PSYCHENCODE 2

# Transcriptomic sex differences in postmortem brain samples from patients with psychiatric disorders

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**Many psychiatric disorders exhibit sex differences, but the underlying mechanisms remain poorly understood. We analyzed transcriptomics data from 2160 postmortem adult prefrontal cortex brain samples from the PsychENCODE consortium in a sex-stratified study design. We compared transcriptomics data of postmortem brain samples from patients with schizophrenia (SCZ), bipolar disorder (BD), and autism spectrum disorder (ASD) with transcriptomics data of postmortem control brains from individuals without a known history of psychiatric disease. We found that brain samples from females with SCZ, BD, and ASD showed a higher burden of transcriptomic dysfunction than did brain samples from males with these disorders. This observation was supported by the larger number of differentially expressed genes (DEGs) and a greater magnitude of gene expression changes observed in female versus male brain specimens. In addition, female patient brain samples showed greater overall connectivity dysfunction, defined by a higher proportion of gene coexpression modules with connectivity changes and higher connectivity burden, indicating a greater degree of gene coexpression variability. We identified several gene coexpression modules enriched in sex-biased DEGs and identified genes from a genome-wide association study that were involved in immune and synaptic functions across different brain cell types. We found a number of genes as hubs within these modules, including those encoding *SCN2A*, *FGF14*, and *C3*. Our results suggest that in the context of psychiatric diseases, males and females exhibit different degrees of transcriptomic dysfunction and implicate immune and synaptic-related pathways in these sex differences.**

## INTRODUCTION

Sex differences are common, well-documented phenomena in psychiatric disorders and include prevalence, age of onset, and response to antipsychotic drugs. These differences have implications for diagnosis and treatment (*1*–*3*). For instance, autism spectrum disorder (ASD) is more frequently diagnosed in boys than girls, with a ratio of approximately 4:1, and girls with ASD often experience more severe symptoms than boys (*4*, *5*). Similarly, schizophrenia (SCZ) is more prevalent in males than females, with a ratio of approximately 1.7:1, and men tend to experience earlier and more acute onset than women (*6*). Although the differences in prevalence are not conclusive for bipolar disorder (BD), females tend to exhibit later onset, more severe symptoms, and greater comorbidity burden than males (*7*–*9*). Furthermore, females are in general at greater risk of adverse drug reactions than males (*10*, *11*). By identifying the specific mechanisms that contribute to sex differences in these disorders, we can develop more targeted and effective interventions that consider sex-specific factors.

It is believed that sex-specific genetic risk associations and molecular profiles may contribute to sex-related phenomena. Carter *et al.* (*12*) developed a genetic liability model to explain sex differences. This model suggests that for a given complex trait, the genetic liability is normally distributed across populations, but the minimum genetic liability required for diagnosis may differ between males and females. Whereas family-based studies have supported this model (*13*–*16*), recent well-powered population-based studies have shown that the genetic liability of common variants is largely similar between males and females for most traits (*17*–*19*). This apparent contradiction may be resolved by the finding that sex-specific environmental factors or genotype-environment interactions may contribute differentially to the observed phenotypic variance in males and females (*20*).

Our previous studies focused on sex-related epigenetic differences, which are influenced by both genetic and epigenetic factors. We observed sex-biased quantitative differences of DNA methylation in SCZ (*21*). In addition, we reported that the up-regulated genes in ASD were hypermethylated in female controls (*22*). Similarly, Werling *et al.* (*23*) found that genes expressed at lower levels in female controls were enriched for genes up-regulated in ASD brains. Hoffman *et al.* (*24*) also observed that gene modules showing significant sex-SCZ interaction effects were enriched for disease risk signatures. These findings suggest that the gene expression changes required for psychiatric diagnoses may differ between sexes, being more pronounced in females than in males. However, further investigation is needed to formally test the hypothesis of transcriptome-based burden differences between males and females using clinical longitudinal data.

Following two recent transcriptome studies that identified opposite molecular signatures for males and females in major depressive

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disorder (MDD) (*24*, *25*), we implemented a sex-stratified study design to detect sex-specific pathology in ASD, BD, and SCZ. Our hypothesis was that the burden of transcriptomic dysfunction in these disorders differs between males and females. This burden would encompass the amount, degree, and organization of gene expression changes, influenced by both genetic and environmental factors. Specifically, we examined two components of the transcriptomic burden: differential gene expression burden and coexpression connectivity burden. To investigate this hypothesis further, we leveraged transcriptomics data from 2160 postmortem prefrontal cortex brain samples from the PsychENCODE project to compare transcriptional profiles associated with SCZ, BD, and ASD between males and females.

## RESULTS

### Higher transcriptomic burden in females than in males

Our study investigated transcriptomic dysfunction in postmortem brain tissue from male and female patients with SCZ, BD, or ASD. Using RNA sequencing (RNA-seq) data from 2160 prefrontal cortex brain samples provided by the PsychENCODE project (Fig. 1A), we explored potential sex differences in transcriptomic burden at the level of individual genes (Fig. 1B) and gene coexpression networks (Fig. 1C).

A sex-stratified approach was used to perform a differential gene expression analysis between cases ($n = 928$) and controls ($n = 1232$) (Fig. 1A). In this approach, transcriptomics data from 2160 postmortem brain samples from both males and females were analyzed separately. The results were compared to identify any differences in the effect sizes, the numbers of differentially expressed genes (DEGs), and the features of genes exhibiting differences in transcriptomic dysfunction burden between the two sexes (Fig. 1B). In addition, we conducted direct expression comparison between males and females in control and case groups separately to obtain a comprehensive assessment of the molecular distinctions across sexes (Fig. 2A).

Larger case-control differences were observed in females (Fig. 2, A to C, and data files S1 and S2). The sex-stratified case-control comparisons in SCZ, BD, and ASD generated the effect size, represented as $\log_2$ fold change ($\log_2$FC), in males and females separately. We then compared the effect size in males and females. The results showed that females had larger case-control differences than did males for all three psychiatric disorders (Fig. 2B). By comparing each condition and sex with its respective controls, the slopes of the female-to-male comparison were 1.7 in SCZ ($P < 2.2 \times 10^{-16}$), 1.7 in BD ($P < 2.2 \times 10^{-16}$), and 8.3 in ASD ($P < 2.2 \times 10^{-16}$) (Fig. 2B). When compared with a control group of the same sex, 1656, 5, and

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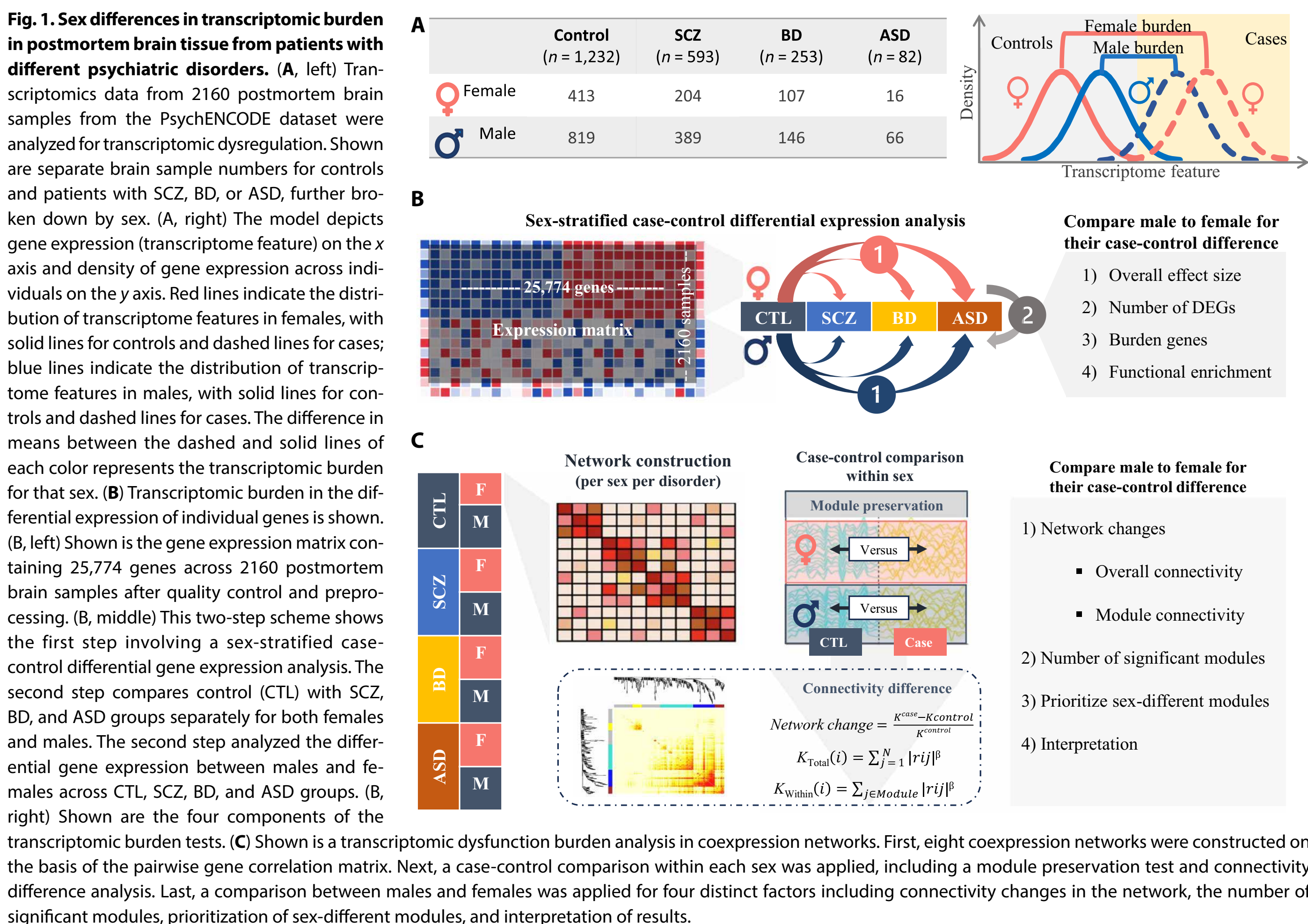


**Fig. 1. Sex differences in transcriptomic burden in postmortem brain tissue from patients with different psychiatric disorders.** (**A**, left) Transcriptomics data from 2160 postmortem brain samples from the PsychENCODE dataset were analyzed for transcriptomic dysregulation. Shown are separate brain sample numbers for controls and patients with SCZ, BD, or ASD, further broken down by sex. (A, right) The model depicts gene expression (transcriptome feature) on the *x* axis and density of gene expression across individuals on the *y* axis. Red lines indicate the distribution of transcriptome features in females, with solid lines for controls and dashed lines for cases; blue lines indicate the distribution of transcriptome features in males, with solid lines for controls and dashed lines for cases. The difference in means between the dashed and solid lines of each color represents the transcriptomic burden for that sex. (**B**) Transcriptomic burden in the differential expression of individual genes is shown. (B, left) Shown is the gene expression matrix containing 25,774 genes across 2160 postmortem brain samples after quality control and preprocessing. (B, middle) This two-step scheme shows the first step involving a sex-stratified case-control differential gene expression analysis. The second step compares control (CTL) with SCZ, BD, and ASD groups separately for both females and males. The second step analyzed the differential gene expression between males and females across CTL, SCZ, BD, and ASD groups. (B, right) Shown are the four components of the transcriptomic burden tests. (**C**) Shown is a transcriptomic dysfunction burden analysis in coexpression networks. First, eight coexpression networks were constructed on the basis of the pairwise gene correlation matrix. Next, a case-control comparison within each sex was applied, including a module preservation test and connectivity difference analysis. Last, a comparison between males and females was applied for four distinct factors including connectivity changes in the network, the number of significant modules, prioritization of sex-different modules, and interpretation of results.

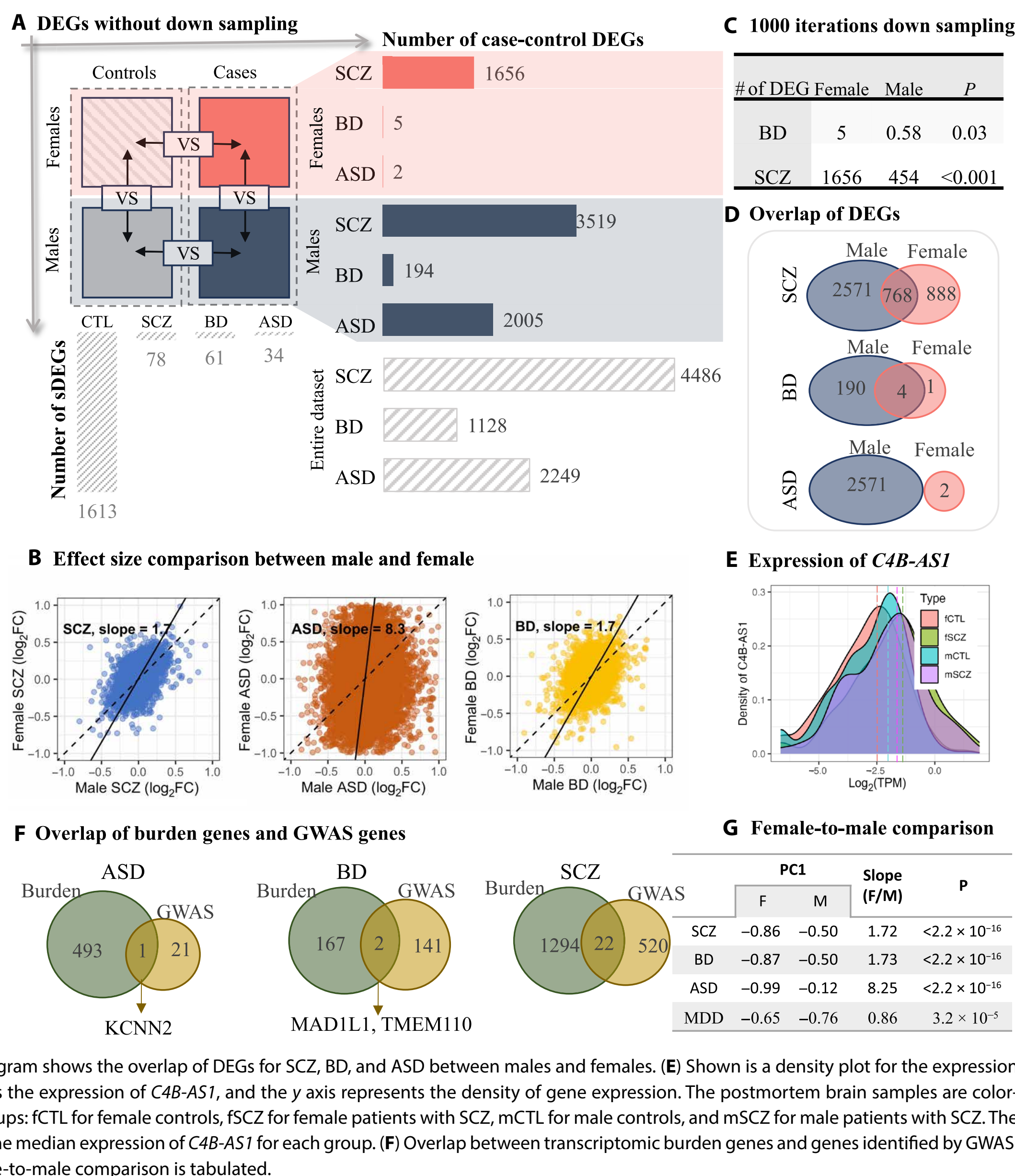


**Fig. 2. Transcriptomic burden of DEGS.** (**A**) Shown is DEG analysis across the three psychiatric disorders and across sex without downsampling. (Top left) The diagram illustrates two types of comparisons: The horizontal comparison contrasts control and case groups, and the vertical comparison differentiates between females and males. In this scheme, each box represents a sample group, with the *x* axis indicating the disease condition (controls or cases). The case groups include SCZ, BD, and ASD, with male and female on the *y* axis. The horizontal bar provides the number of significant DEGs from the case-control comparison. This includes DEGs in SCZ, BD, and ASD for females (top), males (middle), and the entire dataset (bottom). The vertical bar plot illustrates sDEGs (DEGS by sex) in the comparison between males and females in CTL, SCZ, BD, and ASD groups. (**B**) Effect size comparison between males and females in ASD, BD, and SCZ is shown. The effect size ($log_2FC$) was generated from the case-control comparison in males and females separately. Each dot represents a gene; the *x* axis represents the effect size in males, and the *y* axis represents the effect size in females. The slope and *P* values are shown. (**C**) Number of DEGs in the down-sampling analysis is shown. The male groups were down-sampled to match the female sample sizes in BD and SCZ for 1000 iterations. The average number of significant DEGs in males is provided in the second column. (**D**) The Venn diagram shows the overlap of DEGs for SCZ, BD, and ASD between males and females. (**E**) Shown is a density plot for the expression of the *C4B-AS1* gene. The *x* axis displays the expression of *C4B-AS1*, and the *y* axis represents the density of gene expression. The postmortem brain samples are color-coded according to their respective groups: fCTL for female controls, fSCZ for female patients with SCZ, mCTL for male controls, and mSCZ for male patients with SCZ. The dashed lines within the plot represent the median expression of *C4B-AS1* for each group. (**F**) Overlap between transcriptomic burden genes and genes identified by GWAS. (**G**) Statistical information for the female-to-male comparison is tabulated.

2 DEGs were identified in females with SCZ, BD, and ASD, respectively (Fig. 2A). In males, the numbers were higher, with 3519, 194, and 2005 DEGs identified for SCZ, BD, and ASD, respectively (Fig. 2A, fig S1, and data file S1). To address the sample size imbalance, we performed 1000 rounds of down-sampling in the male group, resulting in a smaller average number of DEGs than in females for SCZ (424 versus 1656, $P < 1.00 \times 10^{-3}$) and BD (<1 versus 5, $P = 3.00 \times 10^{-2}$) (Fig. 2C). Because of the limited number of samples in the female ASD group ($n = 16$), down-sampling analysis was not performed. The study also replicated more than 40% of the DEGs in an independent dataset for all three disorders (fig S2).

Although the number of DEGs differed between males and females in each disorder, there were still small but significant overlaps between the two sexes. In SCZ, 768 DEGs were found to be shared between males and females (Fig. 2D, $P = 2.14 \times 10^{-266}$, hypergeometric test). Similarly, in BD, four of five DEGs identified in females were also observed in males (Fig. 2D, $P = 1.55 \times 10^{-8}$, hypergeometric test). However, no overlapping DEGs were found between males and females in ASD, likely because of the small number of DEGs detected in brain tissue of female patients with ASD (only two) (Fig. 2D).

We conducted an analysis of sex differences in gene expression within control and case groups separately. A larger number of sDEGs (DEGs by sex) were identified in the control groups, totaling 1631 genes with differential expression between males and females with a false discovery rate (FDR) < 0.05 (Fig. 2A). In contrast, the ASD group exhibited 34 sDEGs, the BD group had 61, and the SCZ group had 78 (Fig. 2A). To ensure that this finding was not driven by sample size, we down-sampled the control groups to match the sample size of the case groups for 1000 iterations each. After

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down-sampling the control groups to match the number of ASD cases (the smallest disease group with 16 females and 66 males) with 1000 iterations, we observed an average of 382 sDEGs. This number was approximately 11 times greater than the 34 sDEGs identified in the ASD group (permutation test $P = 0.2$).

To define a set of genes that exhibited larger expression changes in postmortem brain tissue from female versus male patients for each disorder, we used permutation-based analysis. Starting with the DEGs that were significant in female, male, or combined analysis (Fig. 2A), we evaluated the distribution of effect sizes from the 1000 down-sampling analysis in males to match the sample size of females. Then, we determined whether the effect size in females exceeded the top 5% of the distribution in males. Genes that met this criterion were identified as transcriptomic burden genes. This analysis resulted in 494 transcriptomic burden genes in ASD, 169 genes in BD, and 1316 genes in SCZ (fig. S3). For example, the *C4B-AS1* gene (*ENSG00000229776*) was identified as a transcriptomic burden gene in SCZ because it showed greater expression changes when comparing female patients with SCZ with controls than when comparing male patients with SCZ with controls (Fig. 2E).

We also checked the overlap between transcriptomic burden genes and sDEGs and found that more burden genes were sDEGs in controls than in cases (fig. S4). Specifically, of 1316 SCZ burden genes, 111 were sDEGs in control groups, but only 4 were sDEGs in patients with SCZ. Among the 169 BD burden genes, 14 were sDEGs in controls, with none identified in BD groups. Similarly, of the 494 ASD burden genes, 62 were sDEGs in controls, and only 2 were noted as sDEGs in ASD groups (fig. S4).

The burden genes in SCZ were found to be significantly enriched for epithelium development ($P = 1.49 \times 10^{-11}$), innate immune responses ($P = 2.09 \times 10^{-6}$), and inflammatory responses ($P = 1.23 \times 10^{-5}$) (table S1). The transcriptomic burden genes in ASD were significantly enriched for oxidative phosphorylation ($P = 5.03 \times 10^{-3}$) and Alzheimer's disease ($P = 6.13 \times 10^{-3}$) (table S1). No enriched functions were detected in female-burden genes in BD (tables S1 and S2). Some burden genes were also significant in genome-wide association study (GWAS) studies (Fig. 2F and data file S3). For instance, *KCNN2* has been identified as significant in both the sex-biased burden tests and ASD GWAS (*26*) (Fig. 2F). Similarly, *MAD1L1* and *TMEM110* were burden genes in BD and were significant in the largest BD GWAS (Fig. 2F) (*27*). Another 22 genes were both burden genes and SCZ GWAS signals (*28*) (Fig. 2F and data files S3 and S4).

Given that MDD is known for its female bias, we examined transcriptomics data from postmortem dorsolateral prefrontal cortex [Brodmann areas 8/9 (BA8/9)] tissue from an MDD cohort. We used log-fold change values derived from the sex-differential analysis conducted by Labonté *et al.* (*29*). A comparison of females and males revealed a higher transcriptomic burden in males, indicated by a calculated slope of 0.86 in the female versus male comparison ($P = 3.2 \times 10^{-5}$) (Fig. 2G). Specifically, there were more DEGs in males than in females, with 1813 DEGs identified in males and 1792 in females (data file S5). In addition, we identified 36 genes that exhibited more pronounced changes in males compared with females.

### Higher transcriptomic burden in gene coexpression connectivity in females than in males

In addition to analyzing transcriptomic burden at the individual gene level, we investigated transcriptomic burden at the network level (also known as connectivity burden) by comparing differences in gene coexpression networks between males and females (Fig. 1C). Using weighted gene coexpression network analysis (WGCNA), we built the coexpression networks on the subset data of each psychiatric disorder, which were further stratified according to sex. Then, we used the module preservation test to assess whether network structures in the case groups were preserved in the control groups by each sex. Network connectivity, representing the cumulative strength of connections with other genes in the network, formed the basis of our analysis. The connectivity burden assessment included overall connectivity changes within sexes between case and control groups, the number of modules with significant connectivity alterations, and the effects of module connectivity changes between males and females. To evaluate the impact of sex and disease on eigengene expression, we constructed the coexpression network on the entire dataset and performed preservation tests on the network constructed using the subset data.

The overall connectivity measurement, kTotal of each gene, a measure of the strength of a gene's connection with all other genes in the network, was calculated separately for each of the eight networks (Figs. 1C and 3A). The kTotal changes between cases and controls were calculated by subtracting the kTotal in the case group from the kTotal in the control group and then dividing it by the kTotal in the control group (see formula in Fig. 1C). Male and female networks were analyzed separately.

We then compared the female-to-male effect size difference, revealing slopes of 2.1 in SCZ, 5.1 in BD, and 18 in ASD (Fig. 3B). The female-male slopes were greater than one in all three disorders, indicating larger changes of overall connectivity in females than in males.

Most modules in the case groups exhibited high preservation, with $Z$ values more than 10, compared with the corresponding control groups of the same sex (Fig. 3C). These results indicated that the fundamental structures of the networks remained largely preserved. We also tested the module preservation between males and females in control groups and observed high preservation, with a $Z$ value greater than 10.

For the module-level analysis, reference modules were created separately using male and female controls. Module differential connectivity (MDC) was used to identify modules with significant connectivity changes (*30*). More modules exhibited significant changes of connectivity in females than in males (Fig. 3D). Specifically, 88.9% of the modules showed significant changes of connectivity in females with SCZ, along with 66.7 and 77.8% of modules in females with BD or ASD, respectively (Fig. 3D). In contrast, 77.8% of the modules showed significant connectivity changes in males with SCZ, with 60.7% in males with BD and 60.7% in males with ASD (Fig. 3D). The comparison of proportions between the two sexes revealed that females had more modules with connectivity changes, which was consistent with the overall connectivity results.

The changes in connectivity for most modules were more severe in females (data files S6 to S8). Specifically, we first fixed the genes in each module using the network constructed in female controls and then compared the module connectivity changes between cases and controls separately for males and females. For modules that displayed significant connectivity changes in both sexes, we then tested whether the changes were significantly different between males and females and identified male-biased and female-biased modules. A male-biased module showed significantly more changes in males than in

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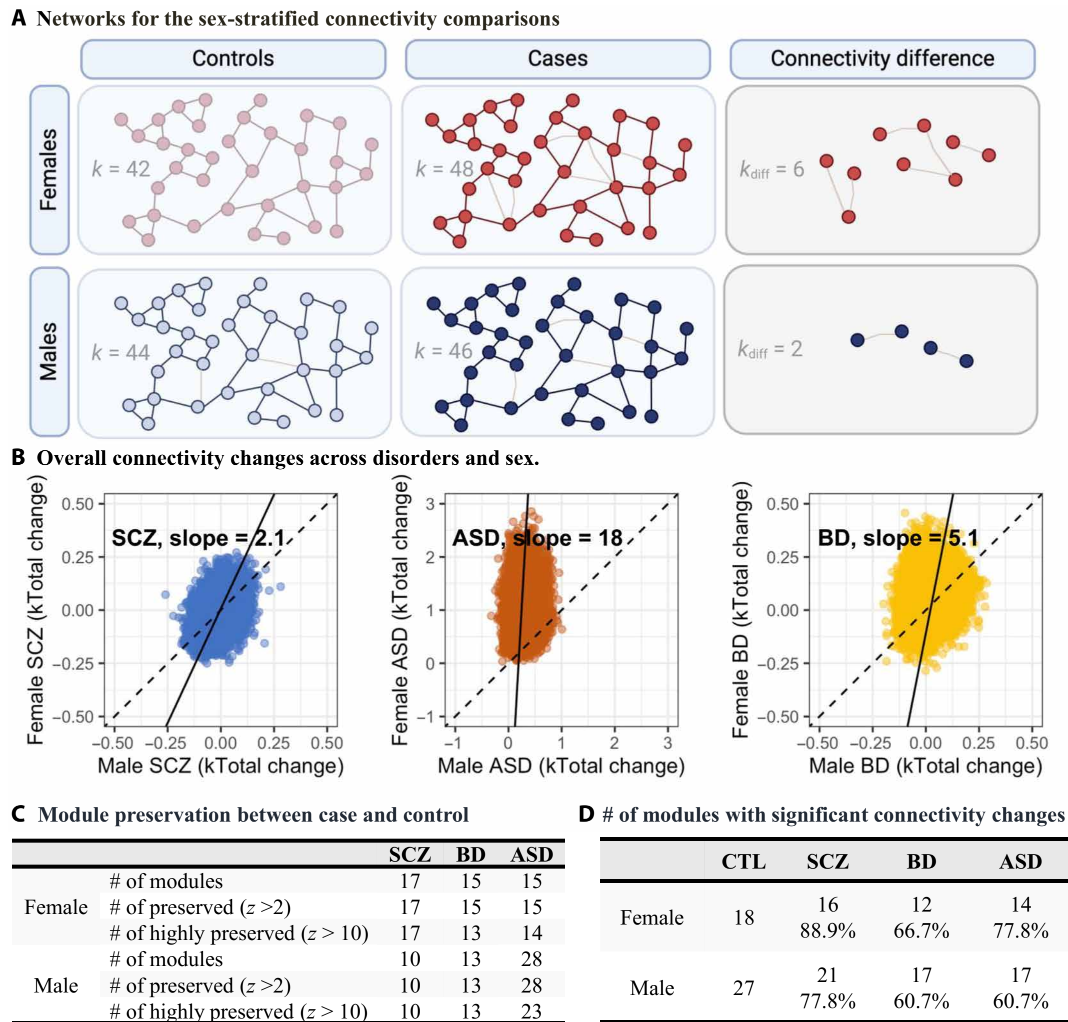


**C Module preservation between case and control**

| | | SCZ | BD | ASD |
|---|---|---|---|---|
| Female | # of modules | 17 | 15 | 15 |
| | # of preserved ($z$ >2) | 17 | 15 | 15 |
| | # of highly preserved ($z$ > 10) | 17 | 13 | 14 |
| Male | # of modules | 10 | 13 | 28 |
| | # of preserved ($z$ >2) | 10 | 13 | 28 |
| | # of highly preserved ($z$ > 10) | 10 | 13 | 23 |

**D # of modules with significant connectivity changes**

| | CTL | SCZ | BD | ASD |
|---|---|---|---|---|
| Female | 18 | 16<br>88.9% | 12<br>66.7% | 14<br>77.8% |
| Male | 27 | 21<br>77.8% | 17<br>60.7% | 17<br>60.7% |

**Fig. 3. Overall connectivity burden in coexpression networks.** (**A**) Conceptual networks for the sex-stratified connectivity comparisons are shown. The networks depict group-averaged connectivity matrices for females and males in the control and case cohorts, as well as the adjacency difference highlighting edges exhibiting significant connectivity changes. (**B**) Overall connectivity changes across the three psychiatric disorders (SCZ, ASD, and BD) and sex are shown. The $x$ axis shows the kTotal change in males, and the $y$ axis shows the kTotal change in females. Each dot represents one gene. The slopes (solid black lines) are plotted for SCZ, ASD, and BD. The dashed black lines represent the slope equal to 1($y = x$), meaning no difference in connectivity changes between males and females. The three psychiatric disorders all have a slope > 1, indicating larger connectivity changes in females than in males. (**C**) Module preservation between case and control groups is shown. For each sex, number of coexpression modules preserved between case and control groups in SCZ, BD, and ASD is presented. (**D**) Number of modules with significant connectivity changes for each sex, and number of coexpression modules with significant connectivity changes between cases and controls is shown. Networks in (A) created with BioRender.com

females, whereas a female-biased module showed significantly more changes in females than in males. In our analysis, 63.6% (7 of 11), 53.8% (7 of 13), and 60.0% (9 of 15) of the modules showed significant female bias in ASD, BD, and SCZ, respectively (data file S6).

In the networks from the entire dataset, a total of 21 modules were identified. Using linear regression with eigengene expression as the outcome, disease group, sex, and the interaction between disease groups and sex as independent variables, we evaluated the contributions of sex and disease to the expression of module eigengene. We only found one module called wM9 (whole dataset coexpression module 9), with the eigengene expression significantly associated with sex (FDR $q = 0.03$), and two modules that exhibited significant interactions between SCZ and sex (wM9 and wM10, $P < 0.05$; data file S8). Most of the genes in wM9 were members of M1 in the network of female controls, whereas most of the genes in wM10 were members of M4 in the network of female controls.

### Modules with greater sex-specific disruption were enriched for genes involved in synapse, immune, and epithelial functions

Coexpression modules that exhibited sex-biased connectivity differences in SCZ, BD, and ASD were prioritized. We incorporated a three-layered analysis using the modules constructed by female controls as the reference (Fig. 4, A and B). At the first layer, sex-stratified MDC was performed to identify modules that displayed sex-biased differences of connectivity. At the second layer, the enrichment for transcriptomic burden genes was assessed to identify modules that carried the greatest number of burden genes (data file S6). At the third layer, GWAS enrichment analysis was performed to identify modules that were enriched for genes implicated in SCZ, BD, and ASD. Modules were ranked on the basis of a composite score of all three layers (data file S7). Another two layers of information were added for functional annotation, including cell-type enrichment and functional enrichment (Fig. 4C and data file S7).

The top-ranked modules enriched in burden genes were M1, M4, M13, and M14 (Fig. 4B). Specifically, the M1 synaptic module contained 3171 genes and exhibited significant connectivity changes in patients of both sexes with SCZ, with a higher average connectivity change in females than in males (−0.15 versus −0.03; $P < 1.21 \times 10^{-200}$) (Fig. 4B). In addition, most genes in wM9, the module showing an SCZ by sex interaction effect in the whole dataset, were members of the M1 module. The M1 module contained 236 female SCZ DEGs with significant overlap [odds ratio (OR) = 1.16, $P = 1.46 \times 10^{-3}$]. The M1 module was also enriched with GWAS genes of SCZ

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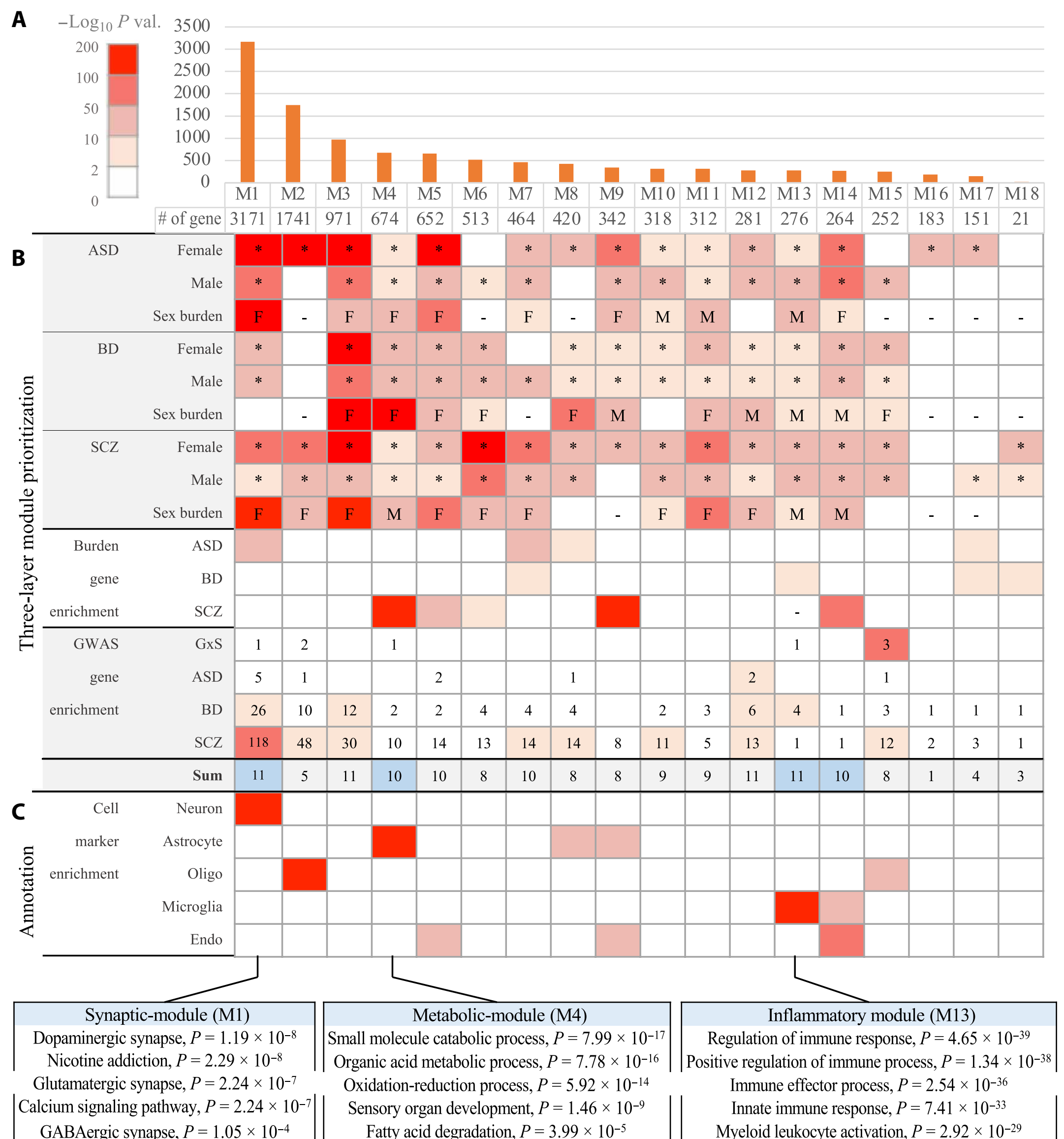


**Fig. 4. Module connectivity differences and module prioritization.** (**A**) Gene distribution across modules is shown. The *x* axis illustrates the modules, and the *y* axis represents the number of genes for each module. (**B**) Three-layer module prioritization is presented. The *x* axis displays each module, and the top nine rows depict sex-stratified MDC results for ASD, BD, and SCZ separately. For each disorder, the "female" row displays MDC in female cases versus female controls, and the "male" row shows MDC in male cases versus male controls. The "sex burden" row assesses the case-control effect size differences between males and females. Colors denote significance levels, with darker shades representing higher significance. An asterisk (*) in the box denotes significant MDC, and a dash (–) represents not tested. "F" indicates more changes in females than males, and "M" indicates more changes in males than females. The three rows titled "burden gene enrichment" show the enrichment of each module with burden genes for ASD, BD, and SCZ. The three "GWAS gene enrichment" rows indicate the enrichment of module genes with genes identified from four GWAS publications for SCZ, ASD, and BD. Numbers in these rows represent the counts of overlapping genes, and the colors show the enrichment *P* values. (**C**) Cell type and function annotation is shown. For each module, the cell marker genes and KEGG pathway enrichment values are provided and are color-coded according to the enrichment *P* values.

(*28*) (overlapping genes = 118, OR = 1.76, $P = 1.80 \times 10^{-10}$), indicating that GWAS genes have sex-biased features in the transcriptome. Cell-type enrichment analysis found that genes in this module were enriched in neurons ($P < 1.00 \times 10^{-270}$) (Fig. 4C). Functional enrichment analysis showed that the M1 module was enriched in synapse-related functions, such as chemical synaptic transmission ($P = 1.46 \times 10^{-46}$), neurotransmitter secretion ($P = 7.65 \times 10^{-17}$), and neurotransmitter release from synapses ($P = 7.65 \times 10^{-17}$), suggesting sex-related biological processes that may be implicated in SCZ risk (table S1 and data files S4 and S7).

Furthermore, module M13 showed enrichment in immune-related pathways (Fig. 4C). This module contained 276 genes and showed significant connectivity changes in ASD, BD, and SCZ compared with controls in both males and females, with the average change of connectivity being higher in males than in females. M13 was enriched in burden genes of BD ($P = 7.55 \times 10^{-3}$) and GWAS genes of BD ($P = 4.00 \times 10^{-2}$) (table S1 and data files S4 and S6). M13 was enriched for genes involved in microglial function ($P = 1.27 \times 10^{-273}$) and regulation of immune responses ($P = 4.65 \times 10^{-39}$), innate immune responses ($P = 7.41 \times 10^{-33}$), and inflammatory responses ($P = 1.25 \times 10^{-23}$) (Fig. 4C).

M4 was the module that contained most of the genes in the wM10, which exhibited SCZ by sex interactions in the whole dataset. M4 contained 674 genes and showed significant connectivity changes in ASD, BD, and SCZ, with an average change of connectivity that was higher in brain tissue from female patients with ASD and BD but higher in brain tissue from male patients with SCZ. M4 was enriched for burden genes for SCZ ($P = 2.20 \times 10^{-209}$) as well as for astrocyte makers ($P < 1.00 \times 10^{-270}$). M4 was also enriched for genes involved in metabolic pathways including small molecular catabolic processes ($P = 7.99 \times 10^{-17}$), organic acid metabolic processes, and others (Fig. 4C and data files S4 and S7).

The M14 module was enriched in genes involved in the inflammatory response ($P = 5.04 \times 10^{-33}$), response to cytokines ($P = 2.04 \times$

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$10^{-28}$), epithelium development ($P = 9.44 \times 10^{-05}$), and endothelial cell migration ($P = 7.13 \times 10^{-04}$) (table S1 and data file S4). With 264 genes, this module exhibited a significant connectivity change in ASD, BD, and SCZ compared with controls, for both males and females. For SCZ and BD, the average change of connectivity was higher in males than in females (0.27 versus 0.17, $P = 5.20 \times 10^{-13}$ in SCZ; 0.27 versus 0.18, $P = 5.55 \times 10^{-5}$ in BD), whereas the opposite trend was observed in ASD (0.65 versus 0.88, $P = 2.71 \times 10^{-8}$) (Fig. 4B and data file S4). In addition, the M14 module was enriched in female up-regulated DEGs and burden genes of SCZ.Cell-type enrichment analysis revealed that M14 was enriched in endothelial cells ($P = 7.15 \times 10^{-70}$) and microglia ($P = 1.78 \times 10^{-25}$) (table S1 and data files S4 and S7).

## DISCUSSION

This study aimed to investigate the sex-specific burden of risk for three common psychiatric disorders—ASD, SCZ, and BD—at the brain transcriptome level. Specifically, we examined the extent of transcriptome dysfunction in prefrontal cortex, focusing on DEGS and coexpression networks. Our analyses revealed disparities between males and females, including differences in the number of DEGs, the effect sizes of specific genes, and the organization of regulatory networks. Our results indicated that females with ASD, SCZ, and BD carry a greater burden of transcriptome dysfunction than do males with these diseases, as evidenced by higher numbers of DEGs and a more disorganized coexpression network.

The current study adds to the growing body of research suggesting that females have a lower risk for ASD and extends this finding to SCZ and BD. The "female protective effect" suggests that females may require a higher genetic and environmental load to develop these psychiatric disorders than males (*31*–*33*). Although the validity of this theory is subject to ongoing debate (*34*), our transcriptome-level analysis of postmortem brain tissue showed that female patients with ASD, SCZ, or BD exhibited a greater burden of DEGs and a larger effect size difference than did male patients. Prior research has shown varying degrees of sex bias in the prevalence of ASD, SCZ, and BD. ASD has the highest male-to-female ratio of 4, followed by SCZ and BD. We found that the slope of gene expression changes (transcriptomic burden in individual genes) and connectivity changes (transcriptomic burden in coexpression networks) for males and females mirrored the pattern of sex bias in the prevalence of these disorders. Furthermore, we validated these transcriptomic burden differences in a secondary dataset that measured gene expression using a microarray platform. In addition, we examined MDD, which is known to have a female bias, and found an increased transcriptomic burden in males compared with females. This observation supported our initial hypothesis that the sex with lower prevalence of these disorders tends to have a higher transcriptomic burden. Together, these results suggest that the differences in gene expression and connectivity between males and females may contribute to the sex differences in the prevalence, age of onset, and symptom severity of ASD, SCZ, and BD.

Our work introduced a transcriptome-based burden model to explain sex bias phenomena in psychiatric disorders, and some of the identified burden genes overlapped with genes pinpointed in GWAS studies. For example, *KCNN2* was identified as significant in both sex-biased burden tests in this study and in a GWAS study of ASD (*26*). Similarly, 22 genes were identified as significant in both burden tests and in a GWAS study of SCZ (*28*).

In our study, females carried a higher level of transcriptomic dysfunction burden at the network connectivity level. In females, larger changes in connectivity were needed to develop an illness, suggesting greater resilience to small disturbances in coexpression network organization. However, this interpretation is only one of several possible explanations for the observed results, and further research is needed. Investigating transcriptome dysfunction at both the individual gene and network levels is important for understanding the burden of specific genes. For example, despite sodium voltage-gated channel alpha subunit 2 (*SCN2A*) being identified as a risk gene for both SCZ and ASD (*35*), individual gene-level analysis did not detect transcriptomic dysfunction burden in females. However, a larger connectivity change was observed in SCZ for this gene (kTotal change: 60.9 in females versus 14.3 in males).

The M1 module, which was enriched for genes involved in synapse function, consistently showed a sex difference in dysfunction burden across the three psychiatric disorders. This was supported by genome-wide significant single-nucleotide polymorphism–by-sex interactions in SCZ, BD, and ASD (*36*). Fibroblast growth factor 12 (*FGF12*) and *FGF14* are hub genes in the M1 module. Both genes play a role in embryonic development and have been associated with SCZ (*37*). The sex-specific functions of *FGF14* were demonstrated in an *Fgf14*-deficient mouse model, where only male $Fgf14^{-/-}$ mice exhibited cognitive deficits and neuronal function changes that mimicked SCZ endophenotypes (*38*). The M1 module also identified several genes encoding voltage-gated calcium channels and gamma-aminobutyric acid type A receptors, including *CACNB2*, *CACNB1*, *CACNA1A*, *GABRB3*, *GABRA3*, *GABRD*, and *GABRA5*. These genes have been well-documented as risk genes and pharmacological targets for psychiatric disorders (*39*), emphasizing the potential need for sex-specific treatment in psychiatric disorders.

Our findings suggest that immune functions exhibited sex-specific effects in these three psychiatric disorders. At the level of individual genes, those with higher transcriptomic burden in females were enriched for immune-related functions. At the network level, immune modules M13 and M14 showed changed connectivity in SCZ, BD, and ASD compared with controls. Unlike most modules that exhibited more connectivity changes in females, M13 and M14 modules in males demonstrated more connectivity changes than in females. This suggested that females may be more vulnerable to disruption of immune-related regulation. Complement 3 (*C3*) (*40*) is an example of a gene that showed greater vulnerability in males at the individual gene level. However, at the network level, as a hub gene in M13, *C3* showed larger connectivity changes for SCZ in males than in females (connectivity change: 5.5 in females versus 45.9 in males), suggesting a higher burden of connectivity for *C3* in males.

The involvement of genes associated with epithelium development in transcriptomic burden pathways in module M14 was intriguing. These genes exhibited sex-biased connectivity changes in the three psychiatric disorders, and this finding was supported by recent sex-dependent GWAS results (*36*). However, the function of the brain-blood barrier endothelium in mediating neuroinflammation in SCZ remains unclear (*36*).

There are limitations to our study. When using a stratified approach for data analysis, there are pros and cons regarding alternative analysis using the sex-by-diagnosis interaction approach (*24*). It

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should be emphasized that the sex-by-diagnosis interaction approach may be prone to type II errors because it requires much larger sample sizes to detect disparities between two nonzero effect estimates (*41*). Another limitation is the small sample size for female patients with ASD, which reduced power to detect case-control DEGs and could be easily confounded with unknown covariates. In addition, the lack of specific clinical information, common in postmortem brain tissue studies, presents a challenge in interpreting the results comprehensively. Further studies are needed to increase the sample size and validate the key findings of our study.

Our study revealed a higher burden of transcriptomic dysfunction in females compared with males for ASD, SCZ, and BD. These findings provide a framework for assessing sex differences in various diseases and traits. Our immune and synaptic pathway data suggest potential pathological differences between males and females with ASD, SCZ, and BD, highlighting that sex-specific treatment approaches may be needed for these disorders.

## MATERIALS AND METHODS

### Study design

This study was designed to investigate differences in transcriptomic dysfunction in postmortem prefrontal cortex brain tissue from male and female patients with SCZ, BD, or ASD. The transcriptome-wide RNA-seq data from postmortem prefrontal cortex brain tissues was provided by the PsychENCODE project. We explored potential sex differences in transcriptomic burden at the level of individual genes and gene coexpression networks.

For the differential analysis of single genes, we first applied sex-stratified differential gene expression analysis to identify disease-associated DEGs in females and males. Next, we compared the sex-stratified DEGs with DEGs in pooled data to identify genes associated with psychiatric disorders. Then, we compared the male and female results to explore shared and sex-specific DEGs. Using several threshold-free methods, we examined the sex differences in a transcriptome-wide manner. Last, we conducted a pathway enrichment test to reveal the function of DEGs in each psychiatric disorder within each sex.

For the coexpression network analysis, we constructed the coexpression modules using the entire dataset and the subset data of each disorder, further stratified by sex. We then calculated the total connectivity of each gene in each group. Next, we performed a case-control comparison separately in males and females and then compared the effect sizes between males and females. After performing this overall comparison, we conducted the MDC analysis using the modules identified in male and female control groups. We highlighted modules with significant module connectivity changes and performed the enrichment test to determine whether they were enriched in burden genes, GWAS genes, and cell marker genes.

### Data acquisition

#### *Discovery data*

We obtained the 2160 transcriptome-wide RNA-seq datasets of postmortem brain tissues from the PsychENCODE project (http://resource.psychencode.org/). Postmortem prefrontal cortex brain tissue was obtained from 593 patients with SCZ, 253 patients with BD, 82 patients with ASD, and 1232 healthy control individuals who did not have a known history of psychiatric disorders. Of the 2160 samples, 559 samples were from BA9, 248 samples were from BA46, and 487 samples were from either BA9 or BA46. There were 866 samples for which the Brodmann areas were not specified.

#### *Replication data*

The BrainEXP-NPD dataset (http://brainexpnpd.org/) (*42*) has 48 human brain transcriptomic datasets from six sources: GEO, ArrayExpress, SMRI, GTEx, ROSMAP, and PsychENCODE (microarray data only). The original brain donors included patients with SCZ ($n = 427$), BD ($n = 312$), and ASD ($n = 53$), as well as controls without these disorders ($n = 6378$). More details about the replication cohort design, methods, and results can be found in the Supplementary Materials.

### Quality control and preprocessing

Following the data processing pipeline of PsychENCODE, we performed quality control including outlier removal, sex checking, and gene filtering. There were 2160 samples with 25,774 genes remaining for the down-stream analysis. The covariances in the final module for the sex-stratified differential gene expression analysis and coexpression analysis consisted of known variables: diagnosis, age, age$^2$, study/batch, postmortem interval (RIN), integrity number (RIN$^2$), brain bank, brain region, sequencing principal components (seqPCs) (1 to 3, 5 to 8, 10 to 14, 16, 18 to 25, 27 to 29), seqPC32, and four unknown variables: surrogate variables (SVs) (1 to 4).

### Differential expression analysis for both case-control and male-female differences

Count-level quantifications were corrected for library size using TMM normalization in edgeR and were transformed as $\log_2(\mathrm{CPM} + 0.5)$. A linear mixed-effects model was implemented using the nlme package in R, and differential gene expression was then calculated between cases and controls for male and female brain tissue samples separately and between males and females in controls and cases separately. The covariates specified in the previous section were included as fixed effects in the model. In addition, we included a random effect term for each unique participant to account for participant overlap across sequencing studies. To control for multiple comparisons, the resulting *P* values were corrected using the Benjamini-Hochberg method.

Overall effect size differences between males and females were evaluated by comparing slopes. To compare the overall case-control differences between males and females, we performed the principal component regression using the $\log_2$ fold change. The female-to-male regression slopes and intercepts were calculated for SCZ, BD, and ASD. For specific genes, we performed the overlap analysis for male and female DEGs and calculated the significance of overlap by hypergeometric distribution using the dhyper function in R.

### Coexpression module analysis

#### *Network construction*

To integrate the findings of individual genes into a comprehensive understanding of their system-wide network structure, we performed WGCNA in two steps including using the entire dataset and the subset data for each disorder, further stratified by sex (*43*). All covariates, except for diagnostic group and sex, were first regressed from our expression dataset. Network analysis was performed with the WGCNA package using signed networks. A soft-threshold power of 5 was used for all studies to achieve approximate scale-free topology ($R^2 > 0.8$). Networks were constructed using the blockwiseModules function. The network dendrogram was created using

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average linkage hierarchical clustering of the topological dissimilarity matrix (1-TOM). Modules were defined as branches of the dendrogram using the hybrid dynamic tree-cutting method. Modules were summarized by their first principal component (ME, module eigengene), and modules with eigengene correlations more than 0.9 were merged. Modules were defined using biweight midcorrelation (bicor), with a minimum module size of 40, deepsplit of 2, merge threshold of 0.1, and negative pamStage.

#### *Modular expression analyses*

For the networks constructed using the entire dataset, eigengene values were obtained through the blockwiseModules function and used as the dependent variable in a linear regression with the lm function, and we used group, sex, and the interaction of group and sex as independent variables. Multiple testing correction was performed using FDR. Modules with original $P$ values less than 0.05 were considered statistically significant and reported.

#### *Module-preservation test*

To assess the presence of unique modules exclusive to the case group, we conducted module preservation testing using the modulePreservation function in WGCNA. The networks constructed within the case groups for each sex were selected as reference networks. Z-summary statistics were computed for assessing the preservation of each module. Z-summary values falling between 2 and 10 were designated as moderate preservation, whereas values surpassing 10 were considered high preservation.

#### *Overall connectivity*

The R function intramodularConnectivity.fromExpr from the WGCNA package was used to calculate the kTotal for each gene. The kTotal was extracted for the eight groups of the expression matrix, including: female control, male control, female SCZ, male SCZ, female BD, male BD, female ASD, and male ASD. We then performed sex-stratified connectivity case-control comparison using the (kTotal in case – kTotal in control)/(kTotal in control) as the effect size estimate. We then calculated the slope and intercepts between the other groups and male SCZ, which served as a reference.

#### *Module connectivity differential analysis*

To quantify differences in transcript network organization between cases and controls in both males and females, we used an MDC metric (*30*). In brief, MDC represents the ratio of the connectivity of all gene pairs in a module from case individuals to that of the same gene pairs from control individuals. The statistics of MDC were computed using the FDR procedure, which can be accessed by permuting the data underlying the two networks. We estimated FDR on the basis of both shuffled samples (networks with nonrandom nodes but random connections) and shuffled gene labels (networks with random nodes but nonrandom connections) and then selected the larger value as the final FDR estimates. Using the module constructed in the control groups in both males and females, we performed the MDC for SCZ, BD, and ASD separately.

### Functional annotation

Enrichment for Gene Ontology (GO; biological process, molecular function, and cellular component) and KEGG pathways was performed using the gProfileR v0.6.4 (*44*). Only pathways containing fewer than 1000 genes were assessed. The background was restricted to brain-expressed genes. An ordered query was used, ranking genes by $\log_2$FC for differential expression analyses or by module eigengene-based connectivity (kME) for coexpression module enrichment analyses.

We used uniformly processed human brain single-cell RNA-seq datasets for cell-type enrichment analysis. These included combined multiple published datasets including newly generated data from PsychENCODE (*45*). Using the single-cell RNA-seq data from Wang *et al.* (*45*), the cell-type specificity was determined by the cluster analysis of the single-cell data. Fisher's exact test was performed for cell-type enrichment in each module. The threshold was FDR $q$ value less than 0.05.

### Statistical analysis

Differential gene expression analysis was performed using the nlme package. Before analysis, raw RNA-seq data were preprocessed with normalization and filtering steps. Differential gene expression analysis was conducted using moderated $t$ tests implemented in limma, with adjustments for multiple testing using the FDR $q$ value. Enrichment analysis was conducted using the hypergeometric distribution as implemented by the dhyper function in the statistics package. WGCNA was performed to identify modules of highly correlated genes. For single testing, a two-tailed $P$ value of less than 0.05 was considered to indicate statistical significance. In cases of multiple testing, the FDR $q$ value was calculated on the basis of the distribution of $P$ values. All statistical analyses were conducted using the R program (version 3.1.3).

## Supplementary Materials

**This PDF file includes:**

Supplementary Materials and Methods
Figs. S1 to S4
Tables S1 and S2

**Other Supplementary Material for this manuscript includes the following:**

Data Files S1 to S8
MDAR Reproducibility Checklist

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