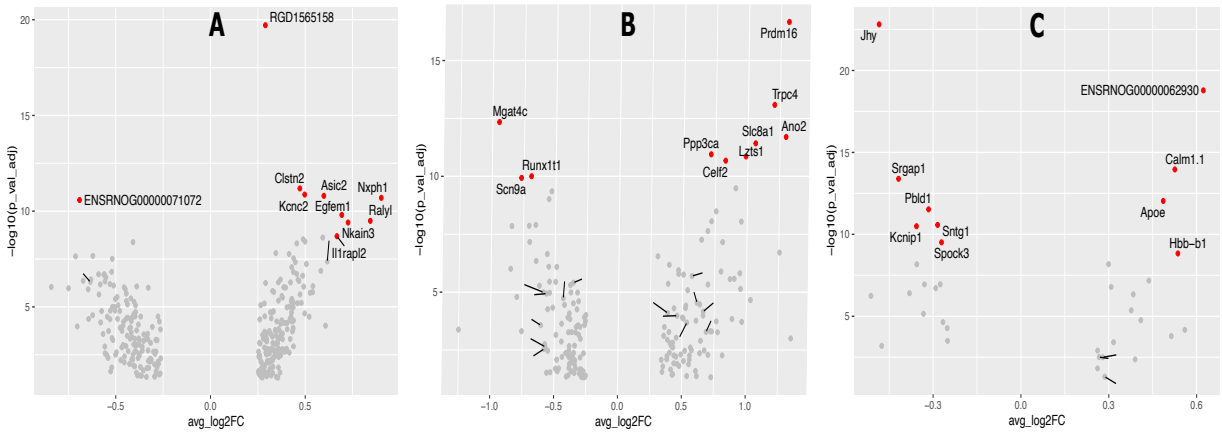

Figure 14: Distribution of the DEGs in Oligodendrocytes and Microglia, Neurons_2 and Oligodendrocytes.

*Since microglia (**A**), neurons_2 (**B**) and oligodendrocytes (**C**) were the cell types with the highest number of DEGs identified in the analysis, the respective distribution of the DEGs per cell type in up- and down-regulated genes is presented in this figure. **A**) GEx analysis in microglia resulted in 333 DEGs with $log_2FC > 0.25$ and FDR<0.05. **B**) GEx analysis in neurons_2 resulted in 187 DEGs with $log_2FC > 0.25$ and FDR<0.05. **C**) GEx analysis in oligodendrocytes resulted in 36 DEGs with $log_2FC > 0.25$ and FDR<0.05. Further information can be drawn from Figure 13 and Table 23-26.*

Table 24: Top 10 DEGs identified in Microglia.

In addition to Figure 14, the Top 10 DEGs dysregulated in PD rats compared to alcohol naïve control rats are represented with the respective foldchange (Avg_log2FC), p value and FDR-corrected p value. DEGs were estimated by the Wilcox test.

Gene	Avg_log2FC	p value	p_adjust
Rgd1565158	0.29	9.18e-25	1.94e-20
Clstn2	0.47	3.07e-16	6.49e-12
Kcnc2	0.49	6.43e-16	1.36e-11
Asci2	0.59	7.46e-16	1.58e-11
Nxph1	0.90	9.52e-16	2.02e-11
Ensrnog00000071072	-0.69	1.24e-15	2.62e-11
Egfeem1	0.69	7.43e-15	1.57e-10
Ralyl	0.84	1.51e-14	3.19e-10
Nkain3	0.73	1.86e-14	3.93e-10
Il1rapl2	0.67	9.59e-14	2.03e-09

Table 25: Top 10 DEGs identified in Neurons_2.

In addition to Figure 14, the Top 10 DEGs dysregulated in PD rats compared to alcohol naïve control rats are represented with the respective foldchange (Avg_log2FC), p value and FDR-corrected p value. DEGs were estimated by the Wilcox test.

Gene	Avg_log2FC	p value	p_adjust
Prdm16	1.31	1.02e-21	2.16e-17
Trpc4	1.21	3.89e-18	8.23e-14
Mgat4c	-0.95	2.14e-17	4.53e-13

Ano2	1.29	9.45e-17	2.00e-12
Slc8a1	1.06	1.79e-16	3.78e-12
Ppp3cA	0.71	5.31e-16	1.12e-11
Lzts1	0.98	6.55e-16	1.39e-11
Celf2	0.83	9.94e-16	2.11e-11
Runxlt1	-0.69	4.69e-15	9.93e-11
Scn9a	-0.77	5.53e-15	1.17e-10

Table 26: Top 10 DEGs identified in Oligodendrocytes.

In addition to Figure 14, the Top 10 DEGs dysregulated in PD rats compared to alcohol naïve control rats are represented with the respective foldchange (Avg_log2FC), p value and FDR-corrected p value. DEGs were estimated by the Wilcox test.

Gene	Avg_log2FC	p value	p-adjust
Jhy	-0.48	7.26e-28	1.54e-23
Ensrnog00000062930	0.62	7.74e-24	1.64e-19
Calm1.1	0.53	5.19e-19	1.09e-14
Srgap1	0.42	1.94e-18	4.1e-14
Apoe	0.49	4.33e-17	9.16e-13
Pbld1	-0.32	1.41e-16	2.98e-12
Sntg1	-0.29	1.26e-15	2.66e-11
Kcip1	-0.36	1.52e-15	3.21e-11
Spock3	-0.27	1.45e-14	3.07e-10
Hbb-b1	0.54	6.94e-14	1.47e-09

GSEA of the DEGs per cell type showed the highest enrichment score for immune pathways and mitochondrial regulatory pathways (e.g., “mitochondrial protein containing complex” (z=-5.29), “mitochondrial electron transport NADH to ubiquinone” (z=-5.17)) in microglia, for neuronal signaling (e.g., “ligand gated anion channel activity” (z=5.51), “serotonin release” (z=4.18)) in neurons_2 and epigenetic and transcriptomic regulation (e.g., “mRNA splicing” (z=6.59), “metabolism of RNA” (z=6.22); “H3K4ME2 & H3K27ME3” (z=-6.71), “H3K4ME3 & H3K27ME3” (z=-6.59)) for oligodendrocytes. In addition, since microglia are the cell type with the highest number of DEGs identified, the top 20 up-regulated pathways were compared across microglia, neurons_2 and oligodendrocytes (Figure 15). The majority of these pathways belong to neuroimmune regulation processes such as toll-like receptor signaling, interferons, and T-cell differentiation. Interestingly, all the shown pathways were dysregulated in the opposite direction in neurons_2. In oligodendrocytes, three pathways

slightly went in the same direction as seen in microglia, seven pathways showed no strong tendency in any direction, and ten pathways are regulated in the opposite direction. In general, when observing the top 20 up-regulated pathways in microglia, it seems like the neurons_2 and the oligodendrocytes cluster are more similar to each other than the microglia cluster.

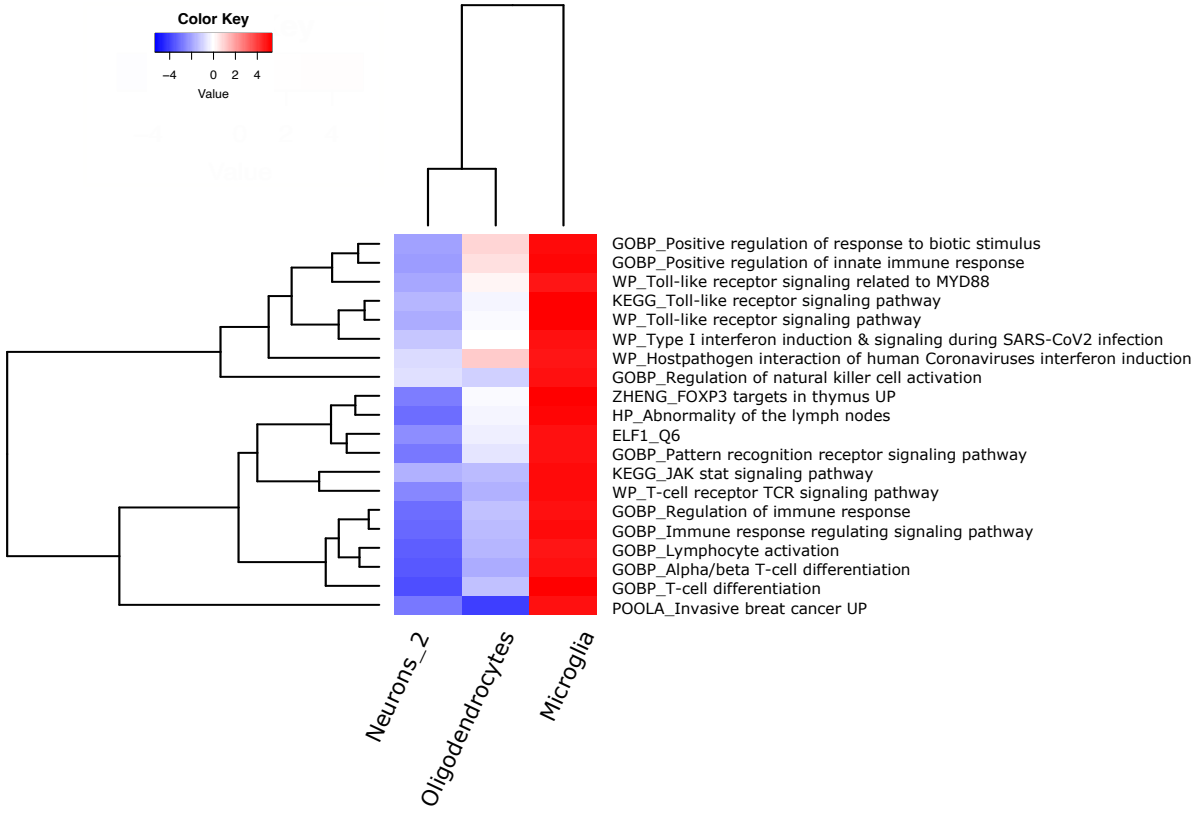

Figure 15: GSEA of the three cell types with the highest number of DEGs.

Since microglia showed the highest number of altered transcripts, for this representation the top up-regulated GSEA results (z-scores) of this cell type are shown as a reference and the corresponding outcome for the respective pathways in neurons_2 and oligodendrocytes are shown beside.

3.4 Study 4: Gene expression of COVID-19-related genes across different ethanol treatment paradigms

As mentioned in the introductory section, the COVID-19 pandemic had a tremendous impact on the global health system. However, the potential correlation between alcohol abuse and COVID-19 infection propensity as well as disease severity has not been extensively studied at this time. Studies 1-3 pointed repeatedly towards a major role of dysregulated immune pathways in AUD, which suggests a potential vulnerability to infectious diseases in general. Therefore, I investigated this research question to contribute further insights into the

potential risk of AUD patients and people with risky alcohol consumption for a severe COVID-19 infection. The findings of this project are also described in Friske et al. (2023). Additionally, we contributed to this topic with a narrative review (Friske & Spanagel, 2023).

3.4.1 Ace2 gene expression is up-regulated in different chronic ethanol treatment paradigms

The cell surface receptor ACE2 is the first interaction point of the SARS-CoV2 infection cascade (Zamorano Cuervo & Grandvaux, 2020). Hence, I tested the three animal models of chronic ethanol intake –repeated intermittent ethanol IP injections (sub-chronic treatment), chronic intermittent ethanol vapor exposure (non-abstinent treatment), and the PD model (abstinent treatment) (additional information about the treatment in Section 2.4)– for *Ace2* GEx in five different organs (Figure 16). In the sub-chronic treatment, organ-specific changes were analyzed using LMM analysis and resulted in a significant group x organ interaction ($F[4,47]=3.596$, $p=0.012$) and the follow-up post-hoc test pointed towards significant up-regulation of Ace2 in the liver ($p=0.002$, $d=1.57$) and lung ($p=0.003$, $d=2.23$) of the treatment group (Figure 15A).

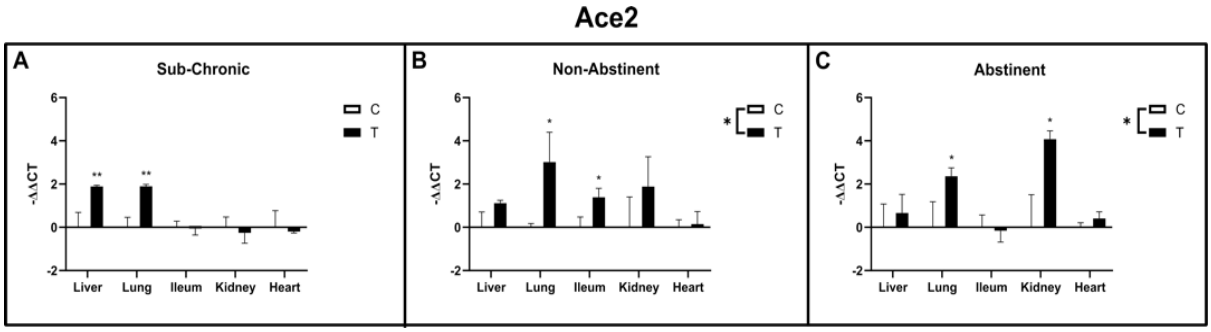

Figure 16: Gene expression of Ace2 across five organs derived from three animal models.

***A**) Sub-chronic model, **B**) non-abstinent model, **C**) abstinent model and respective untreated control rats. Gene expression was measured via RT-qPCR and the organs liver, lung, ileum, kidney and heart were analyzed, based on previous literature describing these organs as particularly vulnerable to SARS-CoV2 infection and showing high levels of Ace2 expression. The analysis was performed using LMM and follow-up post-hoc test. The difference between treatment ("T") and control ("C") group is indicated as mean ± SE. The main finding of this experiment was a consistent significant up-regulation of Ace2 in the lung across all treatment groups.*

In the two treatment groups that received chronic intermittent ethanol vapor for seven consecutive weeks, in the non-abstinence condition, the main effect of group was significant ($F[1,12]=10.624$, $p=0.007$] without interaction, which indicated an overall increase of *Ace2* in the treatment group. A subsequent post-hoc test revealed a significant effect in the lung ($p=0.032$, $d=1.36$) and in the ileum ($p=0.039$, $d=1.31$) (Figure 16B). The PD model (abstinent

treatment) showed a significant group x organ interaction (F[4,30]=3.105, p=0.030) and follow-up post-hoc test resulted in increased *Ace2* GEx in the lung (p=0.046, d=1.10) and the kidney (p=0.005, d=1.52) (Figure 16C).

Taken together, the observation of *Ace2* GEx across three different chronic ethanol treatment paradigms resulted in a consistent up-regulation in the lung regardless of the treatment, which suggests that this organ might be more vulnerable to SARS-CoV2 infection in chronic alcohol consumption. Additionally, some organs showed specific changes in the expression pattern due to the route of administration. Therefore, the diverse pharmacokinetics of IP and vapor/air treatment might cause organ-specific outcomes.

3.4.2 Potential hyper-activation of the ACE2/Ang(1-7)/Mas cascade in abstinence

Due to the complexity of the SARS-CoV2 infection route, ACE2 is not the only important target to focus on. TMPRSS2 is accompanying ACE2 to facilitate cell entry since this protein is cleaving the S2 spike protein to enable the virus to cross the extracellular membrane. As a first fight-back mechanism of the infected cell, the ACE2/Ang(1-7)/Mas cascade is activated to eliminate the virus. Therefore, the above-mentioned animal samples were tested for *Tmprss2* and *Mas* GEx. The sub-chronic treatment as well as the PD model did not show significant differences comparing the treatment and untreated control groups (Figures 17A&C), whereas in the non-abstinence group, *Tmprss2* was significantly up-regulated in the comparison of groups (F[1,14]=5.618, p=0.033), but without any organ-specific effect (Figure 16B). Since the non-abstinence group represents the only treatment where the animals had still significant BACs at time point of death (274 $\pm$ 11 (SE) mg/dl), this effect might contribute to the elevated *Tmprss2* GEx and therefore, acute ethanol intoxication might have a direct impact on the *Tmprss2* levels in the organism. To add further evidence to this hypothesis, single acute ethanol IP injections of low (0.5 g(kg), moderate (1.5 g/kg) or high (3.0 g/kg) concentrations were administered, and the *Tmprss2* GEx was observed in the same five organs. Indeed, *Tmprss2* mRNA expression was organ-specifically affected depending on the administered dose, where the *Tmprss2* gene was increased in the liver of animals with the highest dose and in the lung of animals that received a moderate ethanol challenge.

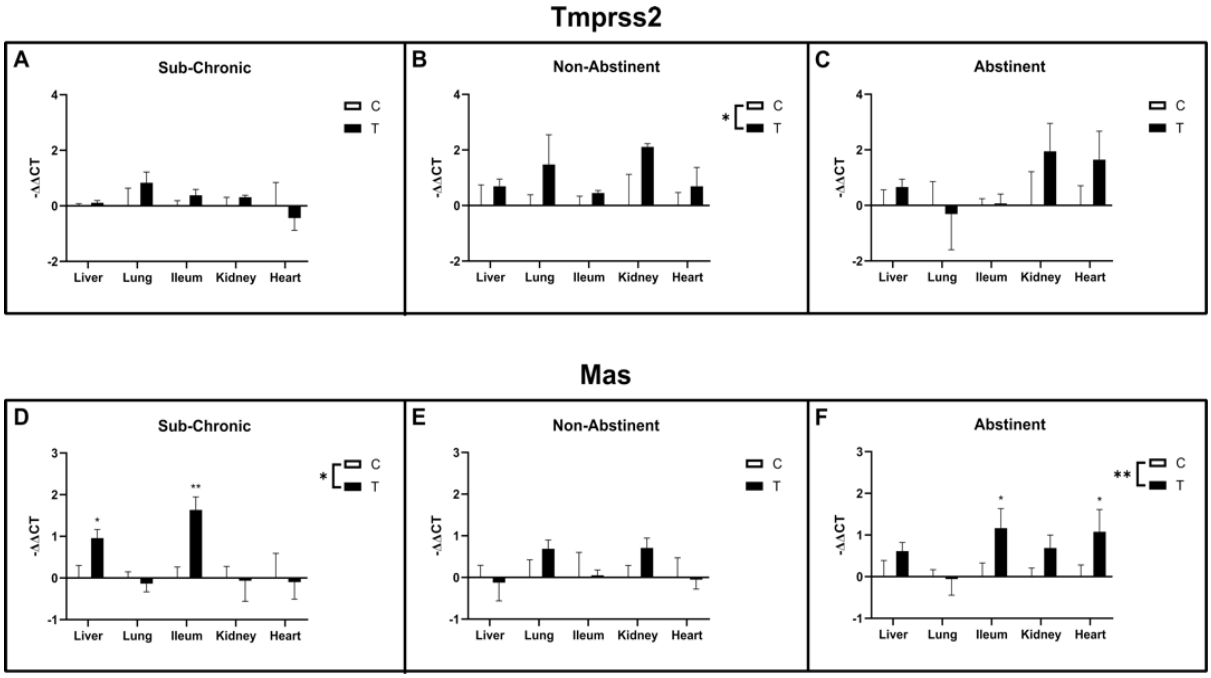

Figure 17: Gene expression of Tmprss2 and Mas across five organs derived from three animal models.

***A**) Sub-chronic model, **B**) non-abstinent model, **C**) abstinent model and respective untreated control rats. Gene expression was measured via RT-qPCR and the organs liver, lung, ileum, kidney and heart were analyzed, based on previous literature describing these organs as particularly vulnerable to SARS-CoV2 infection and showing high levels of Ace2 expression. The analysis was performed using LMM and follow-up post-hoc test. The difference between treatment ("T") and control ("C") group is indicated as mean ± SE. The main finding of this experiment was a treatment-specific, but organ-unspecific up-regulation of Tmprss2 due to absence of abstinence and a treatment-specific up-regulation of Mas due to abstinence in the ileum.*

The GEx of *Mas* showed a significant main effect of group in the sub-chronic (group x organ: F[4,47]=3.029, p=0.026] and the abstinent treatment [group x organ: F[1,12]=20.797, p=0.001), whereby in both treatment groups the ileum was significantly up-regulated (post-hoc test sub-chronic: p=0.001, d=2.23; abstinent: p=0.012, d=1.17). In addition, in the sub-chronic group, the liver was significantly up-regulated (p=0.039, d=1.6), as was the heart in the abstinence groups (p=0.020, d=1.03). No significant differences between the treatment and control group were detected in the non-abstinence vapor treatment (Figure 17D-F). Since the two groups that showed significantly elevated levels of *Mas* GEx included an abstinence phase after long-term ethanol exposure, while long-term ethanol exposure per se does not seem to have significant effects, the detected changes in *Mas* mRNA expression might be related to this abstinence period. These results suggest that during abstinence after long-term alcohol intake, the anti-inflammatory response via the ACE2/Ang(1-7)/Mas cascade might be hyper-activated.

3.4.3 Brain-wide cross-section analyses results in potentially reduced vulnerability for brain tissue to alcohol-induced changes of SARS-CoV2 infection-relevant genes

Due to several previous reports on the long-term consequences of COVID-19 infection on the nervous system (Ellul et al., 2020; Liu et al., 2021; Pajo et al., 2021), we eventually observed the GEx patterns of *Ace2*, *Tmprss2* and *Mas* in animals with long-term ethanol vapor exposure across twenty different brain regions using RT-qPCR and in situ RNA hybridization (Figure 18). Since chronic intermittent ethanol exposure has been shown to result in long-lasting, tremendous molecular changes in brain tissue, we focused this experiment solely on the vapor treatment. Interestingly, the olfactory bulb was the only brain region that showed a treatment-specific effect on the GEx, where we found significant down-regulation of *Mas* mRNA in the non-abstinent vapor animals (group x region interaction: F[1, 10)=9.188, p=0.012; post-hoc test: p=0.002, d=1.68)(Figure 18). Since *Mas* is one of the initial factors in the anti-inflammatory response of the cell, this finding suggests that chronic ethanol intake without abstinence might reduce the ACE2/Ang(1-7)/Mas cascade in the olfactory bulb, which might contribute to a higher risk of anosmia. However, the mRNA expression of the three target genes was unaffected in any other tested condition or brain region. Taken together, these findings suggest that the olfactory is the most important brain region to consider as vulnerable to COVID-19 infection and severity with respect to chronic alcohol intake.

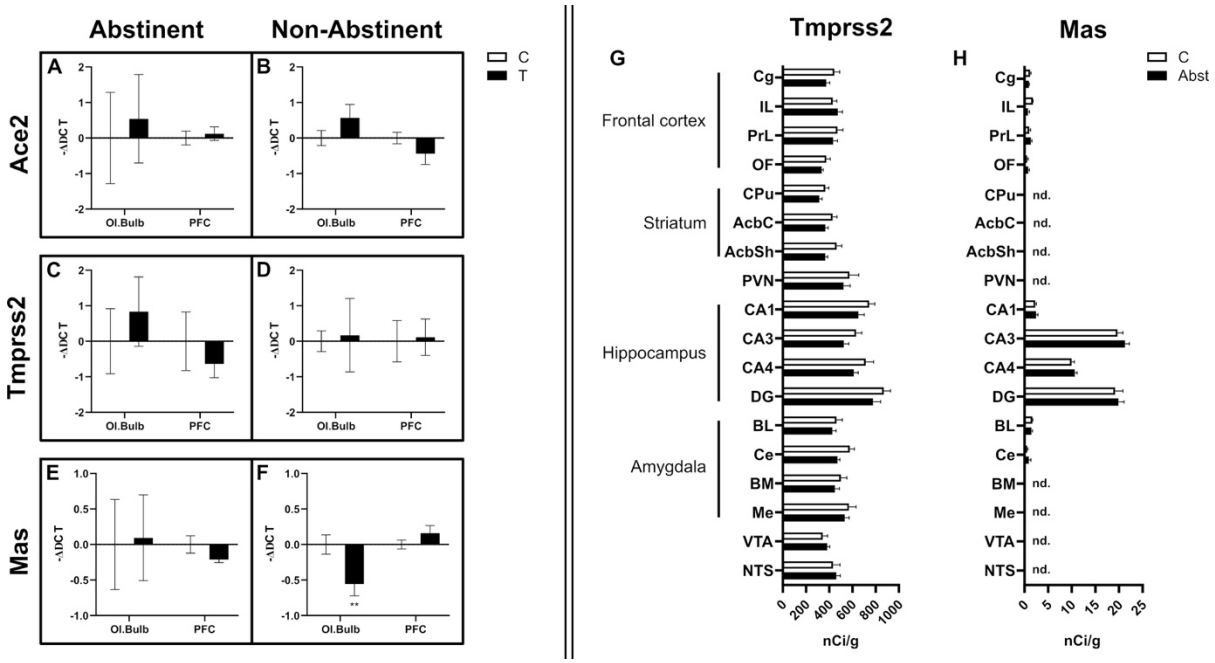

Figure 18: Cross-sectional exploration of Ace2, Tmprss2 and Mas gene expression in the brain.

*RT-qPCR measured expression of the three target genes in olfactory bulb and prefrontal cortex (**A-F**), while in situ hybridization was used to detect mRNA signals of Tmprss2 and Mas in 16 brain regions (**G&H**). Since the detected signal for Ace2 mRNA was too low in the regions captured by in situ hybridization, the analysis was impossible to lead to meaningful results. Expression values for both, RT-qPCR and in situ hybridization are shown as mean ± SE. Solely in the olfactory bulb of non-abstinent vapor treated rats a significant down-regulation of Mas was detected, whereas in all other brain regions and treatment conditions none of the target genes were significantly altered.*

4. Discussion

4.1 Discussion Study 1: Epigenetic and transcriptomic signatures of human post-mortem brain tissue using bulk seq approaches

The aim of this study was to identify dysregulated genes and pathways due to severe AUD in human post-mortem brain tissue on both the epigenomics and transcriptomics levels. Therefore, these two layers were observed and combined to eventually identify interactions between DNA methylation and GEx. For DNA methylation, five brain regions were observed: dlPFC/BA9, ACC, VS, PUT, and CN. RNA-Seq was performed in the three striatal regions VS, PUT, and CN. The main findings of this study are: i) the VS showed the highest impairment on the methylation level with 18 DMSs, while BA9 and PUT did not show any significant alteration; ii) on the level of DMRs, BA9 and PUT did not show any significant difference between AUD cases and controls. iii) On the transcriptional level, CN showed the highest number of DEGs (n=49), while in the PUT one DEG and in the VS, no significantly altered gene was identified. iv) In the multi-omics approach, only the CN showed common significantly dysregulated genes both on the epigenomic and transcriptomic levels.

On the epigenomics level, only VS and CN showed significant alterations in CpG-site methylation patterns.

When observing the identification of DMSs in the included prefrontal and striatal brain regions, the VS resulted in the highest number of DMS (n=18; Table 9). Interestingly, half of these sites were hyper- and half hypomethylated, compared to control individuals. The remaining brain regions showed only a limited number of DMSs, with a range between zero and two sites per brain region. Of these 18 hits in the VS, two sites overlapped with the CN, which are located in *GALNT9* and *PIEZO2*. *GALNT9* is involved in the O-glycosylation process, and previous studies have suggested its expression as a potential prognostic marker for neuroblastoma therapy (Berois et al., 2013). AUD patients are known to have a generally higher propensity for cancer as a comorbidity (Rumgay et al., 2021; Varela-Rey et al., 2013). This correlation of AUD with comorbid cancer diseases is further supported by the findings of the overlap of the DMS between the findings of the CN and peripheral blood, since both *PCAT29* and *KLHL6* have been attributed to cancer in previous research (Choi et al., 2019; Jiang et al., 2022). The *PIEZO2* gene encodes for a stretch-gated ion channel with high

occurrence in somatosensory neurons, and its dysfunction was associated with loss of proprioception (Nagel & Chesler, 2022). Even though, to my knowledge, this gene has not been identified in addiction research before, proprioception is a general phenomenon observed during the alcohol intoxication state. Additionally, the general tendency toward somatosensory impairment in AUD patients has been demonstrated before (Spindler et al., 2021). The most significant findings in the VS were *SLC30A8* (hypermethylated), *FAM20B* (hypermethylated), and *PCAT29* (indistinct). The *SLC30A8* gene belongs to a family encoding for zinc transporters, and this gene in particular has been found to be associated with type 2 diabetes (Bosma et al., 2019), and the *SLC30* genes showed significant expression alteration in Parkinson's disease (Bosomworth et al., 2013). The kinase encoded by *FAM20B* is involved in proteoglycan synthesis, and its function is conserved across species (Worby et al., 2021). Previous studies mostly identified its involvement in dental growth (Lei et al., 2021; Vogel et al., 2012). Hence, the potential impact on AUD patients on a neuronal level requires further investigation. *PCAT29* encodes a long noncoding RNA (lncRNA) that was previously suggested to play a role in neuroblastoma and other tumors (Bao et al., 2021; Mishra et al., 2019) and major depressive disorders (MDD) (Seki et al., 2019). Therefore, this result adds to the already mentioned predisposition of AUD patients to additional comorbidities.

In comparison to a previous methylation study in human AUD blood (Witt et al., 2020), in total three DMSs were overlapping between the CN and the blood samples, which are cg27512762 (*PCAT29*), cg06427508 (*KLHL6*), and cg02849689 (not annotated to any gene). The comparison to blood samples is of high interest since, for molecular diagnostics and studies on the living patient, blood is one of the very few organs that can be observed. Therefore, the overlap between brain samples from human post-mortem brains and blood from alive patients could deliver input for direct prediction of processes in the brain underlying changes in the methylation pattern of the patient, e.g., during treatment trials.

When considering DMRs, CN and VS seem to be the most important regions, while methylation patterns in ACC, BA9 and PUT are nearly unaffected by AUD.

On the level of DMRs, the striatal regions CN and VS represented the highest number of significantly altered methylation regions, while in ACC, one DMR and in BA9 and PUT none were detected as significant for the alcohol dependent phenotype. In the CN, the most significant findings were annotated in *DDAH2*, *CCDC152* and *CAMSAP1* (Table 10). *DDAH2*

encodes for a subtype of dimethylaminohydrolases, which are generally known to regulate the production of nitric oxide. *DDAH2* has previously been suggested to be impaired in a number of psychiatric disorders, such as bipolar disorders and schizophrenia (Kozlova et al., 2022; Pineda-Cirera et al., 2022). Previous studies have additionally reported an association of DDAH2 with sleep disturbances (Amrouni et al., 2011; Xiao et al., 2019), and impaired sleep quality, insomnia, and alterations in the circadian rhythm are well-known phenomena observed in AUD patients (Koob & Colrain, 2020; Ma et al., 2015; Meinhardt et al., 2022; Spanagel et al., 2005). Interestingly, methylation of a SNP in this gene was found to have the opposite effect on its GEx (Pineda-Cirera et al., 2022). However, when we validated this finding using pyrosequencing of the CpG-site in *DDAH2* with the highest significance, we were able to replicate the DMR, but when we followed up on the transcription level, we did not observe any significant alteration of the mRNA transcript of this gene. *CCDC152* is a just recently discovered lncRNA, that is encoded in the antisense region encoding for the *SELENOP* gene (Mita et al., 2021). Expression of this lncRNA results in direct inhibition of the translation of the *SELENOP* protein. *SELENOP* functions as a selenoprotein that is widely expressed in the brain and has been shown to be sensitive to ER stress (Zhang & Song, 2021). In addition, *SELENOP* is involved in neuronal signaling and neuroinflammation and has been associated with neurodegenerative diseases such as Alzheimer's disease (Du et al., 2014; Solovyev, 2020). The *CAMSAP1* gene and the respective protein have a critical role in neuronal differentiation and polarity, and therefore, impairment of this gene has been suggested to lead to neuronal migration disorder, aging, and intellectual disability (Al-Kasbi et al., 2022; Hu et al., 2018; Khalaf-Nazzal et al., 2022; Zhou et al., 2020). The top hits in the VS were assigned to *TMEM232*, *FANCD2OS*, and *HM13*. Also, these three genes have not been associated with AUD or other addictive disorders so far. *TMEM232* was previously studied primarily in context with atopic dermatitis (Han et al., 2023; Shen et al., 2015; Zheng et al., 2021). However, a DMR in this gene has been associated with Alzheimer's disease (Pathak et al., 2019), and a DMS was associated with multiple sclerosis (Souren et al., 2019), which points towards an important role of this gene in CNS-related diseases as well. The gene *FANCD2OS*, also known as *C3orf24*, seems to be not frequently targeted in research. However, a study focusing on multiple system atrophy found a DMS in this gene being associated with this disease (Bettencourt et al., 2020). HM13 has been characterized as a tumor-proliferating factor in glioblastoma (Wei et al., 2017) and was additionally associated with intellectual disability

(Kolarova et al., 2015). In the ACC, one DMR was identified, which is positioned in the *HCG9* gene. This gene encodes for a lncRNA that has been found previously to be linked to psychiatric disorders. A study on post-mortem brain tissue of bipolar as well as schizophrenic patients demonstrated not only alterations in the methylation pattern of this gene but also additional epigenetic modifications, such as epigenetic age effects (Pal et al., 2016). For bipolar disorder, the correlation of *HCG9* hypomethylation has been detected by other laboratories as well (Kaminsky et al., 2012; Kato & Iwamoto, 2014). Considering the high rate of cooccurrence between AUD and bipolar disorders (Nery et al., 2013; Scott et al., 2023), these results might point towards potential genetic commonalities across these psychiatric disorders.

Bottom line, the DMRs identified as most significantly associated with AUD warrant further research in both the field of AUD and in basic neurobiology to fully understand their role in health and disease. However, the findings strengthen the link between AUD and its most common comorbidities, such as neurodegenerative disorders, sleep disturbances, and cancer.

It is apparent that neither BA9 nor PUT show any significant alterations in the methylation pattern due to the alcohol-dependent phenotype. However, when considering another study that focused on smoking-related methylation within the same samples that were also analyzed in this study, these brain regions did not show any DMS and very few DMRs due to smoking (Zillich et al., 2022c), which leads to the conclusion that on the DNA methylation level, the brain regions BA9 and PUT seem to be less vulnerable to alcohol (and smoking)-related methylation pattern changes. Interestingly, when observing DNA methylation patterns in the BA9 due to Cocaine Use Disorder (Poisel et al., 2023), the impact of this drug on DNA methylation seems to be more striking, at least at the level of DMRs. Therefore, the addiction-related impact on the DNA methylation patterns might not only be brain region-specific but also substance-specific.

Epigenomic studies deliver insights into disease-specific methylation patterns to predict their importance in regulatory processes of transcription, but they are also a valuable outcome for molecular diagnostics. Since, especially in psychiatric research, diagnostics are often not straight-forward (Smoller et al., 2019), additional molecular evidence for determining the patient's diagnosis can be helpful. However, there are several concerns about epigenomic studies in general that should be addressed in the experimental design and the interpretation of the results (Longley et al., 2021). First, when considering epigenomic signatures, DNA

methylation is only one part of epigenetics, and therefore, these analyses do not represent the full picture of epigenetic modifications and regulations in AUD. Within the DNA methylation processes, there is an important distinction between methylation and hydroxymethylation, which was not considered in this study. In addition, further epigenetic processes, such as ncRNAs, histone modifications, and transcription factor binding, have been proven to be highly important in addictive behavior (Robison & Nestler, 2011; Stewart et al., 2021). Another interesting epigenetic mechanism that has just recently been discovered is that neurotransmitters, such as dopamine and serotonin, can bind to histones via transglutaminase type 2 to regulate neuronal transcription (Rossin et al., 2023). Especially the so-called dopaminylation seems to play a particular role in addiction, as demonstrated for cocaine and heroin abuse (Blum et al., 2022; Fulton et al., 2022; Lepack et al., 2020; Stewart et al., 2023).

In comparison to previous DNA methylation studies in AUD post-mortem brains, this study adds to the heterogeneity of the findings (Clark et al., 2022; Gatta et al., 2021; Hagerty et al., 2016; Jarczak et al., 2023; Manzardo et al., 2012; Meng et al., 2021; Wang et al., 2016). Nearly none of the previously detected genes have been found in additional AUD-related studies, and therefore, a concrete picture of methylation processes being dysregulated in AUD cannot be drawn yet. This conclusion might be as frustrating as it is comprehensible, since epigenetic signatures can be highly modified by environmental factors (Ladd-Acosta & Fallin, 2016; Law & Holland, 2019; Schiele & Domschke, 2018), and patients' environmental situations might have been quite diverse throughout their lives. Therefore, bigger sample sizes and meta-studies are warranted to combine these heterogeneous findings into a clearer outcome.

Transcriptomic signatures in the striatum reveal a high number of significant DEGs in the CN, while the VS shows no significant alterations in gene expression levels.

RNA-Seq in the three striatal regions VS, CN and PUT revealed 49 DEGs in the CN with the majority of genes being significantly up-regulated and 1 DEG in PUT, that was significantly down-regulated in AUD patients compared to matched control individuals (Table 11). The VS did not show any significant DEGs at a threshold of FDR<0.05. The three genes with the highest significance in the CN are *TTLL4*, *GATA2*, and *CNOT10*. *TTLL4* functions as a polyglutamase, which is mainly involved in post-translational modification (van Dijk et al., 2008) and has previously been associated with neurodegeneration (Mahalingan et al., 2020;

Wu et al., 2022). *GATA2* is a zinc finger transcription factor that is crucial for neuronal development, and patients with *GATA2* deficiency suffer from a variety of pulmonary and immune disorders (Hsu et al., 2015). In a brain-specific manner, this gene has been found to be involved in REM-sleep regulation, Alzheimer's disease, and brain cancer (Abyadeh et al., 2022; Fu et al., 2020; Kirjavainen et al., 2022). As part of the principal deadenylation complex, *CNOT10* is involved in mRNA degradation control (Fadda et al., 2013). However, no association with brain-related diseases has been reported yet, which suggests, that the remaining components of the deadenylation complex might compensate for the dysregulation of *CNOT10*. In the PUT, *DES* was significantly down-regulated. This gene has been reported in context with mesenchymal tumor markers (Sloan et al., 2021; Truong et al., 1990). In context with AUD, it was found altered in the heart tissue of AUD patients (Deliu et al., 2017; Richardson et al., 1998), but so far, no study has reported dysregulation of this gene in the brain tissue of AUD or alcohol-dependent animal models.

In general, it is worth mentioning that the effect sizes of the GEx, measured as log2FC, are comparably low, with most of the DEGs having a log2FC <0.5, while, e.g., in GEx studies in the cancer research field, usually significant genes are considered with a log2FC>2.0 (Gobin et al., 2019; Pido et al., 2021). Nevertheless, these small effect sizes detected in this study are in line with previous transcriptome-wide sequencing studies in AUD (Ferguson et al., 2022a) and respective animal models (Saba et al., 2015; Tabakoff et al., 2009), suggesting that the impact of chronic alcohol consumption on AUD might rather cause alterations of multiple genes simultaneously, which suggests addressing the focus of future research should be more on network and pathway interactions. Rather than target gene studies, to observe the underlying mechanisms of AUD and eventually, identify new target approaches for psychopharmacotherapy. The findings of the GO-term and KEGG-pathway enrichment analyses of this study support this conclusion. Even though focusing on single genes, neither the VS nor the PUT reached a significant number of DEGs, enrichment analyses showed significant results, which were mainly pointing towards an impairment of immune system-related pathways.

Multi-omics data integration in striatal regions shows gene expression regulation on the epigenomic and transcriptomic levels in the CN.

When comparing the methylation and GEx, signatures due to AUD, the CN was the only region of the three striatal brain regions observed that represented an overlap of genes being altered on both levels (Table 12). The 12 overlapping genes are functionally involved in cell adhesion, apoptosis, and basic energy-generating metabolisms, such as gluconeogenesis. Out of these genes, *PFKFB3* has been previously observed in context with chronic ethanol exposure in mice, where its GEx was significantly increased (Morris et al., 2022). Additional studies have identified *PFKFB3* and *HIPK3* with brain tumors and the promotion of glioma progression (Alvarez et al., 2021; Han et al., 2021; Hu & Zhang, 2019). Among these 12 genes, *DYNLT1* and *KLHL7* have been associated with neurodegenerative diseases like Huntington's and Parkinson's disease (Murthy et al., 2017; Rosseto et al., 2021).

In the PUT as well as in the VS, no overlap of genes with altered methylation or transcriptome profiles was observed. When considering the limited to none observed number of genes with significant alterations within one of the two omics layers, this finding does not come as a surprise.

Even though the overlap of the two omics layers was identified in only one of the three brain regions in the striatum, it has to be considered that this study was only focusing on one subtype of epigenomics. Therefore, by considering the additional types of epigenetic sequencing approaches targeting ncRNAs, miRNAs, histone modifications, and DNA hydroxymethylation, a more extensive picture of the dysregulated epigenetic mechanisms and their potential overlap with other omics layers would result. Due to the nature of bulk sequencing approaches, cell type-specific effects might potentially be evened out since a bulk approach considers the tissue as an average of the cell types. Therefore, I strongly suggest further studies in human post-mortem brain tissue on a single cell level to obtain deeper insights with more sensitive data to describe the molecular basis of AUD in the brain. However, a previous study focusing on DNA methylation and the effects of alterations on the respective transcript suggests that mainly DNA hypomethylation and the resulting increase in GEx could be of particular interest to describe the consequences of epigenetic alteration in the alcohol dependent phenotype and its potential as a therapeutic approach (Ponomarev et al., 2012).

4.1.1 Summary

This study investigated the methylation and transcriptomic patterns in human post-mortem brain tissue from several brain regions in both, a separate and integrated manner. DNA-methylation analysis revealed the highest number of DMSs and DMRs in the CN, which is consistent with the findings from RNA-Seq in the same human post-mortem brain tissue. ACC showed moderately altered methylation patterns, while in both BA9 and PUT, no gene was significantly differently methylated. Significant DMSs and DMRs led to alterations in genes relevant for sleep regulation, neurodegenerative diseases, and psychiatric disorders, which is in line with previous research describing comorbidities in AUD patents. In the RNA-Seq approach in the three striatal regions CN, VS, and PUT within the same patients that were included for the DNA methylation analysis, VS and PUT showed limited to no alterations due to the AUD phenotype when focusing on significant differential expression of single genes. The most significant DEGs in the CN were associated with dysregulated immune pathways, neurodegenerative diseases, and cancer-relevant pathways. Multiomics data integration resulted in 12 genes being commonly altered within the CN on both levels, methylation and GEx, while neither PUT nor VS had any overlap across significant alterations on both omics layers. Observing these two omics layers, the PUT seems to be less vulnerable to methylation as well as GEx alterations due to chronic alcohol consumption and AUD. On the other side, the CN showed the highest number of modified methylation sites and regions and also the highest number of significant DEGs, which strongly suggests further research on the CN in terms of additional omics layer observations and alcohol-dependent behavior. In addition, especially the hits from the DNA methylation analysis led to a number of genes that have not been described in the research field of AUD yet, which suggests further investigations to understand the role of these genes in the brain and especially in AUD.

4.2 Discussion Study 2: Meta-analysis of transcriptome-wide sequencing approaches across four species

This study represents the first transcriptome-wide meta-analysis of brain samples derived from AUD patients and PD animals. Especially the translational aspect, including four species –human, rat, mouse, and monkey– and multiple brain regions, provides high evidence not only in a comparative manner but also for each area of these preclinical domains, in its own

right. By considering the increased statistical power as well as the heterogeneity that comes with including individuals from multiple studies, I was able to identify commonly dysregulated genes and pathways across three brain regions that provide high relevance for the AUD research. The main findings of this study describe: i) a high overall heterogeneity across the original studies that were included in the meta-analyses; ii) *Hsd11b1* seems to be relevant for the alcohol-dependent phenotype throughout the investigated species; iii) the NAc is potentially less vulnerable to long-term alcohol intake followed by prolonged abstinence and iv) the transcriptomic signatures in the human AMY warrant an optimized treatment of the rodent PD model to provide translatability in this brain region to the alterations observed in patients.

The observation of the gene expression profiles of the included datasets results in high overall heterogeneity across the original studies.

When observing the DEGs identified by the included datasets from the different studies, I did not identify any gene that was commonly significantly altered across all the datasets, which points towards a high heterogeneity across the datasets. The general aspect of performing meta-analyses is to observe an effect across several original studies, considering potential heterogeneity introduced by, e.g., diverse study designs, and including a larger sample size for the analysis to increase statistical power. One of the major advantages of animal experiments is that multiple paradigms can be controlled throughout the experiment. However, since all the included individuals were outbred animals, the genotype also varied, which might have had an additional influence on the transcriptomic profile (Fotsing et al., 2019; Heap et al., 2009; Philibert et al., 2008). The varying study designs of the included original studies, such as the differences in the alcohol exposure paradigm, including the duration of abstinence before the sacrifice of the animals, might additionally contribute to the heterogeneous DEG distribution among these studies. Also, it is known that mice and rats show differing transcriptomic patterns in response to alcohol exposure (Contet, 2012). Additionally, individuals derived from different breeders can vary genetically and physiologically. It has been shown in the past that the microbiome has an impact on gene expression in the brain via the gut-brain-axis (Margolis et al., 2021) and that the microbiome of laboratory animals is influenced by a variety of factors (Ericsson & Franklin, 2021). Based on the inclusion of datasets derived from different laboratories, the treatment conditions of

the animal experiment, and the sample treatment for the mRNA sequencing approach were diverse, which could have contributed to the high heterogeneity detected in the mRNA expression profiles. Also, the assays to measure transcriptome-wide mRNA expression were heterogeneous. While all the included rodent studies and the majority of the human studies chose microarray as a detection method, the time span of the data collection of the studies is around seven years, which is already long enough to represent the different standards of transcriptome-wide sequencing approaches since this is a very rapidly developing technique with a variety of approaches (Venkatasubbarao, 2004). Nevertheless, the observed heterogeneity across the original studies points out the importance of this meta-analysis: genes identified as significantly altered, despite these inter-study variances, represent targets of particular interest, as their dysregulation was detected regardless of the above-mentioned aspects of heterogeneity. Also, in the clinical situation of human AUD patients and in the human post-mortem brain samples analyzed in pre-clinical experiments, the background of the patients varies in terms of years of alcohol drinking and relapse phases, environment, genotype, microbiome, period of abstinence before death, and psychological aspects that all have an impact on the transcriptomic profile. Therefore, observing transcriptomic changes with a heterogeneous sample background delivers new promising targets that might be representative for a broader population of the alcohol dependent phenotype and might potentially be useful for the development of promising AUD treatments.

The rodent meta-analysis in the PFC resulted in 519 DEGs, of which 124 DEGs were uniquely identified by this approach.

When considering the overlap across the original studies, it is noticeable that only half of the included datasets reported significantly altered transcripts and that these were derived from two studies which were both conducted in the same laboratory, in the same species, and even by the same scientist (Figure 8A, Table 3) (Smith et al., 2016; M. L. Smith et al., 2020). Therefore, the above-mentioned potential resources of heterogeneity were reduced since the laboratory conditions as well as the bias due to the individual scientist and the animal batches were absent.

For the rodent meta-analysis, I included six datasets with a total of 98 individuals. The outcome resulted in 519 DEGs (FDR <0.1) with 124 genes that did not overlap with any of the findings, which represents a unique outcome due to the meta-analytic approach. As a

principle of meta-analysis, an overlap of the identified DEGs with DEGs from the original datasets but also unique DEGs as outcome fits the expectation of a common meta-analysis output, which is aiming to partially replicate previous findings but also identify new genes by increasing sample size and statistical power. GSEA resulted in mostly chromatin-modifying-related terms (Table 16). As identified in Study 1 of this thesis, AUD causes changes in the methylation pattern, which can result in altered chromatin accessibility and structure (Caiafa & Zampieri, 2005; Meng et al., 2015). Therefore, it is not surprising to observe a link between chromatin modification and the GEx pattern, and even further, these results might suggest a connection between DNA methylation, chromatin remodeling, and eventually, altered GEx. As already reported in Study 1 of this thesis, the absolute numbers of the resulting fold-changes are rather low. However, it has been suggested that the threshold set for a fold-change to have biological relevance might differ across research areas, which led to the suggestion to set a generalized threshold for the significance of the fold-change depending on the research question (McCarthy & Smyth, 2009). The small effect sizes in the GEx suggest that even though AUD affects a variety of genes, due to the small effect sizes, it might be more considered targeting gene co-expression networks and pathways than single genes for understanding the molecular pathology of AUD (Ferguson et al., 2019; Ponomarev et al., 2012).

Since all included datasets are derived from microarray experiments, which are known to have reduced sensitivity compared to RNA-Seq –as stated in the Methods section of this thesis– there might be a bias towards lowly abundant genes. Therefore, it is of great importance to include further studies applying RNA-Seq to add additional reliable information regarding lowly abundant transcripts.

Subgroup analysis in the rodent species suggests that the rodent meta-analysis outcome is mainly driven by the mouse species.

Since four out of the six included datasets were derived from C57BL/6J mice and the remaining two datasets studied Wistar rats, I aimed to further explore how these two rodent species influenced the outcome of the meta-analysis. Therefore, I performed a subgroup analysis by splitting the datasets according to the respective rodent species, which resulted in 2 DEGs for the rats and 678 DEGs for the mice (Figure 8B). In comparison to the rodent meta-analysis, the overlap with the rat subgroup analysis was 50%, and the overlap with the

mouse subgroup analysis was 68%. The very limited number of DEGs in the Wistar rats might be explained by the absence of significantly altered transcripts in the included original datasets, while in contrast, in the mouse datasets, three out of four datasets showed multiple significant DEGs. On the genomic level, the mouse and rat species have a more than 90% overlap in nucleotide and amino acid sequence (Wolfe & Sharp, 1993). However, when it comes to alcohol-related behavior, species-specific differences can be seen. E.g., the alcohol deprivation effect (ADE) model, which is a model of relapse behavior, describes the increase in alcohol intake after an abstinence (deprivation) period, and this effect is increasing in intensity with increasing number of deprivation events (Spanagel & Hölter, 1999). This model has persistent findings in rats, while in mice, the ADE seems to be fading with an increasing number of deprivation periods (Vengeliene et al., 2014). In addition, a recent cluster and meta-analysis on stress-induced alcohol consumption in mice and rats showed that stressors enhance home cage alcohol drinking in rats, while in mice, a demonstration of stress-induced elevated alcohol intake was absent (Noori et al., 2014). Generally, the comparability of these two rodent species seems to depend on the treatment paradigm (Gomez et al., 2015; Vengeliene et al., 2009), which suggests that the species should be chosen with respect to the research question and outcome aims.

STRING network analysis resulted in Jun being the highest connected protein in the mouse, as well as the human meta-analysis (Figure 9). Generally, Jun genes are part of the Fos/Jun immediate early gene family that has been associated with neuronal activity and is closely linked to cFos (Morgan & Curran, 1991) – a gene that has been extensively observed in addiction research (Korber & Sommer, 2022). Previous neurobiological studies reported *Jun* to be altered after cocaine intake (McClung & Nestler, 2003; Moratalla et al., 1993; Xu, 2008), as well as after ethanol intake, where the increased expression of *Jun* was associated with attenuation of baroreflex sensitivity (Wang & Abdel-Rahman, 2004). In addition, dysregulation of *Jun* has been found in inflammation, neurodegeneration, and cancer types (Shaulian, 2010), which are comorbid conditions, that are often seen in AUD (de la Monte & Kril, 2014; de Menezes et al., 2013; Mayfield & Harris, 2017).

Human meta-analysis led to 367 DEGs, which were mainly enriched in immune-regulatory pathways.

In total, six datasets resulting in 380 samples were included in the human meta-analysis (Figure 3D, Table 4). When overserving the DEGs of these studies, four datasets detected significant DEGs with the highest number of DEGs in Kapoor et al. (2019) (n=260) (Figure 8C). Since this study also contributed with the highest number of samples, the LOO study (Figure 10) was performed to identify the potential bias that was introduced by this dataset. Since the LOO meta-analysis and the human meta-analysis, including Kapoor et al. (2019), share the majority of DEGs, it was hypothesized that there was a considerably low bias introduced by the Kapoor et al. (2019) dataset.

The human meta-analysis resulted in 367 DEGs with 217 unique altered transcripts. When comparing the total number of identified DEGs across the rodent, human, and monkey species, the human meta-analysis outcome represents the smallest number of significant genes (Figure 8). As described in the study demographics of the human datasets (Table 4), the majority of the samples were derived from the NSWTRC at the University of Sydney, Australia. Since the tissue derived from one individual serves for several laboratories to study, there might be a potential overlap regarding the individuals across the studies, which might have influenced the number of DEGs in the meta-analysis outcome. Unfortunately, to date, it is not possible to estimate the impact of this potential bias since the individual case IDs are not officially reported in the respective studies due to ethical agreements with the brain bank.

Among the top 10 DEGs of the human meta-analysis (Table 14), Angptl4 has been described in AUD research before, where it has been identified as a significant component in the gene x alcohol interaction of alcohol effects on plasma lipid levels (Wang et al., 2020). *FITM3*, which was up-regulated in the meta-analysis outcome, has also been found to be up-regulated in the PFC of schizophrenic patients, and furthermore, it has been attributed to the dysregulation of PFC functions in these patients (Arion et al., 2007). *KCNJ16*, down-regulated in the meta-analysis, has also been found to be down-regulated in a previous study on PTSD cases with comorbid MDD and was positively correlated with RNA age (Zhao et al., 2022). Since the latter two genes are involved in common comorbidities of AUD, such as schizophrenia, PTSD, and MDD (Debell et al., 2014; Gielen et al., 2012), these findings highlight the connection between AUD and other mental health disorders, which is generally estimated to be more than 50% (AshaRani et al., 2022; Yang et al., 2018).

Pathway enrichment analysis pointed towards an enrichment in immune-regulatory pathways, such as the innate immune system, the ER-Phagosome pathway, cytokine

signaling, and IL-10 signaling (Table 17). These findings add further evidence to the importance of dysregulated immune pathways as a driving force in AUD, as described previously (Crews & Vetreno, 2016; Mayfield & Harris, 2017). Cell type enrichment analysis revealed the relevance of astrocytes, endothelial cells, and inhibitory neurons in the investigated tissue. Previous snRNA-Seq studies pointed towards the relevance of astrocytes (Brenner et al., 2020), D1- and D2-MSN (van den Oord et al., 2023), and GABAergic neurons (Dilly et al., 2022) in the alcohol dependent-phenotype. In Study 1 of this thesis, cell-type enrichment analysis of the bulk GEx data also resulted in significant enrichment in astrocytes and endothelial cells in the CN (Zillich et al., 2022b). Therefore, these cell types might have an overarching role in the brains of AUD patients.

Monkey meta-analysis in the PFC resulted in a higher number of DEGs compared to humans and rodents but a lower number of unique genes.

Four datasets comprising 90 individuals were included in the monkey meta-analysis of the PFC. Interestingly, the number of significant DEGs (n=1532 DEGs) was notably higher compared to the human (n= 367 DEGs) and rodent species (n= 519 DEGs), which might be explained by the fact that most of the studies included were sacrificing the monkeys while they were still intoxicated (Table 5). Therefore, these data not only represent the effects of prolonged alcohol consumption, which in the monkeys was designed with duration periods of at least 6 to 12 months, but also the transcriptomic signatures of alcohol intoxication, which are known to differ due to the abstinence phase (Hashimoto et al., 2017; Mayfield, 2017; Mulligan et al., 2017). Hence, the elevated number of significantly altered transcripts in comparison to humans and rodents might be caused by the state of intoxication. In addition, for monkey transcriptomic analysis, internal controls represented by biopsies before the onset of drinking were used to normalize the transcriptomic signals. This method is strikingly different from the usage of external controls as applied in the rodent and human studies, where age matched control individuals were used as comparisons. Previous studies report that the difference between biopsy and autopsy samples per se might be negligible (Bolliger et al., 2010; Castensson et al., 2000). However, the time-dependent component that comes with biopsy sampling before the onset of drinking and the comparison to the necropsy after the end of the treatment (Somel et al., 2006), as well as the potential differences that might come from considering different individuals for normalizing, might still play an important role

in respect to the tissue collection of the other species included in this study. However, even though the number of total DEGs is higher than in the other species included in this study, the number of uniquely identified transcripts is comparably low. Meta-analysis in humans and rodents identified 217 and 124 unique DEGs, respectively, but monkey meta-analysis in the PFC resulted in 35 unique DEGs. While half of the human as well as the rodent original studies reported significant transcripts, in the monkey studies three out of four studies reported significant DEGs (Figure 8). Therefore, the proportion of original datasets, that found significantly altered transcripts, was higher for the monkeys, compared to the other species, and also, the number of commonly identified DEGs across these studies was higher, leading to a higher homogeneity across the datasets. When observing the Top 10 DEGs of the monkey meta-analysis outcome, some prominent genes discovered in, e.g., the cancer field, were represented. In regard to alcohol abuse, the circadian clock gene *BHLHE40* might be the most interesting finding. This gene has been found to be dysregulated in muscle tissue after alcohol bingeing, which contributed to a major impairment in the core molecular clock of the individuals (Tice et al., 2021). Another interesting finding is the down-regulated expression of *SPRY2*, which is a direct inhibitor of *ERK*. A recent study demonstrated that *SPRY2* is directly regulated by miR-27a in human monocytes, which induces monocyte activation and polarization initiated by alcohol (Saha et al., 2015). Another study focusing on MAPKs, including *ERK*, suggested that the MAPKs inhibitor DUSP4, which was up-regulated in the monkey meta-analysis, is interacting with miR-26a to enhance alcohol-induced liver injury (Han et al., 2015). Therefore, both of the latter mentioned genes underline the hypothesis that miRNAs might play a major role in alcohol-induced dysregulation, including liver and brain injury (Gorini et al., 2013; Nunez et al., 2013; Rutledge & Im, 2021). Furthermore, the data derived from this study points towards the involvement of MAPKs in these processes.

When looking at the cross-species overlap, according to the number of common DEGs, the monkey species had a higher overlap with the human species than with the rodents, which is what would be expected from the evolutionary point of these species.

Bottom line, even though the monkey species represented the smallest number of studies and individuals for the meta-analysis, the findings contribute important additional findings to better understanding the alcohol-dependent phenotype.

Cross-species comparison in the PFC suggests *HSD11B1* dysregulation as a conserved phenomenon for the alcohol-dependent phenotype.

When comparing the four species-specific meta-analyses targeting mice, rodents, monkeys, and humans (Figure 8E), it is noticeable that the number of overlapping DEGs across these species is the highest between humans and monkeys, which reflects the closer evolutionary relationship. In addition, one transcript was commonly altered across all four species-specific outcomes and, hence, suggests a conserved target for the alcohol-dependent phenotype, which is Hsd11b1. In humans as well as in rodents and in the mouse-specific analysis, this transcript was found to be significantly down-regulated, while in monkeys, *HSD11B1* was up-regulated. The opposite regulation observed in the monkeys might be caused by the intoxication status of the animals at the time point of death, while the rodents as well as the humans underwent an abstinence period, before they died. *HSD11B1* encodes for the enzyme ß11-HSD1, which is known to catalyze the first reaction step of cortisol anabolism, where cortisone is metabolized to cortisol. Therefore, a reduced mRNA expression of *HSD11B1* might result in down-regulated ß11-HSD1 protein levels, which might eventually lead to lower cortisol levels due to limited anabolism of cortisol to the active glucocorticoid cortisone (Holmes & Seckl, 2006; Wyrwoll et al., 2011). AUD is known to be associated with stressful events that lead to increased cortisol levels in the plasma as well as in several brain regions (Stephens & Wand, 2012), and elevated cortisol concentrations are one of the key aspects of acute and long-term withdrawal (Anderson et al., 2016; Becker, 2017; Heilig & Koob, 2007). Hence, it might be expected that *HSD11B1* would be up-regulated as a potential explanation of the accumulation of cortisol after prolonged alcohol consumption. Furthermore, the up-regulation of this transcript was found in the liver of alcohol dependent individuals (Ahmed et al., 2008; Meng et al., 2013). Generally, *Hsd11b1* expression in the blood and brain tissue of CIE mice was suggested as a predictive factor for the identification of the alcohol-dependent phenotype (Ferguson et al., 2022b). Since the meta-analytic approach selected for this study is based on p value combinations, the direction of dysregulation was considered a secondary outcome. Therefore, I validated the finding of the down-regulated *HSD11B1* mRNA levels by RT-qPCR in the human post-mortem brains of our in-house sample, and I was able to confirm both the significance and the direction of the dysregulation of this transcript (Figure 8F). In addition, since the majority of the human post-mortem studies included in this analysis comprised tissue derived from the NSWTRC at the University of Sydney, Australia, a

potential over-representation of individuals across these datasets might occur. To date, we cannot estimate the exact overlap of individuals across these studies, since the case IDs are not publicly shared. However, I need to assume that a certain degree of bias due to over-represented individuals might be present in my dataset, which underlines the importance of validation experiments of promising target genes in a separate human cohort. Since stress-related processes are mainly mediated by other brain regions, e.g., the extended amygdala, it might be reasonable that *HSD11B1* has a different function in the PFC. Indeed, the ß11-HSD1 enzyme is not only involved in the anabolism of cortisol but also in the generation of active glucocorticoids (Tomlinson et al., 2004). Therefore, even though previous systemic experiments suggest ß11-HSD inhibitors as potential treatment approaches for AUD (Sanna et al., 2016), the function of this transcript in the PFC might differ. Furthermore, the effect of down-regulated *HSD11B1* could be a compensatory mechanism initiated by increasing cortisol levels in the alcohol-dependent phenotype. Eventually, it is not clear what the alterations on the transcriptomic level mean for the functional protein. It might also well be that the decrease in *HSD11B1* mRNA activates post-transcriptional processes, resulting in an over-expression of the ß11-HSD1 protein or an increased activity of the enzyme.

Transcriptomic signatures in the NAc seem to be less vulnerable to long-term alcohol dependence with protracted abstinence in both human and rodent samples.

The NAc is generally known for processing pleasurable feelings and initiating behavior in a goal-directed manner (Floresco, 2015). Therefore, its role in alcohol consumption and the development of addictive behavior has been discussed extensively (Boileau et al., 2003; Chen et al., 2011; Ho et al., 2018; Scofield et al., 2016; Spiga et al., 2014). These studies suggest that mainly during the acquisition phase, where alcohol consumption is driven by its pleasurable and rewarding properties, but also in acute withdrawal, the NAc plays a key role. When it comes to molecular observations on the transcriptome-wide level in long-term abstinent individuals, previous studies have reported only a limited number of significantly altered transcripts in the NAc (Meinhardt et al., 2013; Meinhardt et al., 2015; Osterndorff-Kahanek et al., 2015; X. Rao et al., 2021; Smith et al., 2016; M. L. Smith et al., 2020; Zillich et al., 2022b). Therefore, it is not surprising that in the meta-analysis, both the human meta-analysis and the meta-analysis in the PD rodents, did not result in a high number of significantly altered transcripts, as the included individuals experienced prolonged abstinence

before the time point of death (Table 3). Since the PD rodent model shows comparable outcomes to the human results, this also confirms the translational properties of this animal model to the patient situation on a molecular level. Additionally, these findings are in line with the outcome of Study 1 of this thesis, where the VS –embedding the NAc–showed no significant DEGs as well as no overlapping genes in the data integration of methylome- and transcriptome-wide data derived from human post-mortem brain tissue (Zillich et al., 2022b). However, when considering only methylome-wide data, the VS was among the brain regions with the highest number of DMPs and DMRs (Zillich et al., 2022a), which suggests that these alterations observed at the methylation pattern might have limited impact on transcriptomic alterations. Previous studies reported strong alterations in the transcriptomic profile of the NAc after acute alcohol consumption but loss of these effects after chronic treatment (Contet, 2012; Kerns et al., 2005; Melendez et al., 2012; Repunte-Canonigo et al., 2007). And even further, these studies suggest that the PFC plays an important role in transcriptomic alterations after chronic alcohol treatment, which is in line with the high number of DEGs in the meta-analyses of the PFC (Figure 8). Finally, this leads to the conclusion that on the transcriptome-wide level, the NAc might not be a highly vulnerable brain region of the long-term alcohol dependent phenotype, which was observed in a species-overarching manner. However, the division of NAc core and shell might be taken into consideration when examining alcohol dependent behavior since previously there have been distinct differences described (Barker et al., 2015).

In the AMY, the PD model in rodents seems to lack translatability.

The AMY is a highly stress-sensitive brain region (W. H. Zhang et al., 2021), and AUD is a mental health disorder that is driven by stressful events, especially during the withdrawal and relapse phases but also in the initiation phase, when alcohol is often used as a coping strategy (Flook et al., 2023; Heilig, 2023; Moonat & Pandey, 2012). Hence, the findings in the human post-mortem brain of the AMY fit the expectation of high alterations in the transcriptome of AUD patients (Figure 11B). However, in the rodents, no significant transcript appeared to be significantly impacted, regardless of considering the rodent species as one or analyzing the subgroups of rats and mice separately. This comes as a surprise since the rodent CIE model has been reported to show sensitivity to stress (Heilig & Koob, 2007). However, the alcohol-associated stress mechanisms do not only involve the AMY but also the bed nucleus of the

stria terminalis (BNST) and the HPA axis. In addition, the AMY is a very heterogeneous brain region, consisting of multiple nuclei (AbuHasan et al., 2023), and based on the reported methodologies of the included studies for this meta-analysis, it is not exactly clear which nucleus was extracted for the gene expression analyses. Therefore, the analysis of the rodent AMY datasets might represent an average across several nuclei of the AMY, which might even out potential subregion-specific effects, or the studies might be focusing on a nucleus that might not be affected by alcohol dependence. Since the animals comprised in the AMY analysis were exposed to CIE vapor for 4-8 weeks, with partially 4 days of 16 h exposure per week, this treatment paradigm might not be intense enough to induce long-term alterations of the AMY transcriptomic profile. Therefore, based on the outcome of my analysis, I suggest adjustments to the exposure paradigm to a daily exposure of 16 h per day and an increasing number of exposure days per cycle to ensure consistent effects in the AMY.

Human intra-species comparison resulted in *SERPINA3* being identified as a new potential biomarker for AUD.

Since in the human species, significant DEGs were retrieved for all three brain regions, PFC, NAc and AMY, an intra-species comparison was performed to detect potentially conserved transcripts that are consistently altered in the human brain (Figure 12), which pointed towards a consistent up-regulation of *SERPINA3*. Additionally, previous studies have found *SERPINA3* to be upregulated in the hippocampus as well as the plasma of AUD patients (McClintick et al., 2013; B. Zhang et al., 2021), which suggests *SERPINA3* as a prospective biomarker for AUD. *SERPINA3* has been associated with glioblastoma and other cancer types (Norton et al., 2021; Soman & Asha Nair, 2022), cardiovascular diseases (L. Zhao et al., 2020), Alzheimer's disease (Kenigsbuch et al., 2022; N. Zhao et al., 2020), and progressing Multiple Sclerosis (Fissolo et al., 2021). The corresponding protein SERPINA3 is considered an acute-phase inflammatory component and has the biological functions of inhibiting cellular growth, proliferation, and differentiation as well as anti-apoptotic properties by interacting with, e.g., the MAPK/ERK1/2 pathway (de Mezer et al., 2023). In psychiatric research, *SERPINA3* was mainly associated with schizophrenia, where it was suggested to initiate dysfunctions of the blood-brain barrier and the innate immune system (Horvath & Mirnics, 2014; Murphy et al., 2020; Trepanier et al., 2016; Zhu et al., 2022). This gene is known to be an astrocyte-specific marker (Baker et al., 2007), and the role of astrocytes in AUD is currently under investigation

since the immune-modulatory effects of alcohol are suggested to be at least partially dedicated to the impairment of this cell type in particular (Adermark & Bowers, 2016; Linker et al., 2019). As discussed in the sections above, astrocytes have been repeatedly shown to be relevant in AUD. The consistent up-regulation of *SERPINA3* might lead to a prospective biomarker, which would enhance the molecular tools for AUD diagnosis. Biomarkers can provide a basis for personalized medicine, such as in cancer therapy, where the evaluation of biomarkers is one of the initial steps for the diagnosis and therapy plan (Hristova & Chan, 2019). In AUD research, previous investigations to determine molecular biomarkers have been made. However, there is still not enough data to identify molecular biomarkers for the main important aspects of AUD, such as treatment response prediction, prediction of at-risk individuals, or to subclassify patients (Ferguson et al., 2022a). In direct comparison to cancer research, the limited number of biomarkers and the lack of precision-based therapeutics in AUD might be explained at least partially by limited access to study samples and small funding budgets (Kranzler et al., 2017). As already stated in the introductory section of this thesis, the AUD research area is lacking successful pharmacotherapy, which might be due to the general underrepresentation of personalized medicine approaches and corresponding studies in this research area, which is highly warranted in such a heterogeneous disease phenotype (Lohoff, 2020; Ragia & Manolopoulos, 2017). In addition, according to DSM-V diagnostics, AUD is sub-classified as mild, moderate, and severe (American Psychiatric Association, 2013). Previous studies have shown that the amount of alcohol consumed, level of response to alcohol, and additional paradigms that describe the risk and severity of AUD also lead to distinct transcriptomic patterns, which is further pointing towards the necessity of personalized medicine (Mulligan et al., 2006; Schuckit et al., 2004). Since AUD patients have a high rate of comorbid mental health disorders such as depression, schizophrenia, bipolar disorder, and anxiety, the interaction of these components needs to be addressed to find the best suiting treatment for the individual patient (AshaRani et al., 2022; Castillo-Carniglia et al., 2019; Cornelius et al., 2003; Debell et al., 2014; Yang et al., 2018).

On the level of GSEA, several pathways were commonly dysregulated across the three brain regions, which are mainly related to cytokine signaling, hemostasis, and response to elevated platelet cytosolic Ca^{2+} (Tables 17, 21, 22). However, in respect to the pathway enrichment analysis, the NAc and the AMY show a higher degree of commonality than with the PFC, since additionally, further immune-signaling aspects, mainly with respect to interleukins, are

significantly altered. These results contribute to previous studies that have pointed strongly towards the importance of immune-signaling pathways as the main influencing factors in excessive alcohol consumption and AUD (Mayfield & Harris, 2017).

Transcriptome-wide meta-analysis is a promising tool to predict new targets to study new effective treatment directions for AUD and other diseases.

Meta-analytic approaches are increasingly being implemented in diverse research areas. Not only meta-analyses of binary outcomes derived from clinical studies or preclinical behavioral experiments but also sequencing data from genome-, methylome-, transcriptome-, and proteome-wide approaches are combined to increase statistical power for the identification of new molecular risk factors and mechanisms underlying diverse diseases. Especially, when considering the high degree of heterogeneity across the original datasets, meta-analyses can build a new, reliable basis for future research. This type of analysis is also in line with the 3R principles (Russell & Burch, 1959) and contributes significantly to reducing, replacing, and refining future animal experiments (Avey et al., 2015; Clavert & Duhamel, 2013). Since the methodologies of transcriptome-wide sequencing approaches were established, numerous studies applying these techniques in the addiction research area have been conducted. Mainly microarray, but also more and more RNA-Seq data were obtained to elucidate transcriptional alterations in the brain that might explain the basic principles of alcohol dependence. These findings were predominantly pointing towards immune system-, neuronal dysfunction, and signaling-related pathways (Farris et al., 2015; Murano et al., 2017; Warden et al., 2016). However, the components at the single-gene level of these significantly altered pathways are diverse across these studies. This suggests that even though the general pathways that are affected by alcohol abuse might be solid, the key players in these pathways still require identification. In addition, research on sex-specific effects in AUD is a frequently discussed topic, as there is lacking data from female animals as well as female AUD patients (Agabio et al., 2017; Ruiz & Oscar-Berman, 2013). While in clinical research, the number of studies that include women is drastically increasing, preclinical research is still barely considering both sexes in behavioral and molecular experiments (Li et al., 2021). This is also reflected in the data of this thesis, since in the rodent studies, there was no dataset observing females (Table 3), and in the human post-mortem studies, the number of women was highly limited, as well (Table 4). Considering that women tend to develop AUD faster than men and

are more likely to suffer from the medical consequences of chronic alcohol abuse (Agabio et al., 2017), this points strongly towards sex-specific differences that are required to be understood in detail to provide appropriate treatment in a more personalized way.

4.2.1 Summary

This study does not only represent the first transcriptome-wide meta-analysis in the alcohol-dependent brain but also demonstrates a novel approach to finding conserved signatures across multiple species to provide promising targets to investigate both in preclinical and clinical studies. As the main findings, I found that in the PFC, *HSD11B1* was commonly dysregulated across all four species observed. In addition, the transcriptomic signatures in the NAc seem to be less vulnerable to dysregulation due to AUD and long-term ethanol exposure with protracted abstinence. The results in the AMY referred to a lack of translatability between the human post-mortem brain tissue and the animal model of alcohol dependence, as in the human brain a high number of DEGs were identified, while the animal data showed no altered transcripts. Therefore, the treatment paradigm might need further adjustment to provide translational input to the patient's situation. In the intra-species comparison of the human findings in the PFC, NAc and AMY, *SERPINA3* was commonly up-regulated through all three brain regions. In combination with previous studies from hippocampus and plasma samples, this finding suggests *SERPINA3* as a novel biomarker for AUD. Bottom line, the findings of this study provide a profound basis for follow-up studies on the behavioral as well as the molecular level in both a species-specific a translational cross-species manner to understand the relevance of these targets in AUD in greater detail. However, especially in terms of sex-specific outcomes, additional primary studies, considering both male and female sex, need to be conducted to enable meta-studies with a reliable sex-specific statement since female sex is currently strongly underrepresented in most of the AUD studies.

4.3 Discussion Study 3: Single nuclei RNA sequencing in PD rat tissue

As demonstrated in previous sections of this thesis, the PD rat model is a frequently and long-term studied animal model of alcohol dependence that is induced by CIE, and it has also proven validity in translational aspects of AUD research (Meinhardt & Sommer, 2015). However, when it comes to the latest cutting-edge techniques of molecular analyses, there is

still a big research gap that requires investigation to understand this model in detail. As suggested in Study 1, the CN, which corresponds to the DMS in rats, plays an essential role when it comes to epigenetic and transcriptomic alterations in the human post-mortem brain. Therefore, this study contributes to disentangle the cellular compartments that might play an important role in AUD in the DMS and, furthermore, will deliver potential new routes to investigate the understanding of alcohol dependent behavior. The main findings of this study are: i) the cell types with the highest number of altered transcripts in the PD rats are microglia, neurons, and oligodendrocytes. ii) These DEGs are cell type-specifically enriched in different biological processes, and iii) chronic exposure to alcohol might cause a cell type-specific vulnerability to degradation processes.

First preliminary snRNA-Seq data in the DMS of PD rats suggests a cell type- and treatment-specific vulnerability to degradation as well as an overall reduced stability of nuclei in the PD group.

When it comes to snSeq experiments, the nuclei isolation technique is probably the most delicate step in the sample processing procedure, and a number of nuclei isolation protocols and further adaptations of these have been published, so far (Alvarez et al., 2023; Krishnaswami et al., 2016; Matson et al., 2018). With the development of the nuclei isolation kit, that was released by 10X Genomics in 2022, it was possible to homogenize this step with a bench-marked kit which enabled reproducible outcomes even throughout several sample batches with the additional advantage of decreased sample handling time.

With the establishment of snSeq approaches, not only the sample size as the number of individuals but also the number of cells or nuclei need to be taken into account to interpret the meaningfulness of the outcome. Therefore, the sample size as well as the number of nuclei to aim for strongly depend on the complexity and heterogeneity of the tissue. (Haque et al., 2017). In this first dataset of four PD rats and four matched controls, the average number of nuclei was around 1,800 nuclei per sample (Figure 13), which is considerably low compared to previous studies (Brenner et al., 2020; Dilly et al., 2022). Even though I aimed for a nuclei recovery of 10,000 nuclei per sample, many nuclei were not transformed into GEMs –probably due to degradation– and additionally, a high number of nuclei was filtered out during the QC steps of the analysis due to low fragment reads in general or a low number of unique genes. Interestingly, the overview of the proportion of nuclei per cell type differed

between the treatment groups (Table 23). While oligodendrocytes made up the cell type with the highest proportion of nuclei for both of the treatments, the further distribution of the number of nuclei was diverse. For PD rats, the cell types with the second and third-highest number of nuclei were pyramidal cells and neurons_1. In the control rats, the cell type with the second-highest degree of nuclei was D2-MSN, followed by pyramidal cells. A previous study on isolated mouse oligodendrocytes demonstrated that ethanol, even at high concentrations, does not impair the viability of these cells (Coutts & Harrison, 2015), which might support the finding of no treatment-specific alterations in nuclei degradation within this cell type. In addition, this study suggested that the demyelination processes observed in AUD might be primarily due to acetaldehyde, which occurs during ethanol digestion, rather than ethanol itself.

The reduced number of total nuclei in the PD rats might partially be explained by treatment-specific influences on the overall stability of the nuclei. Chronic alcohol intake has been previously shown to have a tremendous impact on the general composition of the brain tissue by reducing gray and white matter (Monnig et al., 2013; Spindler et al., 2021). Furthermore, the apoptotic effects of alcohol on brain cells have been demonstrated repeatedly (Han et al., 2005; Hernandez et al., 2016; Sokolowski et al., 2014). Therefore, these data add to the previously reported apoptosis-inducing properties of chronic alcohol intoxication and AUD. However, since the overall number of nuclei observed is rather low, an increase in the sample size is required to confirm these preliminary findings.

Microglia, Oligodendrocytes and the neuronal subtype neurons_2 show the highest degree of altered transcripts across all identified clusters.

GEx analysis in the identified cell types pointed towards a particular relevance for microglia, oligodendrocytes and neurons_2, since these clusters revealed the highest number of significantly altered transcripts (Tables 23-26, Figure 14). When observing the Top 10 genes within these cell types, some genes have already been proven to have relevance in addiction. The pain-associated gene *Scn9a*, which was down-regulated in the neuron_2 cluster (FC=-0.77, p_{adj}=1.17e-10) (Table 25, Figure 14B), was shown to be strongly associated with the alcohol dependent phenotype across several animal models of alcohol dependence (Muir et al., 2023). *Ppp3ca*, which was up-regulated in neurons_2 (FC=0.71, p_{adj}=1.12e-11), is involved in brain plasticity and has been found in a recent cross-species meta-analysis of genome-wide

expression studies (GWES) on cocaine addiction, including human, mouse, and rat datasets, to be significantly associated with cocaine misuse (Forero & Gonzalez-Giraldo, 2020). Furthermore, the relevance of TRP channels in psychiatric disorders has been discussed previously, and *Trpc4*, which was significantly up-regulated in the top 10 DEGs of the neurons_2 cluster in this study (FC=1.21, p_{adj}=8.23e-14), has been associated with anxiety and depressive disorders (Naziroglu & Demirdas, 2015). In oligodendrocytes, the *ApoE* gene, which encodes for an apolipoprotein, was significantly up-regulated (FC= 0.49, p_{adj}=9.16e-13) (Table 26, Figure 14C). This gene has been examined in AUD before; however, to my knowledge, its expression has been solely measured in plasma so far. Nevertheless, the plasma expression of *ApoE* was positively associated with alcohol consumption (Wilkens et al., 2023), and up-regulated *ApoE* in AUD patients undergoing early abstinence was linked to cognitive impairments (Escudero et al., 2023). For the top 10 DEGs identified in microglia, no transcript has been previously reported to be associated with AUD or other addictive disorders.

As already discussed in Study 1 and 2 of this thesis, the fold-changes of the significantly altered transcripts are comparably low. Since Study 1 and 2 focus on bulk expression data, it is worth mentioning, that the same phenomenon can be seen at the single cell level as well. Furthermore, the previously published snRNA-Seq data in pre-clinical AUD research reported rather small effect sizes (Brenner et al., 2020; Dilly et al., 2022), which is demonstrating that the overall influence of alcohol on particular transcripts is rather small in both bulk and single cell resolution.

GSEA of the three cell types with the highest number of DEGs displayed dysregulated pathways in a cell type-specific manner. Microglia were negatively enriched for mitochondrial regulation pathways and positively enriched for immune responses. Neurons_2 were mainly associated with dysregulated neuronal signaling pathways, which supports previous findings on the impairment of neurotransmitter release and neuronal activity in AUD (Li et al., 2019; Ward et al., 2009). Interestingly, in oligodendrocytes, GSEA suggests a down-regulation of epigenetic processes and an up-regulation of transcriptional processes. Therefore, this finding does not only support the findings from Studies 1 & 2, but is also in line with the general knowledge of biology, describing the potential influence of decreased methylation levels on increased transcriptional processes (Migliori et al., 2010).

The majority of the top 20 up-regulated pathways in microglia are neuroimmune regulation processes, which highlights the importance of microglia in the neuroimmune response to chronic alcohol abuse and furthermore supports the previous findings, also described in Study 1 & 2 of this thesis, that AUD is causing an impairment of the neuroimmune system.

These first preliminary data from the PD rat model contribute to previous findings from snRNA-Seq in the addiction field.

As stated in the introductory section of this thesis, single cell techniques are becoming the leading, cutting-edge method in molecular analysis of GEx. However, in the field of addiction research, only a limited number of scSeq studies have been conducted so far. But still, cell type-specific dysregulation has been determined, e.g., from bulk data, by observing the transcripts that are known to have a cell type-specific expression pattern. As already shown in Study 1, cell type enrichment analysis pointed towards an important role for astrocytes and endothelial cells in the CN of deceased AUD patients. In the snRNA-Seq dataset derived from the DMS of PD rats, a small degree of altered transcripts was detected in astrocytes but none in endothelial cells. However, the overall number of both of these cell types, but especially of endothelial cells, was very limited, which does not provide a fundamental basis to draw final conclusions on the potential involvement of these cell types in the DMS of the PD model. Study 2 suggested that mainly inhibitory neurons but also astrocytes and endothelial cells, might play a role in the transcriptomic alterations seen in AUD. Therefore, the findings from Study 1 & 2 are somehow overlapping. However, the additional involvement of inhibitory neurons shown in Study 2 might underline the findings of the neurons_2 cluster that was found to have the second-highest number of DEGs in the snRNA-Seq experiment.

The cell types with the highest number of detected nuclei and altered transcripts were microglia, oligonucleotides, and neurons. A previous study examining the interaction between epigenetic and transcriptomic regulatory mechanisms on the bulk expression level, highlighted an activation of microglia led by an up-regulation of microglia-specific marker genes (Ponomarev et al., 2012). Microglia and their function in the AUD-specific impairment of neuroimmune regulation have been repeatedly demonstrated (Holloway et al., 2023; Wei et al., 2023). Furthermore, the potential role of microglia dysfunction in alcohol-induced neurogenesis and neurodegeneration has been discussed repeatedly (Anand et al., 2023; Kalinin et al., 2018). Therefore, the high number of DEGs observed in neurons and microglia

in the dataset of this study gives additional evidence to the interaction between these two cell types in AUD. Previous snRNA-Seq studies on the alcohol-dependent phenotype also point out the particular relevance of neuronal cells, mainly GABAergic neurons, as well as microglia (Brenner et al., 2020; Dilly et al., 2022). However, these studies were conducted in the PFC and AMY, and the cell type composition changes across brain regions. In the NAc, so far two studies have been conducted studying snRNA-Seq in an addiction context. Both of these reported high alterations in D1- and D2-MSN as well as microglia and oligodendrocytes (Savell et al., 2020; van den Oord et al., 2023). Since van den Oord et al. (2023) were focusing on human post-mortem brain tissue, they were using the second human dataset of the PFC derived from Brenner et al. (2020) to find potential overlaps across the brain regions, and it was possible to replicate DEGs within the same cell type across these datasets. Savell et al. (2020) conducted the snRNA-Seq data as the starting point of the study to then follow up the behavioral relevance of the determined DEGs in MSNs of cocaine intake. This elegant study demonstrated on a sex-specific level that immediate early genes in MSNs might drive the response to acute cocaine intake in both male and female rats. However, in astrocytes, the authors found sex-specific signatures, leading to the conclusion that sex differences might be observed in a cell type-specific manner.

Oligodendrocytes are glia cells that are involved in myelination formation and maintenance at neuronal axons (Simons & Nave, 2015). Therefore, the repeatedly demonstrated transcriptomic alteration in this cell type, which is also supported by the dataset of this study (Brenner et al., 2020; Dilly et al., 2022; van den Oord et al., 2023), might contribute to the proneness to neurodegenerative processes, eventually leading to neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Chandrashekar et al., 2023; Sanna et al., 2023; Zhang et al., 2022).

Bottom line, these preliminary snRNA-Seq data of the DMS from PD rats point towards a pronounced role of microglia, neurons, and oligodendrocytes, which adds further evidence to the so far limited amount of published scSeq data. However, the expansion of the sample size in regard to number of individuals but also the number of nuclei is of great importance to give evidence to these preliminary findings and be able to identify the cell clusters with greater confidence.

4.3.1 Summary

This study describes the first preliminary data of snRNA-Seq in the DMS of PD rats. This model is frequently used to examine alcohol-dependent behavior, which highlights the importance of obtaining cell type-specific outcomes to understand this phenotype to a greater extent. The main findings of this study constitute the highest number of DEGs in microglia, neurons, and oligodendrocytes. The majority of the top 20 up-regulated pathways are neuroimmune regulation processes, which emphasizes the importance of microglia in the neuroimmune response to chronic alcohol abuse and furthermore supports the previous findings, also described in Study 1 & 2 of this thesis, that AUD is causing an impairment of the neuroimmune system.

Since, during the analysis, a much lower number of nuclei was obtained than expected, I additionally observed how specific cellular populations are affected by this degradation process. Indeed, there was a treatment- and cell type-specific effect of vulnerability to cellular degradation discovered. This effect was suggestively pronounced to be strongest in neurons and pyramidal cells. Therefore, this preliminary dataset does not only provide cell types and target genes to follow up on but also suggests that chronic exposure to alcohol decreases cellular stability and therefore increases the vulnerability to degradation processes in a cell type-specific manner.

4.4 Discussion Study 4: Gene expression of COVID-19-related genes across different ethanol treatment paradigms

Alcohol consumption and AUD per se have been previously shown to reduce the activity and capability of the immune system and were suggested as a risk factor for several diseases affecting the immune system (Ferguson et al., 2022b; Warden et al., 2020; Zuluaga et al., 2016). In regard to the COVID-19 pandemic, the current state of research does not deliver a clear-cut conclusion whether above-average drinking and AUD should be considered risk factors. Therefore, this study aimed to contribute important data to determine above-average alcohol consumption and AUD as potential risk factors for COVID-19. The main findings of this study are: (i) the observed SARS-CoV2 infection-relevant genes *Ace2*, *Tmprss2*, and *Mas* are dysregulated organ-specifically in peripheral organs where *Ace2* is consistently up-regulated in the lung throughout all observed treatment paradigms, which might lead to

increased levels of ACE2 protein and therefore an increased stochastic probability of the virus entering the cells in the lung. (ii) Up-regulation of *Mas* GEx due to abstinence in the treatment paradigm may lead to a hyper-activated anti-inflammatory response initiated by the ACE2/Ang(1-7)/Mas cascade, which may be interpreted as a protective factor for COVID-19 disease severity. (iii) The olfactory bulb was the only brain region showing significant alterations in the GEx pattern of the target genes, where *Mas* expression levels significantly declined. This opposite effect to the observations in the peripheral organs suggests a potential increased risk for anosmia during and after SARS-CoV2 infection.

Increased *Ace2* mRNA expression might lead to a higher probability of virus entry due to a rising ACE2 receptor presence on the cell surface.

Previously, substance use disorders (SUDs) such as opioid use disorders and smoking have been in focus during the COVID-19 pandemic, where increased infection probability as well as a higher risk of worse prognosis and mortality have been reported (Baillargeon et al., 2021; Patanavanich & Glantz, 2021; Wang et al., 2021). These SUD patient studies have shown up-regulated *Ace2* expression levels in several organs (Althobaiti et al., 2020; J. C. Smith et al., 2020; Wei & Shah, 2020). Therefore, our findings are in line with those of other SUDs, which indicates that AUD and chronic alcohol consumption might be considered risk factors for severe COVID-19 as well. In addition, most of the AUD patients also consume cigarrets on a regular basis (Bobo & Husten, 2000). Since smoking has already been classified high-risk factor for severe COVID-19 early in the pandemic, AUD patients are at even higher risk considering their comorbid smoking. In addition, the lung is the most vulnerable organ for SARS-CoV2 infection since it is the first point of contact with the virus via its main route of infection (Jiang et al., 2020), and increased expression levels of *Ace2* in specific organs have been shown to lead to an increased infection probability in these organs (He et al., 2021). However, as soon as the cells are infected by the SARS-CoV2 virus, a decline in *Ace2* expression levels takes place due to the internalization process during virus entry, which also affects the ACE2 receptor as it is a membrane surface protein. These reduced ACE2 levels induce an imbalance in the ACE2/Ang(1-7)/Mas cascade and an increased activitiy of its counterplayer pathway, the ACE/AngII/AT1R cascade, which causes inflammation and oxidative stress and, in the worst case, can lead to acute respiratory distress sysndome (Richardson et al.), systemic organ failure and death (Kaseb et al., 2021). The expression levels of *Ace2* were interpreted

controversely in the literature when it came to COVID-19 risk factors. Therefore, in risk groups of severe COVID-19 such as diabetic and obese patients, *Ace2* expression levels have been shown to be increased, induced by pro-inflammatory states (Gkogkou et al., 2020). However, increased *Ace2* expression levels could provide a molecular explanation for epidemiological findings resulting in a higher infection propensity and worsened prognosis in AUD patients. Interestlingly, the number of alcoholic drinks per week has previously been correltated to ARDS during and after COVID-19 (Lassen et al., 2021). In addition, AUD patients have been reported to have an increased risk of hospitalization due to COVID-19 (Allen et al., 2021), prolonged hospital stays, and complications (Varela Rodriguez et al., 2021). The well-known general impairment in the innate and adaptive immune system due to long-term alcohol consumption (Kershaw & Guidot, 2008; Simou et al., 2018) certainly delivers additional evidence to conclude that above-guideline drinking in a chronic manner could represent a risk factor for infectious diseases such as COVID-19.

***Tmprss2* expression levels are consistently up-regulated across the models, which might result in accelerated virus infiltration.**

The detected overall up-regulation of *Tmprss2* GEx in the absence of abstinence suggests that expression of this gene is dysregulated by the presence of blood alcohol concentration. The up-regulation of *Tmprss2* mRNA might lead to accelerated virus infiltration since this gene is involved in the first step of virus entry, where SARS-CoV2 binds to the cell membrane and S2 spike proteins are cleaved to enable the virus to enter (Hoffmann et al., 2020). To underline the importance of this gene even further, previous research has suggested TMPRSS2 inhibitors as therapeutic approaches against COVID-19 (Hoffmann et al., 2020; McKee et al., 2020; Zununi Vahed et al., 2020).

The *Mas* mRNA is opposingly dysregulated in peripheral organs and brain tissue.

Once SARS-CoV2 has entered the cells and replicated, the anti-inflammatory ACE2/Ang(1-7)/Mas cascade is activated, and *Mas* is one of the key players in this pathway (Gheblawi et al., 2020). Therefore, up-regulation of *Mas* has been suggested as a potential protective factor against severe complications during COVID-19. In our study, we identified a treatment-specific up-regulation of *Mas* due to the abstinence period after chronic alcohol intake, which appeared consistently in the ileum. The digestive system, including the ileum, has been shown

to be particularly vulnerable to COVID-19 consequences since patients with SARS-CoV2 infection often report nausea, vomiting, and diarrhea, which occur mainly at the beginning of the disease (Gu et al., 2020; Kotfis & Skonieczna-Zydecka, 2020). These studies also suggest the gastrointestinal tract as an alternative route of infection for COVID-19. Hence, this finding of up-regulation of *Mas* mRNA in the sub-chronic and PD cohorts suggests partial protection against certain side effects from SARS-CoV2 affecting the gastrointestinal tract.

In the brain, *Mas* was specifically downregulated in the olfactory bulb, while any other brain region did not show significant differences in the GEx of the detected target genes. This effect might lead to decreased activity of this anti-inflammatory response to the virus infection in the olfactory bulb, and therefore represents a potential predisposition to anosmia. Previous studies support the finding that the olfactory bulb is strongly impaired in rodents with prenatal or neonatal alcohol exposure (Akers et al., 2011; Burke et al., 2016; Chen et al., 1999), as well as adult rodents with a history of chronic alcohol intake (Collins et al., 1998; Gericke et al., 2006; Penland et al., 2001). Overall, the observations in 18 brain regions suggest that brain tissue in general, except the olfactory bulb, seems to be less vulnerable to COVID-19 infection. The rodent brain generally has low expression levels of the observed COVID-19 marker genes, despite a high expression of *Ace2* in the olfactory bulb, which supports the findings of this study (https://mouse.brain-map.org/gene/show/45849).

4.4.1 Summary

This study focused on the expression of SARS-CoV2 infection-relevant genes *Ace2*, *Tmprss2*, and *Mas* in diverse chronic ethanol treatment groups in six different organs: the brain, liver, kidney, lung, ileum, and heart. While *Ace2* was consistently up-regulated in the lung across all treatment paradigms, *Tmprss2*, and *Mas* showed treatment-specific outcomes. The main findings of this study suggest an increased propensity for COVID-19 infection and severe disease progression, including anosmia, in patients with long-term alcohol intake. In addition, these patients might suffer from additional side effects of COVID-19, depending on their blood alcohol concentration during the progression of the infection. These findings are in line with epidemiological data that reported an increased risk of COVID-19 infection in general as well as complications and mortality in AUD patients and patients with a history of above-guideline drinking. Nevertheless, additional studies need to be conducted to fully understand the impact of chronic alcohol intake on infectious diseases such as COVID-19, since the

observed AUD cohorts are usually statistically underrepresented in the reported studies. Especially the sex-specific aspect needs to be further elucidated. However, it has been shown that during the pandemic, some people increased their alcohol consumption, which will have a long-lasting effect on the global health system.

5. Concluding remarks

The aim of this thesis was to contribute molecular examinations on a transcriptome-wide level in human post-mortem brain tissue as well as in the brain of the PD rat model by applying cutting-edge techniques, such as RNA-Seq, transcriptome-wide meta-analysis, and snRNA-Seq on thirteen brain regions in total. Commonly identified DEGs, pathways, and networks strongly suggest targets for further investigation. Since this thesis was observing transcriptomic changes across a variety of brain regions, this data points towards brain regions that should be further investigated but also brain regions that might not be highly relevant for the alcohol-dependent phenotype describing long-term alcohol abuse and prolonged abstinence.

On the bulk expression level, Studies 1 & 2 pointed out the limited relevance of the ventral striatum, embedding the NAc, for the long-term abstinent phenotype, as both human post-mortem brain and tissue derived from the PD rodent model did not yield a notable number of altered transcripts. Furthermore, the CN, which was analyzed in Study 1 on both the methylation and the transcription level was identified as the major striatal region that is dysregulated in this specific phenotype on both levels of investigation. Dysregulated genes in this brain region suggested dysregulated immune pathways, as well as an increased potential for neurodegenerative diseases and cancer. Since, especially on the DNA methylation level, a number of genes were observed that have not been reported in context with AUD before, these findings point towards further functional investigations on relevant features of the alcohol-dependent phenotype such as craving and relapse.

Study 2 examined the transcriptome-wide alterations in human post-mortem brain and the PD animal model in a meta-analytic approach. Here, the PFC was the brain region with the highest number of altered transcripts for all the examined species: rat, mouse, human, and monkey. Furthermore, *HSD11B1* has been identified as commonly dysregulated in the PFC across all these species, suggesting that this gene might play a particular role for the alcohol-

dependent phenotype. The intra-species comparison in humans comparing the meta-analysis findings of PFC, NAc and AMY, led to a new potential biomarker, namely *SERPINA3*, which was found to be up-regulated consistently across these regions and in addition has also been reported to be up-regulated by previous studies focusing on the hippocampus and plasma of AUD patients.

When I further investigated the CN, which is referred to as the DMS in rodents, in Study 3 by applying snRNA-Seq in the DMS of PD rats, oligodendrocytes, microglia, and a neuronal subtype were suggested to play a particular role in the transcriptomic signatures that endure protracted abstinence. GSEA showed distinct patterns of dysregulated pathways in a cell type-specific way. The DEGs in microglia were enriched for neuroimmune processes, which added evidence to the alterations in the CN of the human post-mortem brain detected in Study 1. Additionally, DEGs in oligodendrocytes were positively enriched in transcriptional processes and negatively enriched in epigenetic processes, pointing towards an interfering mechanism between epigenetic and transcriptomic processes, as already seen in the CN in Study 1.

In Studies 1-3 pathway analyses repeatedly resulted in dysregulated immune functions, which led to the question whether AUD might represent a potential risk factor for SARS-CoV2 infection, especially since this study was conducted during the COVID-19 pandemic. Indeed, Study 4 demonstrated that *Ace2* gene expression was consistently up-regulated in the lungs of three rat models of long-term alcohol intake, which suggested that this organ is particularly vulnerable to SARS-CoV2 infection, when alcohol has been consumed in a sub-chronic or chronic manner. Since this virus is primarily entering the system via the lungs, this finding was interpreted as a potential risk factor for an increased SARS-CoV2 infection rate and severe disease progression. Furthermore, *Mas*, the gene involved in the initiation of the anti-inflammatory response cascade to oppose the viral infection, was down-regulated in the olfactory bulb, which might point towards an increased risk for long-term consequences of COVID-19, such as anosmia.

In summary, this multi-modal study, including a variety of techniques to investigate transcriptional regulation underlying AUD across multiple brain regions, represents a comprehensive overview of altered transcripts, networks, and pathways that might build the basis for follow-up studies on a molecular as well as the behavioral level.

6. Outlook

The findings of this thesis form the basis for several follow-up studies on different levels. The meta-analysis results suggest immune-regulatory processes as well as *HSD11B1* in the PFC are significantly altered by AUD. Therefore, further studies that will look into addiction-relevant behaviors in PD rats when targeting these molecules might deliver further functional insights and will also reveal their potential therapeutic role. Additionally, *SERPINA3* was identified as a molecular biomarker for AUD since it appeared to be consistently up-regulated in all observed human post-mortem brain regions, as well as in other previous studies focusing on blood samples and the hippocampus of deceased AUD patients. Since the diagnostics of mental disorders in general is facing limitations, such as, e.g., subjective assessment tools in the most common diagnostic tools DSM and ICD, and their overall lacking sensitivity (Gauld, 2022; Norris et al., 2016), molecular biomarkers are of great importance to support the diagnostic process. Therefore, this study contributes to improving the diagnostic conditions of AUD in clinics by suggesting *SERPINA3* as a potential molecular biomarker.

Especially the findings derived from the comparative meta-analyses in different species and brain regions can serve as an excellent basis for follow-up studies. The robustness of this study, by including a significantly higher number of individuals than single laboratory experiments can afford and by considering the heterogeneity that comes from diverse experimental designs and conditions, provides a profound basis to explore the behavioral meaning of these transcriptomic changes. In addition, this study also provides insights into the translatability of the animal models to the patient situation and can provide suggestions for optimization of the models. Hence, we hope that this study will serve as a lively dataset that will be extended over time to potentially identify even more commonly dysregulated transcripts across species.

Appendix

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