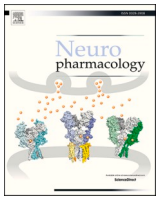
Neuropharmacology 295 (2026) 110995

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm



Region- and sex-dependent single-cell transcriptomic signatures of neurons and glia in the prefrontal cortex and nucleus accumbens in a rat model of alcohol relapse

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ARTICLE INFO

Keywords:
Alcohol use disorder
Addiction
Single nuclei RNA sequencing
Dopamine receptor
Phosphodiesterase
Synaptic plasticity
Neuroinflammation

ABSTRACT

Alcohol use disorder (AUD) is characterized by problems controlling alcohol drinking despite adverse consequences, development of tolerance and/or withdrawal symptoms. Notably, sex differences in alcohol consumption patterns and susceptibility to relapse are well documented but remain poorly understood at the molecular level.

In this study, we performed single-nuclei RNA sequencing (snRNA-seq) of the medial prefrontal cortex (mPFC) and nucleus accumbens (NAcc) from male and female outbred RccHan Wistar rats exposed to the alcohol deprivation effect (ADE) paradigm, a well-established model of relapse-like drinking behaviour. Comparing high- to low-drinking rats, we found pronounced transcriptional changes across different cell types, with the highest number of differentially expressed genes observed in GABAergic medium spiny neurons (MSNs) of the NAcc and glutamatergic neurons of the mPFC associated with relapse. Importantly, we also identified sex- and region-dependent transcriptional alterations, including differential expression of dopamine receptors and phosphodiesterase family genes, which have previously been associated with AUD in humans, as well as alterations in the transcription of genes associated with synaptic plasticity and neuroimmune signalling. Finally, we found induction of immune-related genes in microglia and sex-dependent activation of immune- and myelination-related genes in astrocytes and oligodendrocytes.

These findings highlight cell type-, region-, and sex-dependent molecular signatures associated with alcohol relapse drinking, which may provide new therapeutic targets for AUD.

1. Introduction

AUD is a chronic, often relapsing disease characterized by problems controlling alcohol consumption despite adverse consequences. Alcohol addiction arises from a multifaceted interaction of biological, psychological and social factors, and results in significant health and economic burdens (Gautier et al., 2021; Kendler et al., 2008). Globally, harmful alcohol use contributes to 8.6% of the disease burden in men and 1.7% in women, indicating significant sex differences. Although a range of environmental and genetic contributors to alcohol consumption and addiction have been recognized, a deeper understanding of how these influences vary between sexes is still missing (Becker et al., 2017; Reilly et al., 2017).

Neurobiological investigations have highlighted the involvement of

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https://doi.org/10.1016/j.neuropharm.2026.110995
Received 22 August 2025; Received in revised form 19 April 2026; Accepted 26 April 2026
Available online 27 April 2026

the medial prefrontal cortex (mPFC) and nucleus accumbens (NAcc) in AUD. The mPFC regulates executive function and is involved in reward processing (Ball et al., 2011), playing a key role in the escalation of alcohol drinking and the development of alcohol dependence (Abernathy et al., 2010; Heilig et al., 2017). The NAcc, in the ventral striatum, is the main input structure of the basal ganglia and is crucial for reward-related processes; it regulates the environmental control of alcohol drinking and relapse (Chen et al., 2011). Dysregulation of these two brain regions contributes to the development of alcohol dependence through changes in neuronal circuits, neurotransmitter systems, and inflammatory responses (Clarke and Adermark, 2015; de Zavalia et al., 2021; Hong et al., 2019; Ji et al., 2017; Lu et al., 2019).

To investigate the mechanisms driving alcohol addiction and relapse, we employed the alcohol deprivation effect (ADE) paradigm, a well-established preclinical model for studying relapse (Spanagel and Hölter, 1999; Vengeliene et al., 2014). In this model, animals have prolonged access to alcohol, interrupted by repeated phases of deprivation. Upon re-exposure to alcohol following a withdrawal period, rodents exhibit a transient but significant increase in alcohol intake, known as the ADE, along with a progressive preference for solutions containing higher concentrations of alcohol (Vengeliene et al., 2014; Wolffgramm, 1991). Moreover, research using alcohol-preferring rat strains demonstrated that repeated cycles of deprivation can drive compulsive-like drinking behaviours, where consumption persists even when alcohol is adulterated with aversive agents like quinine (Giuliano et al., 2021; Spanagel et al., 1996; Vengeliene et al., 2014; Villarín Pildaín et al., 2013).

Sex differences in alcohol consumption and in the development of alcohol dependence are well-documented. In humans, males generally show higher alcohol consumption and dependence rates than females (Becker and Koob, 2016; Becker et al., 2017; Ceylan-Isik et al., 2010; Flores-Bonilla and Richardson, 2020; Wilsnack et al., 2009). However, females experience more severe adverse effects of alcohol use, including physical health complications, cognitive deficits and mental health issues (Becker and Koob, 2016; Becker et al., 2017; Ceylan-Isik et al., 2010; Flores-Bonilla and Richardson, 2020; Wilsnack et al., 2009). In contrast, rodent models often show the opposite pattern, with females displaying higher alcohol intake than males (Mittal et al., 2020; Sneddon et al., 2019; Tambour et al., 2008). We recently showed that female outbred RccHan Wistar rats had higher alcohol consumption than males in the ADE paradigm, and greater sensitivity to drug-paired cues in the Pavlovian Conditioned Approach (Hakus et al., 2025). These sex-dependent differences in alcohol consumption highlight the importance of investigating sex-dependent molecular mechanisms in AUD.

Here, we performed snRNA-seq of the mPFC and NAcc from high-drinking (HD) and low-drinking (LD) female and male ADE rats without an alcohol-naïve (water) control. The results provide insights into the cell-specific molecular adaptations underlying region and sex differences in alcohol relapse drinking.

2. Materials and methods

2.1. Animals

All experimental procedures complied with the European Communities Council Directive 2010/63/EU and were approved by the local state authority (Landesamt für Gesundheit und Soziales (LaGeSo), Berlin; approval no. G0102/19). Experiments were conducted on postnatal day 60 (PND60) using six female and six male RccHan Wistar rats (Envigo, Netherlands). The animals were housed individually to facilitate precise measurement of individual alcohol intake and kept under a 12:12-h light/dark cycle. Ambient conditions were controlled with a temperature range of 20-24 °C and relative humidity maintained between 45 and 65%. Rats were provided with standard laboratory chow (Ssniff, Soest, Germany) and water ad libitum for the entire study duration. All efforts were made to minimize animal suffering and reduce the number of animals used.

2.2. ADE procedure

At PND60, rats were exposed to a 4-bottle free-choice paradigm, providing continuous ad libitum access to tap water and ethanol solutions at concentrations of 5%, 10%, and 20% (v/v), respectively, prepared by diluting 96% ethanol (TechniSolv, VWR Chemicals, France) in tap water. To prevent spillage and evaporation, specialized bottle caps (Zoonlab GmbH, Germany) were used, and bottle positions were rotated regularly to avoid location bias.

Rats underwent six cycles of ADE. After an initial 8-week phase of continuous ad libitum alcohol access, a 2-week deprivation period was introduced, followed by reintroduction of access to alcohol. Over the next 11 months, the animals were subjected to repeated deprivation (2-5 weeks) and reintroduction phases (6-8 weeks). Ethanol consumption was recorded daily for 5 days before and after each deprivation phase. Baseline ethanol intake (BL) was defined as the average daily ethanol consumption in grams of pure ethanol per kilogram of body weight (g/kg/day) across the 5 days preceding a deprivation period. Post-deprivation ethanol consumption was measured daily and compared to baseline levels. During the first 24 h of the 6th reintroduction phase, 0.1 g/l quinine was added to all ethanol solutions (5%, 10%, and 20%) to test aversion. Rats were classified as HD or LD based on their alcohol consumption during ADE5 using sex-specific cutoffs set at 7 g/kg for females and 4 g/kg for males.

2.3. Tissue preparation

At the conclusion of the 6th ADE cycle, all animals were killed by decapitation under isoflurane anaesthesia (5% isoflurane in 2% O_2). Brains were quickly extracted and snap-frozen in methyl butane (Sigma-Aldrich Ltd.) for 2 min, then cooled to −40 °C with liquid nitrogen, before being stored at −80 °C. Using a cryotome set at −16 °C, 1-mm-thick coronal brain slices were prepared. Micropunches were taken from the mPFC and the NAcc on a cold plate maintained at −20 °C. The tissue samples were immediately stored in DNA LoBind® Tubes (Eppendorf) and preserved at −80 °C for subsequent nuclei isolation.

2.4. Nuclei isolation

Frozen micropunches were lysed in NP-40 Lysis Buffer (containing 10 mM Tris-HCl pH 8.0, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, 1 × protease inhibitor cocktail, and RNase inhibitor) and homogenized using a pestle. The homogenates were filtered through a 70 µm pre-separation strainer and centrifuged at 500×*g* for 5 min at 4 °C. Pellets were washed twice using Wash Buffer (PBS supplemented with 1% BSA and RNase inhibitor) and incubated on ice. After the final wash, nuclei were resuspended in Wash Buffer, stained with DAPI, and incubated for 2 min on ice. The suspension was then filtered through a 40 µm Flowmi cell strainer into Sort Buffer (PBS with 1% BSA and RNase inhibitor) and sorted by FACS at the BIH Core facility.

2.5. RNA sequencing

Sequencing libraries were equimolarly pooled based on Qubit concentration measurements and TapeStation size distributions. The loading concentration of the pool was determined using a qPCR assay (Roche, #7960573001). Libraries were then sequenced on the Illumina NovaSeq 6000 platform, on one lane of SP flowcell in 28 + 10+10 + 90 sequencing mode, with a target of 200 million reads per library.

2.6. snRNA-seq data processing

2.6.1. Single-cell pre-processing and alignment

Cell Ranger (10x Genomics, version 7.1.0) (Zheng et al., 2017) was

used to construct the reference genome, align, and quantify the counts from single-cell samples adding –include-introns flag. Genome sequence and gene annotations files for *Rattus novergicus* mRatBN7.2 assembly were downloaded from the Rat Genome Database. Reference sequence was prepared using the cellranger mkref function. Subsequently, the cellranger count function was used for alignment and quantification of reads. Resulting count matrices were used as input for downstream analysis.

2.6.2. Pre-processing and quality control

Count matrices were loaded and analyzed with Scanpy (version 1.9.1) (Wolf et al., 2018). Quality control metrics were computed, including total number of detected genes per cell, Unique Molecular Identifier (UMI) counts per cell, and percentage of mitochondrial counts. Cells with fewer than 500 detected genes, more than 20,000 total UMI counts, or greater than 1.5% mitochondrial counts were excluded from further analysis.

2.6.3. Normalization, dimensionality reduction, and clustering

Gene expression counts for each cell were log-normalized and scaled by a factor of 10,000 (Satija et al., 2015). Principal component analysis (PCA) was conducted using the top 500 most variable genes. The first 30 principal components (PCs) were then used to construct the neighbourhood graph. Clustering was carried out using the Leiden algorithm (Traag et al., 2019) with a resolution setting of 0.3. To visualize the clustering results in a lower dimensional space, Uniform Manifold Approximation and Projection (UMAP) was applied. The gene expression level shown in the UMAP density plots represents the per-cell normalized and log-transformed expression value across all cells in the dataset. The relative expression scale is defined globally across all clusters and is not recalculated within individual clusters or tissues. Each point corresponds to a single cell's expression of the indicated gene/-genes, and the kernel density estimate visualizes regions in the UMAP space with expression-weighted cell densities (Luecken and Theis, 2019).

Differentially expressed genes in each cluster were identified using the Wilcoxon rank-sum test.

2.6.4. Differential expression and pathway analysis

The analysis of differentially expressed genes (DEGs) was performed using the *"rank_genes_groups"* function with Wilcoxon rank-sum test. Genes with an adjusted p-value <0.05 were marked as significant. GO term enrichment analysis was performed with the ClusterProfiler package (version 4.14.4) (Wu et al., 2021; Xu et al., 2024; Yu, 2024; Yu et al., 2012). The "score" in the supplemental tables represents the strength and direction of the rank-based test used for differential expression analysis. Specifically, for each gene and group, Scanpy performs a Wilcoxon rank-sum test comparing expression values in the group against all other cells. The raw Wilcoxon statistic is then converted into a Z-score, which is reported as the "score."

2.6.5. Weighted Gene Co-expression Network Analysis (WGCNA)

To address potential variability associated with the use of an outbred strain, we performed a Weighted Gene Co-expression Network Analysis (WGCNA) (Langfelder and Horvath, 2008; Tian et al., 2020) on pseudobulk single-cell RNA-seq data using the pyWGCNA package (version 2.2.1). The analysis aimed to investigate the relationship between gene expression modules and alcohol consumption dose while accounting for relevant biological and technical covariates. Briefly, single-cell expression data were aggregated into pseudobulk profiles at the cluster level to enhance signal robustness and reduce cell-to-cell variability. Expression matrices were normalized and filtered to include genes with sufficient expression across samples. Co-expression networks were constructed using soft thresholding to approximate scale-free topology, and module eigengenes were correlated with experimental traits, including sex, cluster identity, alcohol consumption dose, and brain region. To mitigate confounding effects of sex, the analysis was performed in a sex-stratified manner, and module-trait relationships were evaluated separately for males and females (Supp. Figs. 11 and 12).

3. Results

3.1. Clustering and cell annotation

We performed snRNA-seq on the mPFC and NAcc of outbred RccHan Wistar rats exposed to the ADE paradigm (Fig. 1A). Our dataset comprised transcriptomic profiles from 204,347 nuclei collected from 6 male and 6 female animals grouped into HD and LD ($n = 3$ per group) based on their individual daily ethanol drinking profile (g/kg/day) at the 5th cycle. This time point was selected as the drinking phenotype is well established after repeated cycles of alcohol access and deprivation, and the paradigm is generally accepted to be fully established after approximately eight months of total alcohol exposure, which corresponds to this cycle. Sex-specific cut-offs of 7 g/kg for females and 4 g/kg for males were applied, consistent with our previous findings showing higher alcohol consumption in female RccHan Wistar rats under the same ADE paradigm (Hakus et al., 2025; Supp. Fig. 1).

Clustering analysis revealed 12 clusters with distinct marker gene profiles (Fig. 1B and C). Based on canonical markers commonly identified in transcriptomic studies, we annotated the major brain cell types, including GABAergic neurons, glutamatergic neurons, oligodendrocytes, oligodendrocyte progenitor cells, astrocytes, microglia, radial-glia–like progenitors, and endothelial cells. Four clusters mapped to GABAergic neurons, three to glutamatergic neurons, and the remaining clusters each represented a unique non-neuronal cell type (Fig. 1B). Among the different clusters of GABAergic neurons, we specifically identified medium spiny neurons (MSNs) in cluster 0 based on the expression of *Pde10a, Bcl11b, Drd1* and *2* markers (Reiner et al., 2024) (Supp. Fig. 2).

Cell type proportions were in line with prior snRNA-seq studies (Brenner et al., 2020; Reiner et al., 2022, 2024), and neurons were overrepresented compared to non-neuronal populations (Supp. Fig. 3). In line with this, the highest numbers of DEGs were detected in cluster 0 MSNs of the NAcc and in cluster 1 glutamatergic neurons of the mPFC (Extended Data 1 and 2). Importantly, we observed no differences in cell-type proportions in NAcc and mPFC between HD and LD rats, consistent with findings from human post-mortem studies (Brenner et al., 2020; Kapoor et al., 2019). Finally, WGCNA analysis showed that gene expression modules were associated with cell type and alcohol dose (Supp. Fig. 11, Extended Data 9), as well as with brain region, in a sex-stratified manner (Supp. Fig. 12, Extended Data 9), rather than reflecting variability due to strain differences or technical noise.

3.2. Sex- and region-dependent transcriptional changes of dopamine receptors and phosphodiesterases in NAcc MSNs and mPFC glutamatergic neurons

Alcohol exposure is known to affect dopaminergic neurotransmission, particularly within the NAcc, the so-called dopaminergic reward pathway (Heinz, 2002). The NAcc is primarily composed of GABAergic MSNs, which largely express either D1 or D2 dopamine receptors (Gerfen and Surmeier, 2011; Reiner et al., 2024). In our analysis, we specifically identified MSNs within GABAergic neurons of cluster 0 (Supp. Fig. 2) and focused our investigation on this population. Our regional comparison between the mPFC and NAcc confirmed significantly higher expression of the dopamine receptor genes *Drd1* and *Drd2* in the NAcc (Supp. Fig. 4A, Extended data 3).

Previous studies have demonstrated that long-term alcohol intake leads to a post-synaptic downregulation of dopamine D2 receptors, which may represent an adaptation to prevent overstimulation of the striatal dopaminergic system (Hietala et al., 1994; Kuikka et al., 2000; McBride et al., 1993; Stefanini et al., 1992; Tupala et al., 2001; Volkow

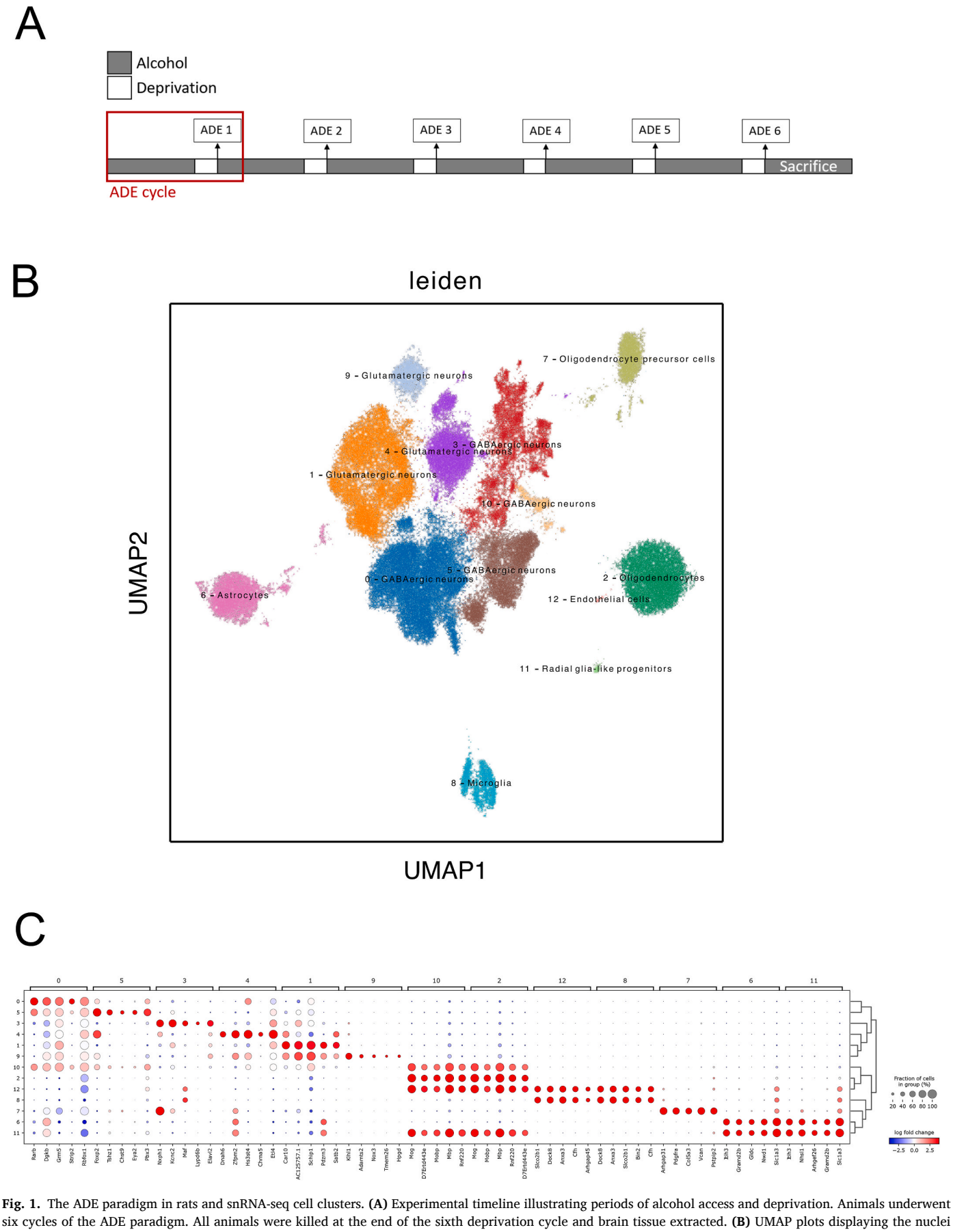


Fig. 1. The ADE paradigm in rats and snRNA-seq cell clusters. **(A)** Experimental timeline illustrating periods of alcohol access and deprivation. Animals underwent six cycles of the ADE paradigm. All animals were killed at the end of the sixth deprivation cycle and brain tissue extracted. **(B)** UMAP plots displaying the nuclei

divided into 12 distinct clusters after annotation with canonical cell-type-specific marker genes. **(C)** Dot plot showing the top five marker genes expressed by each cell cluster.

et al., 1996).

In our dataset, we observed sex-specific transcriptional changes in the dopamine receptor genes *Drd1* and *Drd2* when comparing HD versus LD rats (Fig. 2A, Extended Data 1 and 2). We also performed a differential expression analysis based on alcohol consumption dose (Extended Data 8). In female HD rats, both *Drd1* and *Drd2* transcripts were downregulated in NAcc MSNs, with estimated decreases of 20% and 9%, respectively, based on log2 fold changes, while in mPFC glutamatergic neurons, both genes showed a 47% estimated reduction relative to LD rats (Fig. 2A). In contrast, in male HD rats, both *Drd1* and *Drd2* were upregulated in glutamatergic neurons of the mPFC, with estimated increases of 37% and 82%, respectively, while in MSNs of the NAcc, *Drd2* expression was increased by an estimated 20%, whereas *Drd1* levels remained unchanged compared to LD rats (Fig. 2A). Direct comparison of sexes, within the HD and LD subgroups, further highlighted sex and region differences (Supp. Fig. 4B, Extended data 3). These results suggest that dopamine receptor regulation in the ADE paradigm varies by both sex and brain region, which may contribute to sex-specific responses to alcohol.

Phosphodiesterase (PDE) proteins are closely linked to dopaminergic signalling and have been implicated in the regulation of alcohol consumption (Wen et al., 2018). By modulating levels of the second messengers cyclic AMP (cAMP) and cyclic GMP (cGMP), PDEs have been recognized as interesting therapeutic targets in AUD. In our dataset, *Pde* genes showed extensive dysregulation in a sex- and region-dependent manner (Fig. 2B, Extended Data 1 and 2). In NAcc MSNs in HD female rats, *Pde4b* mRNA was the most significantly downregulated gene with an estimated reduction of 35%, based on log2 fold change, whereas *Pde1a* mRNA was the most significantly upregulated gene with an estimated increase of 57% (Supp. Fig. 5). In NAcc MSNs in HD males, *Pde1a* mRNA was similarly upregulated, showing an estimated increase of 29%, whereas *Pde4b* mRNA levels remained unchanged (Fig. 2B). In glutamatergic neurons of the mPFC, *Pde4b* mRNA was downregulated in HD females by an estimated 29% but upregulated in HD males by an estimated 60% (Fig. 2B, Extended Data 1 and 2). Similarly, *Pde1a* mRNA showed upregulation in mPFC glutamatergic neurons in HD males but

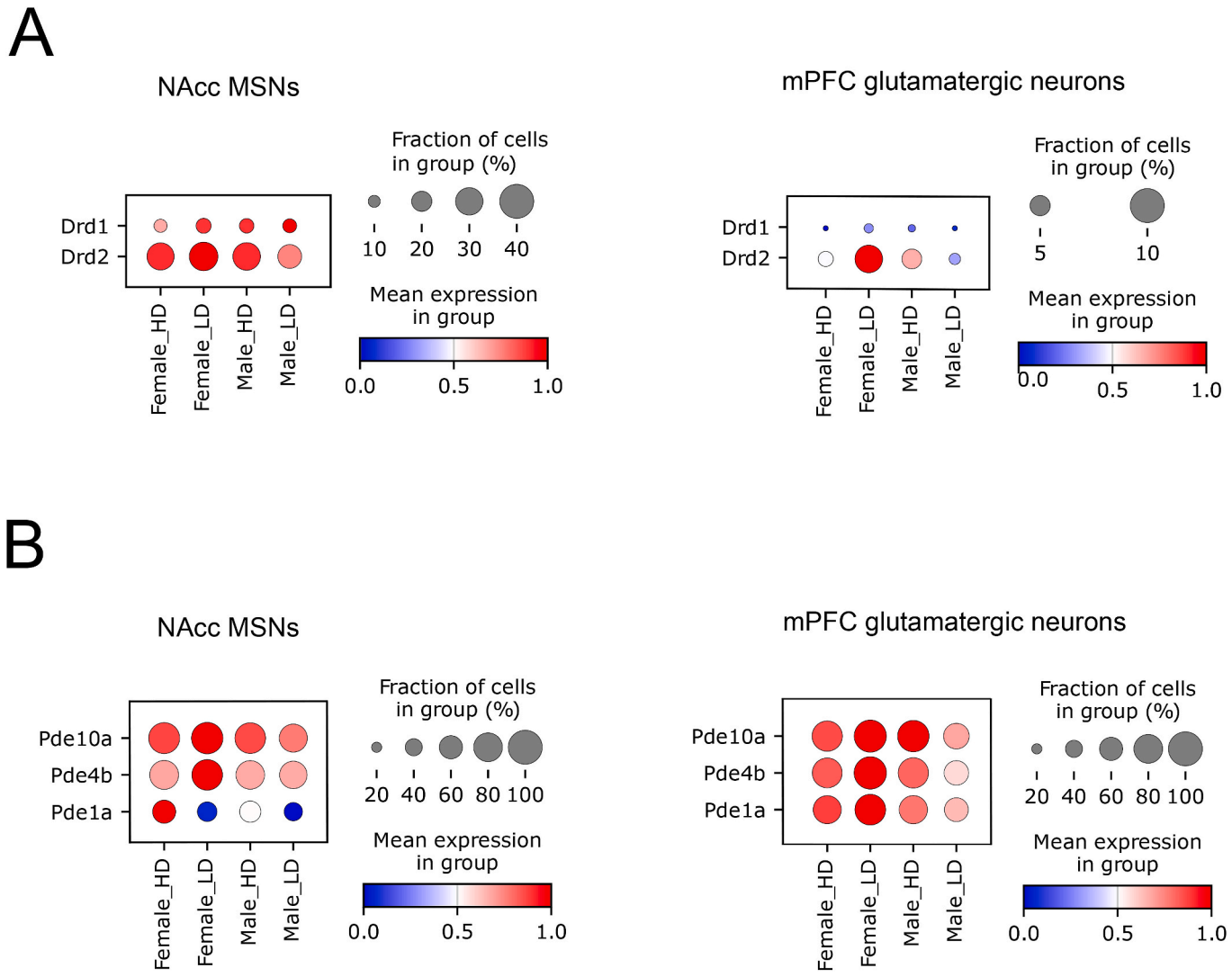


Fig. 2. Sex- and region-dependent expression of dopamine receptors and phosphodiesterases genes in HD and LD rats across neuronal subtypes and sexes. Dot plots showing the expression of **(A)** *Drd1* and *Drd2* and **(B)** *Pde10a, Pde4b* and *Pde1a* genes in female and male HD and LD rats in NAcc MSNs and mPFC glutamatergic neurons. Dot size represents the proportion of cells expressing the gene within each group and colour indicates the average gene expression level. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

downregulation in HD females (Fig. 2B, Extended Data 1 and 2). Additionally, we identified differential expression of *Pde10a* which has emerged as a key modulator of excessive alcohol consumption (Wen et al., 2018). In female HD rats, *Pde10a* mRNA was downregulated in glutamatergic neurons and MSNs by an estimated 22% and 18%, respectively. In contrast, *Pde10a* mRNA was upregulated by an estimated 76% in mPFC glutamatergic neurons in HD males (Fig. 2B, Extended Data 1 and 2). Overall, these findings are in line with previous microarray studies that identified *Pde1a, Pde1b, Pde4b* and *Pde10a* as differentially expressed genes in the brains of AUD rodent models. For example, *Pde4b* and *Pde10a* mRNAs were altered in whole-brain samples from alcohol-naïve high-versus low-alcohol-preferring mice (Mulligan et al., 2006). Moreover, *Pde1a* mRNA was dysregulated in the frontal cortex following a single 4-h alcohol intake session (Mulligan et al., 2011), whereas *Pde1b* and *Pde10a* mRNAs showed changes in response to chronic intermittent ethanol intake (Osterndorff-Kahanek et al., 2013).

3.3. Transcriptional changes and sex-dependent alterations of synaptic plasticity pathways in NAcc MSNs and mPFC glutamatergic neurons in HD rats

Chronic ethanol exposure has a strong impact on synaptic plasticity, particularly through its modulation of both GABAergic and glutamatergic neurotransmission (Abrahao et al., 2017; Lovinger and Abrahao, 2018; Lovinger and Roberto, 2013; Morisot and Ron, 2017; Zorumski et al., 2014). Previous work has shown that long-term alcohol intake leads to changes of excitatory and inhibitory synapses, with recent reviews outlining complex and persistent effects on synaptic strength and connectivity (Abrahao et al., 2017; Lovinger and Abrahao,

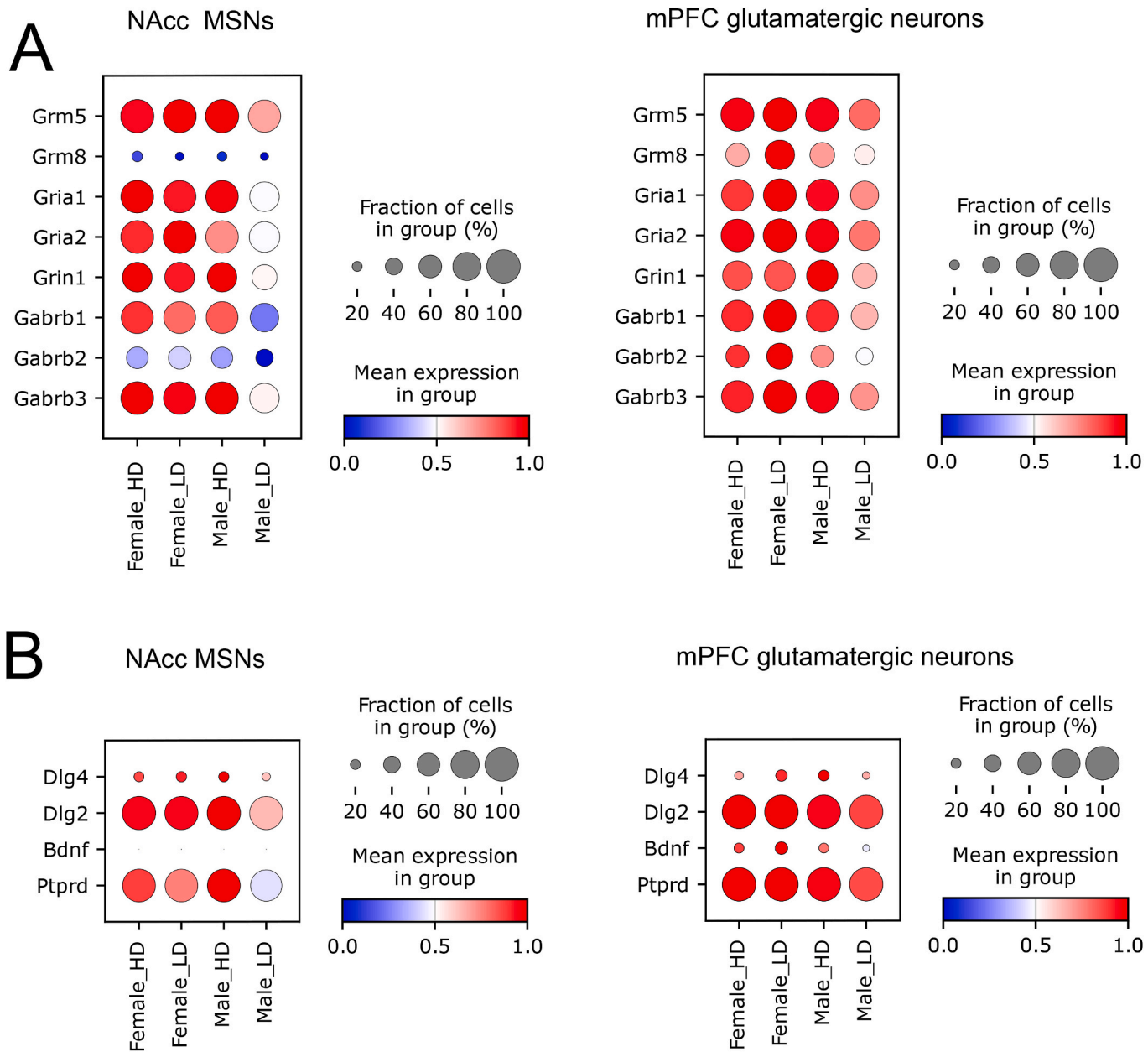


Fig. 3. Sex- and region-dependent expression of synaptic plasticity associated genes in HD and LD rats across neuronal subtypes and sexes. Dot plots showing the expression of **(A)** glutamatergic and GABAergic receptor subunits and **(B)** *Dlg4, Dlg2, Bdnf* and *Ptprd* genes in female and male HD and LD rats in NAcc MSNs and mPFC glutamatergic neurons. Dot size represents the proportion of cells expressing the gene within each group and colour indicates the average gene expression level. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2018; Lovinger and Kash, 2015; Morisot and Ron, 2017).

Our analysis revealed marked transcriptional changes in genes associated with glutamatergic signalling, a key pathway involved in synaptic plasticity, learning and memory. Among metabotropic glutamate receptors, *Grm5* mRNA was downregulated in mPFC glutamatergic neurons and NAcc GABAergic MSNs by an estimated 10% and 3.5%, respectively, based on log2 fold change, in HD female rats. In contrast, *Grm5* expression was consistently upregulated in HD males, with estimated increases of 41% in glutamatergic neurons and 42% in MSNs (Fig. 3A, Extended Data 1 and 2). *Grm8* mRNA levels were elevated in HD males, showing estimated increases of 46% in glutamatergic neurons and 27% in MSNs. In HD females, *Grm8* was also upregulated in NAcc MSNs by an estimated 46% but significantly downregulated in mPFC glutamatergic neurons by an estimated 50% (Fig. 3A, Extended Data 1 and 2). In the ionotropic receptor family, we observed sex-dependent alterations of *Gria1* and *Gria2* mRNAs, encoding AMPA receptor subunits (Extended data 1 and 2). Specifically, *Gria1* mRNA was downregulated in mPFC glutamatergic neurons in HD females but upregulated in HD males, while expression was increased for both sexes in NAcc MSNs (Fig. 3A). Additionally, *Grin1* mRNA, a core NMDA receptor subunit, was upregulated in both HD males and females (Fig. 3A).

Beyond glutamatergic signalling, our data also revealed widespread dysregulation of GABAergic gene expression in HD rats, including several GABA receptor subunits (*Gabr*), suggesting broader alterations in excitatory-inhibitory balance in the ADE paradigm (Fig. 3A, Extended Data 1 and 2).

In line with the observed sex-dependent changes in glutamatergic and GABAergic signalling, we also detected differential expression of key postsynaptic scaffold proteins that govern synaptic structure and plasticity. *Dlg4* mRNA encoding PSD-95 was markedly downregulated in mPFC glutamatergic neurons of HD females, and upregulated in HD males (Fig. 3B), while *Dlg2* mRNA encoding PSD-93 exhibited a similar sex-dependent expression pattern in GABAergic MSNs of HD rats (Fig. 3B, Extended data 1 and 2).

While PSD proteins play a crucial role in stabilizing synaptic structures, the regulation of synaptic plasticity also heavily depends on neurotrophic factors, like brain-derived neurotrophic factor (BDNF), which influence activity-dependent changes and support long-term synaptic modifications. In our dataset, *Bdnf* mRNA expression, although very low in MSNs, exhibited sex-specific differences. In HD females, *Bdnf* was downregulated by an estimated 10% in mPFC glutamatergic neurons and by 81% in NAcc MSNs. In contrast, HD males exhibited an estimated 43% upregulation of *Bdnf* in mPFC glutamatergic neurons, while expression in NAcc MSNs remained unchanged (Fig. 3B, Extended data 1 and 2).

Notably, one of the most significantly altered gene, when comparing HD versus LD rats, was *Ptprd*, a receptor-type protein tyrosine phosphatase known to govern synapse formation and stability, particularly at excitatory synapses. In mPFC glutamatergic neurons, *Ptprd* mRNA was slightly downregulated by an estimated 3% in HD females but significantly upregulated in HD males by an estimated 32% (Fig. 3B, Extended data 2). In contrast, *Ptprd* mRNA was upregulated in NAcc MSNs of both HD males and females by an estimated 44% and 8%, respectively, suggesting a potential role in network reorganization in the ADE paradigm (Fig. 3B, Supp. Fig. 5, Extended data 1).

Finally, we identified differential expression of several cell adhesion and synaptic organization genes, such as *Nrg1, Nrg3, Nrxn1, Nrxn3*, and *Nlgn1* (Supp. Fig. 7, Extended data 1 and 2). These genes are integral to synapse specification, stabilization, and signalling, and their changes may reflect compensatory plasticity or maladaptive neuroadaptations in response to prolonged ethanol exposure.

In addition to the differential expression analysis, when comparing HD and LD rats, Gene Ontology (GO) analysis revealed genes associated with the following pathways: learning, memory, cognitive processes, synaptic transmission, regulation of synapse organization, synapse assembly, dendrite development and morphogenesis, axonogenesis, postsynapse organization in both NAcc MSNs and mPFC glutamatergic neurons (Fig. 4).

3.4. Sex- and region-dependent transcriptomic signatures of cell damage in NAcc MSNs and mPFC glutamatergic neurons in HD rats

Our results revealed robust alterations in genes associated with neuronal injury, oxidative stress, inflammation and cellular stress responses, many of which exhibit distinct region- and sex-specific patterns. Notably, genes implicated in apoptosis and neuronal damage, such as *Apoe*, *Bax, Bcl2, Atf4, Tp53, Hspa1a* and various caspase family components (*Casp*), were differentially expressed across groups (Extended data 1 and 2). More specifically, *Casp 3, Casp8ap2 and Casp9* mRNAs were upregulated in mPFC glutamatergic neurons and NAcc MSNs in HD males, while in HD females, *Casp8ap2 and Casp9* mRNAs were downregulated in mPFC glutamatergic neurons (Fig. 5A). Expression of the *Apoe* gene was slightly upregulated in both glutamatergic neurons and GABAergic MSNs of HD females but downregulated in HD males (Fig. 5A, Supp. Figs. 5 and 6). In contrast, *Bcl2* mRNA, an anti-apoptotic gene, was downregulated in NAcc MSNs and mPFC glutamatergic neurons in HD females but upregulated in NAcc MSNs in HD males. *Atf4* mRNA, a stress-responsive transcription factor linked to neuronal injury, was elevated mPFC glutamatergic neurons of HD males, but suppressed in HD females (Fig. 5A). Markers of oxidative stress and mitochondrial dysfunction, including *Sod1*, *Sod2*, *Mt-nd1*, and *Mt-co1*, also displayed changes in gene expression (Fig. 5A, Extended data 1 and 2). Both *Sod1* and *Sod2* mRNAs were downregulated across neuron subtypes in the mPFC and NAcc, regardless of sex, indicating a shared oxidative stress signature. In contrast, mitochondrial genes showed sex-specific differences. Thus, *Mt-nd1* and *Mt-co1* mRNAs were reduced in mPFC glutamatergic neurons in HD females but upregulated in HD males. Interestingly, *Mt-co1* mRNA exhibited the opposite pattern in NAcc MSNs (Fig. 5A, Extended data 1 and 2).

Finally, we identified several DEGs in HD rats associated with neuroimmune signalling, apoptosis, and cancer-related pathways that have been associated with AUD in a recent systematic review and meta-analysis (Friske et al., 2025) (Fig. 5B, Extended data 1 and 2). Among these are the neuroimmune-related genes, *Fosl1* and *Mmp19,* whose expression was upregulated in HD males but downregulated in females. Particularly, in mPFC glutamatergic neurons *Fosl1* and *Mmp1*9 mRNAs were reduced by an estimated 45% and 52%, respectively, in HD females, whereas in HD males, their expression increased by an estimated 27% and 54% (Fig. 5B). Genes involved in cell proliferation and apoptotic pathways, including *Mavs*, *Cd28*, and members of the *Mapk* family, were also differentially expressed. Notably, *Mavs* and *Cd2*8 mRNAs were upregulated in mPFC glutamatergic neurons in HD males but downregulated in HD females (Fig. 5B). Furthermore, genes linked to cancer-related processes, such as *Atl1* and *Spdef*, were downregulated in mPFC glutamatergic neurons in HD females but upregulated in HD males. *Atl1* mRNA showed increased expression in NAcc GABAergic neurons in HD males, while *Spdef* mRNA expression was downregulated in HD females (Fig. 5B). Together, these findings highlight complex, cell type-, sex-, and region-dependent molecular responses in the ADE paradigm, suggesting potentially different mechanisms of neuronal vulnerability.

3.5. Neuroinflammatory signalling in glial cells of HD rats

Glial cells serve as the brain's principal immune regulators, playing a central role in orchestrating neuroimmune signalling and maintaining CNS homeostasis (Ransohoff and Brown, 2012). Increasing evidence highlights neuroinflammation as a key driver of alcohol-induced neurodegeneration, affecting individuals across all developmental stages, including neonates, adolescents, and adults (Yang et al., 2014). AUD has been associated with significant changes in glial gene expression, reflecting pathological neuroimmune adaptations (Bazzi et al., 2025;

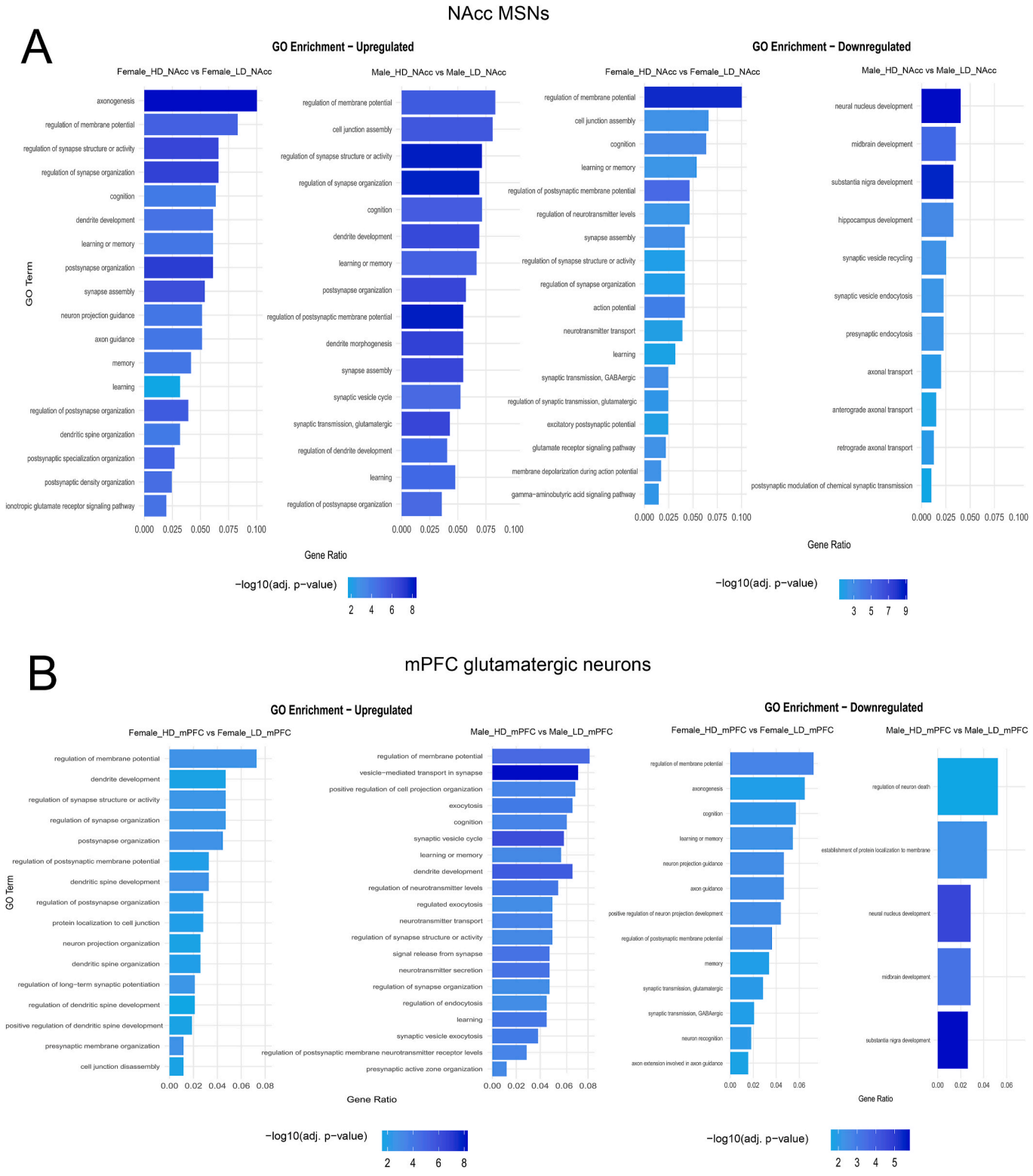


Fig. 4. GO enrichment analysis of DEGs from HD vs LD female and male rats across neuronal subtypes. Top biological processes related to synaptic plasticity significantly enriched in DEGs in **(A)** NAcc MSNs and **(B)** mPFC glutamatergic neurons from HD vs LD female and male rats.

Blednov et al., 2012; Warden et al., 2025). In particular, dysfunction of the microglia, the resident brain macrophages, has been implicated in both the initiation and progression of AUD (Henriques et al., 2018). Consistent with these observations, our data revealed robust upregulation of genes involved in inflammatory signalling pathways in microglia. The most significantly upregulated genes and pathways in our dataset, when comparing HD versus LD rats, were associated with immune function and cellular activation, consistently observed across both sexes and brain regions (Fig. 6, Extended data 4). This included genes such as *Nrp1, Cd74, Plac8, Card 11* along with components of the complement system (*C1qa, C1qb, C1qc, C3, C4a*) and major histocompatibility complex (MHC) (*Rt1-da, Rt1-ba, Rt1-db1, Rt1-ce16*) (Fig. 6A, Supp.

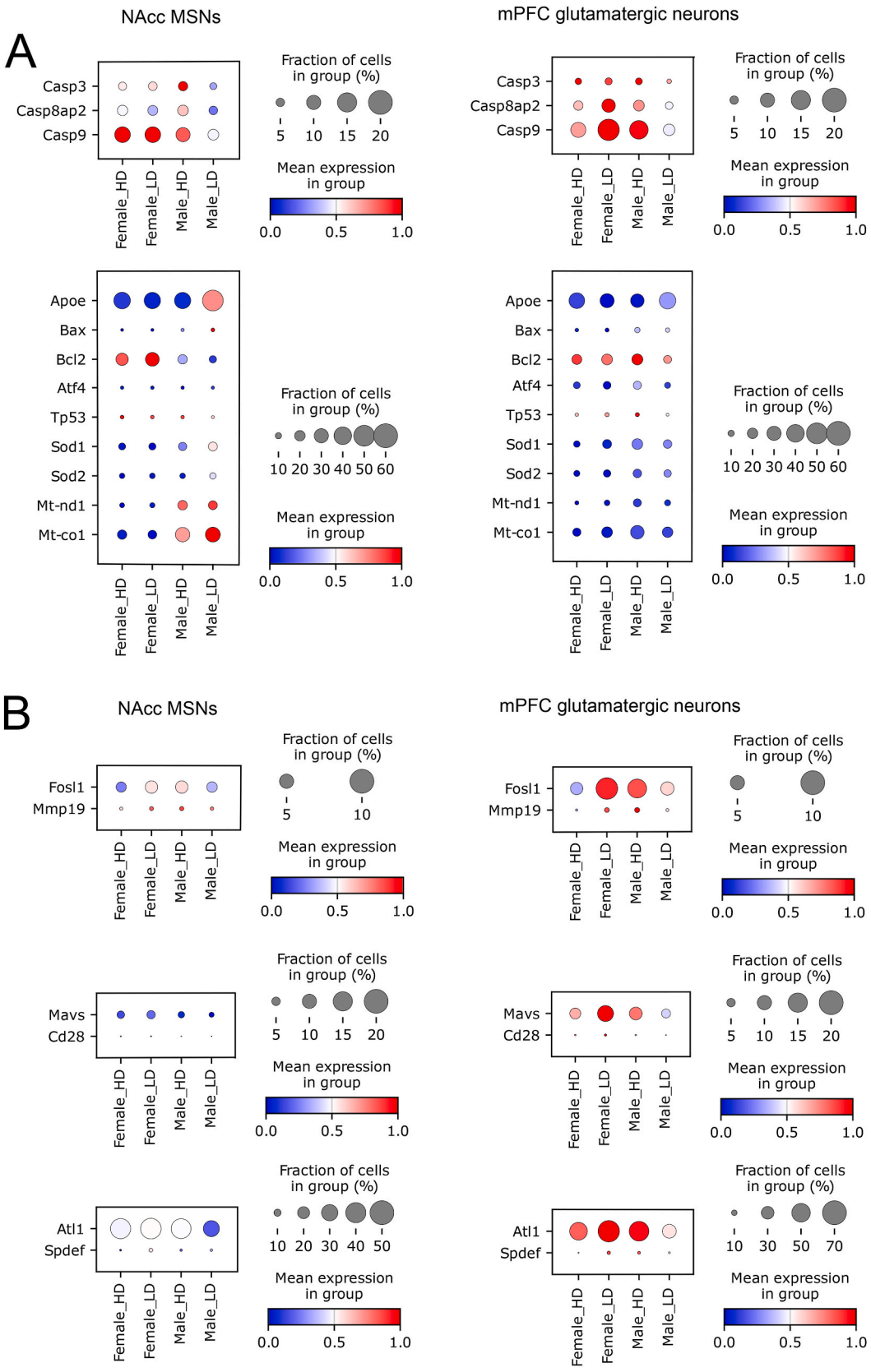


Fig. 5. Sex- and region-dependent expression of neurotoxicity genes in HD and LD rats across neuronal subtypes and sexes. **(A)** Dot plots showing the expression of genes related to apoptosis and neuronal damage and **(B)** neuroimmune, apoptosis, and cancer-related genes associated with AUD (Friske et al., 2025) in female and male, HD and LD rats in NAcc MSNs and mPFC glutamatergic neurons. Dot size represents the proportion of cells expressing the gene within each group, and colour indicates the average gene expression level. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

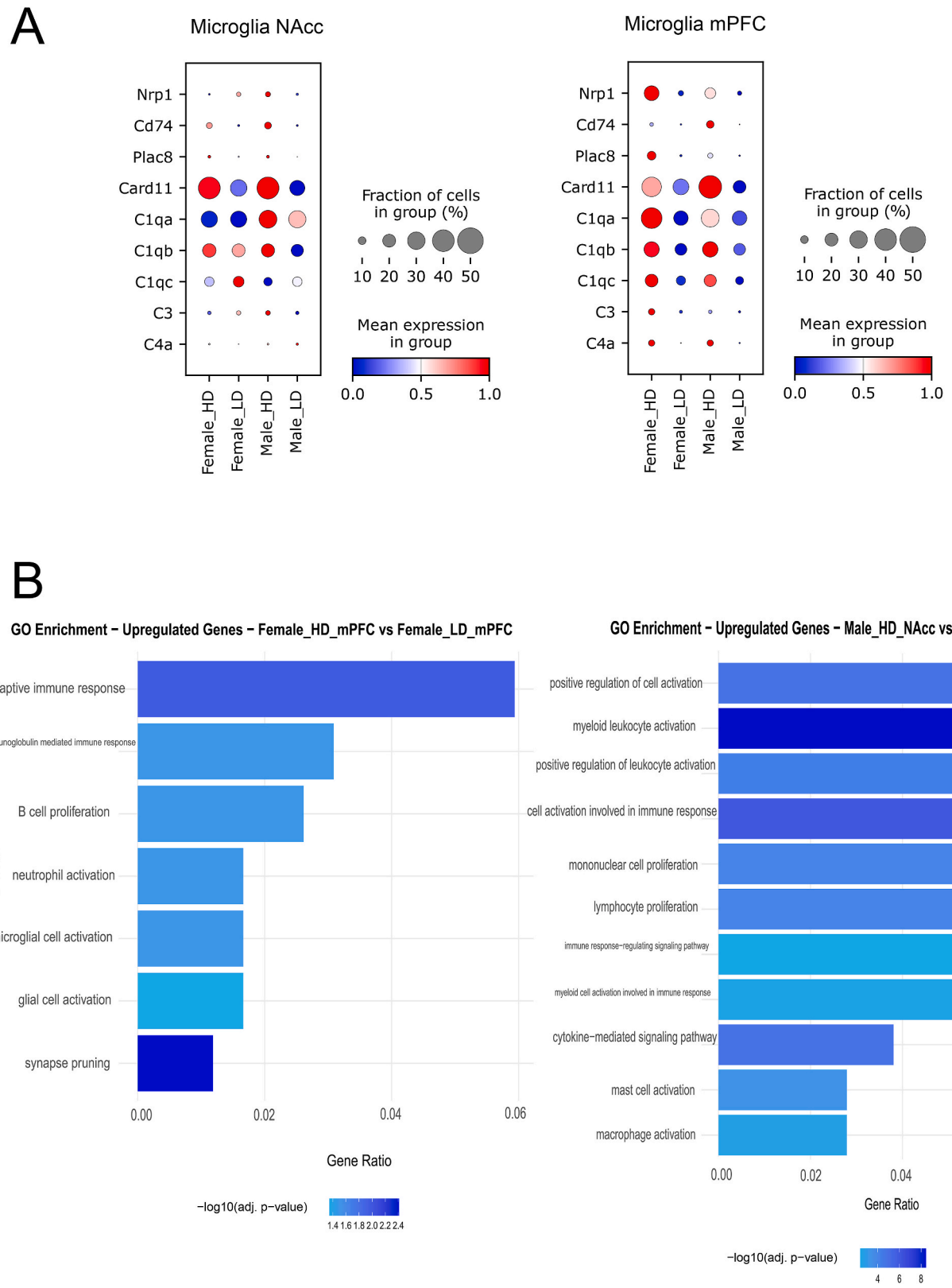


Fig. 6. Expression of neuroinflammatory genes in microglia of HD and LD rats across brain regions and sexes. **(A)** Dot plots showing the expression in microglia of inflammatory genes in NAcc and mPFC, female and male, HD and LD rats. Dot size represents the proportion of cells expressing the gene within each group, and colour indicates the average gene expression level. **(B)** Top biological processes related to immune activation significantly enriched in upregulated genes in microglia from HD vs LD male and female rats across brain regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 8, Extended data 4). Among these, the type I transmembrane protein, Neuropilin-1 (*Nrp1*), which is expressed by microglia in response to CNS inflammation and injury (Sherafat et al., 2021), was markedly upregulated in NAcc and mPFC of HD males, as well as in mPFC of HD females (Fig. 6A, Supp. Fig. 8). Similarly, *Cd74*, also known as the MHC class II invariant chain, is a well-established marker of reactive microglia (Potru and Spittau, 2023). Increased expression of *Cd74* has been implicated in Alzheimer's disease (Mathys et al., 2019; Sala Frigerio et al., 2019), multiple sclerosis (Masuda et al., 2019), and ischemic brain injury (Hwang et al., 2017). In our dataset, *Cd74* mRNA was significantly upregulated in microglia from mPFC and NAcc in HD males and from NAcc in HD females, suggesting a potential involvement in alcohol-related neuroimmune responses (Fig. 6A, Supp. Fig. 8). When comparing HD and LD rats, GO analysis linked upregulated DEGs found in microglia to biological pathways such as synapse pruning, adaptive immune response, immunoglobulin-mediated immune response, microglial activation, and cell activation involved in immune response (Fig. 6B). Notably, previous studies suggested a regulatory role of microglia in synapse elimination in AUD (Lacagnina et al., 2017; Warden et al., 2025).

In addition to microglia, astrocytes also exhibited pronounced gene expression changes associated with neuroinflammatory responses (Fig. 7, Extended data 5). Previous human snRNA-seq studies identified astrocytes as the glial subtype with the highest number of DEGs in response to chronic alcohol exposure, highlighting their key role in AUD (Brenner et al., 2020; Warden et al., 2025). Among the DEGs in astrocytes, we observed a prominent induction of several genes linked to neuroimmune function and the complement system specifically in female HD rats, including *C1qa*, *C1qb*, *C1qc*, *C3* and *Cd74*, in NAcc and mPFC. Notably, *C1qa*, *C1qb*, *C1qc*, and *C3* were specifically upregulated in astrocytes from the mPFC of female HD rats, while *Cd74* was selectively upregulated in astrocytes from the NAcc of both sexes (Fig. 7A). Additionally, we observed an elevated expression of the glutamate transporters, *Slc1a3* (also known as *GLAST*) and *Slc1a2* (GLT-1), in HD

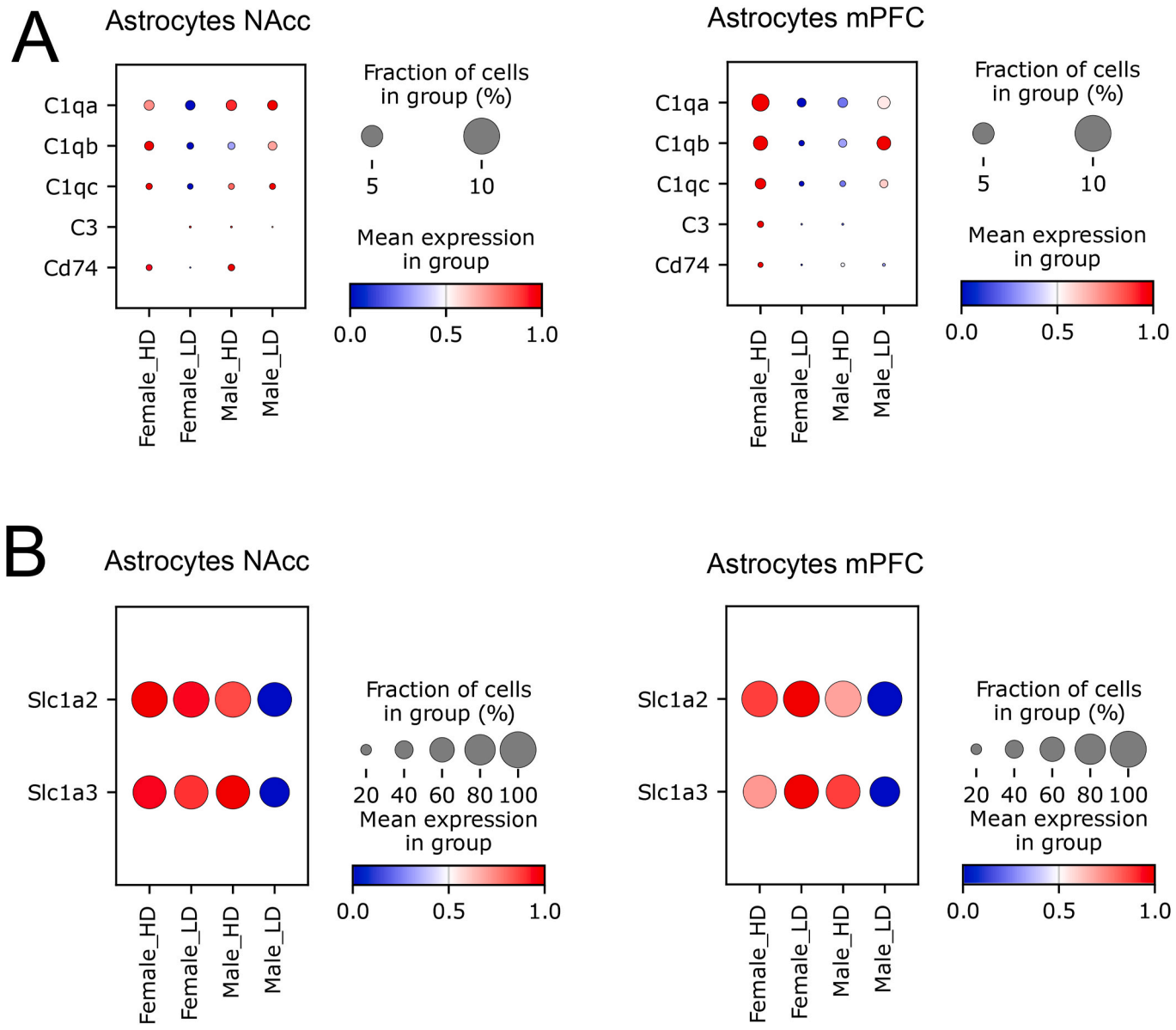


Fig. 7. Expression of neuroinflammatory and glutamate transporters genes in astrocytes of HD and LD rats across brain regions and sexes. Dot plots showing the expression of genes related to **(A)** neuroinflammation and **(B)** glutamate transporter in NAcc and mPFC, female and male, HD and LD rats. Dot size represents the proportion of cells expressing the gene within each group, and colour indicates the average gene expression level. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

males but not in females across both brain regions examined (Fig. 7B). In HD female rats, *Slc1a3* mRNA was downregulated in the mPFC. These transporters play crucial roles in supporting neuronal survival by preventing excitotoxicity and oxidative stress through the active clearance of extracellular glutamate (Gupta et al., 2013; Hardingham and Lipton, 2010). Their upregulation in males may reflect a compensatory response to alcohol-induced glutamatergic imbalance, a key feature of neuroinflammatory states (Aroeira et al., 2014; Hansson and Rönnbäck, 2003; Malarkey and Parpura, 2008).

Interestingly, astrocytes also exhibited pronounced sex-specific alterations in the expression of circadian rhythm-associated genes. In both the NAcc and mPFC, *Rora* and *Per3* were significantly upregulated in male but downregulated in female HD rats (Supp. Fig. 9A). A similar trend was observed for *Rorb*, although its expression remained unchanged in the NAcc of HD females (Supp. Fig. 9A). Given the well-established link between alcohol consumption, circadian clock gene regulation and neuroinflammation (de Zavalia et al., 2021), these findings may reflect sex-dependent interactions between alcohol and the molecular circadian machinery.

Lastly, we identified significant differential gene expression in oligodendrocytes in HD rats (Figs. 8 and 9, Extended data 6). Oligodendrocytes are critical for the production of myelin sheaths. Due to the high metabolic demands of myelination, oligodendrocytes are especially vulnerable to environmental insults, including alcohol exposure (Bazzi et al., 2025). Our data revealed a marked sex-dependent transcriptional response within these cells, with particularly prominent changes observed in female HD rats. These rats exhibited upregulation of several inflammatory genes, including *C1qa*, *C1qb*, *C1qc*, *Cd74*, *Zeb1* and *Marchf1* (Fig. 8A), as well as increased expression of genes related to myelination, such as *Tf*, *Mobp*, *Mbp*, and *Mag* in NAcc and mPFC (Fig. 8B, Supp. Fig. 10, Extended data 6). Specifically, the immune-related genes *C1qa*, *C1qb*, *C1qc*, and *Zeb1* were upregulated in females in the mPFC, *Cd74* was upregulated in the NAcc in both sexes, and *Marchf1* was elevated in females in both brain regions (Fig. 7A). Among the myelin-associated genes, *Tf* emerged as the most significant upregulated gene in HD females while it was downregulated in the NAcc of HD males and showed no change in the male mPFC (Fig. 8B, Supp. Fig. 10, Extended data 6). Interestingly, *Mobp* and *Mbp*, both key components of the myelin sheath, were consistently induced in HD rats, with the exception of *Mbp*, which, in the mPFC, showed no change in HD male rats (Fig. 8B). GO analysis further supported these findings, revealing enrichment in pathways related to myelin formation and oligodendrocyte function when comparing HD and LD rats among the different groups (Fig. 8C). While alterations in myelin-related gene expression have been reported in several studies (Bazzi et al., 2025; Lewohl et al., 2000), to our knowledge, this is the first study to specifically highlight sex-dependent differences in individual transcripts within oligodendrocytes.

Interestingly, similar to astrocytes, oligodendrocytes also displayed differential expression of circadian rhythm-related genes, specifically in female HD rats in the NAcc (Supp. Fig. 9B). This was characterized by a marked upregulation of *Arntl* and *Clock*, accompanied by downregulation of *Per 2*, *Per3*, *Rora*, *Rorb*, *Cry 1*, and *Cry 2* (Supp. Fig. 9B).

Finally, we identified *Pde4b* as a significantly dysregulated module within oligodendrocytes in HD animals (Fig. 9A, Extended data 6) (Salem et al., 2024). The PDE4 enzyme family, known for its strong expression in both neurons and immune cells, plays critical roles in modulating inflammation and in promoting remyelination in oligodendrocytes (Schepers et al., 2023). In our study, expression of *Pde4b* mRNA was reduced in oligodendroglia in the mPFC of HD females but increased in oligodendroglia in the NAcc of HD males (Fig. 9A). When comparing sexes, within the HD and LD subgroups, *Pde4b* mRNA expression was generally higher in females relative to males across both brain regions (Fig. 9B, Extended data 7). Furthermore, *Pde4b* mRNA levels were increased in the NAcc compared to the mPFC regardless of sex (Fig. 9C, Extended data 7). Given that non-selective PDE4 inhibitors have been shown to reduce ethanol consumption in mice (Blednov et al., 2014, 2018, 2020, 2022), rats (Franklin et al., 2015; Wen et al., 2018), and non-treatment seeking individuals with AUD (Grigsby et al., 2023), our findings provide new insight into potential region- and sex-specific mechanisms that may underlie the therapeutic effects of this drug class.

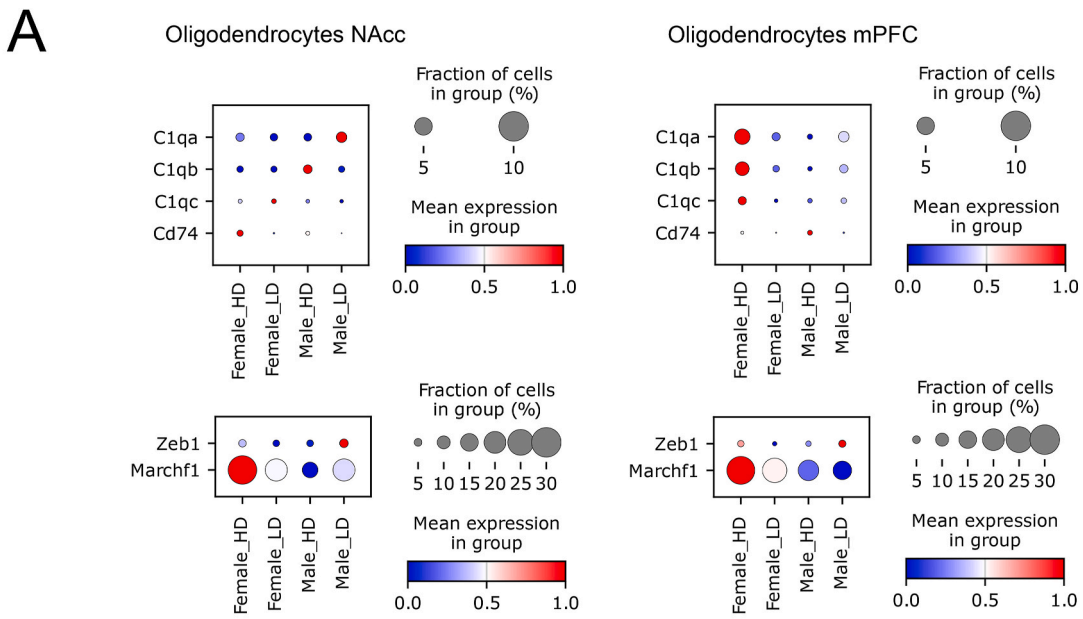
4. Discussion

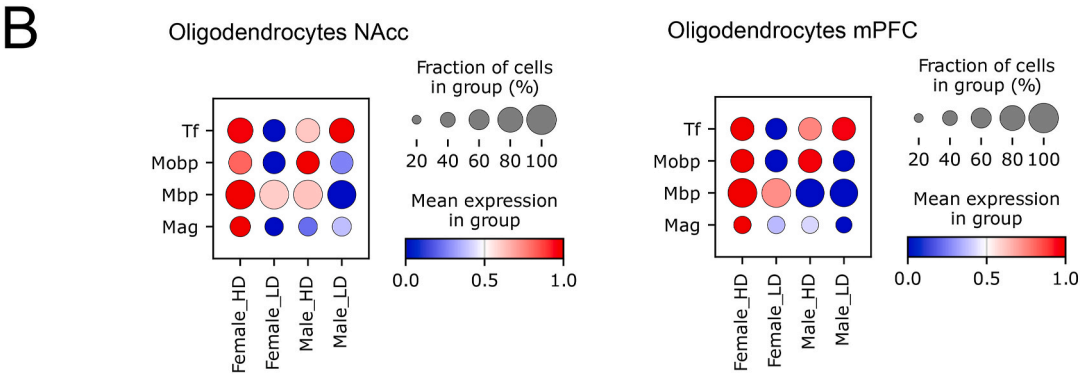
Alcohol is a potent neurotoxic agent capable of crossing the blood-brain barrier and exerting widespread effects in the CNS (Alfonso-Loeches and Guerri, 2011). Acute and chronic alcohol exposure leads to transcriptional alterations in pathways involved in neurotoxicity, synaptic plasticity and neuroimmune signalling, underscoring alcohol's extensive impact on brain function and cellular homeostasis (Farris et al., 2015; Warden et al., 2025). Importantly, emerging evidence suggests that the transcriptional alterations induced by alcohol are both region- and sex-dependent, with distinct cellular populations contributing differentially to the development of alcohol dependence and relapse (Erickson et al., 2021; Warden et al., 2020, 2021, 2025). However, the precise molecular pathways underlying these responses remain poorly understood. In this study, we employed snRNA-seq to examine the transcriptomic landscape associated with excessive alcohol consumption in two key brain regions involved in addiction and reward processing, the mPFC and NAcc, in both female and male outbred RccHan Wistar rats exposed to the ADE paradigm. This outbred rat strain is characterized by a high degree of inter-individual variability in voluntary alcohol intake, making it a valuable translational model for capturing the diversity observed in human drinking behaviour (Momeni et al., 2015).

Notably, we previously showed significant sex differences in alcohol consumption in outbred RccHan Wistar rats exposed to the ADE paradigm with higher alcohol intake and greater sensitivity to drug-paired cues in females (Hakus et al., 2025). These behavioural differences are paralleled by differential gene expression in neurons and glia from the NAcc and mPFC of female and male rats, providing novel insight into the molecular mechanisms underlying sex differences in the vulnerability to alcohol effects. In general, females are known to be more susceptible than males to the neurotoxic and harmful effects of chronic alcohol consumption (Cruz et al., 2023). When consuming similar amounts of alcohol, women with AUD face higher risks than males of developing cancer, liver damage, and cardiovascular disease (Åberg et al., 2017; Flores-Bonilla and Richardson, 2020; Mann et al., 2005). Sex differences in alcohol-related behaviours may be attributed to intrinsic differences in brain organization and to the influence of gonadal steroid hormones on brain function (Almeida et al., 1998; Becker et al., 2001). These hormones are known to modulate a wide range of neural processes, including hypothalamus-driven social behaviour, hippocampal and prefrontal cortex functions related to learning, memory and cognition, amygdala-mediated stress responses, dopamine-regulated reward pathways, and overall synaptic plasticity (Flores-Bonilla and Richardson, 2020; Hamson et al., 2016; Hyer et al., 2018). Interestingly, neonatal exposure to estrogen in female rats has been shown to induce male-type patterns of alcohol consumption (Almeida et al., 1998).

In this study we show pronounced region- and sex-dependent transcriptional alterations across neuronal and glial populations, with significant modulation of genes involved in dopaminergic and phosphodiesterase signalling, synaptic plasticity and neuroinflammation. These dysregulated networks may represent either a harmful consequence of alcohol exposure or a compensatory response aimed at restoring normal CNS function.

Alcohol is well known to influence dopamine release, particularly in the NAcc, where it plays a key role in mediating the rewarding effects of alcohol and reinforcing drinking behaviour (Boileau et al., 2003; Di Chiara and Imperato, 1988). In line with this, alcohol withdrawal is associated with reduced dopamine release in the striatum (Rossetti et al., 1992). Both clinical and preclinical studies emphasize the





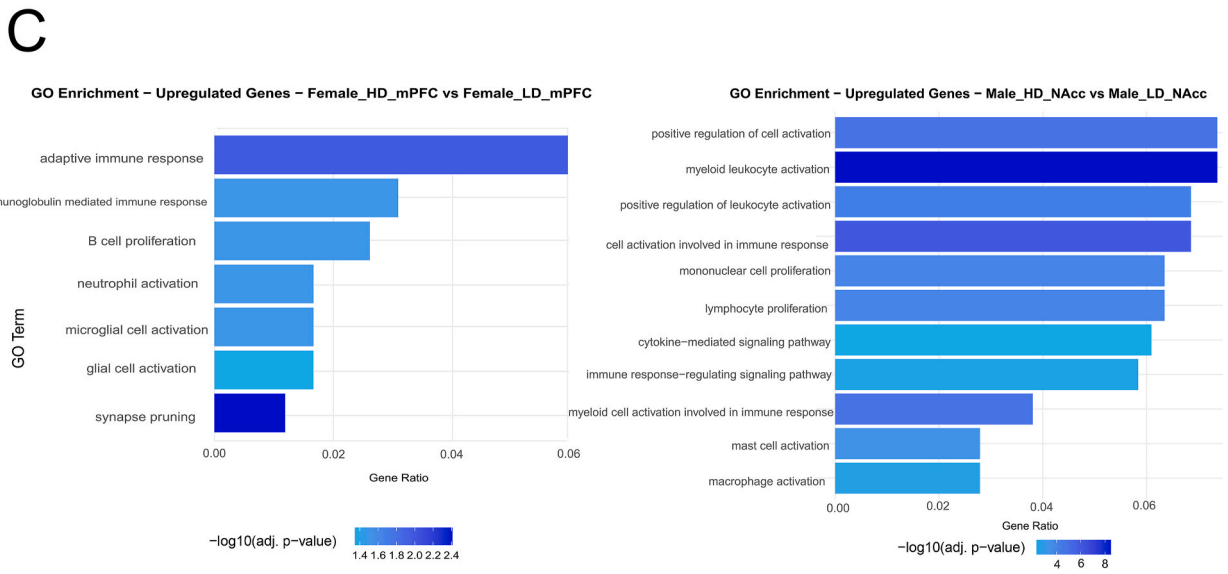


Fig. 8. Expression of neuroinflammatory and myelination genes in oligodendrocytes of HD and LD rats across brain regions and sexes. **(A)** Dot plots showing the expression in oligodendrocytes of genes related to neuroinflammation and **(B)** myelination in female and male HD and LD rats in NAcc and mPFC. **(C)** Top biological processes related to myelination and oligodendrocytes function significantly enriched in upregulated genes in oligodendrocytes from HD vs LD female and male rats across brain regions.

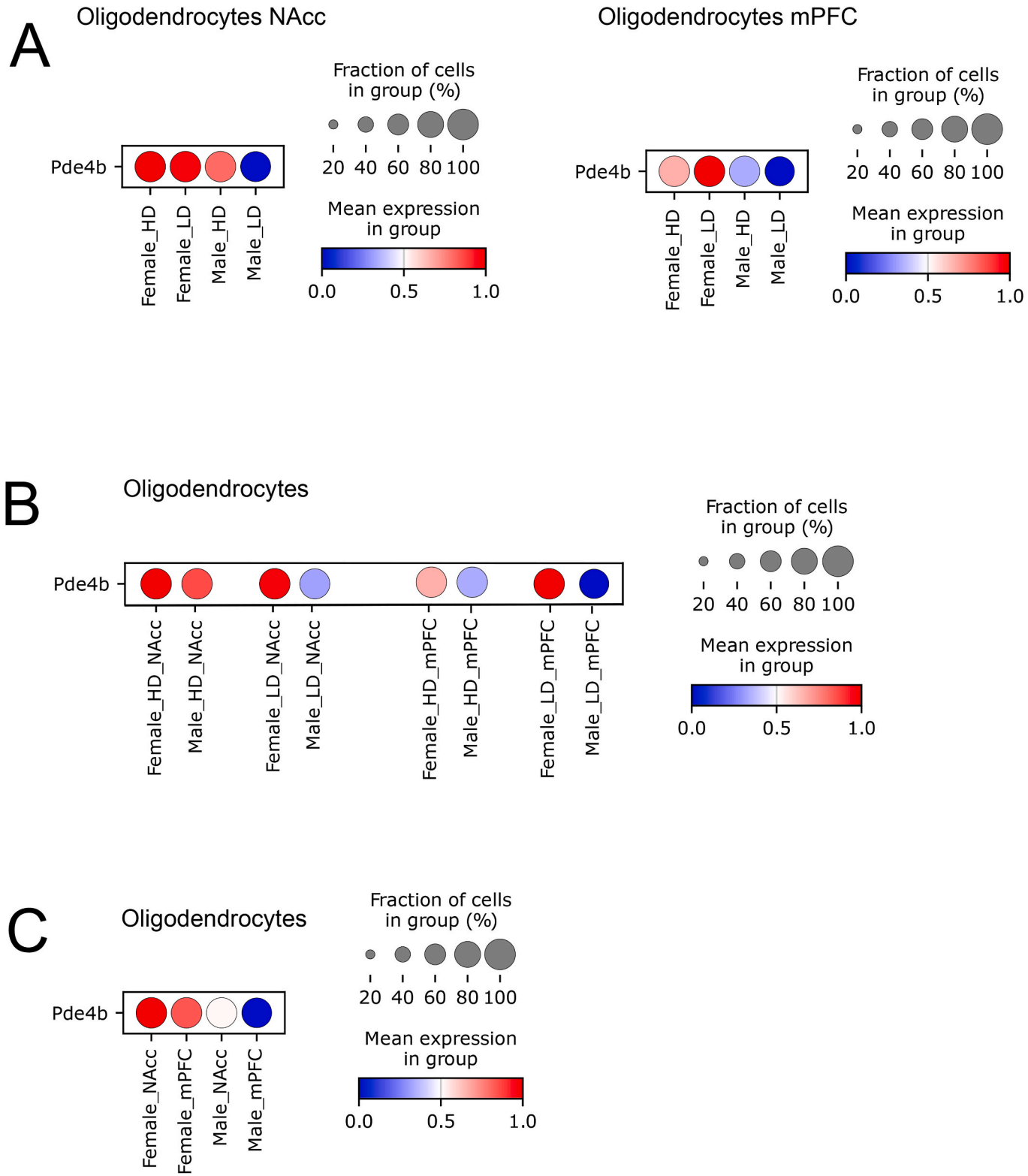


Fig. 9. Expression of *Pde4b* gene in oligodendrocytes of HD and LD rats across brain regions and sexes. **(A)** Dot plots showing the expression in oligodendrocytes of *Pde4b* in female and male HD and LD rats in NAcc and mPFC. **(B)** Sex and **(C)** region specific expression of *Pde4b* in oligodendrocytes. Dot size represents the proportion of cells expressing the gene within each group, and colour indicates the average gene expression level. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

importance of D2 receptors in AUD. Decreased levels of D2 receptors were found in alcohol-preferring rodents (McBride et al., 1993; Stefanini et al., 1992), as well as in humans with AUD (Hietala et al., 1994; Tupala et al., 2001; Volkow et al., 1996). In our study using the ADE paradigm, female HD rats showed lower expression of *Drd1* and *Drd2* mRNAs in NAcc MSNs and mPFC glutamatergic neurons compared to LD rats. In contrast, male HD rats showed higher expression of *Drd1* mRNA in mPFC glutamatergic neurons and NAcc MSNs, and higher expression of *Drd2*

mRNA in NAcc MSNs compared to LD rats. These results suggest that dopamine receptor regulation in the ADE paradigm varies by both sex and brain region. The functional implications of the differential expression of dopamine receptors remain to be further explored. In general, preclinical findings on the role of D2 receptors in AUD are inconsistent. While pharmacological inhibition of D2-like receptors in the NAcc was found to reduce alcohol intake (Czachowski et al., 2001; Kaczmarek and Kiefer, 2000), increased D2R expression in the striatum can lead to reductions in alcohol preference and consumption (Thanos et al., 2001).

Consistent with our findings on dopamine receptor gene expression, we observed transcriptional changes in the expression of *Pde* genes that were sex- and brain region-dependent. PDEs are particularly interesting as potential therapeutic targets for reducing excessive alcohol consumption since they regulate cAMP/cGMP signalling pathways that have been shown to suppress alcohol intake (Logrip, 2015). Notably, *Pde1a* mRNA was upregulated in mPFC glutamatergic neurons in HD males but downregulated in HD females compared to LD rats. The expression of *Pde10a*, a key modulator of excessive alcohol consumption (Wen et al., 2018), was upregulated in mPFC glutamatergic neurons in HD males but downregulated in glutamatergic neurons and MSNs in female HD rats compared to LD rats. *Pde4b* emerged as a particularly interesting gene in our dataset, showing altered expression not only in neurons but also in oligodendrocytes, consistent with previous studies (Salem et al., 2024). In NAcc MSNs, *Pde4b* mRNA was the most significantly downregulated gene in HD female rats. Interestingly, non-selective PDE4 inhibitors have been shown to reduce alcohol consumption in mice (Blednov et al., 2014, 2018, 2020, 2022), in rats (Franklin et al., 2015; Wen et al., 2018), and in non-treatment seeking individuals with AUD (Grigsby et al., 2023). Our results provide new insights into potential cell type-, region- and sex-dependent mechanisms that may underlie the therapeutic effects of this promising class of drugs in AUD.

Chronic alcohol exposure is known to induce synaptic plasticity at both GABAergic and glutamatergic synapses (Lovinger and Kash, 2015; Abrahao et al., 2017; Morisot and Ron, 2017; Lovinger and Abrahao, 2018). In particular, receptors such as GABRA3, GABBR1, GABBR2, GRIA1, and GRIN2B are widely recognized to affect alcohol-induced behaviours (Farris et al., 2015). Consistent with this, we observed differential expression of several genes associated with synaptic plasticity in the ADE paradigm, showing upregulation in male HD rats in mPFC and NAcc, as well as region-dependent changes in female HD rats compared to LD rats. Interestingly, we found increased expression of *Grin1* mRNA, encoding a key NMDA receptor subunit, in both male and female HD rats compared to LD rats. NMDA receptors are well-established as key molecular targets of ethanol, and the induction of glutamatergic and GABAergic synapses has been described previously for chronic alcohol consumption (Gass and Olive, 2008; Henriksson et al., 2008). Notably, enhanced glutamatergic signalling in the mPFC may contribute to the persistent impairment in cognitive control of goal-directed behaviours often observed in individuals with addiction (Koob and Volkow, 2010). Ethanol's impact on glutamatergic signalling may also be mediated by glial cells, in particular astrocytes, which play a key role in maintaining synaptic homeostasis via their expression of glutamate receptors (Aroeira et al., 2014; Gupta et al., 2013; Malarkey and Parpura, 2008). In this study, we observed an induction of genes encoding GLAST and GLT-1 specifically in astrocytes from the NAcc and mPFC of male HD rats. Interestingly, increased expression of GLT-1 has previously also been detected in the NAcc of rats following voluntary ethanol consumption (Alhaddad et al., 2014; Das et al., 2015; Sari et al., 2016). Moreover, the expression of the GLAST protein was found to be higher in post-mortem cortical tissue from humans with AUD compared to controls (Kashem et al., 2019).

Finally, our findings implicate neuroinflammatory processes in glial cells as a key feature of alcohol dependence and relapse. Specifically, we observed upregulation of neuroinflammatory genes in microglia in both sexes, while astrocytes and oligodendrocytes showed similar inductions predominantly in HD female rats. Recent snRNA-seq studies in AUD identified immune changes in glia, but did not fully take into consideration region- and sex-dependent effects (Brenner et al., 2020; van den Oord et al., 2023; Warden et al., 2025). Microglia were identified as critical modulators of alcohol neurotoxicity (Henriques et al., 2018). In fact, alcohol can directly activate microglia, alter their morphology and immune activity (Warden et al., 2020, 2021, 2025). Alcohol exposure also triggers the release of proinflammatory cytokines and chemokines, which, in turn, can modulate alcohol-related behaviours (Blednov et al., 2005, 2012). Moreover, chronic alcohol exposure promotes microglial proliferation, a phenomenon observed both in rodent models of AUD and in post-mortem brain tissue from individuals with AUD (He and Crews, 2008; Siemsen et al., 2021). Inhibition of microglia activation using the antibiotic minocycline has been shown to influence several alcohol-related behaviours, including altering alcohol-induced motor deficits (Wu et al., 2011), lowering voluntary alcohol intake in mice (Agrawal et al., 2011), and diminishing both anxiety and relapse-like drinking during withdrawal in rats (Gajbhiye et al., 2018). Moreover, previous research has shown that microglia depletion can reduce the expression of proinflammatory genes following acute withdrawal from binge alcohol exposure (Walter and Crews, 2017). However, recent studies using RNA sequencing suggested that microglia may act in conjunction with astrocytes to regulate acute and chronic voluntary alcohol behaviours (Warden et al., 2021).

Besides neuroinflammatory signals in glial cells, our analysis revealed an upregulation of myelination-related genes in oligodendrocytes from female HD rats, suggesting enhanced myelin-associated transcriptional activity in response to high alcohol intake. This observation is in line with recent transcriptomic findings in cultured mature oligodendrocytes from C57BL6/J mice (sex not determined), reporting elevated expression of *Plp1*, *Mbp* and *Mag* mRNAs after exposure to high alcohol concentrations (Bazzi et al., 2025). Interestingly in humans, post-mortem studies in male patients with AUD showed a down-regulation of myelin-related gene expression (Farris et al., 2015; Lewohl et al., 2000; Liu et al., 2016).

Finally, this study comes with limitations. One limitation is the absence of an alcohol-naïve control group, which precludes direct assessment of transcriptional changes induced solely by alcohol exposure or deprivation. Consequently, the observed differences between HD and LD animals should be interpreted as relative molecular adaptations associated with relapse-like drinking behaviour, rather than absolute up- or downregulations compared to baseline. In this context, the HD vs LD comparison looks at expression differences in an alcohol-sensitized background and the resulting set of DEGs at relapse are potentially reporting better on this epigenetic scar than comparisons to drug naïve animals (Heilig et al., 2017). Our experimental design was focused on identifying molecular differences between individuals exhibiting varying degrees of relapse-like drinking within the ADE model, an approach that reflects the behavioural and molecular variability observed among alcohol-experienced subjects in clinical settings. Finally, variability across samples is another limitation, especially given the use of an outbred strain such as RccHan Wistar rats. Although this biological variability may increase differences in gene expression, it also reflects natural diversity in alcohol-related behaviours, making the model more representative of human populations.

Despite the high prevalence and widespread impact of AUD, effective treatment options are still limited and typically result in only modest reductions of harmful drinking. This study contributes to the identification of potentially relevant target networks by highlighting cell type-, region-, and sex-dependent molecular signatures associated with alcohol relapse drinking in rats.

Funding

This work was supported by grants from the German Research

Foundation (SFB/TRR265 B04 to J.P. and C.W., SFB/TRR167 B07 to J. P., and SFB/TRR167 Z01 to M.B., B.A.) and the German Federal Ministry of Education and Research (German Center for Mental Health 01EE2503B to J.P.). M.B. acknowledges funding from the DFG within CRC1160 (Project ID 256073931-Z02), CRC1453 (Project ID 431984000-S1), CRC1479 (Project ID: 441891347- S1), TRR 359 (Project ID 491676693-Z01), TRR 353/1 (Project ID 471011418-SP02), and FOR 5476 UcarE (Project ID 493802833-P7). Additional funding was received from the German Federal Ministry of Education and Research (BMBF) within the Medical Informatics Funding Scheme, PM4Onco–FKZ 01ZZ2322A (M.B.).

CRediT authorship contribution statement

Ludovica Rigat: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Bismark Appiah:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Aileen Hakus:** Methodology, Writing – review & editing. **Asude Zülal Gül:** Methodology, Writing – review & editing. **Christine Winter:** Conceptualization, Resources, Supervision, Writing – review & editing. **Melanie Boerries:** Conceptualization, Resources, Supervision, Writing – review & editing. **Josef Priller:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

We confirm that this manuscript is not under consideration elsewhere and will not be submitted for publication elsewhere until a final decision is made. The manuscript is free from fabrication, fraud, or plagiarism. Additionally, we declare that we have no conflicts of interest to disclose.

Acknowledgements

We thank Tanja Specowius, Thomas Conrad and the MDC/BIH Genomics Technology Platform at the Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association (MDC) and Berlin Institute of Health (BIH), Berlin, Germany (https://www.mdc-berlin.de/de/technologie-plattformen) for technical support and assistance in this work. We are grateful to Matthias Groβer for his critical reading of the revised manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2026.110995.

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