Sex and cell-specific gene expression in corticolimbic brain regions associated with psychiatric disorders revealed by bulk and single-nuclei RNA sequencing

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eBioMedicine
2023;95: 104749
Published Online xxx
https://doi.org/10.1016/j.ebiom.2023.104749

Summary

Background There are sex-specific differences in the prevalence, symptomology and course of psychiatric disorders. However, preclinical models have primarily used males, such that the molecular mechanisms underlying sex-specific differences in psychiatric disorders are not well established.

Methods In this study, we compared transcriptome-wide gene expression profiles in male and female rats within the corticolimbic system, including the cingulate cortex, nucleus accumbens medial shell (NAcS), ventral dentate gyrus and the basolateral amygdala (n = 22–24 per group/region).

Findings We found over 3000 differentially expressed genes (DEGs) in the NAcS between males and females. Of these DEGs in the NAcS, 303 showed sex-dependent conservation DEGs in humans and were significantly enriched for gene ontology terms related to blood vessel morphogenesis and regulation of cell migration. Single nuclei RNA sequencing in the NAcS of male and female rats identified widespread sex-dependent expression, with genes upregulated in females showing a notable enrichment for synaptic function. Female upregulated genes in astrocytes, Drd3+MSNs and oligodendrocyte were also enriched in several psychiatric genome-wide association studies (GWAS).

Interpretation Our data provide comprehensive evidence of sex- and cell-specific molecular profiles in the NAcS. Importantly these differences associate with anxiety, bipolar disorder, schizophrenia, and cross-disorder, suggesting an intrinsic molecular basis for sex-based differences in psychiatric disorders that strongly implicates the NAcS.

Funding This work was supported by funding from the Hope for Depression Research Foundation (MJM).

Keywords: RNA sequencing; Sex difference; Psychiatric disorder; Nucleus accumbens

Introduction

There are well-established sex differences in the prevalence of psychiatric disorders.[1,2] Affective disorders, in particular, are more common in women.[3,4] Nevertheless, a wide range of common psychiatric disorders show sex-differences in the age of onset, symptoms, severity, and treatment outcomes. The breadth of these differences suggests considerable differences in the activity of key

Abbreviations: DEGs, differentially expressed genes; RNA-seq, RNA sequencing; snRNA-seq, single-nuclei RNA sequencing; NAcS, the nucleus accumbens shell; CGC, the cingulate cortex; BLA, the basolateral nucleus of the amygdala; vDG, the dentate gyrus of the ventral hippocampus; CCAC, the Canadian Council on Animal Care; FACC, Facility Animal Care Committee; SNPs, single nucleotide polymorphisms; GWAS, genome-wide association studies; GO, gene ontology; UMAP, Uniform Manifold Approximation and Projection; MSNs, medium spiny neurons; GTEx, the gene tissue expression

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Research in context

Evidence before this study

There are well-established sex differences in physiology and behavior, as well as in the development of psychiatric disorders such as depression. Depression is more common in women than men. Pre-clinical research models using rodents have traditionally focused primarily on males.

Added value of this study

In this study, we explored sex-specific patterns of gene expression in different brain regions that have been associated with psychiatric disorders in rats using RNA-seq and snRNA-seq. We used sophisticated bioinformatics techniques to identify relevant genes and networks associated with distinct sex-transcriptomic profiles in corticolimbic regions.

Implications of all the available evidence

Our study provides comprehensive evidence of region-specific patterns of sex differences in gene expression in rat corticolimbic regions and demonstrates their potential relevance for human psychopathology. The results together contribute to our understanding of sex-differences in brain regions relevant to psychiatric disorders and underscore the inclusion of sex as a biological variable in future studies.

molecular systems that underlie vulnerability for mental disorders.

Pre-clinical research models, even those modelling affective disorders, have traditionally focused on males.[5] Indeed, male-biased research in neuroscience is particularly prevalent compared to other fields[6] and there is a notable scarcity of studies examining sex differences at the molecular level.[7] The sex bias has been justified, in part, by the idea that cycling female reproductive hormones contribute unwanted 'noise'.[8] The evidence for this assumption was never compelling. Studies in rats, for example, reveal substantial fluctuations in testosterone occur in adult males, which correspond to variation in neural function and behaviour.[9,10] Thus variations in pituitary-gonadal hormones are a normal feature of adult life and common in both males and females. Importantly, many differences in behaviours between males and females are not associated with gonadal hormone differences. Some of these differences are mediated by genes located on X and Y chromosomes, or by neurochemistry as well as brain structure.[11,12]

Funding agencies now justifiably insist on the inclusion of both male and female subjects, with the obvious exception of research into reproductive function, and have emphasized the importance of sex as a biological variable.[13] While there are well-established sex differences in physiology and behaviour, our knowledge of even fundamental sex-specific molecular mechanisms remains extremely limited and thus the biological dimensions of sex in neuroscience research are largely unexplored. Recent studies report sex-specific patterns of gene expression in the brain,[14] but transcriptomic differences in specific neural circuits commonly associated with psychiatric disorders are unknown. For instance, while corticolimbic regions are consistently linked with psychiatric disorders, we know little about sex-specific patterns of gene expression in these areas. Alterations in gene expression are a fundamental component of psychiatric disorders[15–17] and epidemiological evidence suggests sex-specific genetic risk factors underly psychiatric disorders.[18] Furthermore, transcriptome wide association studies (TWAS) have elegantly demonstrated GWAS signal acts through gene expression to confer risk for various psychiatric disorders.[19–21] Therefore, understanding baseline differences in the transcriptome will likely be informative in understanding risk for psychiatric disorders.

To address these existing gaps, the present study carried out detailed transcriptomic analysis using RNA sequencing (RNA-seq) and single-nuclei RNA sequencing (snRNA-seq) in male and female rats. We focused on corticolimbic regions associated with mood disorders and also emphasized in current rodent models of depression and anxiety.[2,22–28] These regions included the medial shell of the nucleus accumbens (NAcS), the cingulate cortex (CGC), the basolateral nucleus of the amygdala (BLA) and the dentate gyrus of the ventral hippocampus (vDG). We note these regions have also been implicated in both schizophrenia and autism spectrum disorders.[29,30]

We aimed to (1) identify sex-specific gene expression patterns and sex-related co-expression networks; (2) characterize cell type specific patterns of sex-dependent gene expression using snRNA-seq; and (3) assess the relevance of these genes to human psychiatric disorders.

Methods

Animals

All procedures were performed in accordance with the guidelines established by the Canadian Council on Animal Care (CCAC) with protocols approved by the Douglas Research Centre (FACC) and the McGill University Animal Care Committee (UACC). Long-Evans rats were purchased from Charles River and bred at the Douglas Mental Health University Institute animal facility. Food and water were provided *ad libitum* and bedding was changed weekly. Rats were kept in a colony room with temperature set at 22° ± 1 °C, relative humidity levels between 30 and 50% and they were on a 12:12 light dark cycle (lights on at 8 am) throughout the

study except for the mothers who were transferred to a maternal room one week prior to delivery and were on a 12:14 h light dark cycle with lights on at 9 am until the pups reached postnatal day 22. Pups were pair housed in groups of the same sex. All animals were sacrificed between postnatal day 90–100, between 10:00 am and 12:00 pm in light phase. Male and female cages were counterbalanced during sacrifice on each day. Rat heads were rapidly decapitated using guillotine and placed on ice. Rat brains were rapidly removed, flash frozen using cold isopentane and stored at –80 °C. 24 male and 23 female sibling rats from 12 mothers were used to permit comprehensive and robust bioinformatic analysis such as weighted gene co-expression network analysis (WGCNA) for RNA sequencing.

Tissue collection

Rat brains were rapidly removed, flash frozen using cold Isopentane and stored at –80 °C. Tissue was sliced coronally at 200 µm thickness until bregma –3.4 mm. Brains were then removed and remounted for horizontal slicing. Tissue samples were obtained in all depots using a 0.8 mm diameter tissue puncher, with tissue accessed through the following criteria; CGC, coronally slicing from bregma 3.7 mm to bregma 2.7; NAcS, coronally slicing, from bregma 2.7 mm to bregma 1.0 mm; BLA, coronally slicing, from bregma –1.8 mm to bregma –3.4; vDG –200 µm horizontally slicing from interaural 2.2 mm to interaural 3.4 mm.[31]

Bulk RNA sequencing

Tissue samples were obtained using puncher after brain slicing and RNA was extracted using the Qiagen RNA Mini kit (Qiagen, Cat# 74104) as per the manufacturer's instructions with on-column DNase I treatment (See Supplementary Materials). RNA quality was examined using a Bioanalyzer 2100 (Agilent technologies, Santa Clara, USA). RNA libraries were prepared using the Illumina TruSeq Stranded Total RNA LT set (Cat# RS-122-2301, Illumina Canada Ulc.). Libraries were prepared together, with 15 different indexes placed in each sequencing flow cell. Paired-end, 100 bp read-length RNA-seq was carried out one time to avoid batch effect, using the HiSeq 4000 at a depth of 25 M reads per sample at the McGill University and Genome Quebec Innovation Centre. RNA-seq data were aligned to the Rattus Norvegicus rn6 genome (NCBI accession GCF_000001895.5) with the STAR aligner.[32] Counts of reads aligned to rat genes were calculated in the same process by STAR with the "quantMode GeneCounts" option.

RNA-seq differential gene expression analysis

Read counts from the STAR alignment of bulk RNA-seq data were used to compute differential expression statistics (fold change, p-value, false discovery rate (FDR)) between male and female samples with the exactTest function of the edgeR package,[33] which tests genewise for the differences in means between two groups of negative-binomially distributed counts, following the method of Robinson and Smyth (2008).[34] These differential expression calculations between male and female groups were conducted for each brain region separately. Prior to the differential expression calculation, genes with fewer than one read per million mapped reads in at least 5 samples were excluded from the analysis. False discovery rate values for multiple testing correction were computed by edgeR with the Benjamini–Hochberg method. Multidimensional scaling plots (MDS) were generated in edgeR with the plotMDS function on normalized counts using the default "logFC" method in which the computed distances between samples are based on $\log_2$ fold change.

RNA-seq was validated using quantitative reverse transcription-polymerase chain reaction (qRTPCR) using the Roche LightCycler 480 SYBR Green I master mix (Roche Molecular Systems, Inc.). 500 ng of RNA was converted to cDNA using maxima first-strand cDNA synthesis kit (Cat# K1642, Thermo Scientific.) for qRTPCR expression quantification using Roche LightCycler 480.

Rank-rank hypergeometric overlap analysis (RRHO)

Overlaps between the differential expression of two independent RNA-seq comparisons were visualized and measured with a rank-rank hypergeometric overlap analysis.[35] The full threshold-free lists of differential expression data were first ranked by increasing log fold change. The RRHO2 "stratified" method (https://rdrr.io/github/RRHO2/RRHO2/) was used to detect the overlap between genes differentially expressed in the same or opposite directions,[36] where the bottom right and top left quadrant display overlap of genes with concordant differential expression, and the top right and bottom left discordant overlap. Rat genes were converted to human orthologs using the Biomart package in R. The hypergeometric probability of overlap was computed with the nematode genome comparison browser (https://nemates.org) and 19,827 genes were used for the number of human coding genes (https://useast.ensembl.org/Homo_sapiens/Info/Annotation).

Single nuclei RNA sequencing (snRNA-seq)

Frozen tissue (50 mg) from 4 animals of the same sex in a new cohort were pooled before nuclei were isolated and captured as previously reported.[37] Libraries were prepared using 10X genomics kits (see Supplementary Materials). Paired-end sequencing, with a read length of 100 base pairs, was carried out using NovaSeq 6000 at a sequencing depth of 625 M. Single nuclei RNAseq data were analysed and bulk tissue cell type deconvolution was performed to estimate cell type proportions in bulk RNA-seq data. To establish reliability, four

independent biological pooled NacS samples (2 rats of same sex) were obtained and new snRNA-seq was performed as mentioned above, except the nuclei isolation was done using 10X genomics Chromium nuclei isolation kit with Rnase inhibitor (Cat# 1000494, 10X genomics.) and nuclei was counted using countess II FL automated cell counter after staining with Hoeschst 33342 staining dye solution (Abcam, Cat# ab228551). In total 41,778 nuclei with an average of 85,665 reads per nucleus sequenced across all runs, with 29,357 nuclei retained after quality control and filtering.

Filtered matrices from Cell Ranger were analysed using Seurat v4.3[38,39] in R v3.6.1. Nuclei with a proportion of mitochondrial genes >5%, ribosomal genes >5% or >500 genes were filtered from the dataset. Following filtering 29,357 nuclei remained and were log normalized and integrated using Seurat.[40] Dimensional reduction was carried out using the first 25 principal components, with clusters identified using K-nearest neighbours at a resolution of 0.1. Specific cluster markers were identified using the built-in Seurat function FindALLMarkers, with a minimum log fold change of 0.25 and expression in >25% of cells. Clusters were annotated using common marker genes from the literature and previously published datasets.[41–43] We used an iterative clustering method in which clusters composed of putative doublets (using DoubletFinder or those cluster expressing marker genes for non-overlapping cell types) or those composed of only a subset of samples were removed. Differential expression between males and females within each cluster was carried out using the Wilcoxon rank-sum test and Bonferroni posthoc adjustment. An adjusted p-value threshold of 0.05 with an absolute log fold change greater than 0.25 were used to identify differential expression.[38,39]

Bulk tissue cell type deconvolution

MuSiC (MUlti-subject Single Cell deconvolution) is a deconvolution method that utilizes cross-subject single-cell RNA-seq (scRNA-seq) to estimate cell type proportions in bulk RNA-seq data.[44] We performed cell type deconvolution using the pseudo bulk RNA-seq from our snRNA-seq and our bulk RNA-seq, using our snRNA-seq dataset as a reference, for male and female rats in the 4 brain regions. Cell type proportions were estimated by clustering the cell types into 8 cell clusters (Astrocyte, Cholinergic, Endothelial, GABAergic, Microglia, MSN, Oligo, OPCs) and using marker genes from the snRNA-seq dataset. A marker gene was defined as one which was expressed in greater than 90% of cells in a given cluster.

We further performed cell deconvolution analysis in human bulk RNA-seq data obtained from GTExV8 (https://gtexportal.org/home/datasets) using the same cluster markers identified in our snRNA-seq dataset. Human basal ganglia (nucleus accumbens) RNA-seq data were extracted and mapped to rat genes using BioMart,[45] which included 14,808 genes in 246 samples that were expressed in more than 90% of samples. The marker gene set selected for deconvolution was defined as genes expressed in more than 85% of cells in a given cluster.

Pseudo bulk RNA-seq analysis

Pseudo bulk RNA-seq analysis was performed on each snRNA-seq sample using the AggregateExpression() function in Seurat. We then used DESeq2 to perform principal component analysis on the log transformed data using the plotPCA() function.[46] The first 2 principal components comprising 81% and 11% of the variance, respectively, were used for plotting without further transformation. Differential expression was performed on the pseudobulked samples to compare the $\log_2$ fold change with that from the bulk RNA-seq data.

Gene ontology (GO) analysis

We functionally annotated DEGs with GO analysis using the Clarivate analytics Metacore program using rat RNA-seq data as reference genes (http://portal.genego.com). The output also showed DEG enrichment networks of characterized canonical pathways. Genes within network modules were characterized using the anRichment package in WGCNA (https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/GeneAnnotation/Tutorials/anRichment-Tutorial). After correction for multiple comparisons using an FDR adjustment, the 5 enriched terms in biological process were retained. Gene ontology enrichment analysis for the snRNA-seq differentially expressed genes was conducted using clusterProfiler v4.2.2 using all genes expressed in the snRNA-seq dataset as background.[47]

MAGMA

Genome-wide association studies (GWAS) enrichment for genes differentially expressed in the bulk RNA-seq and snRNA-seq dataset was conducted using MAGMA v1.10. A window of ± 20k around each gene was used to annotate genes from the respective GWAS using SNP annotations from the 1000 genome European dataset and gene annotations from the NCBI build 38 annotation.[48] Supplementary Table S18 holds the information concerning GWAS summary statistics used in these analyses.

Statistics

For qRTPCR, gene expression fold changes were calculated using the [delta][delta]Ct method. Statistical testing was performed either with GraphPad Prism 9 or with R v4.1.1 and Rstudio v1.4.1717. The specific statistical method used for each comparison is stated in the results section. A minimum alpha of 0.05 is used throughout the manuscript. We used the Benjamini–Hochberg method where necessary to correct for multiple comparisons.

Role of funders
The funder had no role in the study design, data collection, data analysis, interpretation, and writing the report.

Results

Region-specific sex differences in the cortico-limbic transcriptome

We performed RNA-seq to investigate somatic gene transcriptional profiles, excluding genes from sex chromosomes and mitochondria, in the NAcS, BLA, CGC, and vDG (Supplementary Fig. S1a). For each region male and female (n = 22–24/sex) samples derived from 12 mothers were analysed to create data sets sufficiently large to permit comprehensive and robust bioinformatic analysis. Bulk tissue RNA-seq was performed using the Illumina Hiseq 4000 with a sequencing depth of 25 M reads/sample. Multidimensional scaling plots (MDS) revealed a clear separation between male and female samples in the NAcS, in contrast to the other brain regions. Supplementary Fig. S1b shows the full MDS plots for each brain region, and Fig. 1a presents scatter plots of the positions of points along the first component of those MDS plots (Fig. 1a and Supplementary Fig.S1b). DEG analysis was performed between male and female samples using the edgeR package[33] with a conservative false discovery rate cut-off (absolute fold change >1.5, FDR <0.01) was used to classify genes as differentially expressed. We found remarkable, region-specific effects of sex on the transcriptome. The NAcS showed the most marked differences, with 3418 somatic DEGs. We also found 328, 144, and 171 somatic DEGs in the BLA, CGC and vDG, respectively (Fig. 1b, and Supplementary Fig. S1c–S1f, and Supplementary Tables S1–S4).

We found only 20 DEGs (0.5%) that were common across the 4 regions (Fig. 1c), suggesting sex-specific patterns of gene expression in the rat brain are region-specific. GO analysis revealed that these 20 common DEGs were enriched for terms related to "metabolic process" (Fig. 1d). Furthermore, many of these genes were immediate early genes (e.g., *Arc*, *Fos*, *Gadd45b*, *Ier5l*) and were upregulated in female rats (Fig. 1e), suggesting increased basal neuronal activity in the corticolimbic system of our female rats at the time of sacrifice.

When analysing genes from sex chromosomes and mitochondria, we also observed that the NAcS showed the most prominent differences between males and females, with 136 DEGs located on the X-chromosome in the NAcS compared with 16, 11, and 11 in the BLA, vDG and CGC (Fold change >1.5, FDR <0.01), respectively (Supplementary Fig. S2a). There were 6 common X-chromosome genes differentially-expressed across all 4 tissues (*Gm27733*, *AABR070393*, *LOC680227*, *Eif2S3*, *Ddx3x*, *Kdm6a*) (Supplementary Fig. S2b). Gene ontology (GO) analysis using Metacore (Clarivate Analytics) revealed X-chromosome DEGs in the NAcS were enriched for GO terms related to "histone H2A monoubiquitinations" ($p = 5.46 \times 10^{-4}$), "histone H2A ubiquitination" ($p = 1.08 \times 10^{-3}$) and "presynapse assembly" ($p = 1.25 \times 10^{-3}$) (Supplementary Fig. S2c). There were 7, 7, 1 and 0 mitochondrial genes differentially expressed in the NAcS, BLA, vDG and CGC, respectively (absolute Fold change >1.5, FDR <0.01, Supplementary Fig. S2d, Supplementary Tables S5–S8).

For validation using qRT-PCR across the sequenced samples, we randomly selected 19 candidate DEGs including immediate early genes and transcription factors. Comparison of mRNA fold change between RNA-seq and qRT-PCR was performed using simple linear regression that examines the relationship between two quantitative variables by fitting a line to the observed data.

The results showed significant associations between the RNA-seq and the qRT-PCR across all 4 brain regions (NAcS, beta = 0.265, 95% CI = (0.228–0.301), $R^2 = 0.933$, $p < 0.0001$; BLA, beta = 0.282, 95% CI = (0.256–0.309), $R^2 = 0.968$, $p < 0.0001$; vDG, beta = 0.210, 95% CI = (0.145–0.276), $R^2 = 0.744$, $p < 0.0001$; CGC, beta = 0.329, 95% CI = (0.299–0.358), $R^2 = 0.974$, $p < 0.0001$) (Supplementary Fig. S3a–S3e, Supplementary Table S9). The linear regression assumptions of normality and homogeneity of variance of residuals were satisfied; there was no deviation from linearity (CGC, p = 0.5; vDG, p = 0.76; NAcS, p = 0.36; BLA, p = 0.97). We then assessed enrichment for the bulk DEGs in GWAS from 9 psychiatric disorders (ADHD, anxiety disorder, ASD, bipolar disorder, cross-disorder, MDD, PTSD, schizophrenia and Tourette syndrome). Several gene lists were positively enriched at a nominal p-values <0.05, but only the enrichment of DEGs from the NAcS in the cross-disorder GWAS passed correction for multiple comparisons (Fig. 1f).

NAcS genes with sex-specific expression in rat and human

Labonté et al. used bulk RNA-seq to assess sex differences of gene transcription across multiple brain regions in humans, including the nucleus accumbens (NAc). We compared the sex-dependent DEGs in the rat NAcS to the human dataset (13 males and 9 females; Supplementary Table S11 from Labonté et al.)[16] to identify conserved sex-dependent expression patterns. The rank-rank hypergeometric overlap (RRHO) algorithm[35] using the "stratified" RRHO2 method of Cahill et al., 2018,[36] was used to generate the heatmap (Fig. 2a) after matching Ensembl gene names between the two studies. The significant region overlap across species was in genes upregulated in females vs males (upper right quadrant of Fig. 2a). Conversion of rat DEGs to human orthologues identified conserved genes between species. Between the two studies, 303 were common sex-dependent DEGs (hypergeometric test, $p < 4.86 \times 10^{-29}$,

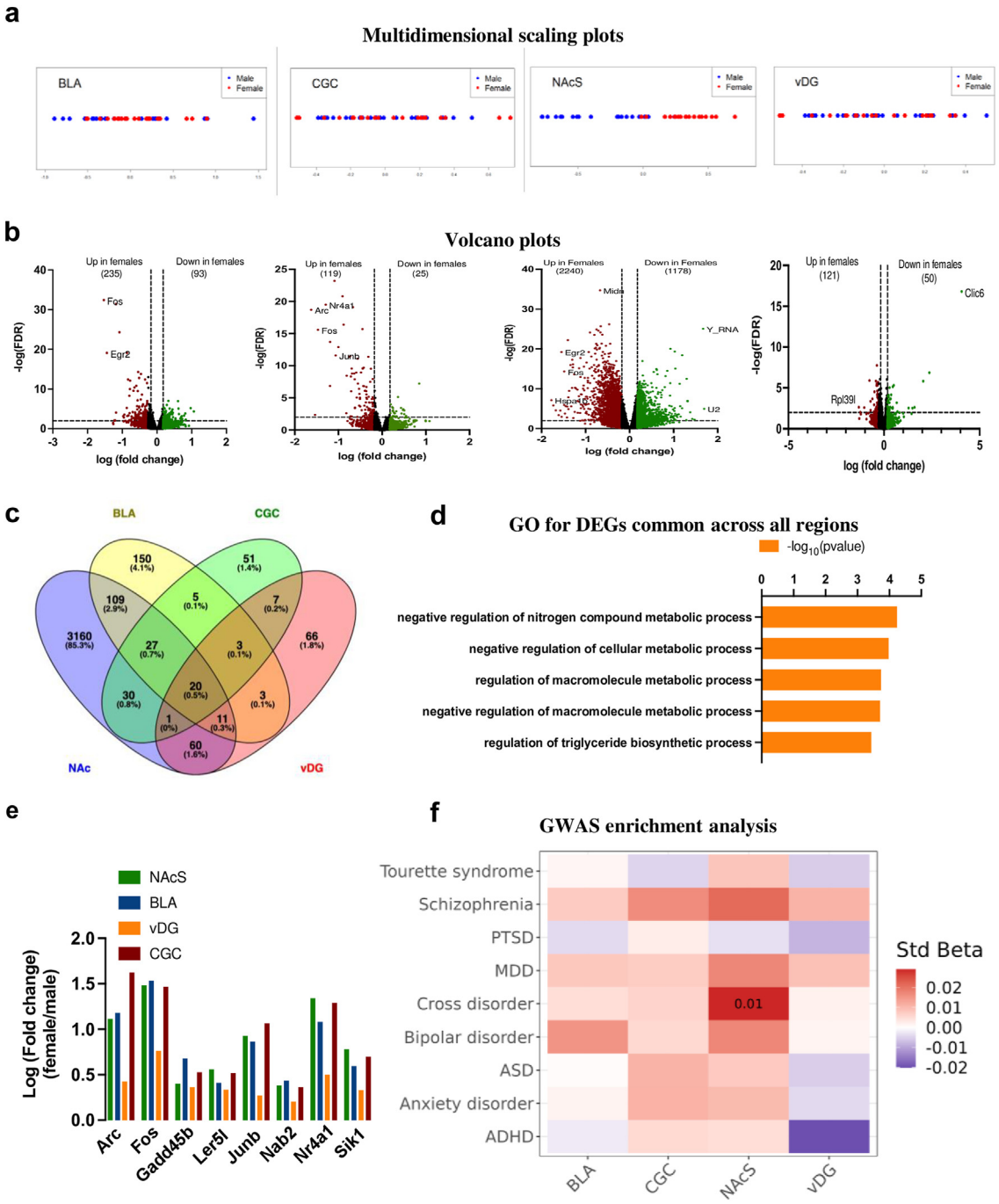

Fig. 1: **Sex-dependent transcriptome alterations in the rat limbic system.** (a) Scatter plots showing the positions of points along the first principal component of multidimensional scaling plots (MDS), showing strong separation between male (blue) and female (red) samples in the NAcS. n = 22–24. (b) Volcano plots show genes differentially expressed between males and females in all 4 brain regions. (c) Venn diagram shows the overlap in DEGs across the four brain regions. (d) Gene ontology (GO) enrichment of the 20 common DEGs across the four brain regions. (e) Common DEGs include many immediate early genes which are upregulated in females compared to males. (f) GWAS enrichment analysis for the association between genes differentially expressed between males and females across 4 rat corticolimbic brain regions and 9 psychiatric disorders. Results are displayed according to standardized beta (Std Beta), for associations passing correction for multiple comparisons at an FDR p-value of 0.05 the corresponding FDR p-value is also presented.

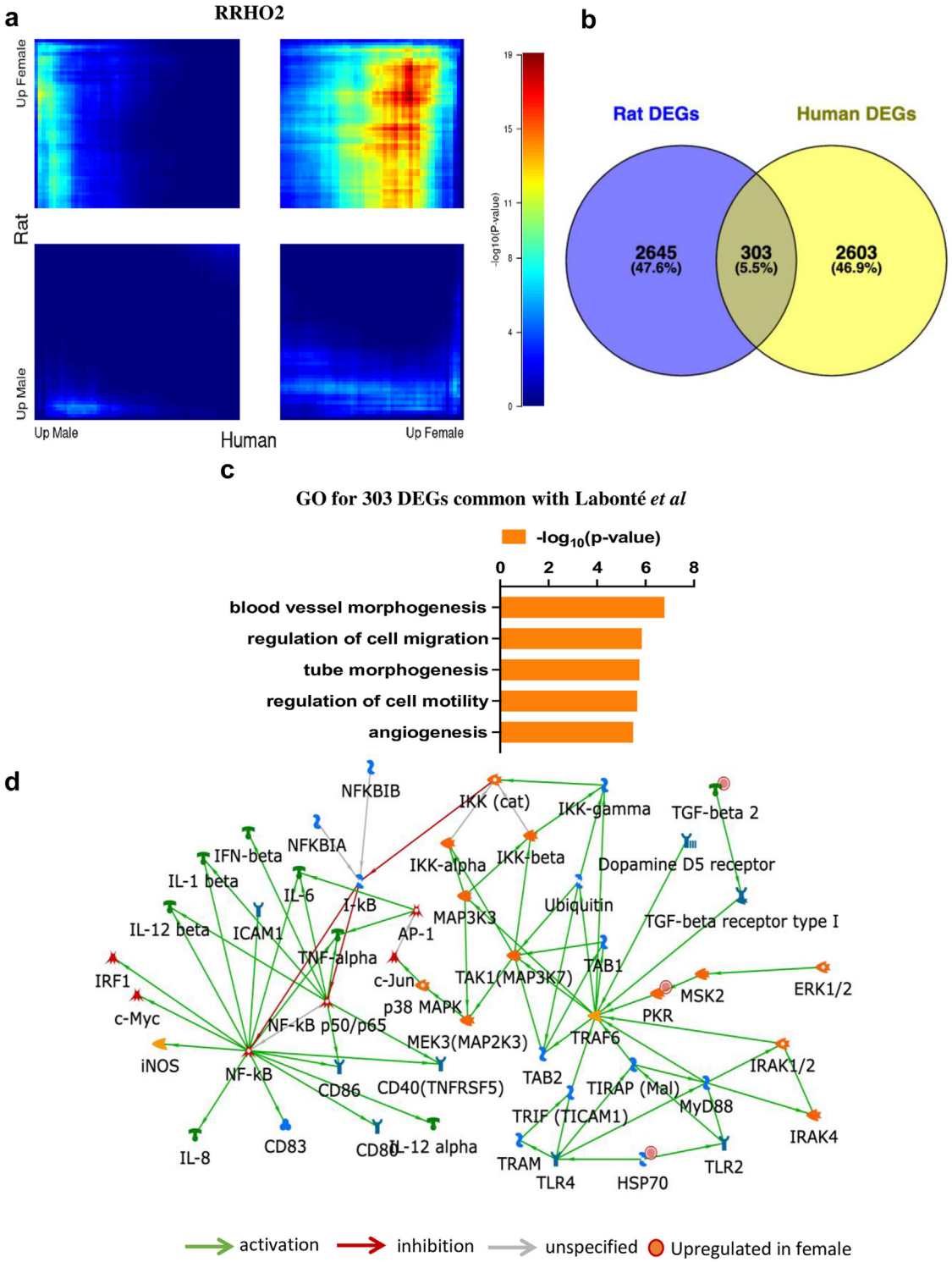

***Fig. 2:* Overlap of NAc sex DEGs between rats and humans.** (a) Rank-rank hypergenometric overlap (RRHO2) map showing the threshold-free overlap of the male vs female differential expression comparisons in rat and human. Pixels represent the overlap between the transcriptome of each comparison, and are colour coded with the significance of overlap ($-\log_{10}$(p-value) of a hypergeometric test). Genes along each axis are sorted from most up-regulated in males vs females (lower left) to most down-regulated in males vs females (top right). (b) Venn diagram shows overlap of sex-dependent DEGs between rats and humans (hypergeometric test, $p < 4.86 \times 10^{-29}$, representation factor 0.6). (c) GO analysis of overlapping DEGs between rats and humans. (d) Gene network of the overlapping DEGs generated using Metacore.

representation factor 0.6; Fig. 2b, Supplementary Table S10) between rat and human dataset from Labonté et al.[16] These 303 DEGs were enriched for GO terms related to "Blood vessel morphogenesis" ($p = 1.64 \times 10^{-7}$), "regulation of cell migration" ($p = 1.44 \times 10^{-6}$) and "tube morphogenesis" ($p = 1.80 \times 10^{-6}$) (Fig. 2c). Interestingly, sex-specific changes in both blood vessels and at the blood brain barrier have been observed in both affective disorders and animal models of chronic stress.[49] Among these common genes between rat and human we identified a gene network with *NF-kB*, *TRAF6*, *TAK1 (MAP3K7)*, *RelA (p65 NF-kB subunit)*, *MyD88*, and *I-kB* as hubs (Fig. 2d). This network was also involved in the *I-kappaB kinase/NF-kappaB* signalling and response to cytokine signalling. The transcription factors *AP-1*, *c-Jun*, *NF-kB* family genes, *TGF-β receptor 1*, *TGF-β receptor 2* and *TGF-β2*, which were present in the network, were upregulated in the NAcS of female rats. *TGF-β1* showed the greatest difference in expression between males and females in the network. Interestingly, *TGF-β1* was also differentially expressed in both our dataset and Labonté et al.[16] at comparable levels (in human, logFC = 0.73, $p = 0.03$; in rats, logFC = 0.76, $p = 4.6 \times 10^{-8}$, FDR = 6.3×10^{-7}).

Single nuclei RNA sequencing (snRNA-seq)

We next used snRNA-seq to investigate cell-specific gene expression patterns underlying our results (Fig. 3a). After alignment, stringent quality control and data integration (Supplementary Fig. S5a–S5c) we identified 19 distinct clusters from 29,357 nuclei (Supplementary Table S12). Among these clusters were 7 clusters of medium spiny neurons (MSN); three of which were Drd1-positive (Drd1+), two Drd2-positive (Drd2+) one Grm8-positive (Grm8+) and one Drd3-positive (Drd3+) (Fig. 3b–d; Supplementary Fig. S5d; Supplementary Table S13). We also identified a population of activated MSNs, which expressed high levels of immediate early genes. Previous studies in humans and mice[50,51] have demonstrated subclusters of MSNs represent distinct spatial and functional subsets, with enrichment for distinct disorders. As such, we chose to retain a level of clustering that reflects this MSN diversity. We also identified a population of GABAergic neurons that did not express canonical MSN markers (e.g., *Ppp1r1b*) or dopamine receptors, as previously reported by Savell et al.[43]

We identified 2521 genes as differentially expressed between males and females. There were no differences in cell type proportions between males and females (Fig. 4a). Most of these DEGs were shared across clusters (Fig. 4b; Supplementary Table S14), with the most DEGs found in oligodendrocytes (390 genes), GABAergic neurons (324 genes), Drd3 MSNs (286 genes) and the Astrocyte A (279 genes) clusters. Interestingly these were also among the clusters with the strongest expression of sex-hormone receptors (Fig. 4c). We next used MAGMA to assess the enrichment of these genes in GWAS from 9 psychiatric disorders (ADHD, anxiety disorder, ASD, bipolar disorder, cross disorder, MDD, PTSD, schizophrenia and Tourette syndrome). We partitioned this analysis by genes that were upregulated in males or in females, and found a striking enrichment of genes upregulated in females within several psychiatric disorder GWAS (Fig. 4d). In total 10 associations of DEGs passed correction for multiple comparisons and all were upregulated in females. These included 3 associations with DEGs from the Astrocyte A cluster (anxiety, cross disorder and schizophrenia), 3 associations with Drd3 DEGs (bipolar disorder, cross disorder and schizophrenia), 3 associations with oligodendrocyte DEGs (bipolar disorder, cross disorder and schizophrenia) and 1 association with Grm8 MSNs (bipolar disorder). We then used gene ontology enrichment analysis to functionally annotate these female upregulated DEGs and found an enrichment for synaptic related terms in the Astrocyte A, Drd3 MSN and oligodendrocyte clusters (Fig. 5a, c, and e; Supplementary Table S15). The Grm8 MSN female upregulated genes were not enriched for any GO terms.

Subsequent gene network analysis of these DEGs implicated YAP1 (Yes1 associated transcriptional regulator) as putative regulators in Astrocyte A cluster (Fig. 5b). Notably, synaptotagmin was upregulated in females compared to males. This network is involved in mitochondrial ATP synthesis coupled electron transport and regulation of calcium ion-dependent exocytosis.

We also identified *c-Src*, *Ephrin-A receptor*, *Ephrin-B receptor*, and *FAK1* (*focal adhesion kinase 1*) as regulators of the Drd3 MSN network. Many Ephrin receptor family members such as Ephrin-A receptor 6, Ephrin-A receptor 5 were upregulated in females compared to males (Fig. 5d). While in the oligodendrocyte network, *c-Raf-1*, *c-Src*, *ERK1*, *ERK1/2*, *ERK2 (MAPK1)*, *G-protein alpha-q/11*, and *VEGFR-2* were identified as hub regulators, which is involved in regulation of cell communication (Fig. 5f). Together these results illustrate baseline sex difference in the rat nucleus accumbens shell center on synaptic function and are enriched for several human psychiatric disorders.

To validate the results in cell type proportions, we used cell-type deconvolution analysis in our bulk RNA-seq dataset, using cluster markers identified in our snRNA-seq dataset (Supplementary Table S16). Consistent with our snRNA-seq data, deconvolution of our bulk RNA-seq NAcS data revealed cell type populations varied as a function of brain regions but not as function of sex in rats (Supplementary Fig. S6a). Deconvolution of human basal ganglia RNA-seq data, from the GTEx study (n = 246), revealed no significant difference in transcriptomic signature associated with astrocyte, cholinergic, endothelial, GABAergic, microglia, total MSNs, oligodendrocyte, or OPCs clusters

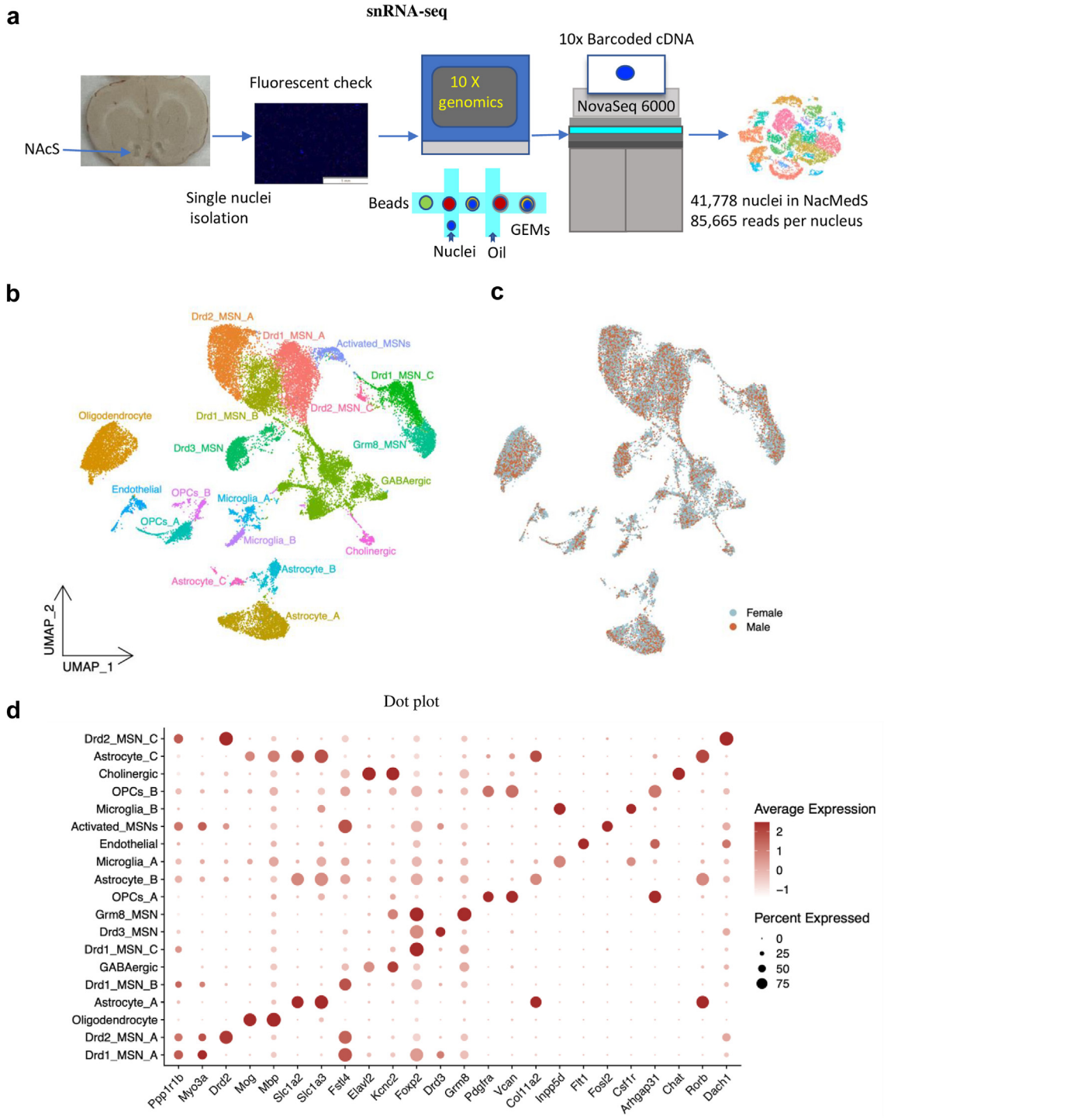

***Fig. 3:* Single nucleus RNA sequencing in the rat nucleus accumbens medial shell.** (a) Schematic of the tissue isolation and single nucleus RNA sequencing in the nucleus accumbens medial shell. (b) UMAP of 29,357 nuclei recovered from the rat NAc medial shell coloured by their annotated cluster. (c) UMAP coloured by male (11,590) and female (17,767) nuclei. (d) Dotplot displaying marker genes for each annotated cluster. A darker colour indicates a higher average expression of the gene, with a larger dot size indicating the gene is present in a larger percent of nuclei within that cluster.

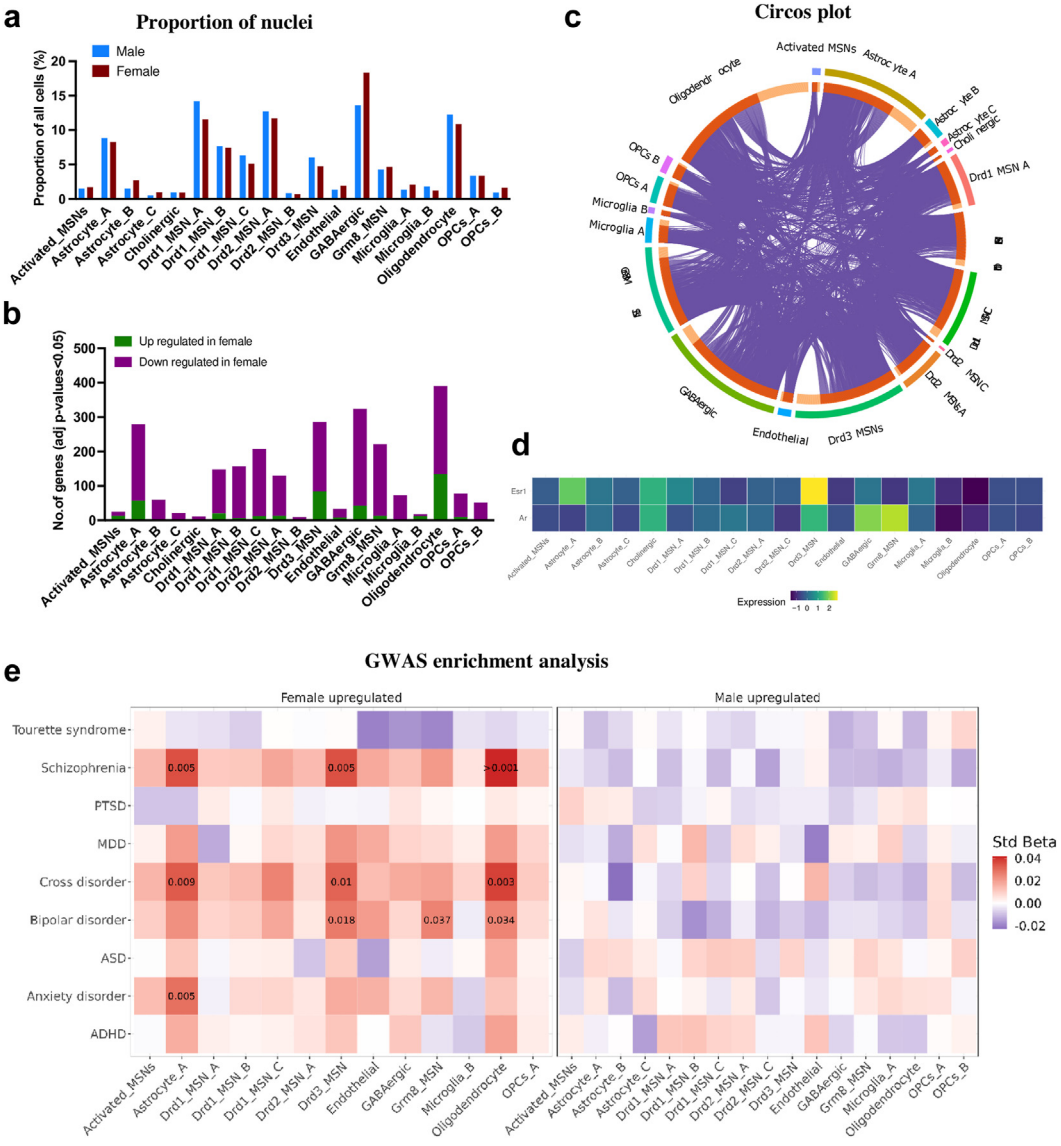

Fig. 4: **Differential expression in snRNA-seq clusters and GWAS enrichment.** (a) Proportion of nuclei male and female nuclei across all clusters. (b) Number of genes differentially expressed in each cluster. Green indicates up regulated and purple indicates down regulated. (c) Circos plot showing all genes differentially expressed between male and female samples across all clusters. The outer bar is proportionate to the number of differentially expressed genes with that cluster. The lighter orange hue within this bar indicates that gene was uniquely differentially expressed in that cluster, with a darker orange hue illustrating that gene was also differentially expressed in another cluster. The commonly differentially expressed genes are linked with a purple connector. (d) Heatmap showing the scaled expression of the sex hormone receptors Esr1 and Ar across all the identified clusters. Yellow indicates the highest level of expression and dark blue indicating the lowest level of expression. (e) GWAS enrichment analysis for the association between genes differentially expressed between males and females in various cell types of the rat Nucleus accumbens shell and 9 psychiatric disorders. Results are displayed according to standardized beta (Std Beta), for associations passing correction for multiple comparisons at an FDR p-value of 0.05 the corresponding FDR p-value is also presented.

between males and females. We also found no significant difference in transcriptomic signatures associated with activated MSNs as well as Drd MSNs in the basal ganglia of males compared to females (Supplementary Fig. S6b, Supplementary Table S17). We performed pseudobulk analysis on the snRNA-seq samples to assess correlation with the bulk RNA-seq findings. Similar to the bulk RNA-seq, the snRNA-seq showed distinct separation along the second principal component. Furthermore, the log fold change for expression changes between male and female samples, between the bulk and pseudobulk comparisons was significant

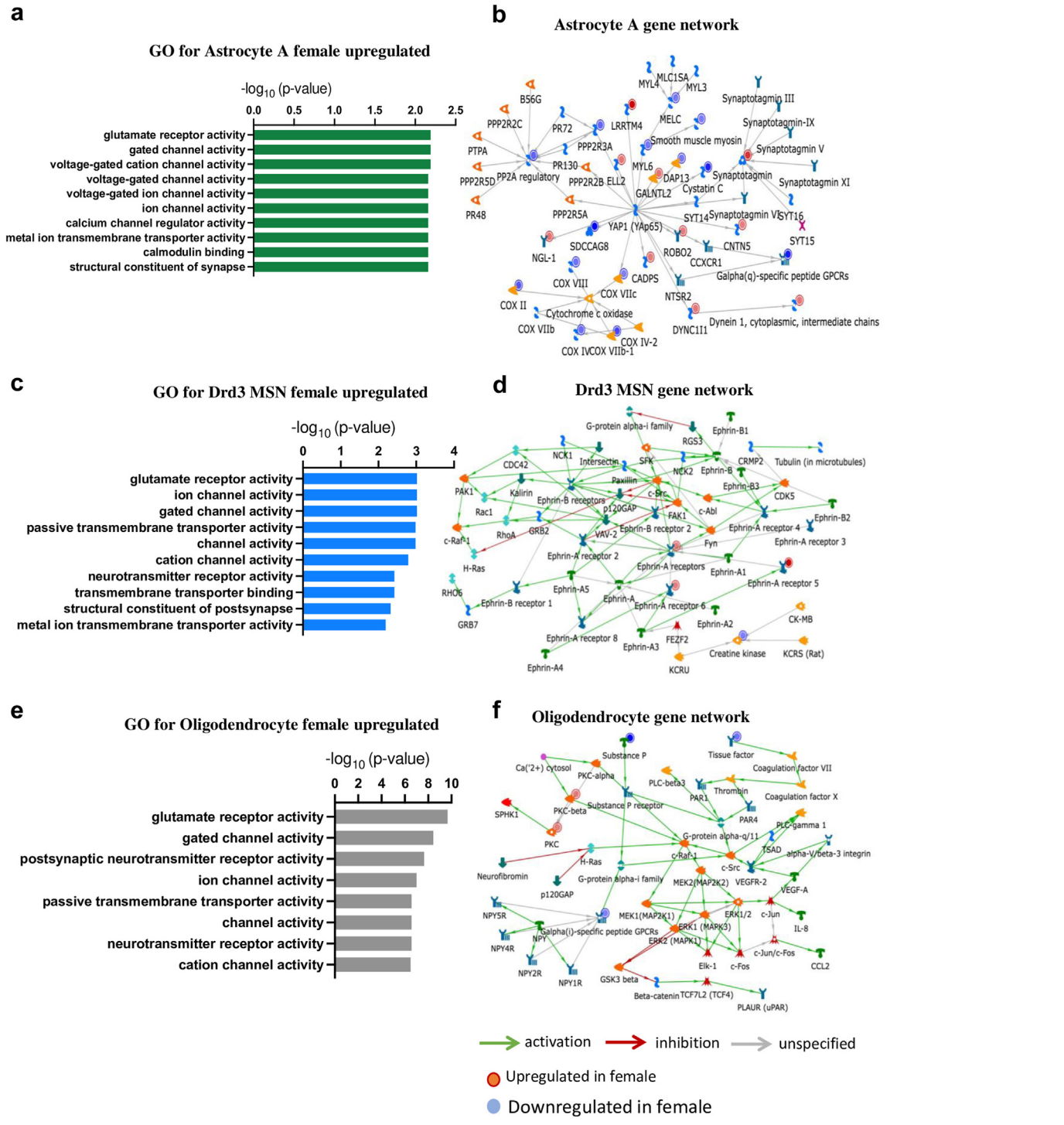

Fig. 5: **Gene ontology and network analysis for genes differentially expressed in Astrocyte A, Drd3 MSNs and oligodendrocytes.** Gene ontology enrichment analysis for genes upregulated in females within the astrocyte (a), Drd3 MSNs (c) and oligodendrocyte (e) clusters and gene regulatory networks for genes differentially expressed within the astrocyte (b), Drd3 MSNs (d) and Oligodendrocyte clusters (f). Genes are positioned with respect to their respective protein localizations. The arrow indicates direction of regulation, with red, blue or grey indicating negative, positive or an undefined pattern of regulation, respectively. A red or blue dot indicates significant upregulation or down regulation in females.

($p < 2.2 \times 10^{-16}$ and $r = 0.34$), suggesting reproducibility in these datasets (Supplementary Fig. S6c and S6d). Finally, gene set enrichment analysis (GSEA) showed enrichment of synaptic related GO terms in both bulk and pseudobulked datasets, supporting the idea of baseline sex differences in synapse-related expression patterns within the rat NAcS (Supplementary Fig. S6e).

Discussion

We investigated sex-specific patterns of gene expression within corticolimbic areas of the rat that are commonly associated with psychopathology in humans. Despite employing a conservative FDR value (0.01) we detected more than 4000 DEGs across all brain regions, likely as a function of the sample size and thus statistical power. The relatively small number of DEGs common across regions is consistent with previous reports showing that sex-dependent gene expression patterns are region dependent.[16,52] Remarkably, we found over 3000 DEGs in the NAcS alone. Our informatic analyses revealed compelling evidence for the relevance of these sex dependent DEGs in the NAcS for human psychopathology. The commonly sex-dependent DEGs between rats and humans identified a pro-inflammatory gene network linked to TGFβ as a candidate mechanism for sex differences in NAc-associated psychopathology. Finally, we used snRNA-seq with nuclei derived from the NAcS to show sex-dependent patterns of gene expression, which were particularly prevalent in astrocyte, Drd3 MSNs and oligodendrocyte cell populations associated with sex differences in the development of psychiatric disorders.[53–56] These findings suggest that the NAcS is a key brain region underlying sex differences in vulnerability for psychopathology.

Our findings are in general agreement with the recent GTEx study that described sex-specific patterns of gene expression across 44 human tissues.[14] Sex-specific gene expressions were observed in thousands of genes but typically with small fold change. Similarly, we found >3000 DEGs within the NAcS but with moderate effect sizes. GTEx is an extensive map of genotype-gene expression relationships in more than 800 postmortem human participants in different tissues,[57] including participants from 20 to 70 years of age. Though this is an essential dataset for functional genomics and a gigantic contribution to the field, the wide age range of the participants should be considered with caution, as age influences brain gene expression.[58] In non-human models, brains are immediately frozen after collection, all the animals have about the same age and had been exposed to a standard environment throughout their lives, therefore the gene expression changes between sexes reported here may refer to actual baseline differences. These findings suggest that sex differences in neural functions are likely to accrue through the coordinated effects of multiple sex-specific transcripts each with only a modest effect. This conclusion is consistent with the described polygenic architecture of psychiatric disorders revealed through recent GWAS.[59,60] In support of our findings, evidence from animal models has indicated that changes in the transcriptome/proteome can manifest sex-specific neuropsychiatric endophenotypes, exhibiting male or female specific vulnerability impacting the brain, including corticolimbic regions.[61–63]

NAcS showed high levels of sex differences in gene expression

The NAc core and shell differ in morphology, cell types, molecular components, neural connections and subserve different functions.[64,65] The NAc core is involved in goal-directed action and motor coordination, whereas the NAcS is more directly involved in the regulation of emotion. The NAcS is thus strongly implicated in depression in both pre-clinical and clinical studies, including those using targeted deep brain stimulation.[28,56,66–72] There is compelling evidence for the importance of projections from both the ventral dentate gyrus as well as the ventral tegmental area to the NAcS for stress-induced depression-like behaviours in rodent models.[26,70] These same circuits are associated with individual differences in susceptibility and coping behaviors.[66,72–74] The NAcS is also a major component of the mesocorticolimbic dopamine system that mediates the compulsive reward seeking that defines addictions. Sex differences in dopaminergic input and activity at the level of the NAcS is implicated in the development of addictions.[75,76] In humans, cocaine-dependent women show greater stress-induced activity in the ventral striatum, which includes the NAcS.[77] Therefore, our study provides further evidence for the role of the NAcS in driving sex dependent patterns of behaviour.

Conserved TGF family genes are upregulated in female NAcS

We found several members of the TGF family were upregulated in female NAcS, which was also seen in human data from Labonté et al.[16] TGF signalling regulates dopamine neuron firing and modulates GABAergic neuron transmission.[78–80] We note that our snRNA-seq analyses revealed differences in Drd+MSNs, as well as GABAergic neurons on the basis of expression patterns. Members of the TGF family are implicated in depression[81] and pain,[82,83] both of which exhibit sex differences in their prevalence.[84,85] In addition, sex differences in neuroinflammation profiles are seen in psychiatric disorders[86] and the TGF-β1 is upregulated in individuals with depression.[87] TGF-β dependent MAPK pathway activation downregulates the expression of tight and adherens junctions.[88] We also describe upregulation of NF-kB family genes in the NAcS of females and network analysis revealed a putative interaction between the TGF family and NF-kB families of transcription factors. NF-kB members have important regulatory roles in inflammatory and stress responses,[89,90] both of which

are closely associated with mood disorders and schizophrenia.[91–94] As such, these pathways are intriguing candidates for future experimental evaluation of sex difference in psychiatric disorders.

Sex- and cell-specific patterns of synaptic gene expression

Several sets of genes upregulated in females in the snRNA-seq dataset were enriched for synapse-related GO terms. These findings align with previous studies reporting sex differences in dendritic spine structure and an increased female spine density within the NAc.[95,96] The NAcS has the highest density of Drd3 receptors in the brain.[97] Genetic and positron emission tomography studies elegantly illustrate the importance of Drd3 signalling in the ventral striatum for MDD, pain, impulsivity and addiction[98–100]; all of which, as previously discussed, display marked sex differences in presentation and epidemiology. Our study provides a strong basis for future interrogation of Drd3+MSN function as a mechanism for dopamine-related, sex-specific traits and disorders.

In our snRNA-seq dataset, we found high levels of differential expression in mature oligodendrocytes, with these genes enriched for synaptic markers. Expression of synaptic genes in oligodendrocytes has been associated with neuronal induction of myelination.[101] This is of importance as myelination and oligodendrocyte function has an increasingly appreciated role in MDD.[102] Furthermore, in a recent snRNA-seq study, Nagy et al. demonstrated high levels of differential expression of oligodendrocyte precursor cells (OPCs) and changes in oligodendrocyte differentiation, in individuals with MDD.[37] Together with these results, our study designates the oligodendrocyte lineage as a promising candidate for future investigation into sex dependent risk associated with MDD.

In summary, we describe strikingly region-specific patterns of sex differences in gene expression in corticolimbic regions of the rat and demonstrate their potential relevance for human psychopathology. The putative mechanisms for sex differences of transcriptomes in the NAc appear to be involved mainly in synaptic function, inflammation, and dopaminergic signalling. These results contribute to our understanding of sex differences in brain regions and highlighted specific biological processes associated with them in the NAc, which are relevant for future investigation. Interestingly, sex specific changes have also been reported in animal models of neurodevelopmental disorders and chronic stress.[28,61,62] Our study also underscores the inclusion of sex as a biological variable in future studies which is crucial for understanding the sex differences in vulnerability to diseases. Our study will also act as a resource for future mechanistic inference of sex differences in psychiatric disorders. In particular studies manipulating Drd3+MSNs and TGF signalling will be important for future mechanistic inference to the sexual dimorphisms in psychiatric disorders.

Limitations

The current study bears limitations. First, we only examined adult rats. Our findings might be better interpreted in relation to conditions more prevalent in females and appearing later in life. Future studies will need to investigate sex differences in early development as well as the effects of hormonal surges in the adolescent period that may reflect mechanisms for conditions more prevalent in males, such as autism and attention deficit hyperactivity disorder (ADHD) that emerge at younger ages. Moreover, the timing of the appearance of tissue-specific sex differences in the transcriptome will also inform on mechanism. Second, our analyses are limited to basal levels of transcription. The issue of activated transcription in response to environmental triggers relevant for the onset of psychiatric morbidity will be a critically important avenue for future studies. Furthermore, future studies considering the interaction between sex-chromosome genes and those from somatic chromosomes will be an exciting future avenue in the investigation of sex differences within the brain. Third, expanding on our findings using orthogonal methods, such as spatial transcriptomics, will be important to further our understanding of sex dependent processes. Furthermore, multimodal approaches which integrate cellular transcriptomes, epigenomes and electrophysiological properties with hormonal measurements will be important to expand our understanding of sex differences in corticolimbic regions. Further expanding these methods to include other corticolimbic regions such as the medial amygdala will be important future steps for the field. Considering the temporal specificity of many psychiatric disorders and the risk associated with measures of adiposity future work investigating the transcriptomic effects of age and weight in corticolimbic regions might be informative. Fourth, our manuscript focuses on psychiatric disorders. This is because they have well characterized sex dependent characteristics and have been widely implicated in the four regions we studied due. However, these regions are also involved in a diverse range of traits which are not related to psychiatric disorders and future studies integrating our findings with a more diverse set of traits and disorders will likely be highly informative. In summary, we describe strikingly region-specific patterns of sex differences in gene expression in corticolimbic regions of the rat and demonstrate their potential relevance for human psychopathology. Our study will act as a resource for future mechanistic inference of sex differences in psychiatric disorders. In particular studies manipulating Drd3+MSNs and TGF signalling suggest an important target for future mechanistic inference to the sexual dimorphisms in psychiatric disorders. Finally, we rely

on enrichment of gene ontology terms for functional inferences of our differentially expressed genes, which suffer from both publication biases and false positive associations are difficult to exclude using these analyses.

Contributors

EF analysed snRNA-seq data and wrote the manuscript. DMA performed GWAS enrichment and reviewed the manuscript. MJS analysed RNA-seq data, performed WGCNA analysis and cell type deconvolution analysis of bulk RNA-seq data. NO analysed RNA-seq data. XW performed RNA extraction, prepared RNA-seq libraries and qRTPCR validation work. CN performed the snRNA-seq experiment. SM analysed RNA-seq data. KC took care of animals. PPS supervised the GWAS enrichment analysis and reviewed the manuscript. ARN supervised snRNA-seq data analysis. JD supervised the project. MJM supervised the project and with TYZ wrote the initial draft of the manuscript. TYZ designed the experiment, collected tissue, and developed the technique to do RNA-seq, performed snRNA-seq, data analysis, and with MJM wrote the initial draft of the manuscript. MJM and PPS verified the underlying data. All authors read and approve the final manuscript.

Data sharing statement

All authors agree to share the data related to current study. The bulk RNA-seq and snRNA-seq data are available through the Gene Expression Omnibus (GSE162419). The accession number for the snRNA-seq data is GSE168599.

Declaration of interests

The authors have no competing interests to declare.

Acknowledgements

We thank the McGill University and Genome Quebec Innovation Centre for performing RNA sequencing.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104749.

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