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Sex-Differential Gene Expression in Developing Human Cortex and Its Intersection With Autism Risk Pathways

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ABSTRACT

BACKGROUND: Sex-differential biology may contribute to the consistently male-biased prevalence of autism spectrum disorder (ASD). Gene expression differences between males and females in the brain can indicate possible molecular and cellular mechanisms involved, although transcriptomic sex differences during human prenatal cortical development have been incompletely characterized, primarily due to small sample sizes.

METHODS: We performed a meta-analysis of sex-differential expression and co-expression network analysis in 2 independent bulk RNA sequencing datasets generated from cortex of 273 prenatal donors without known neuropsychiatric disorders. To assess the intersection between neurotypical sex differences and neuropsychiatric disorder biology, we tested for enrichment of ASD-associated risk genes and expression changes, neuropsychiatric disorder risk genes, and cell type markers within identified sex-differentially expressed genes (sex-DEGs) and sex-differential co-expression modules.

RESULTS: We identified 101 significant sex-DEGs, including Y-chromosome genes, genes impacted by X-chromosome inactivation, and autosomal genes. Known ASD risk genes, implicated by either common or rare variants, did not preferentially overlap with sex-DEGs. We identified 1 male-specific co-expression module enriched for immune signaling that is unique to 1 input dataset.

CONCLUSIONS: Sex-differential gene expression is limited in prenatal human cortex tissue, although meta-analysis of large datasets allows for the identification of sex-DEGs, including autosomal genes that encode proteins involved in neural development. Lack of sex-DEG overlap with ASD risk genes in the prenatal cortex suggests that sex-differential modulation of ASD symptoms may occur in other brain regions, at other developmental stages, or in specific cell types, or may involve mechanisms that act downstream from mutation-carrying genes.

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Autism spectrum disorder (ASD) is a pervasive neurodevelopmental disorder defined by deficits in social communication and repetitive, restrictive behaviors that currently affects about 1 in 36 children in the United States (1). Despite this high prevalence, the underlying biological mechanisms are not fully understood and effective therapeutic interventions are lacking. Both common inherited and rare genetic variants have been shown to contribute to ASD symptoms, including several common variant loci (2) and over 100 ASD-associated genes identified by exome sequencing (3–8). Another key contributor to ASD diagnosis is sex: ASD is consistently diagnosed 3 to 5 times more frequently in males than in females, which makes ASD one of the most sex-skewed neuropsychiatric conditions (9). This strong male-biased prevalence suggests the existence of male-specific risk and/or female-specific protective mechanisms (10,11). To delineate such mechanisms and advance our knowledge of sex bias in ASD, it is imperative to understand baseline, or neurotypical, biological differences between males and females in the brain, particularly during early development and before ASD symptom onset.

Although male and female human brains are highly similar, there are some exceptions, and sex-specific differences have been observed in total brain volume, connectivity, and the cytoarchitecture of certain brain regions (e.g., amygdala, hypothalamus) (12–15), as well as behaviors (e.g., aggression, social behaviors, and reproductive behaviors) (10,14–16) and the prevalence of neuropsychiatric disorders (e.g., ASD, schizophrenia, major depressive disorder) (9,17,18). Studies of human postnatal brain tissue have revealed sex-biased expression of sex chromosome genes and, to a lesser extent, autosomal genes (13,19–21). Sex differences in gene expression may drive or indicate sex differences in brain function and development and may begin to take shape as early as midgestation due to the effects of prenatally secreted sex hormones (12) or the functions of sex chromosome genes (10,12,15). These early-life programming events impact neural structure, function, and behavioral phenotypes later in life (13,14,16,19,20,22,23).

To date, the delineation of transcriptomic sex differences during human prenatal neurodevelopment, particularly in regions like the prefrontal cortex implicated in human

neuropsychiatric conditions (24), have been limited by small sample sizes. A 2016 study of transcriptome expression by microarray (25) of the prenatal neocortex from just 8 donors (4 females, 4 males, 16–22 postconception weeks) reported prenatal, male-biased enrichment for genes related to glial/immune functioning, paralleling transcriptomic changes reported in ASD brains (26,27). A subsequent, expanded analysis of samples from the BrainSpan resource again showed male-skewed expression of gene sets annotated to astrocyte and microglial function, particularly during prenatal development (28,29). Notably, ASD-associated risk genes from exome sequencing studies were not preferentially sex-differentially expressed in these reports, which suggests that sex-differential mechanisms may act on pathways that are altered by risk gene variants as opposed to direct sex-differential regulation of risk genes' transcription. In contrast, analysis of whole fetal human brain revealed sex-biased expression of a small number of autosomal ASD risk genes, with 7 genes exhibiting higher expression in males and 5 showing higher expression in females (30). The brain region(s) or cell type(s) that drive these differences in the whole brain are not yet known. Collectively, reported patterns highlight potential interactions between sex-influenced regulatory factors and ASD risk genes and/or specific cell types, although with the caveats of small donor number, microarray-based quantifications, or nonspecific tissue dissections (25–27,29,30). Additionally, sex differences in co-expression network structure have not yet been examined in human prenatal cortex. Such analyses would identify individual genes or groups of genes that show differing expression correlation patterns in male and female brains, which could indicate sex differences in the functional activities of these genes.

Here, we validate and refine understanding of prenatal sex-differential gene expression patterns by utilizing larger, RNA sequencing (RNA-seq) datasets derived specifically from human cortex. We have quantified transcriptome-wide sex-differential expression levels and co-expression differences in 2 large datasets of prenatal cortex tissue derived from 273 donors [BrainVar, dorsolateral prefrontal cortex (DLPFC), 87 donors (31) and University of California, Los Angeles (UCLA), cortex, 186 donors (32)]. Meta-analysis of both datasets identified 101 sex chromosome and autosomal sex-differentially expressed genes (sex-DEGs), which do not overlap with ASD risk genes and are not enriched for ASD-dysregulated modules. We also observed limited evidence for sex-specific gene co-expression in prenatal tissue, finding 1 male-specific module present only in the BrainVar dataset. These results define a set of genes that are prenatally sex-differentially expressed in human cortex from a large sample of individual donors, thus providing a resource for the study of sex-influenced developmental conditions. Furthermore, the observation that known ASD risk genes are not sex-differentially expressed in prenatal cortex tissue suggests that sex-differential risk mechanisms may influence the expression of ASD risk genes in other biological contexts (other brain region(s), ages, in specific cell types) or by influencing pathways affected by mutations in risk genes as opposed to direct regulation of risk genes' expression in the prenatal cortex.

METHODS AND MATERIALS

Prenatal RNA Sequencing Data

RNA-seq data from human donors age 14 to 21 post-conception weeks were used, including DLPFC samples from the BrainVar project (31) (Table S1 in Supplement 2) and whole cortex samples from a UCLA-based collection (32) (Table S2 in Supplement 2). Preprocessing steps are detailed in Supplement 1. Processed data included 15,649 genes and 87 samples (41 males, 46 females) for BrainVar, and 16,193 genes and 186 samples (101 males, 85 females) for UCLA.

Cell Type Composition

Cell type proportion estimates for neural cell types were calculated by dtangle (version 2.0.9) (33) using cell type signatures from prenatal single-nuclei RNA-seq (34). Male and female estimates per cell type were compared within each dataset using 2-sided Wilcoxon tests. Cell proportion estimates were further applied to deconvolve and estimate gene expression associated with specific cell types per sample by bMIND (35), and limma voom (36,37) was used to test for sex differences in estimated cell type-specific expression (additional details are provided in Supplement 1).

Differential Expression and Meta-Analysis

Differential expression analyses were performed separately in the 2 datasets using limma voom (version 3.52.4) (36,37) on expressed genes under a regression model including age, RNA-seq batch, RNA integrity number, postmortem interval, estimated intermediate progenitor cell proportion, and surrogate variables (Supplement 1). Differential expression results from both datasets were subsequently combined for meta-analysis using MetaRNASeq (38). We defined sex-DEGs as genes with Benjamini–Hochberg false discovery rate (FDR) $\leq .1$. Within each dataset, we also defined sex-specific age-DEGs by running age-differential expression analysis separately in male and female samples and by testing for sex by age interactions. Additional details are included in Supplement 1.

Functional Annotation

We tested sex-DEGs for enrichment of ASD risk genes as implicated by rare and common variants and by transcriptome-wide association studies (TWASs), genes with ASD-dysregulated expression in the brain, genes associated with neuropsychiatric phenotypes, cell type markers, and databases of biological functions using 2-sided Fisher's exact tests and Gene Set Enrichment Analysis [GSEA; (39); additional details are provided in Supplement 1 and Table S3 in Supplement 2].

Co-expression Analyses

We applied weighted gene co-expression network analysis (WGCNA, version 1.71) (40) separately to male and female samples from each dataset and then compared female and male modules using module preservation analysis in the WGCNA R package (40,41). In BrainVar, we also assessed module similarity across sexes by directly comparing gene content and sample clustering by eigengene values of male-

and female-identified modules; overlapping and clustering modules were merged (see [Supplement 1](#)). Sex-differential and grouped co-expression modules were tested for functional enrichment of ASD risk genes, ASD-dysregulated genes, neuropsychiatric phenotype risk genes, cell type marker genes, databases of biological functions, and sex-DEGs identified here.

To identify individual genes that contribute to sex-differential co-expression, we applied sparse leading eigen-gene driven to expressed genes from each dataset (42–44). Next, we prioritized 2 sets of genes with nonzero leverage in the co-expression network: 1) primary genes, which cumulatively accounted for 90% of leverage in the network; and 2) secondary genes, which accounted for the remaining 10%. We compared primary and secondary genes to all sex-specific WGCNA modules and functionally annotated with gProfiler (45). Additional information is provided in [Supplement 1](#).

RESULTS

Sex-Differential Expression in the Prenatal Human Cortex

First, we quantified sex-differential gene expression in 2 independent datasets from midfetal (14–21 postconception weeks) cortex tissue from de-identified donors without known neurological or psychiatric disorders: BrainVar, including 87 DLPFC samples from 41 male and 46 female donors (31), and UCLA, including 186 cortical tissue samples from 101 male and 85 female donors (32). Estimates of the proportions of 10 prenatal cortical cell types in the bulk tissue samples by dtangle ([Methods and Materials](#)) (34) showed no significant sex differences in either dataset, but highlighted a systematic difference between datasets in cell type representation, likely stemming from differences in cortical dissection depth (BrainVar: cortical plate; UCLA: whole cortical wall; [Figure 1A, B](#)). To adjust for this, we included intermediate progenitor cell proportion as a covariate for differential expression together with age, RNA-seq batch, RNA integrity number, postmortem interval, and surrogate variables ([Methods and Materials](#)).

To determine the compatibility of BrainVar and UCLA data for meta-analysis of sex-differential expression, we assessed sex-DEGs that were identified in 1 dataset for their expression differences in the other dataset by estimating π_0 and π_1 ([Supplement 1](#)). Of sex-DEGs ($FDR \leq .1$) from BrainVar, an estimated fraction of 0.883 also showed association in UCLA, and of sex-DEGs from UCLA, an estimated fraction of 0.832 also showed association in BrainVar, supporting the application of meta-analysis for sex-DEG discovery. Direct comparison of sex-DEGs from the BrainVar and UCLA datasets identified 49 genes (28 X chromosome, 19 Y chromosome, 2 autosomal) that met $FDR \leq .1$ independently in both datasets and common functional enrichments driven by sex chromosomal genes (Y-linked inheritance, X-linked inheritance, estrogen metabolism, and sulfuric ester hydrolase activity; see [Supplement 1](#)).

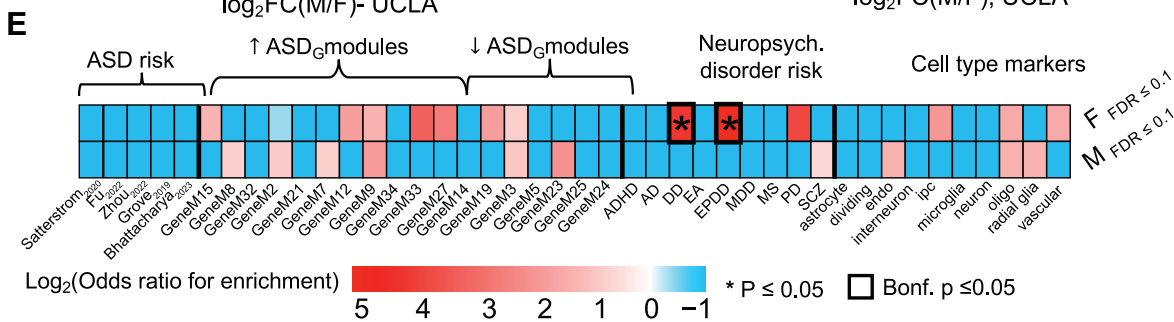
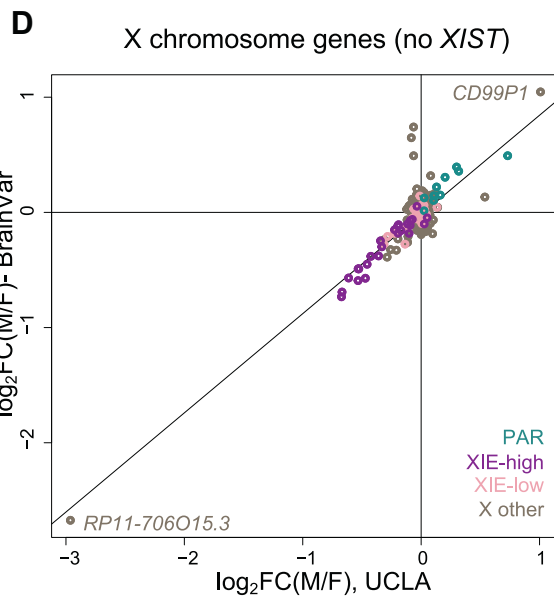
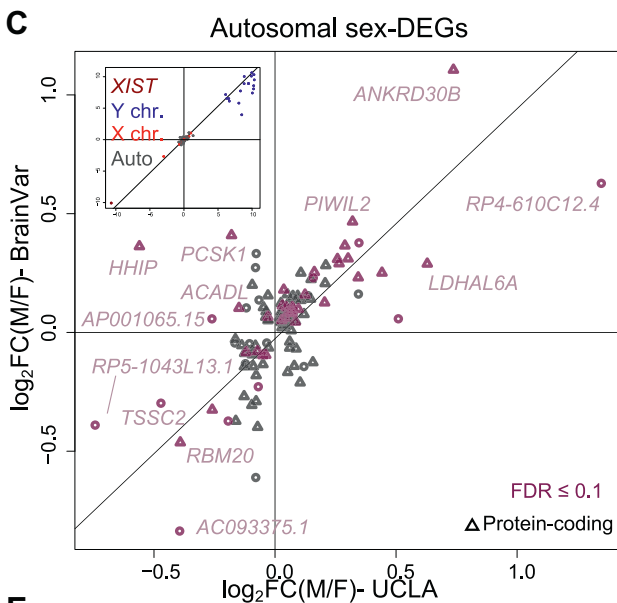
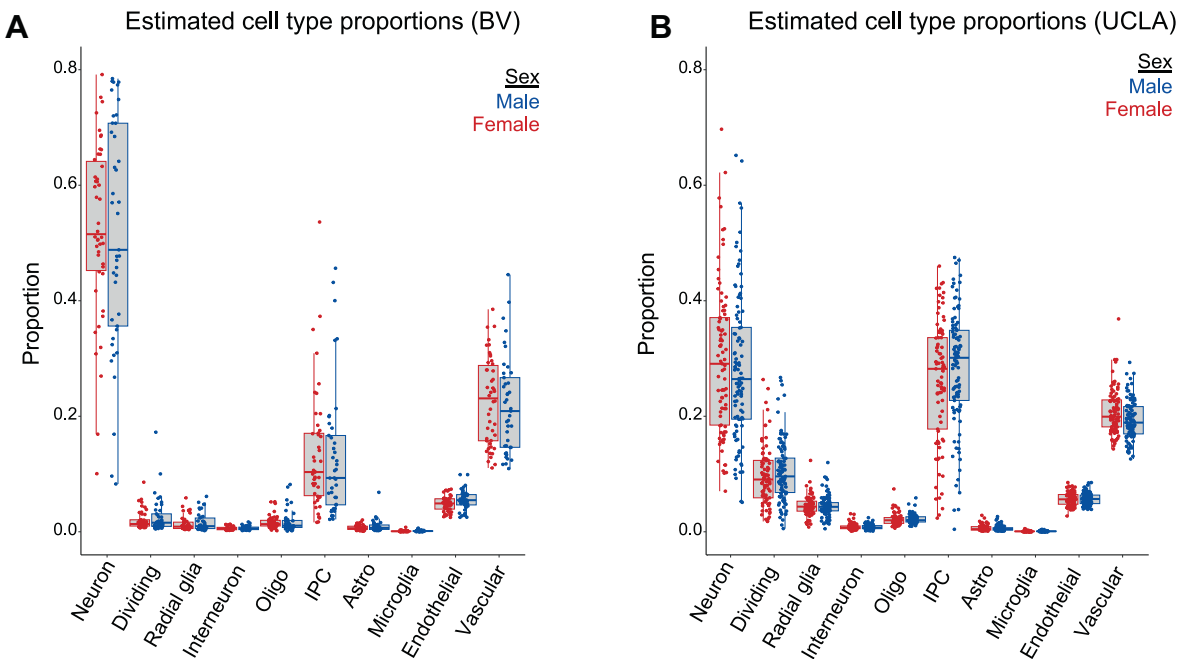
Meta-analysis of sex-differential expression in the BrainVar and UCLA datasets identified 106 sex-DEGs at an $FDR \leq .1$, 101 of which had consistent effect directions in both datasets (43 X chromosome, 18 Y chromosome, 40 autosomal) ([Table S4](#) in [Supplement 2](#)). Significant sex-DEGs exhibited

consistent sex effects between datasets, as evidenced by highly correlated \log_2 fold change (FC) estimates for all sex-DEGs (Pearson $r = 0.98$; 95% CI, 0.977–0.990; $p = 1.19 \times 10^{-76}$) and autosomal sex-DEGs (Pearson $r = 0.83$; 95% CI, 0.701–0.908; $p = 3.21 \times 10^{-11}$) ([Figure 1C](#)). Sex effects for all tested X-chromosome genes were also strongly correlated (Pearson $r = 0.88$; 95% CI, 0.860–0.899; $p = 9.83 \times 10^{-167}$) ([Figure 1D](#)).

Among the 101 sex-DEGs with consistent effect directions, Y-chromosome genes and *XIST* showed the largest expression differences while autosomal effects were more subtle ([Figure 1C, D](#)). Sex chromosomes encode a total of 49 protein-coding sex-DEGs ($FDR \leq .1$), including 38 X-chromosome genes (28 female-biased, 10 male-biased) and 11 on the Y chromosome (all male-biased). Genes with previous evidence of X inactivation escape (XIE) (46) showed higher expression in females (21 XIE-high probability and 5 XIE-low probability genes at $FDR \leq .1$) ([Supplement 1](#); [Table S5](#) in [Supplement 2](#)) while several genes in the pseudoautosomal regions (PARs) showed male-biased expression (8 PAR genes at $FDR \leq .1$), consistent with an extension of X inactivation epigenetic marks into the PAR and partial transcription repression in chromosomal females. Sex-DEGs include several non-PAR, non-XIE X-chromosome genes (X-other), including female-biased genes *CXORF36*, *GYG2*, *PIN4*, *CA5BP1*, and *RP11-706O15.3* and male-biased genes *RBBP7* and *REPS2*. These genes may escape X-chromosome inactivation (female-biased) or respond to sex-specific expression activation mechanisms in the prenatal cortex.

Autosomal, protein-coding sex-DEGs include 6 female-biased and 25 male-biased genes. Three female-biased genes (*ITGA8*, *COCH*, and *BAG2*) are associated with known nervous system functions such as neurite outgrowth in sensory neurons and neuronal response to heat stress. Male-biased genes include *PTPN5*, *NDRG2*, *NAPB*, and *PRPH2*, which encode proteins involved in neuron and dendrite growth, and *NKAPL* and *RBPJ*, which are associated with the Notch-mediated signaling pathway. *NAPB* and *NKAPL* have also been associated with phenotypes of global developmental delay (47–49) and genome-wide association study (GWAS) signals for ASD and schizophrenia (50,51). Noncoding autosomal sex-DEGs consist of 2 pseudogenes, 6 long intergenic noncoding RNA genes, and 1 antisense gene ([Table S4](#) in [Supplement 2](#)).

We further reasoned that age might interact with sex-differential mechanisms during this midfetal developmental window, when gonadal testosterone levels in males are rising (22). However, we observed largely consistent age effects on gene expression in males and females in both the BrainVar (union of sex-specific age-DEGs, Pearson r for beta coefficients = 0.78; 95% CI, 0.765–0.794; $p < 2.2 \times 10^{-16}$) and UCLA (union of age-DEGs, Pearson $r = 0.92$; 95% CI, 0.922–0.927; $p < 2.2 \times 10^{-16}$) datasets ([Figure 2A, B](#); [Tables S6, S7](#) in [Supplement 2](#)). Two age-DEGs showed opposing directions of effect in males and females in both BrainVar and UCLA datasets: protein-coding *CRISPLD1* expression rose in females and fell in males across development, and long intergenic noncoding RNA gene *CTD-2587M2.1* fell in females and rose in males ([Figure 2A, B](#)). We also detected 28 genes (1 X chromosome, 27 autosomal) with



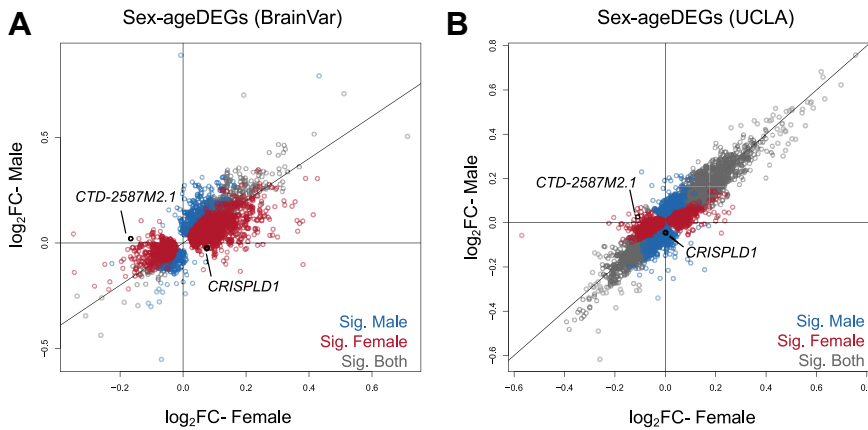


Figure 2. Sex-shared and sex-specific age-DEGs in the prenatal cortex. **(A, B)** Scatterplot of \log_2 FCs from females (x-axis) and males (y-axis) for age-DEGs with $FDR \leq .1$ in BrainVar **(A)** and UCLA **(B)**. Point color denotes the sex(es) in which each gene reached $FDR \leq .1$ for the effect of age. Age-DEGs with opposing direction of effect in males and females from both datasets are noted on plots. Sig. Both indicates genes with $FDR \leq .1$ for age in both sexes; Sig. Female indicates genes with $FDR \leq .1$ for age only in females; Sig. Male indicates genes with $FDR \leq .1$ for age only in males; DEG, differentially expressed gene; FC, fold change; FDR, false discovery rate; Sig, significant; UCLA, University of California, Los Angeles.

a significant age by sex interaction in the UCLA dataset ($FDR \leq .1$), whereas 0 genes reached significance in the BrainVar dataset (Tables S6, S7 in Supplement 2).

Functional Enrichment of Sex-DEGs

Consistent with cell type proportion estimates (Figure 1A, B), neither male- nor female-biased sex-DEGs were enriched for cell type markers (Figure 1E). We also did not find significant enrichment for rare variant-associated ASD risk genes from multiple exome sequencing studies (6–8), common variant-associated ASD risk genes from GWAS (2), or ASD-associated genes from a TWAS derived from prenatal cortex expression (52); in fact, 0 sex-DEGs overlapped with any of these gene sets (Figure 1E; Table S8 in Supplement 2). This pattern is consistent with previous studies of the human cortex (25,29) and suggests that, as a group, the expression of currently known ASD risk genes is not directly regulated by sex-differential mechanisms in the prenatal human cortex, at least not as is detectable in bulk tissue. We also found limited evidence for sex-differential expression of ASD risk genes within specific cell types as estimated from bulk tissue data by bMIND (Methods and Materials; Tables S9, S10 in Supplement 2): risk genes *GIGYF1* (6–8) and *PPP1R9B* (6) had significantly male-biased expression in neurons from UCLA only, and *MAPT* (2) and *PPP1R9B* and *PTK7* (6) had significantly female-biased expression in endothelial cells from BrainVar only.

An alternative hypothesis is that sex-differential mechanisms may regulate an independent set of genes, which

then interact with ASD-impacted neurobiology downstream from mutation-carrying risk genes. Gene expression changes in the brains of people with ASD provide a molecular signature of such downstream effects of risk factors and disorder pathology. Therefore, we compared sex-DEGs with ASD-impacted co-expression modules defined in all lobes of the postmortem human cortex (53) or in the frontal and temporal cortex (26). We did not find significant enrichment for female- or male-biased sex-DEGs in any ASD-dysregulated modules (Figure 1E; Table S8 in Supplement 2).

Beyond ASD, we observed significant female-biased enrichment of risk genes for developmental delay (DD) and DD with epilepsy (DD odds ratio = 19.32, $p = 6.7 \times 10^{-4}$, $p_{adj} = .013$, 3 genes overlap; DD with epilepsy odds ratio = 36.14, $p = .0017$, $p_{adj} = .034$, 2 genes overlap) (Figure 1E; Table S8 in Supplement 2). Female-biased DEG overlap with *SMC1A* and *DDX3X*, 2 X-chromosome genes reported to escape XCI (46), drive these results. Additionally, *DDX3X* and *USP9X* overlap with rare variants that show replicable, female-preferential DD risk in females (54). Risk genes for other sex-influenced diagnoses, such as schizophrenia, major depressive disorder, multiple sclerosis, and Alzheimer’s disease, were not significantly enriched for sex-DEGs (Figure 1E).

Threshold-free enrichment testing for sex-skewed expression of gene sets of interest by GSEA (39) revealed nominal enrichment for female-biased expression of ASD-upregulated module M27 (normalized enrichment score [NES] = -0.5304 ,

Figure 1. Sex-differential patterns of gene expression in the prenatal human cortex. **(A, B)** Estimated proportions of 10 cell types in male (blue) and female (red) cortex in the BrainVar dataset **(A)** and UCLA dataset **(B)**. Each dot represents a specific cell type estimate for an individual sample. **(C)** Scatterplot of \log_2 FCs from individual datasets for autosomal genes within the top 200 differentially expressed genes ranked by p value from meta-analysis (Pearson $r = 0.62$, p value = 1.01×10^{-16}). Point color denotes significance at $FDR \leq .1$ (deep red). Point shape denotes protein coding genes (triangle) and noncoding genes (circle). Inset: FCs from all top 200 differentially expressed genes (Pearson $r = 0.98$, p value = 3.57×10^{-151}). **(D)** Scatterplot of \log_2 FCs from individual data sets for all X-chromosome genes (Pearson $r = 0.88$, p value = 9.83×10^{-167}). Colors indicate specific groups of X genes. **(E)** Patterns of sex-DEG enrichment from meta-analysis results for 5 ASD risk gene sets (2,6–8,52), ASD-associated modules (ASD_A) (53), 9 disease risk gene sets (31), and 10 cell types (34) using 2-sided Fisher’s exact tests. F_{FDR} indicates female-biased $FDR \leq .1$; M_{FDR} indicates male-biased $FDR \leq .1$. AD, Alzheimer’s disease; ADHD, attention-deficit/hyperactivity disorder; ASD, autism spectrum disorder; Astro, astrocyte; BV, BrainVar; DD, developmental delay; EA, educational attainment; EPDD, epilepsy and developmental delay; FC, fold change; FDR, false discovery rate; IPC, intermediate progenitor cell; MDD, major depressive disorder; MS, multiple sclerosis; Oligo, oligodendrocyte; PAR, pseudoautosomal region; PD, Parkinson’s disease; SCZ, schizophrenia; sex-DEG, sex-differentially expressed gene; UCLA, University of California, Los Angeles; XIE, X inactivation escape.

$p = .038$, $FDR = .187$); interneuron markers ($NES = -0.413$, $p = .094$, $FDR = .389$); and DD with epilepsy risk markers ($NES = -0.576$, $p = .033$, $FDR = .244$). We did not observe enrichments for any tested gene set toward male-skewed expression (Table S11 in Supplement 2).

Sex-Specific Co-expression Patterns in the Prenatal Cortex

Using WGCNA (40), we defined 14 co-expression modules in females and 11 modules in males from BrainVar (Figure 3A; Figure S1 in Supplement 1; Table S12 in Supplement 2) and then grouped genes from similar male and female modules based on gene content overlap and expression similarity across samples (Methods and Materials; Supplement 1; Figure 3A; Figures S2, S3 in Supplement 1; Table S13 in Supplement 2). Modules that did not have a clear counterpart in the network of the other sex included female modules mod22_F, mod16_F, mod12_F, and mod11_F and male module mod20_M. Sex-shared module group 7 was enriched for male-biased sex-DEGs, ASD risk genes, and vascular cell marker genes, suggesting a potential connection between male sex and ASD risk as related to prenatal vascular cell function (Figure 3A). However, consistent with sex-DEGs, no sex-specific modules were enriched for ASD risk genes (Figure 3C), while mod11_F was enriched for the ASD-elevated, ribosomal function-associated GeneM27 module (odds ratio = 106.37, $p_{adj} = 1.07 \times 10^{-64}$) (Figure 3C; Table S15 in Supplement 2). Module preservation analysis comparing male and female network structure identified mod20_M as poorly preserved in females, indicating male-specific co-expression for mod20_M genes ($Z_{summary} = 8.7$) (Figure 3B). Notably, mod20_M genes showed substantially lower average expression than genes in other modules (Figure S4 in Supplement 1). Mod20_M did not show any cell type marker or ASD-associated module enrichments but was enriched for Gene Ontology terms related to immune responses and signaling (Figure 3C, D; Tables S14 and S15 in Supplement 2). Top hub genes within mod20_M include transcription factors *HOXC4* and *PRDM13* (55) and 2 protein-coding genes with rising expression across prenatal development (31), *SLC5A9* and *TUBA4B*.

We further applied sparse leading eigengene driven (42) to identify individual genes with strong influence on co-expression structure differences between sexes (Methods and Materials). In BrainVar, we identified 915 genes with nonzero leverage on network sex differences, including 409 primary genes that collectively accounted for 90% of the variability in network correlation structure between sexes (Table S16 in Supplement 2). Primary genes overlapped substantially with the mod20_M module (396 of 409 genes) and were enriched for Gene Ontology terms related to cytokine production and immune signaling (Table S16 in Supplement 2).

Co-expression patterns differed in the UCLA dataset (Figure S5 in Supplement 1). Module preservation analysis for 20 modules defined in females and 21 modules defined in males did not identify any sex-specific modules (Figure S6 in Supplement 1). Sparse leading eigengene driven identified 1103 genes with nonzero leverage on network sex differences, including 354 primary genes. UCLA primary genes did not preferentially overlap with the BrainVar mod20_M module, and

top Gene Ontology enrichments point to protein binding and biosynthetic and metabolism processes (Table S17 in Supplement 2). These differences in co-expression structure may stem from discrepancies in cortical dissection between BrainVar (DLPFC cortical plate) and UCLA (whole cortex vs. cortical wall).

DISCUSSION

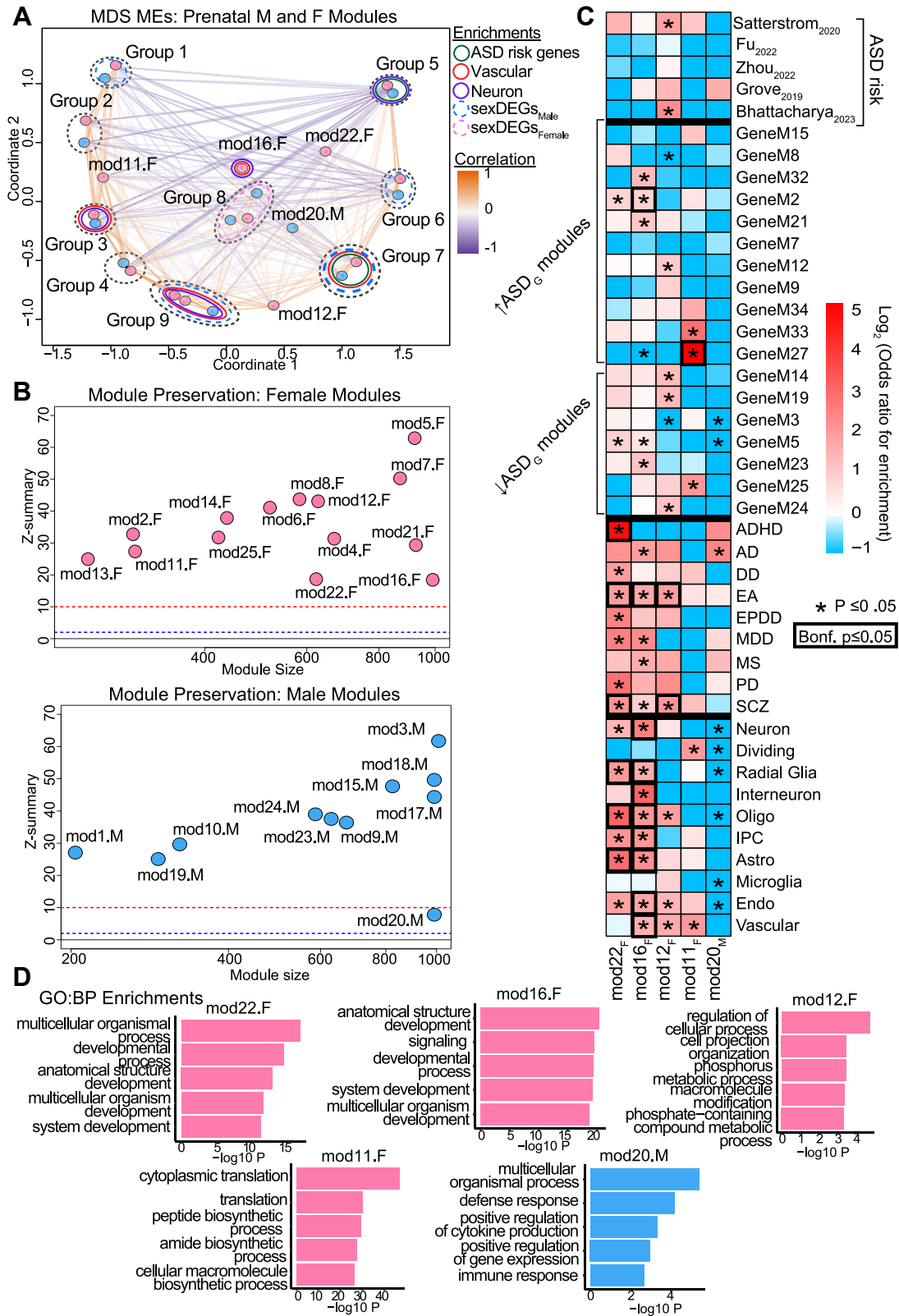
ASD is one of the most sex-biased disorders, with a consistently male-biased diagnosis rate, suggesting the existence of male-specific risk and/or female-specific protective mechanisms. To unravel these sex-differential mechanisms, it is crucial to first understand baseline biological differences between developing male and female brains before ASD onset. Toward this end, we investigated patterns of sex-differential gene expression in the prenatal human cortex, a brain region implicated in human cognitive processes and neurodevelopmental disorders, including ASD.

As expected, based on copy number and previous studies (30,46), sex chromosome genes were strongly differentially expressed. Genes that escaped X inactivation exhibited consistent female-biased expression, and Y chromosome and several PAR genes showed male-biased expression. Sex differences in the expression of autosomal and other X chromosome genes were also identifiable with our large (combined) sample size and meta-analysis approach, although the magnitudes of the expression differences were modest. Our findings support previous studies that have reported predominantly sex-neutral expression patterns in the developing human cortex (25,29).

We also revisited the following 2 hypotheses regarding the interaction of sex-differential biology and ASD risk (25), now with larger datasets: 1) ASD risk genes are sex-differentially regulated; and 2) sex differences interact with ASD etiologic pathways that are affected downstream from variants in risk genes. Consistent with previous research, we found that ASD risk genes, implicated by either rare or common variants, did not show significant enrichment for sex-skewed expression levels in prenatal cortex tissue. In contrast to previous reports, male-biased DEGs were not significantly enriched for ASD-upregulated gene sets, and female-biased DEGs were not significantly enriched for ASD-downregulated modules.

We note several challenges and limitations regarding comparison of prenatal sex-DEGs to ASD risk genes and ASD-dysregulated modules. First, currently known ASD risk genes from exome sequencing are entirely autosomal due to challenges related to statistical power for gene discovery on the X chromosome in a male-skewed condition like ASD, where the majority of cases have only 1 maternally inherited X chromosome, while de novo variants that drive gene-based associations arise primarily on paternal chromosomes. GWAS for ASD have not found genome-wide significant signals on the X chromosome and have similarly focused on autosomes (2). Sex chromosome genes including Y, XIE, and PAR genes consistently show sex-differential expression, and it is possible that a subset of these genes exerts a sex-differential influence on disorder-associated pathways. Sex chromosome genes have been understudied in human genetics research (56), and future research focusing on the sex chromosomes may identify

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additional ASD risk genes, at which point the hypotheses tested here will need to be reevaluated.

Regarding ASD-dysregulated gene expression, previous studies have reported enrichment for male-biased DEGs from prenatal and postnatal cortex in co-expression modules with elevated expression in ASD (25,28); in this study, meta-analysis of BrainVar and UCLA data did not replicate this pattern. Further exploration revealed significantly male-skewed expression of glia- and immune-annotated, ASD-elevated module *ctx.m19* (26) by GSEA in the BrainVar dataset only (NES = 2.07, FDR = 1.7×10^{-3}), although adjustment for surrogate variables eliminated this signal. This suggests that dataset features and choices related to data handling can impact the patterns observed, particularly for small and variable effects like sex differences. Larger samples and consistent tissue dissection and processing will be key for identifying replicable sex effects. It is also possible that a different set of genes may have altered expression in the prenatal ASD brain and that comparison of prenatal sex-DEGs to postnatal ASD-altered expression patterns does not capture the key processes involved. A TWAS based on prenatal cortex expression can impute prenatal ASD-DEGs, although we found that ASD-DEGs from a developmental TWAS (52) also did not overlap with prenatal sex-DEGs.

In BrainVar, genes in the male-specific *mod20_M* co-expression module were enriched for functions related to immune/inflammatory responses and signaling. There were no sex differences in estimated microglial or astrocyte cell proportion, which suggests that this male-biased neuroimmune enrichment may instead reflect augmented neuroimmune gene expression and signaling in neurotypical male cortical tissue during fetal development. This pattern is mirrored in ASD cortex (26,53). Estimated cell type-specific sex differences from prenatal bulk cortex do not demonstrate strong sex differences in microglia or astrocytes, although the estimated low abundance of these cell types likely impacts power. We also note that *mod20_M* module genes were very lowly expressed, and a comparable module is not evident in the UCLA dataset. Mechanisms such as variable transcriptional silencing across brain regions and dynamic changes in inflammatory/immune state during development may contribute to these noisy signals. Nonetheless, this putative pattern of male-skewed neuroimmune biology is consistent with several recent lines of evidence implicating the role of the immune system and inflammation in the male-skewed development of ASD-related behaviors (57–64).

A key limitation that pertains to the evaluation of both ASD risk and ASD-dysregulated gene sets in this study is that our observations of sex-differential expression are limited to prenatal, midgestation cortex bulk tissue. Prenatal sex differences in gene expression may be more pronounced, show different directions of effect, implicate other functional processes, or intersect with ASD-associated genes in other brain regions (14,19,20,53,65–67), such as the hypothalamus and amygdala (65). ASD-associated genes may also be sex-differentially expressed within specific cortical cell types. Although we found several ASD risk genes with estimated sex-differential expression in neurons and endothelial cells by cell type deconvolution, these patterns were not consistent between datasets and require direct measurement in single or sorted cells. A recent single-cell RNA-seq analysis of developing brain found that ASD risk genes with male-biased expression were predominantly expressed in microglia, while those with female-biased expression were mostly observed in neuronal and astrocytic cells (68). Validation in independent data is needed to corroborate this pattern and/or the estimated patterns from bulk tissue. ASD-associated genes may also be sex-differentially expressed at ages that were not assayed here or transiently sex-differentially regulated within the tested midgestation window. Finally, sex-differential factors may influence ASD etiology via mechanisms that were not addressed here, such as by sex-differential splicing regulation, translation regulation, or posttranslational modifications of ASD risk genes.

Conclusions

Definition of transcriptome-wide sex differences in gene expression level and co-expression structure in the midfetal human cortex is fundamental to understanding baseline sex differences in the human brain and necessary to delineate biological processes that contribute to sex-biased conditions like ASD. Future studies of sex-differential expression of genes and isoforms in additional brain regions, at different developmental stages, and using single cells or nuclei will be valuable and may point to pathways, cell types, and functions that link sex-differential neurobiology to neuropsychiatric disorder etiology. Collectively, such research will advance our understanding of mechanisms of sex-differential risk for ASD and other neurodevelopmental disorders and may provide avenues for future therapeutic development.

Figure 3. Co-expression patterns in the male and female prenatal DLPFC from BrainVar. **(A)** Module clustering by multidimensional scaling of module eigengenes. Module colors denote male (blue) or female (pink) WGCNA. Line color indicates the direction and degree of correlation between module eigengenes. Groups include modules identified in males and females that are grouped based on gene content similarity. Modules not grouped include label of sex after module number. See Figure S3 in Supplement 1 for grouped module IDs. Enrichments for sex-DEGs, ASD risk genes, vascular cell markers, and neuronal markers with adjusted p value $\leq .05$ by 2-sided Fisher's exact test are noted by group or module outline. **(B)** Module preservation in males for modules identified in females (top) and preservation in females for modules identified in males (bottom). **(C)** Enrichments for ASD risk gene sets (2,6–8,52), ASD-associated modules (ASD_G) (53), disease risk gene sets (31), and cell type (34) markers for nongrouped, sex-differential modules by 2-sided Fisher's exact tests. **(D)** Top Gene Ontology biological process terms for each nongrouped, sex-differential module of interest. AD, Alzheimer's disease; ADHD, attention-deficit/hyperactivity disorder; ASD, autism spectrum disorder; DD, developmental delay; DLPFC, dorsolateral prefrontal cortex; EA, educational attainment; Endo, endothelial cell; EPDD, epilepsy and developmental delay; GO:BP, Gene Ontology biological processes; IPC, intermediate progenitor cell; MDD, major depressive disorder; MS, multiple sclerosis; Oligo, oligodendrocyte; PD, Parkinson's disease; SCZ, schizophrenia; sex-DEG, sex-differentially expressed gene; WGCNA, weighted gene co-expression network analysis.

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