# Defining Patterns of Sex-Differential Expression in the Human Cortex During Prenatal Development and the Intersections with Autism Spectrum Disorder

By

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ABSTRACT

Autism spectrum disorder (ASD) is a pervasive neurodevelopmental disorder with a consistently male-skewed prevalence, implicating a prominent role for sex-differential biology in the risk and presentation of ASD. Although genome-scale analyses have identified patterns of gene expression and risk variants associated with ASD, these studies have primarily focused on differences between cases and controls without directly examining the additional variable of sex. Thus, the mechanistic interactions of sex and ASD neurobiology remain largely unknown and warrant investigation to advance our understanding of the strong sex bias in ASD. To elucidate sex-differential ASD risk and identify future therapeutic targets, thorough characterization of baseline sex-differential neurobiology during development and changes in disorder state is essential. Therefore, this thesis work aims to quantify genome-scale expression patterns in the human brain sensitive to biological sex and identify transcriptomic points of convergence with ASD-associated neurobiology, likely representing mechanisms that may be involved in sex-differential modulation on ASD etiology. First, we identified robust sex-differential transcriptomic signatures and associated functional pathways during human prenatal cortical development using two large, independent RNA-sequencing studies, comprising an order of magnitude more donors than previous studies. In agreement with prior work, we do not find evidence of sex-differential expression of currently known ASD risk genes in the prenatal cortex from this large sample, suggesting that sex-differential modulation may occur downstream of mutation-causing risk genes or that sex-differential regulation of risk genes is not evident in bulk cortex tissue. In the postnatal cortex, we defined sex-differential gene expression patterns and changes within the context of ASD brain. Here, we observe an attenuation of autosomal sex-differential expression effects in the ASD brain. We also identified genes with transcriptomic signatures reflective of sex-differential risk profiles that implicate female-skewed expression of neuronal cell markers and ASD risk genes in protection against ASD and male-skewed expression of neuroimmune and glial cell markers in vulnerability to ASD. Overall, this thesis work motivates future experiments at the cellular level

and sex-attenuated genes, protective genes, and vulnerability genes identified here are high priority targets for experimental studies, providing avenues for future therapeutic development.

## INTRODUCTION

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# Neural Transcriptomic Analysis of Sex Differences in Autism Spectrum Disorders: Current Insights and Future Directions

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**Abstract**

Autism spectrum disorder (ASD) is consistently diagnosed 3-5 times more frequently in males than females, a dramatically sex biased prevalence that suggests the involvement of sex-differential biological factors in modulating risk. The genomic scale of transcriptomic analyses of human brain tissue can provide an unbiased approach for identifying genes and associated functional processes at the intersection of sex-differential and ASD-impacted neurobiology. Several studies characterizing gene expression changes in the ASD brain have been published in recent years with increasing sample size and cellular resolution. These studies report several convergent patterns across data sets and genetically heterogeneous samples, including elevated expression of gene sets associated with glial and immune function, and reduced expression of gene sets associated with neuronal and synaptic functions, in ASD brain. Assessment of neurotypical cortex tissue has reported parallel patterns by sex, with male-elevated expression of overlapping sets of glial/immune-related genes and female-biased expression of neuron-associated genes, suggesting potential roles for these cell types in sex-differential ASD risk mechanisms. However, validating and further exploring these mechanisms is challenged by the available data, as existing studies of ASD brain include a limited number of female ASD donors and focus predominantly on cortex regions not known to show pronounced sex-differential morphology or function. With this review, we summarize convergent findings from several landmark studies of the transcriptome in ASD brain and their relationship to sex-differential gene expression, and discuss limitations and remaining questions regarding transcriptomic analysis of sex differences in ASD.

**Introduction**

Autism spectrum disorder (ASD) is a pervasive neurodevelopmental disorder that currently affects about one in 59 children in the United States (1). With a consistent 3- to 5-fold excess of diagnosed males relative to females (1,2), ASD is among the most sex-skewed neuropsychiatric conditions. Despite this difference in prevalence, large-scale studies comparing the clinical phenotype of ASD in affected males and females have described reduced restricted and repetitive behaviors in females (3–6) and variable sex effects on social communication traits (4,5), but similar overall severity between the sexes (3,5). Both common, inherited variants (7,8) and rare, *de novo*, highly deleterious variants (9,10) contribute to ASD risk. Though several X chromosome loci are implicated, including *FMR1* (Fragile X Syndrome (11)), *MECP2* (Rett Syndrome (12)), *NLGN3*, and *NLGN4X* (13), the majority of known risk variants are autosomal (8–10). Several autosomal ASD risk genes are preferentially associated with developmental disorder in one sex, including male-biased incidence in ASD cases of rare, deleterious variants in *CHD8*, *MBD5*, and *SYNGAP1* (14), but the mechanisms behind these differences are not fully understood.

By generating an approximate readout of molecular function in tissues and cells at a genomic scale, transcriptomic analyses in brain are useful for exploring the neurobiology of brain disorders and provide information that is complementary to gene discovery work. With the application of appropriate statistical methods, the genomic scale of transcriptome analyses allows unbiased discovery of the biological processes involved in a condition, and facilitates bioinformatic comparison across genome-scale data sets to identify points of convergence. Transcriptome studies can also be applied to characterize sex differences in a specific condition or trait, including differences between affected males and females and sex-differential risk mechanisms. Identification of male-female differences in the disorder state, which may be suggestive of sex-differential etiology or response to a disorder, requires sufficiently powered, balanced samples of affected males and females (Figure 1A). Investigation of sex-differential

risk requires the integration of case-control comparisons with the characterization of baseline, neurotypical sex differences to identify genes and associated biological processes that are influenced by both sex and disorder biology (Figure 1B).

Such genes can be further classified by their direction of effect on risk. Genes with high(er) expression in the disorder state and in the more frequently affected sex (males, for ASD) are more likely to be involved in, or tagging, sex-differential vulnerability mechanisms of disorder pathobiology (Figure 1C). Genes with high(er) expression in the healthy state and in the less frequently affected sex (females, for ASD) are likely involved in, or tagging, sex-differential protective mechanisms (Figure 1D). A major goal of transcriptomic studies exploring sex-differential risk mechanisms for ASD is to find these genes whose functions are involved in the amplification of male vulnerability, and/or the reduction of female risk.

With this goal in mind, this review aims to delineate key results from landmark studies of transcriptomic patterns in ASD brain tissue and to describe how ASD-associated changes intersect with neurotypical sex differences. We also discuss implications of these patterns for sex-differential risk mechanisms, limitations of currently available data sets, and remaining questions for future investigation.

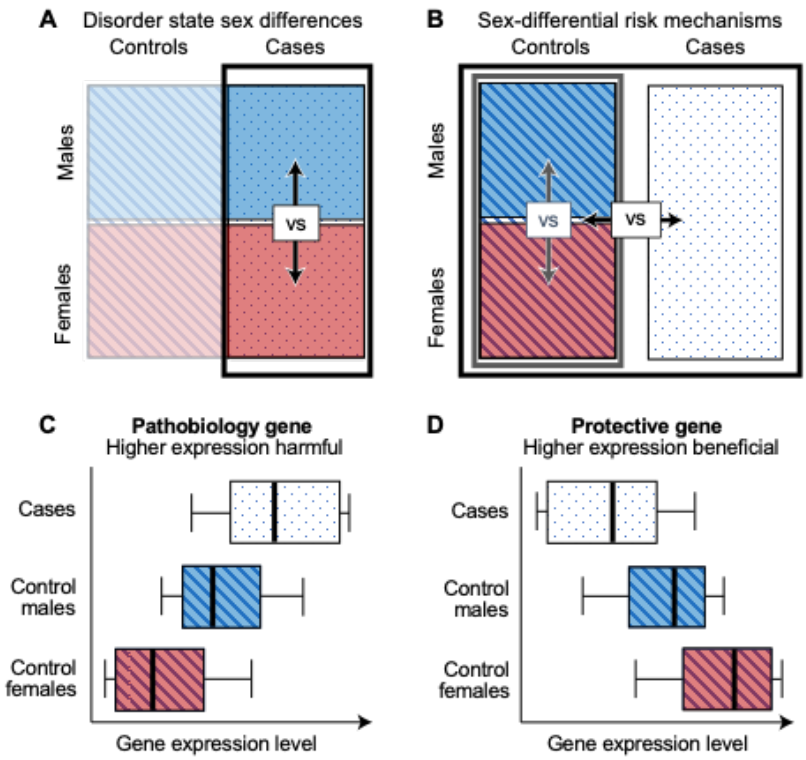


**Figure 1: Study designs and outcomes for transcriptomic analysis of sex differences.** A) Characterization of sex differences in the disorder state requires comparisons of male and female cases. B) Identification of genes and processes involved in sex-differential risk requires comparison of neurotypical male and female controls and comparison of controls to case cohorts. C) Putative pathobiology genes are predicted to show elevated expression in the disorder state and in the more frequently affected sex. D) Putative protective genes are predicted to show reduced expression in the disorder state and elevated expression in the less frequently affected sex.

**Availability of transcriptome data from the ASD brain**

Several landmark studies characterizing transcriptomic differences in ASD vs. control brain tissue and cells have been published to date (15–19). However, available brain tissue from ASD donors is sparse, and so these studies have relied on limited sample sizes (Table 1), ranging from just 6 ASD donors in an early gene expression study (15) to 48 ASD donors in the most recent report on bulk brain tissue transcriptomics (18). Critically, study samples were not fully independent. In total, tissues assayed across these five studies were derived from 160 unique donors: 69 ASD and 91 controls. These are relatively small numbers with which to tackle

the known genetic and phenotypic heterogeneity of ASD, and they also lag behind transcriptome data generation for other neuropsychiatric disorders such as schizophrenia, for which a recent publication from the CommonMind Consortium included 353 cases (20). Tissue availability limits brain transcriptome studies of both conditions relative to genetic analyses, where recent genome-wide association studies (GWAS) included 18,381 ASD (8) and 36,989 schizophrenia cases (21), and rare variant analyses included 11,986 ASD (10) and 3,444 schizophrenia cases (22).

Consistent with ASD's male-skewed prevalence, all data sets are strongly male-dominated: of the 69 total ASD donors, just 14 are female. Appropriately, all case and control groups are sex-matched, reducing the likelihood of confounding sex with case-control status, but directly limiting the number of female brain transcriptomes available for analysis. This paucity of data from case and control females presents significant challenges for characterizing differential expression patterns 1) between male and female ASD cases, such as has been observed for major depressive disorder (23,24) and post-traumatic stress disorder (25), 2) between female cases and controls, to determine how the female ASD brain diverges from sex-specific expectation, and 3) to identify sex-by-diagnosis interaction effects, in which ASD transcriptomic changes differ in magnitude or direction of effect between the sexes.

**Table 1. Tissue Donors and Brain Regions Assayed in Transcriptomic Studies of ASD Brain**

| | | | ASD | | | Controls | | | Region | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Study | Data type | Total donors | Total | Males | Females | Total | Males | Females | Frontal cortex | Temporal cortex | Occipital cortex | Cerebellum |
| Garbett et al, 2008 | Microarray, bulk tissue | 12 | 6 | 4 | 2 | 6 | 4 | 2 | No | STG | No | No |
| Voineagu et al, 2011 | Microarray, bulk tissue | 36 | 19 | 14 | 5 | 17 | 16 | 1 | BA9 | BA22, BA41-42 | No | Yes |
| Gupta et al, 2014 | RNA-seq, bulk tissue | 73 | 32 | 24 | 8 | 41 | 32 | 9 | BA10, BA44 | No | BA19 | No |
| Parikshak et al, 2016 | RNA-seq, bulk tissue | 96 | 48 | 39 | 9 | 48 | 40 | 8 | BA9 | BA22, BA41-42 | No | Vermis |
| Velmeshev et al, 2019 | RNA-seq, single-cell | 31 | 15 | 12 | 3 | 16 | 12 | 4 | PFC, ACC | No | No | No |
| Total unique donors | | 169 | 75 | 59 | 16 | 94 | 74 | 20 | | | | |

Total number of donor individuals and the brain regions assayed in each study are listed. Abbreviations: BA, Brodmann Area; STG, superior temporal gyrus; PFC, prefrontal cortex; ACC, anterior cingulate cortex.

**Transcriptomic changes in the ASD brain**

Despite relatively small sample sizes, studies of bulk brain tissue have successfully described alterations in the ASD neural transcriptome. One of the more consistently observed patterns is the elevated expression of genes associated with astrocyte, microglial, immune, and inflammatory function, which was first reported by Garbett and colleagues (15). Differential expression analysis of microarray data from six ASD-control pairs from the Autism Tissue Program (now Autism BrainNet, www.autismbrainnet.org) revealed increased expression of genes enriched for antigen-specific and cell-specific immune response, inflammation, autoimmunity, and immune-mediated cell death Gene Ontology (GO) categories in the ASD superior temporal gyrus. Subsequent, larger studies of bulk tissue using microarray (16) or RNA-seq (17,18) have recapitulated this pattern via the discovery and functional annotation of gene co-expression networks, or modules. Specifically, Voineagu and colleagues (16) identified a module, titled M16, that was enriched for genes associated with the functions of astrocytes and microglia and up-regulated in the ASD brain relative to controls. A 2014 study from Gupta and colleagues (17) used a larger sample and signed co-expression networks to resolve M16

into separate astrocyte- (Mod7) and activated microglia-associated modules (Mod5), though only the ASD-elevated expression of Mod5 was significant after multiple testing correction. The latest and largest study by Parikshak et al. (18) further expanded the resolution of this signal by identifying three modules with significantly elevated expression in ASD, including one with clear enrichment for astrocyte markers (CTX.M9) and another enriched for microglia markers (CTX.M19).

It is not known whether these putative changes in glial involvement are secondary consequences of an upstream risk exposure or whether astrocytes and microglia are involved in the primary, symptom-associated pathology of ASD. None of the studies described here observed enrichment of ASD genetic risk factors, including rare or common variants, within any of the ASD-elevated, glia/immune-associated modules, suggesting that the functional changes driving these elevated expression patterns are likely downstream from the immediate effects of genetic risk variants. Regardless of whether these changes are downstream from causal factors, it remains possible that they are upstream from (and contribute to) ASD symptoms, which would have implications for the utility of glial/immune pathways as treatment targets. Future experimental work is needed to address this possibility.

Bulk tissue transcriptomic analyses have also identified modules associated with neuronal and synaptic functions that generally show reduced expression in ASD cortex. Voineagu et al. (16) reported an ASD-down-regulated neuronal module (M12) associated with synaptic function, vesicular transport, and neuronal projection, and Gupta et al. (17), resolved M12 into three signed modules, all enriched for synaptic transmission function. Of these three, Mod6 was significantly up-regulated in ASD, while Mod1 genes, found to be associated with inhibitory ion channel activity related to synaptic transmission, were significantly down-regulated. Parikshak et al. (18) later identified three ASD-down-regulated modules (CTX.M4, CTX.M10, and CTX.M16) that also overlap with the M12 module and are enriched for neuronal

markers and synapse genes, and implicated neuronal firing rate as a key feature in the CTX.M10 and CTX.M16 modules.

In contrast to the up-regulated glial/immune genes, down-regulated neuronal/synaptic genes show some evidence of overlap with ASD genetic risk, potentially suggesting that changes in these genes' expression may be closer to ASD's causal roots. Using a permutation test to assess gene set enrichment for common variant association signal from an early ASD GWAS (26), Voineagu et al. (16) observed that M12 genes showed significant enrichment for ASD common variant risk. However, findings from Gupta et al. (17) and Parikshak et al. (18) have not supported this initial genetic risk enrichment, instead finding rare variant risk gene enrichment for ASD only in modules that did not show significant expression changes in ASD, and weak enrichment for common variant ASD GWAS signal in the ASD-*up*-regulated CTX.M20 module. These contrasting findings suggest that upstream genetic risk and downstream transcription changes affecting neurons may involve separable gene sets and functions.

Other expression changes reported in bulk tissue analyses of ASD brain include differences between brain regions, similarities between cases of different genetic etiology, and variation across individual donors. Studies that evaluated both cortex and cerebellar tissue (16,18) observed larger-magnitude expression changes in cortex, leading to a substantially greater number of significant ASD-differentially expressed genes in cortex versus cerebellum (444 vs. 2 genes (16) and 1,142 vs. 0 genes (18)), demonstrating far greater sensitivity of the postnatal cortex to transcriptional changes in ASD. Differential expression analysis comparing frontal and temporal cortex tissue found a reduced number of regionally differentially expressed genes in ASD (510 (16) and 551 genes (18) in controls, versus 8 (16) and 51 genes (18) in ASD), suggesting that cortical patterning is less distinct, and may be disrupted, in ASD. Analysis of data from nine patients with 15q11.2-13.1 duplication syndrome (dup15q) alongside samples from idiopathic ASD cases reported striking similarities in transcriptomic changes between these two groups of cases (18), suggesting convergent neurobiological changes downstream from

heterogeneous genetic risk exposures. Expression changes in individual samples, however, are more variable than group averages may suggest. While tissue from ASD donors is more likely than controls to show relatively reduced expression of neuronal/synaptic genes and elevated expression of glial/immune genes, this is not universal across all ASD samples (Figure 2). Critically, these directional expression changes do not appear to be male-specific, as female samples are similarly likely as male samples to show these same patterns.

Although differentially expressed genes from bulk tissue sequencing can be annotated to the functions of particular cell types, bulk tissue analyses are unable to definitively tease apart the cellular source(s) of these expression changes, nor can they definitively distinguish effects of cell number versus cell type-intrinsic expression changes. Single-cell RNA-seq can begin to address this unknown. Velmeshev et al. (19) published the first such data set in ASD, generating single-nucleus RNA-seq data from prefrontal and anterior cingulate cortex from ASD cases and controls aged 4-22 years. Out of 17 cell type clusters identified, protoplasmic astrocytes were the only cell type found to be relatively more prevalent in ASD than controls, suggesting that altered cell type composition may contribute to the elevated glial/immune gene expression seen in ASD brain tissue. Cell type-specific differential expression analyses run within each cluster further showed that the genes most significantly elevated in ASD were predominantly observed in protoplasmic astrocytes or microglia, while genes most significantly reduced in ASD were predominantly observed in cortical layer 2/3 excitatory neurons and VIP-positive interneurons. This study also reported enrichment for rare variant-associated ASD risk genes among differentially expressed genes that was significantly greater than expected in layer 4 and layer 2/3 excitatory neurons, and VIP- and SST-positive interneurons. Similarly, an independent analysis in amygdala (27) reported down-regulation of ASD risk genes in amygdala interneurons, but did not observe up-regulation of ASD-associated genes in microglia and astrocytes of the amygdala, suggesting that this pattern may be region-specific. These early findings are promising, and future data generation from a larger number of individual donors and

a wider range of brain regions will provide valuable insight into the molecular changes characterizing ASD at the cellular level, and how they relate to genetic susceptibility and overall ASD pathology.

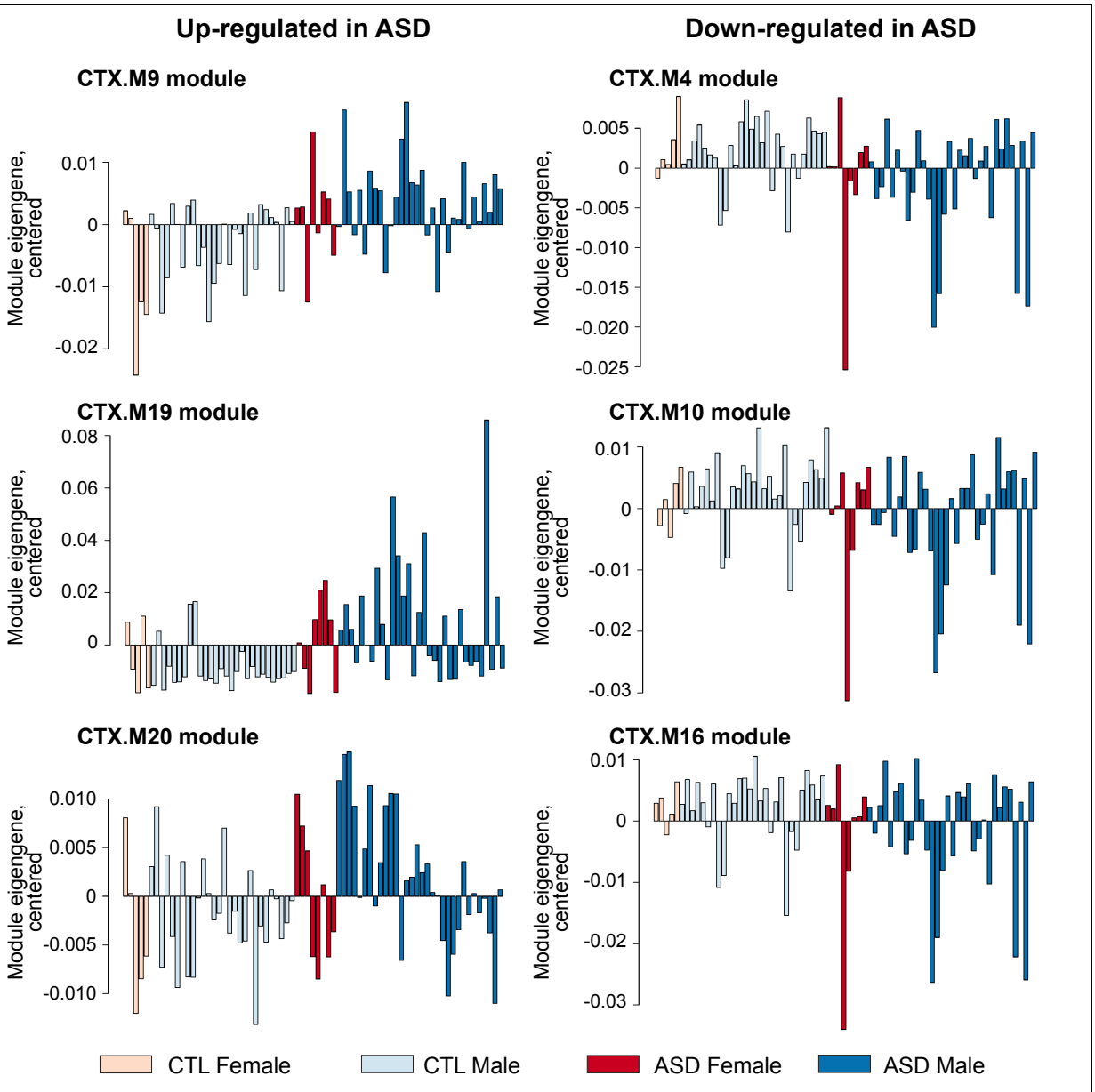


**Figure 2: ASD-associated gene co-expression modules in the temporal cortex.** Visualization of module eigengene values (*y*-axis) across temporal cortex samples (*x*-axis); within each sex-by-diagnosis group, samples are displayed in ascending age order. Modules up-regulated in ASD (CTX.M9, CTX.M19, CTX.M20) are shown in the left column and modules down-regulated (CTX.M4, CTX.M10, CTX.M16) in ASD are shown in the right column (18). Abbreviations: CTL, Control.

**Neurotypical sex-differential expression as related to ASD-control differences**

Transcriptomic alterations in ASD brain tissue as compared with controls define changes associated with the disorder state, and to elucidate sex-differential risk mechanisms for ASD, it is necessary to understand how these disorder-associated patterns intersect or interact with neurotypical sex differences (Figure 1B). A 2016 study (28) addressed this question directly, by comparing the results of a series of sex-differential expression analyses from neurotypical cortex tissue to ASD-associated gene expression changes and risk genes. Specifically, Werling and colleagues applied a linear mixed effects model to characterize sex-differential expression in three data sets: an adult discovery data set, consisting of 58 cortex samples from five male and five female donors aged 13-40 years from BrainSpan, a prenatal data set of 86 cortex samples from four male and four female donors of 16-22 post-conception weeks, and an adult replication data set of 13 cortex samples from five male and five female donors aged 16-56 years.

Genes belonging to either of the ASD-elevated, glial/immune modules defined at the time (M16 and Mod5) (16,17) were significantly more likely to show male-biased than female-biased expression in both the adult discovery and prenatal data sets, and M16 genes were also significantly male-skewed in the adult replication data. Male-biased genes in the adult and prenatal cortex also showed significant enrichment for astrocyte and microglial marker genes defined by multiple sources (29,30). Conversely, genes belonging to the ASD-down-regulated, neuronal/synaptic modules (M12, Mod1) were significantly more likely to show female-biased expression in the adult discovery data, though this pattern was not observed in the prenatal or adult replication sets. ASD risk genes, including candidate genes from the SFARIGene database (31) and genes with rare *de novo* protein-truncating or missense variants in ASD cases (32), were not enriched for sex-differential expression in any data set, consistent with a working model that the mechanisms driving ASD's sex-biased prevalence operate largely downstream from genetic risk variation (28).

A subsequent analysis of neurotypical sex-differential expression spanning the spatiotemporal range of the BrainSpan data set incorporated 594 samples from 40 donors (23 male, 17 female) aged 8 post-conception weeks to 40 years (33). Assessment of sex-differential expression patterns in cortex tissue across developmental time for the six ASD-dysregulated modules reported by Parikshak et al. (18) corroborates prior observations: all neuron-associated modules show largely sex-neutral expression across development, while astrocyte- and microglia-associated modules show male-biased expression in mid-late fetal development, with CTX.M9 additionally male-biased in adulthood (Figure 3). Notable male-biased, astrocyte-associated genes in CTX.M9 include *APOE*, as well as genes like *RERG* and *SLCO1C1* which are associated with neural responses to hormones. Consistent with CTX.M19 enrichment for microglial functions, top male-biased genes in CTX.M19 include *LYN*, *B2M*, and *RHBDF2*, which are implicated in regulation and responses to immune cell signals and processes. Further addressing prenatal sex differences, an additional study characterizing transcriptomic patterns in whole brains from 120 second trimester human donors also found 2,756 genes with sex-differential expression (34). In contrast to the findings from BrainSpan described above, these differentially expressed genes showed significant enrichment for high-confidence ASD risk genes, though the direction of sex effects was split, with 7 male-biased and 5 female-biased risk genes.

These ASD- and sex-differential expression patterns seen in cortex tissue suggest the existence of parallel shifts in neurobiological features in neurotypical males (relative to females) and in ASD (relative to controls). Male-skewed expression of astrocyte/microglial/immune genes, both separate from and linked to ASD-up-regulated expression is consistently observed, while ASD-down-regulated, neuronal/synaptic genes are variably female-skewed across data sets. In either functional category, neurotypical tissue from the more frequently affected sex (males) is transcriptionally closer to ASD than females, suggesting that glial/immune and/or neuronal biology may be involved in sex-differential risk mechanisms. Based on the direction of

effect in ASD cases versus controls, we hypothesize that increased glial function and/or cell number are associated with ASD pathobiology, while the maintenance or relative elevation of neuronal function or cell number may be protective (Figure 1C, D). Further research, including more extensive single-cell transcriptomics, is needed to determine how cell type composition and molecular function contribute to the sex differences seen in bulk tissue, and neurobiological experiments will be required to investigate the putative pathobiological and protective mechanisms involving these cell types. Importantly, the sex-balanced expression of genes linked to ASD genetic risk in cortex tissue suggests that, at least in the cortex, sex-differential risk-modulatory mechanisms operate downstream from genetic risk factors, instead of as upstream regulators of risk gene expression (28). For a condition as genetically heterogeneous as ASD, this is a hopeful observation, as it suggests that interventions targeting downstream pathology may be able to modulate symptoms in patients of diverse genetic etiologies.

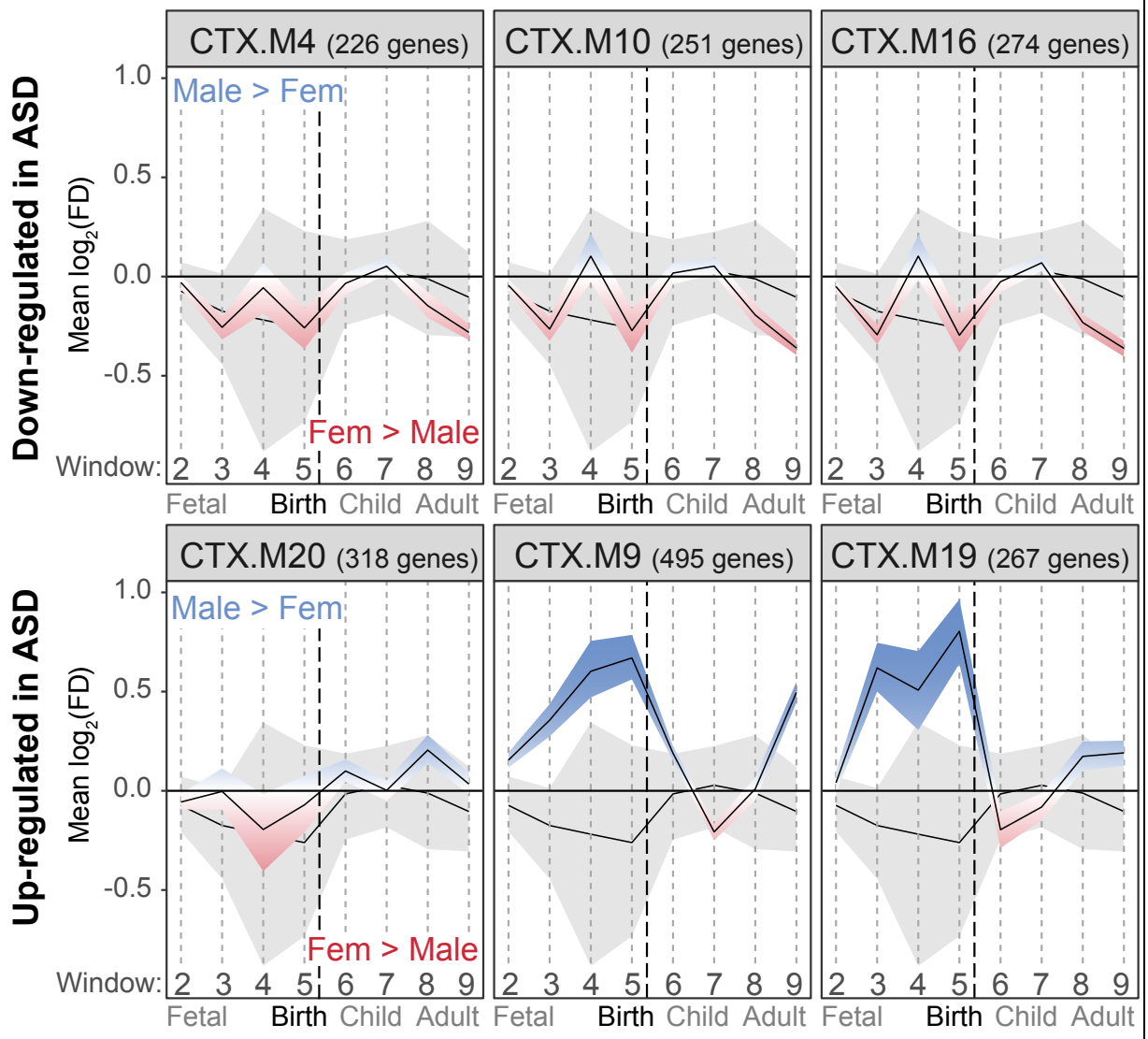


**Figure 3: Sex-differential expression of ASD-dysregulated module genes across developmental time.** The average $log_2$ fold difference (black line) observed in frontal and temporal neocortex samples from the BrainSpan resource (33) for protein-coding genes belonging to the six ASD-down- (top) or up-regulated modules (bottom) identified by Parikshak et al. (18) are displayed at each of eight developmental windows (vertical gray dashed lines). Red-blue shading notes the 99% confidence interval around the mean $log_2$ fold difference for each gene set. The median and interquartile range of the $log_2$ fold difference for all 17,222 protein-coding genes expressed in the frontal and temporal cortex samples are displayed in gray behind each modules' sex-differential expression.

**Conclusions, limitations, and future directions**

Although the studies reviewed here offer insights into ASD neurobiology and sex-differential risk mechanisms, these findings are limited by sample availability across multiple

dimensions, including sex, age, brain region, and cell type. With currently available data from only 14 female ASD donors across major transcriptome studies, we are significantly hindered in our ability to characterize sex differences between affected males and females, to assay female-specific shifts from sex-matched norms, and to identify interaction effects between sex and ASD.

This characterization effort is further complicated by age. As a neurodevelopmental but lifelong disorder, gene expression changes in the ASD brain are not static across development. Sex-differential biology is similarly dynamic, unfolding and shifting across defined stages including early sexual differentiation, puberty, and menopause. Thorough understanding of sex-differential risk and neuropathology in ASD will require making sense of the interactions between these two developmentally moving targets. Regarding early development, transcriptomic data from (presumably) neurotypical human tissue is relatively most available in the mid-fetal stage, a time when ASD risk genes are also strongly expressed and co-expressed (35,36). However, human tissue samples from late fetal and early childhood stages is sparse, and access to prenatal, presumably ASD tissue would require fetal genetic diagnoses for already rare conditions. The ASD study samples described here (Table 1), and developmentally focused data sets like BrainSpan (33) and BrainVar (37) include samples that span puberty, but all lack phenotypic information regarding donors' pubertal status and lack sufficient numbers of males and females for a well powered comparison at this stage. Adulthood is well covered by large-scale data sets such as GTEx (38), though GTEx skews elderly such that a majority of the included females are likely peri- or post-menopausal. Further challenging our ability to thoroughly characterize baseline sex differences in brain transcriptomes, females donors are nearly universally underrepresented across these data sets: for nervous system tissues, the male:female ratio is approximately 2.5:1 in GTEx, 1.3:1 in BrainSpan, and 1.4:1 in BrainVar overall, with 2.4:1 for postnatal samples.

Existing transcriptome data for ASD brain is also regionally limited, focused predominantly on the cortex and cerebellum. ASD risk genes are strongly expressed in cortex tissue (35,36) and cortical neurons (10), and transcriptomic changes are evident in ASD (15–19) and neurotypical male (28) cortex, but ASD-associated changes have not yet been characterized in many subcortical brain regions. Therefore, it is not known whether ASD etiology and sex-differential biology interact directly in the cortex, or if other brain regions play a larger role in sex-differential risk mechanisms. A rich body of neuroendocrinology and neurobehavioral work has detailed cellular and morphological sex differences in subcortical brain regions such as the hypothalamus and the bed nucleus of the stria terminalis (BNST) (39). These subcortical regions could also be directly and robustly involved in ASD-associated pathology, or ASD risk could be modulated through neural circuits that physically connect sex-differential regions to ASD-affected regions. However, bulk tissue transcriptomics in these regions have been largely deprioritized in the past due to their anatomical complexity (e.g. multiple small and functionally distinct hypothalamic nuclei). Single-cell and spatial transcriptome technology are now facilitating the characterization of these challenging regions (40) and can be applied to characterize, or rule out, their involvement in sex-differential risk for ASD. Whether in subcortical regions or cortex, single-cell analyses will be essential to better delineate the cell types and/or cellular states that are directly involved in ASD pathology and sex-differential biology, and the likely mechanisms by which these cell types contribute to phenotype. Already, single-cell transcriptome data in cortex has suggested possible roles for both cell type composition and cell type-specific changes in ASD-associated gene expression patterns (19), but additional data generation is needed to validate and refine these findings.

Moving forward, *in vivo* and *in vitro* model systems will also be critical tools for exploring the transcriptomic, functional, and behavioral consequences of ASD-associated genetic variants in both sexes, at precisely selected developmental stages, and in multiple brain regions. Comparable to human cortex, transcriptomic characterization of several mouse models of ASD

risk loci, including *Chd8*, *Arid1b*, *Shank3b*, and 16p11.2 deletion, finds downregulation of gene modules associated with neuronal functions of axon guidance and glutamatergic neurotransmission (41–43). Heterozygous mutant lines for *Chd8,* a top ASD risk gene, also consistently show reduced expression of other known ASD risk genes (43–45), and one study observed enrichment of immune response-associated, ASD-up-regulated genes in genes up-regulated in *Chd8*$^{+/-}$ mutants (44), while another observed enrichment of ASD-down-regulated, neuron-associated genes among *Chd8*$^{+/-}$-down-regulated genes (45). These patterns validate, in part, changes reported in human brain, and demonstrate the potential of animal models for linking specific genetic risk variants to broader downstream transcriptomic consequences. However, these studies have so far focused on disorder-associated changes independent from sex effects, which must be directly addressed in future analyses. Animal work also has yet to demonstrate if elevated expression of glial genes is cause or consequence of ASD pathology. Further experimental work that disrupts “hub genes” central to the altered co-expression networks, or that targets the function of the specific cell types linked to these modules, could help to determine if increased glial function (or decreased neuronal function) contributes to, or is simply correlated with, phenotypic changes in brain function and behavior.

Human induced pluripotent stem cells (hiPSCs) derived from ASD patient samples are another useful model, with distinct advantages of being human cells, more readily available than brain tissue, and able to model very early developmental stages. Findings from transcriptomic analyses of hiPSCs differentiated into excitatory and inhibitory neurons follow those from post-mortem brain, including dysregulation of gene networks involved in neuronal differentiation, development of neuronal projections and patterning, and synaptic signaling (46,47). However, the neural cell types that can be derived from hiPSCs are currently limited, and it is not yet known which dimensions of sex-differential biology can be recapitulated in a cell culture system, either with or without the application of sex steroid hormones. Exploration of the experimental

conditions required for astrocyte- or microglia-neuronal co-cultures, and for appropriately modeling sex differences, can advance the utility of hiPSCs for experimental work in this area.

Across the board, from human studies to model systems, limited availability of female ASD samples and limited attention to sex as a biological variable have hindered our ability to describe and understand sex differences in ASD pathology and risk. Further characterization of sex differences in the ASD disorder state and in the general population will be important for ASD and for other neuropsychiatric and neurological disorders with sex-differential prevalence or presentation, and will better set the stage for experimental work delineating mechanisms. Toward this goal, we encourage individual laboratories, funding agencies, and journals to promote or enforce the inclusion of female samples in all human and model work, and to push toward the analysis of sex-balanced sample sets where possible, even for conditions that are sex-skewed. Focused attention on this question for transcriptomic studies specifically, and across preclinical and clinical research domains more generally, is important, as understanding sex differences in disorder state and risk has great potential for uncovering fundamental aspects of disorder pathology and for the design of therapeutics to benefit both male and female patients.

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Lee T. Kissel and Donna M. Werling declare that they have no biomedical financial interests or potential conflicts of interest to disclose.

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**CHAPTER I**

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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, though 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 two independent bulk RNA-seq data sets generated from cortex of 273 prenatal donors without known neuropsychiatric disorders. To assess 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 identify 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, do not preferentially overlap with sex-DEGs. We identify one male-specific co-expression module enriched for immune signaling that is unique to one input data set.

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

**Introduction**

Autism spectrum disorder (ASD) is a pervasive neurodevelopmental disorder defined by deficits in social communication and repetitive, restrictive behaviors and currently affects about 1 in 36 children in the United States (1). Despite this high prevalence, underlying biological mechanisms are not fully understood and effective therapeutic interventions are lacking. Both common inherited and rare genetic variants have been demonstrated to contribute to ASD symptoms, including several common variant loci (2) and over 100 ASD-associated genes identified by exome sequencing (2–6). Another key contributor to ASD diagnosis is sex: ASD is consistently diagnosed 3 to 5 times more frequently in males than females, making ASD one of the most sex-skewed neuropsychiatric conditions (7). This strong male-biased prevalence suggests the existence of male-specific risk and/or female-specific protective mechanisms (8,9). 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, prior to 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 cytoarchitecture of certain brain regions (e.g., amygdala, hypothalamus) (10–13), behaviors (e.g., aggression, social behaviors, and reproductive behaviors) (8,12–14), and prevalence of neuropsychiatric disorders (e.g., ASD, schizophrenia, major depressive disorder) (7,15,16). Studies of human postnatal brain tissue have revealed sex-biased expression of sex chromosome genes and, to a lesser extent, autosomal genes (11,17–19). 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 mid-gestation, due to the effects of prenatally secreted sex hormones (10) or

to the functions of sex chromosome genes (8,10,13). These early life programming events impact neural structure, function, and behavioral phenotypes later in life (11,12,14,17,18,20,21).

To date, the delineation of transcriptomic sex differences during human prenatal neurodevelopment, particularly in regions implicated in human neuropsychiatric conditions like the prefrontal cortex (22), have been limited by small available sample sizes. A 2016 study of transcriptome expression by microarray (23) of the prenatal neocortex from just 8 donors (4 females, 4 males, 16-22 post-conception weeks (PCW)) reported prenatal, male-biased enrichment for genes related to glial/immune functioning, paralleling transcriptomic changes reported in ASD brains (24,25). A subsequent, expanded analysis of samples from the BrainSpan resource again observed male-skewed expression of gene sets annotated to astrocyte and microglial function, particularly during prenatal development (26,27). Of note, ASD-associated risk genes from exome sequencing studies were not preferentially sex-differentially expressed in these reports, suggesting 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 found 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 (28). The brain region(s) or cell type(s) driving these differences in 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, though with the caveats of small donor number, microarray-based quantifications, or nonspecific tissue dissections (23–25,27,28). 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) data sets derived specifically from human

cortex. We have quantified transcriptome-wide sex-differential expression levels and co-expression differences in two, large data sets of prenatal cortex tissue derived from 273 donors (BrainVar, dorsolateral prefrontal cortex (DLPFC), 87 donors (29) and UCLA, cortex, 186 donors (30)). Meta-analysis of both data sets identifies 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 observe limited evidence for sex-specific gene co-expression in prenatal tissue, finding one male-specific module present only in the BrainVar data set. These results define a set of genes that are prenatally sex-differentially expressed in human cortex from a large sample of individual donors, providing a resource for the study of sex-influenced developmental conditions. Further, the observation that known ASD risk genes are not sex-differentially expressed in prenatal cortex tissue suggests that sex-differential risk mechanisms may influence ASD risk genes' expression 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**

**Prenatal RNA-sequencing data**. RNA-seq data from human donors aged 14-21 PCW were used, including DLPFC samples from the BrainVar project (29) (**Table S1**) and whole cortex samples from a UCLA-based collection (30) (**Table S2**). Preprocessing steps are detailed in the Supplement. 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 (v2.0.9) (31), using cell type signatures from prenatal single nuclei RNA-seq (32). Male and female estimates per cell type were compared within each data set using the two-sided Wilcoxon test. Cell proportion estimates were further applied to deconvolve and estimate gene

expression associated with specific cell types per sample by bMIND (33), and limma voom (34,35) was used to test for sex differences in estimated cell type-specific expression. Further details in Supplement.

**Differential expression and meta-analysis.** Differential expression analyses were performed separately in both data sets using limma voom (v3.52.4) (34,35) on expressed genes under a regression model including age, RNA-seq batch, RIN, post mortem interval (PMI), estimated intermediate progenitor cell (IPC) proportion, and surrogate variables (SVs; Supplement). Differential expression results from both datasets were subsequently combined for meta-analysis using MetaRNASeq (36). We defined sex-DEGs as genes with Benjamini-Hochberg false discovery rate (FDR)≤0.1. Within each data set, 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 interaction. Additional details included in Supplement.

**Functional annotation.** We tested sex-DEGs for enrichment of ASD risk genes as implicated by rare and common variants, genes with ASD-dysregulated expression in brain, genes associated with neuropsychiatric phenotypes, cell type markers, and databases of biological functions using two-sided Fisher's exact tests and Gene Set Enrichment Analysis ((37); further details in Supplement and **Table S3**).

**Co-expression analyses.** We applied Weighted Gene Co-Expression Network Analysis (WGCNA, v1.71) (38) separately to male and female samples from each data set, and then compared female and male modules using module preservation analysis in the WGCNA R package (38,39). 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). 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 contributing to co-expression differences between sexes, we also applied the sparse leading eigengene driven (sLED) test to expressed genes from each data set (40–42). We then prioritized two sets of genes with non-zero leverage in the co-expression network: 1) primary genes, which cumulatively account for 90% of leverage in the network; and 2) secondary genes, which account for the remaining 10%. We compared primary and secondary genes to all sex-specific WGCNA modules and ran functional annotation with gProfiler (43). Additional information in Supplement.

## Results

**Sex-differential expression in the prenatal human cortex.** We first quantified sex-differential gene expression in two independent data sets from mid-fetal (14-21 PCW) cortex tissue from de-identified donors without known neurological or psychiatric disorders: BrainVar, including 87 DLPFC samples from 41 male and 46 female donors (29) and UCLA, including 186 cortical tissue samples from 101 male and 85 female donors (30). Estimates of the proportions of 10 prenatal cortical cell types in the bulk tissue samples by dtangle (Methods) (32) showed no significant sex differences in either data set, but highlighted a systematic difference between data sets in cell type representation, likely stemming from differences in cortical dissection depth (BrainVar: cortical plate; UCLA: whole cortical wall; **Fig. 1A & B**). To adjust for this, we included IPC proportion as a covariate for differential expression alongside age, RNA-seq batch, RIN, PMI, and surrogate variables (Methods).

To determine the compatibility of BrainVar and UCLA data for meta-analysis of sex-differential expression, we assessed sex-DEGs identified in one data set for their expression differences in the other data set by estimating pi0 and pi1 (Supplement). Of sex-DEGs (FDR≤0.1)

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 BrainVar and UCLA identifies 49 genes (28 X chr, 19 Y chr, 2 autosomal) that meet FDR≤0.1 independently in both data sets, and common functional enrichments driven by sex chromosomal genes ('Y-linked inheritance', 'X-linked inheritance', 'estrogen metabolism' and 'sulfuric ester hydrolase activity'; see Supplement).

Meta-analysis of sex-differential expression in BrainVar and UCLA identifies 106 sex-DEGs at FDR≤0.1, of which 101 genes have consistent effect directions in both data sets (43 X chr, 18 Y chr, 40 autosomal; **Table S4**). Significant sex-DEGs exhibit consistent sex effects between data sets, as evident by highly correlated $\log_2$FC (fold change) estimates for all sex-DEGs (Pearson r=0.98, 95% CI 0.977-0.990, p=1.19E-76) and autosomal sex-DEGs (Pearson r=0.83, 95% CI 0.701-0.908, p=3.21E-11; **Fig. 1C**). Sex effects for all tested X chromosome genes are also strongly correlated (Pearson r=0.88, 95% CI 0.860-0.899, p=9.83E-167; **Fig. 1D**).

Among the 101 sex-DEGs with consistent effect directions, Y chromosome genes and *XIST* show the largest expression differences while autosomal effects are more subtle (**Fig. 1C, D**). On the sex chromosomes, there are a total of 49 protein-coding sex-DEGs (FDR≤0.1), including 38 X chromosome genes (28 female-biased, 10 male-biased) and 11 on the Y chromosome (all male-biased). Genes with prior evidence of X inactivation escape (XIE) (44) show higher expression in females (21 XIE-high probability and 5 XIE-low probability genes at FDR≤0.1; Supplement, **Table S5**) while several genes in the pseudoautosomal regions (PARs) show male-biased expression (8 PAR genes at FDR≤0.1), consistent with an extension of X chromosome 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 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 (45–47) and GWAS signals for ASD and schizophrenia (48,49). Noncoding autosomal sex-DEGs consist of 2 pseudogenes, 6 lincRNA genes, and 1 antisense gene (**Table S4**).

We further reasoned that age may interact with sex-differential mechanisms during this mid-fetal developmental window, when gonadal testosterone levels in males are rising (20). However, we observed largely consistent age effects on gene expression between males and females in both BrainVar (union of sex-specific age-DEGs, Pearson r for beta coefficients =0.78, CI 0.765-0.794, p<2.2E-16) and UCLA (union of age-DEGs, Pearson r=0.92, CI 0.922-0.927, p<2.2E-16) (**Fig. 2A, 2B; Table S6,S7**). Two age-DEGs showed opposing directions of effect in males and females in both BrainVar and UCLA: protein-coding *CRISPLD1* expression rises in females and falls in males with increasing developmental age, and lincRNA *CTD-2587M2.1* falls in females and rises in males (**Fig. 2A, 2B**). We also detected 28 genes (1 X chr, 27 autosomal) with significant age-by-sex interaction in UCLA (FDR≤0.1), while 0 genes reached significance in BrainVar (**Table S6, S7**).

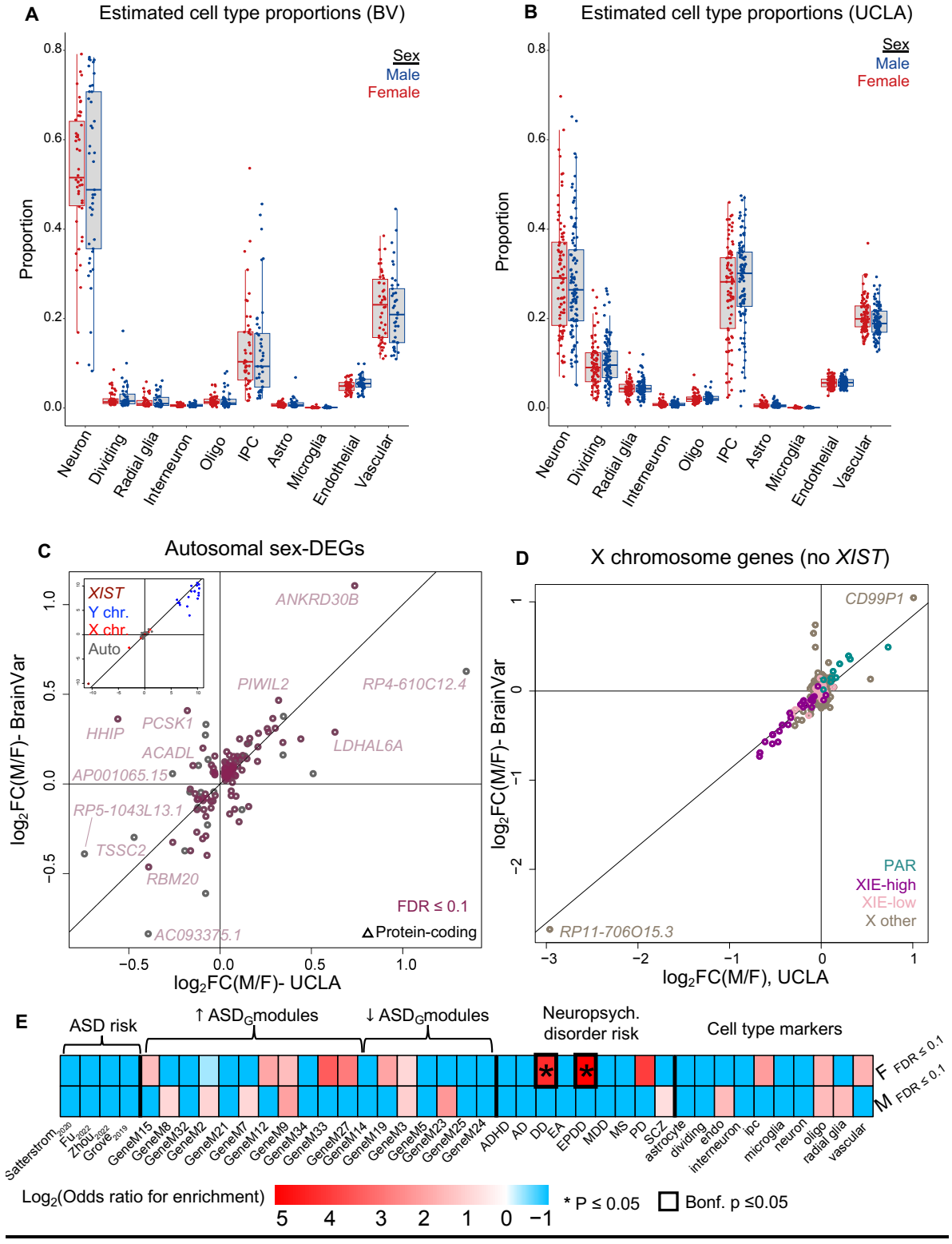


Fig 1: Sex-differential patterns of gene expression in the prenatal human cortex. (A-B) Estimated proportions of ten cell types in male (blue) and female (red) cortex in the BrainVar data set (A) and UCLA data set (B). Each dot represents a specific cell type estimate for an

individual sample. (C) Scatter plot of log2 fold changes from individual data sets for autosomal genes within the top 200 differentially expressed genes ranked by p-value from meta-analysis using the full model (Pearson r=0.62, p-value =1.01E-16). Point color denotes significance at FDR≤0.1(deep red). Point shape denotes protein coding genes (triangle) and noncoding genes (circle). Inset: Fold changes from all top 200 differentially-expressed genes (Pearson r=0.98, p-value =3.57E-151). (D) Scatter plot of log2 fold changes from individual data sets for all X chromosome genes (Pearson r=0.88, p-value =9.83E-167). Colors indicate specific groups of X genes. (E) Patterns of sex-DEG enrichment from meta-analysis results for 4 ASD risk gene sets (2,6,50,51), ASD-associated modules (52), 9 disease risk gene sets (29), and 10 cell types (32) using two-sided Fisher's exact test. $F_{FDR}$ = Female-biased FDR≤0.1; $M_{FDR}$ = Male-biased FDR≤0.1; BV = BrainVar; Oligo = oligodendrocyte; IPC = intermediate progenitor cell; Astro = astrocyte; sex-DEGs = sex-differentially expressed genes; PAR = pseudoautosomal region; XIE = X inactivation escape; ASD = autism spectrum disorder; DD = developmental delay; ADHD = attention deficit hyperactivity disorder; EPDD = epilepsy and developmental delay; EA = educational attainment; SCZ = schizophrenia; MDD = major depressive disorder; MS = multiple sclerosis; PD = Parkinson's disease; AD = Alzheimer's disease; $ASD_G$ modules = autism spectrum disorder- associated modules (52).

**Functional enrichment of sex-DEGs.** Consistent with cell type proportion estimates (**Fig. 1A, 1B**), neither male- or female-biased sex-DEGs are enriched for cell type markers (**Fig. 1E**). We also do not find significant enrichment for rare variant-associated ASD risk genes from multiple exome-sequencing studies (6,50,51) nor for common variant-associated ASD risk genes from GWAS (2); in fact, 0 sex-DEGs overlap with any of these gene sets (**Fig. 1E**, **Table S8**). This pattern agrees with previous studies of human cortex (23,27) 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 as detectable in bulk tissue. We also find limited evidence for sex-differential expression of ASD risk genes within specific cell types as estimated from bulk tissue data by bMIND (Methods; **Tables S9, S10**): risk genes *GIGYF1* (6,50,51) and *PPP1R9B* (6) have significantly male-biased expression in neurons from UCLA only, and *MAPT* (2) and *PPP1R9B* and *PTK7* (6) have 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 ASD brain provide a molecular

signature of such downstream effects of ASD risk factors and disorder pathology. To assess this second hypothesis, we compared sex-DEGs to ASD-impacted co-expression modules defined in all lobes of post-mortem human cortex (24,52) or in frontal and temporal cortex (24,52). We do not find significant enrichment for female- or male-biased sex-DEGs in any ASD-dysregulated modules (**Fig. 1E, Table S8**).

Beyond ASD, we observe a significant female-biased enrichment of risk genes for developmental delay (DD) and DD with epilepsy (EPDD) (DD OR=19.32, p=6.7E-04, $p_{adj}$=0.013, 3 genes overlap; EPDD OR=36.14, p=0.0017, $p_{adj}$=0.034, 2 genes overlap; **Fig. 1E**, **Table S8**). Female-biased DEG overlap with *SMC1A* and *DDX3X*, two X chromosome genes reported to escape XCI (44), drive these results. Additionally, *DDX3X* and *USP9X* overlap with rare variants that show replicable, female-preferential DD risk in females (53). Risk genes for other sex-influenced diagnoses, such as schizophrenia, major depressive disorder, multiple sclerosis, and Alzheimer's disease, are not significantly enriched for sex-DEGs (**Fig. 1E**).

Threshold-free enrichment testing for sex-skewed expression of gene sets of interest by GSEA (37) finds nominal enrichment for female-biased expression of ASD-upregulated module M27 (Normalized Enrichment Score (NES)=-0.5304, p=0.038, FDR=0.187), interneuron markers (NES=-0.413, p=0.094, FDR=0.389), and EPDD risk markers (NES=-0.576, p=0.033, FDR=0.244). We do not observe enrichments for any tested gene set toward male-skewed expression (**Table S11**).

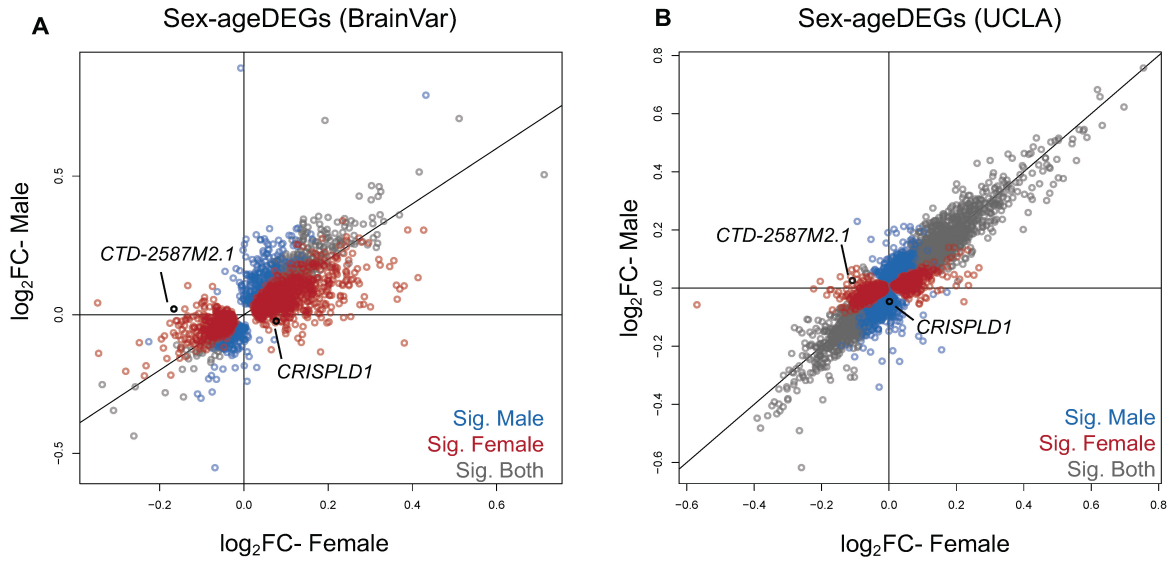


Fig 2: Sex-shared and sex-specific age-DEGs in the prenatal cortex. (A-B) Scatter plot of log2 fold changes from females (x-axis) and males (y-axis) in two individual data sets (BrainVar: A; UCLA: B) of age-DEGs (FDR≤0.1). Point color denotes the sex(es) in which each gene reached FDR≤0.1 for the effect of age. Sig. Male = Genes with FDR≤0.1 for age only in males. Sig. Female = Genes with FDR≤0.1 for age only in females. Sig. Both = Genes with FDR≤0.1 for age in both sexes.

**Sex-specific co-expression patterns in the prenatal cortex.** Using WGCNA (38), we defined 14 co-expression modules in females and 11 modules in males from BrainVar (**Fig. 3A, Fig. S1 Table S12**), and then grouped genes from similar male and female modules based on gene content overlap and expression similarity across samples (Methods, Supplement; **Fig. 3A, Fig. S2, S3, Table S13**). Modules that did not have a clear counterpart in the network of the other sex included female modules $mod22_F$, $mod16_F$, $mod12_F$, $mod11_F$ and male module $mod20_M$. Sex-shared module group 7 enriches 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 (**Fig. 3A**). However, consistent with sex-DEGs, no sex-specific modules were enriched for ASD risk genes (**Fig. 3C**), while $mod11_F$ is enriched for the ASD-elevated, ribosomal function-associated GeneM27 module (**Fig. 3C, Table S15**; OR=106.37, $p_{adj}$=1.07E-64). Module preservation analysis comparing male and female network structure

identifies mod20$_M$ as poorly preserved in females, indicating male-specific co-expression for mod20$_M$ genes ($Z_{summary}$=8.7; **Fig. 3B**). Of note, mod20$_M$ genes show substantially lower average expression than genes in other modules (**Fig. S4**). Mod20$_M$ does not show any cell type marker or ASD-associated module enrichments but is enriched for GO terms related to immune responses and signaling (**Fig. 3C, D; Tables S14, S15**). Top hub genes within mod20$_M$ include transcription factors *HOXC4* and *PRDM13* (54), and two protein-coding genes with rising expression across prenatal development (29), *SLC5A9* and *TUBA4B*.

We further applied the sLED algorithm (40) to identify individual genes with strong influence on co-expression structure differences between sexes (Methods). In BrainVar, we identify 915 genes with non-zero leverage on network sex differences, including 409 “primary” genes that collectively account for 90% of the variability in network correlation structure between sexes (**Table S16**). Primary genes overlap substantially with the mod20$_M$ module (396 of 409 genes) and are enriched for GO terms related to cytokine production and immune signaling (**Table S16**).

Co-expression patterns differ in the UCLA data set (**Fig. S5**). Module preservation analysis for 20 modules defined in females and 21 modules defined in males does not identify any sex-specific modules (**Fig. S6**). sLED identifies 1,103 genes with non-zero leverage on network sex differences, including 354 primary genes. UCLA primary genes do not preferentially overlap with the BrainVar mod20$_M$ module, and top GO enrichments point to protein binding and biosynthetic and metabolism processes (**Table S17**). These differences in co-expression structure may stem from discrepancies in cortical dissection between BrainVar (DLPFC cortical plate) and UCLA (whole cortex, cortical wall).

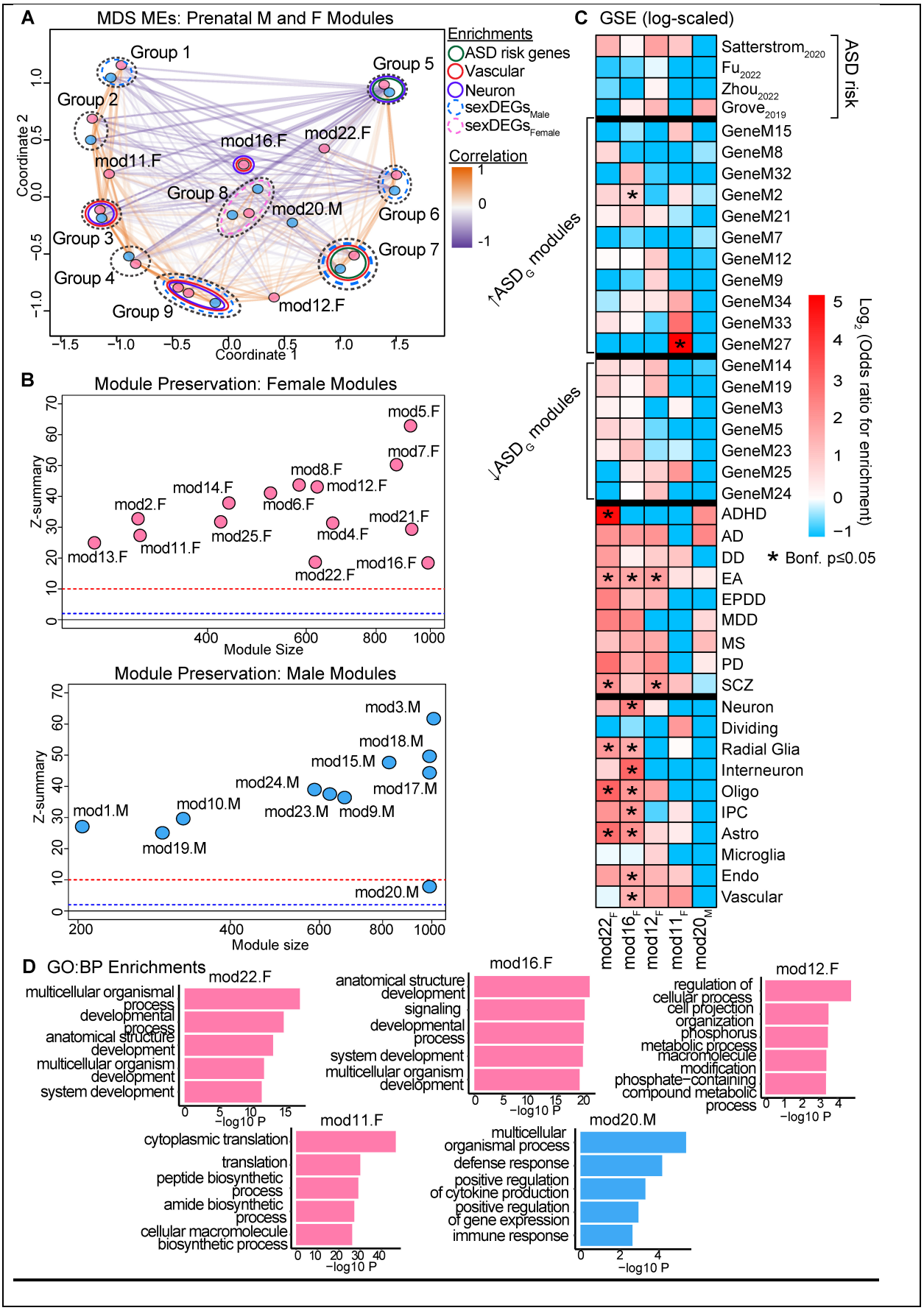
A
MDS MEs: Prenatal M and F Modules
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
mod11.F
mod16.F
mod22.F
mod20.M
mod12.F
Coordinate 1
Coordinate 2
Enrichments
ASD risk genes
Vascular
Neuron
sexDEGs Male
sexDEGs Female
Correlation
B
Module Preservation: Female Modules
Z-summary
Module Size
mod5.F
mod7.F
mod8.F
mod12.F
mod14.F
mod6.F
mod2.F
mod21.F
mod25.F
mod4.F
mod11.F
mod13.F
mod22.F
mod16.F
Module Preservation: Male Modules
Module size
mod3.M
mod18.M
mod15.M
mod17.M
mod24.M
mod10.M
mod23.M
mod9.M
mod1.M
mod19.M
mod20.M
C
GSE (log-scaled)
Satterstrom2020
Fu2022
Zhou2022
Grove2019
ASD risk
↑ASDG modules
↓ASDG modules
GeneM15
GeneM8
GeneM32
GeneM2
GeneM21
GeneM7
GeneM12
GeneM9
GeneM34
GeneM33
GeneM27
GeneM14
GeneM19
GeneM3
GeneM5
GeneM23
GeneM25
GeneM24
ADHD
AD
DD
EA
EPDD
MDD
MS
PD
SCZ
Neuron
Dividing
Radial Glia
Interneuron
Oligo
IPC
Astro
Microglia
Endo
Vascular
mod22F
mod16F
mod12F
mod11F
mod20M
Log2 (Odds ratio for enrichment)
* Bonf. p≤0.05
D
GO:BP Enrichments
mod22.F
multicellular organismal process
developmental process
anatomical structure development
multicellular organism development
system development
−log10 P
mod16.F
anatomical structure development
signaling
developmental process
system development
multicellular organism development
mod12.F
regulation of cellular process
cell projection organization
phosphorus metabolic process
macromolecule modification
phosphate−containing compound metabolic process
mod11.F
cytoplasmic translation
translation
peptide biosynthetic process
amide biosynthetic process
cellular macromolecule biosynthetic process
mod20.M
multicellular organismal process
defense response
positive regulation of cytokine production
positive regulation of gene expression
immune response

Fig. 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 Fig. S3 for grouped module IDs. Enrichments for sex-DEGs, ASD risk genes, vascular cell markers, and neuronal markers with adjusted p-value ≤ 0.05 by two-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, ASD-associated modules, disease risk gene sets, and cell type markers for non-grouped, sex-differential modules by two-sided Fisher's exact test. (D) Top Gene Ontology Biological Process terms for each non-grouped, sex-differential module of interest. Oligo = oligodendrocyte; IPC = intermediate progenitor cell; Astro = astrocyte; Endo = endothelial cell; sex-DEGs = sex-differentially expressed genes; ASD = autism spectrum disorder; DD = developmental delay; ADHD = attention deficit hyperactivity disorder; EPDD = epilepsy and developmental delay; EA = educational attainment; SCZ = schizophrenia; MDD = major depressive disorder; MS = multiple sclerosis; PD = Parkinson's disease; AD = Alzheimer's disease; GO:BP = Gene Ontology Biological Processes; $ASD_G$ modules = autism spectrum disorder- associated modules (52).

**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 underlying mechanisms, it is crucial to first understand baseline biological differences between developing male and female brains, prior to 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 (28,44), sex chromosome genes are strongly differentially expressed. Genes that escape X chromosome inactivation exhibit consistent female-biased expression and Y chromosome and several PAR genes show male-biased expression. Sex differences in expression of autosomal and other X chromosome genes are also identifiable with our large, combined sample size and meta-analysis approach, though the magnitude of expression differences are modest. Our findings support prior studies reporting predominantly sex-neutral expression patterns in the human developing cortex (23,27).

We also revisited two hypotheses regarding the interaction of sex-differential biology and ASD risk (23), now with larger data sets: (1) ASD risk genes are sex-differentially regulated; and (2) Sex differences interact with ASD etiological pathways that are affected downstream from variants in risk genes. Parallel to previous research, we find that ASD risk genes, implicated by either rare or common variants, do not show significant enrichment for sex-skewed expression levels in prenatal cortex tissue. However, in contrast to prior reports, we do not find male-biased DEGs to be significantly enriched for ASD-upregulated groups of genes nor female-biased DEGs enriched for ASD-downregulated modules.

We note several challenges and limitations regarding comparison of prenatal sex-DEGs to ASD risk gene sets and ASD-dysregulated modules. First, currently known ASD risk genes from exome sequencing are entirely autosomal, due to challenges in statistical power for risk gene discovery on the X chromosome in a male-skewed condition like ASD, where the majority of cases have only one, maternally inherited X chromosome, while *de novo* variants that drive gene-based association results arise primarily on paternal chromosomes. GWAS studies for ASD do not find genome-wide significant signals on the X and similarly focus on autosomes (2). Sex chromosome genes including Y, XIE, and PAR genes, show consistently sex-differential expression, and it is possible that a subset of these genes exert sex-differential influence on disorder-associated pathways. Sex chromosome gene have been understudied in human genetics research (55), and future research focusing on the X and Y chromosomes may identify additional ASD risk genes, at which point the hypotheses tested here will need to be re-evaluated.

Regarding ASD-dysregulated gene expression, previous studies report enrichment for male-biased DEGs from prenatal and postnatal cortex in co-expression modules with elevated expression in ASD (23,26); here, meta-analysis of BravinVar and UCLA does not replicate this pattern. However, further exploration finds significantly male-skewed expression of glia- and immune-annotated, ASD-elevated module ctx.m19 (24) by GSEA in BrainVar only (NES=2.07, FDR=1.7E-03), though adjustment for SVs eliminates this signal. This suggests that data set

features and choices in data handling can challenge interpretation of the patterns reported, particularly for small and variable effects like sex differences. Increasingly larger sample sizes with consistent tissue dissection and processing will be key for identifying replicable sex effects. It is also possible that a distinct set of genes may have altered expression in the prenatal ASD brain, and that the comparison of prenatal sex-DEGs to postnatally defined ASD-altered expression patterns does not capture the key biological processes at play.

In BrainVar, genes in the male-specific $mod20_M$ co-expression module are enriched for functions related to immune/inflammatory responses and signaling. We find no sex difference in estimated microglial or astrocyte cell proportion between males and females, suggesting 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 (24,52). Estimated cell type-specific sex differences from prenatal bulk cortex do not demonstrate strong sex differences in microglia or astrocytes, though the estimated low abundance of these cell types likely impacts power. We also note that $mod20_M$ module genes are very lowly expressed and a comparable module is not evident in the UCLA data set. Mechanisms such as variable transcriptional silencing across brain regions and dynamic changes in inflammatory/immune state during development could contribute to these noisy signals. Nonetheless, this putative pattern of male-skewed neuroimmune biology aligns with several recent lines of evidence implicating the role of the immune system and inflammation in the male-skewed development of ASD-related behaviors (56–63).

A key limitation pertaining to the evaluation of both ASD risk and ASD-dysregulated gene sets is that our observations of sex-differential expression are limited to prenatal, mid-gestation 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 (12,17,18,64–67), such as the hypothalamus and amygdala (65). ASD-associated genes may also be sex-differentially expressed within specific

cortical cell types. Though we find several ASD risk genes with estimated sex-differential expression in neurons and endothelial cells by cell type deconvolution, these patterns are not consistent between data sets and require direct measurement in single or sorted cells. A recent single cell RNA-seq analysis of developing brain finds that ASD risk genes with male-biased expression are predominantly expressed in microglia, while those with female-biased expression are 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 not assayed here, or transiently sex-differentially regulated within the tested mid-gestation window. Finally, sex-differential factors may influence ASD etiology via mechanisms not addressed here, such as by sex-differential splicing regulation, or by translation regulation or post-translational modifications of ASD risk genes.

Definition of transcriptome-wide sex differences in gene expression level and co-expression structure in the mid-fetal human cortex is fundamental to understanding baseline sex differences in the human brain, and necessary to delineate biological processes contributing to sex-biased conditions like ASD. Future studies of sex-differential expression of genes and isoforms in additional brain regions, developmental stages, and 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.

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**Author Contributions**

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**CHAPTER II**

# Sex-differential gene expression in the ASD brain and relationship to risk-associated mechanisms

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**Introduction**

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). ASD is also consistently diagnosed 3 to 5 times more frequently in males than females, suggestive of a prominent role for sex-differential biology in the risk and presentation of ASD (2). Despite its high prevalence, current understanding of the underlying biological mechanisms of ASD has not yet led to the development of effective therapeutic interventions. Common inherited, rare, and *de novo* genetic variants have been identified as associated risk factors, implicating over 100 individual genes in risk of ASD, which converge on common neurodevelopmental pathways (3–5). Comparisons of brain tissue samples from ASD and non-ASD control donors in bulk RNA sequencing studies have revealed patterns of reduced expression of neuronal/synaptic genes, several of which are also risk-implicated variant genes, and elevated expression of inflammatory/immune-related genes in the ASD cortex (6–8). Furthermore, single nucleus sequencing of ASD brain tissue detects an overlap of ASD-associated risk genes and down-regulated expression of neuronal genes in excitatory and interneuron populations in the ASD brain (9), further implicating dysregulation of neuronal cells and signaling in ASD.

Given the sex difference in ASD prevalence, factors specific to male or female biology may modulate the impact of genetic or other risk factors for ASD, leading to relatively greater vulnerability in males and ultimately higher ASD prevalence among males (10–13). Based on this hypothesis, identification of points of convergence where sex-influenced phenomena intersect with disparities between ASD cases and control individuals could unveil mechanisms driving the pronounced sex bias in ASD. Genomic data sets provide an unbiased framework for comparing sex effects and ASD effects. Previous work comparing independent analyses of ASD effects and of sex differences identified overlaps of neurotypical male-skewed and ASD-

upregulated genes related to glial cells and functions, and female-skewed expression of ASD-downregulated genes associated with neuronal cells in the human cortex (14,15).

These findings suggest a hypothesis that neurotypical males may be more phenotypically similar to ASD individuals than are neurotypical females, and that this male-ASD similarity may stem from the functions of specific cortical cell types. This pattern aligns with the general concept of the “extreme male brain theory” (16), in that the expression level of certain cell type-associated genes in ASD cortex is an exaggeration of the expression difference in males relative to females: some genes elevated in males are further elevated in ASD, and some genes with reduced expression in males (elevated in females) are further reduced in ASD. We hypothesize that genes showing increased expression levels among male controls compared to females that are further elevated in ASD may be linked to mechanisms of male-skewed vulnerability and increased risk for developing ASD. Conversely, genes displaying heightened expression levels among female controls that are reduced in ASD may be linked to mechanisms of female-skewed protection against an ASD phenotype.

Among ASD individuals, neurotypical sex differences may persist or be altered. Neuroimaging studies investigating sex-differential neuroanatomy have observed attenuation of neurotypical sex differences in ASD consistent with a shift toward male-typical neural anatomy and connectivity in ASD females (“masculinization”; auditory cortex, cerebellum, frontal-temporal white matter pathways (17–19)), and with a shift toward female-typical neural anatomy and connectivity in ASD males (“feminization”; thalamus, parietal cortex (BA6), somatosensory-motor network (17–19)), depending on the region. It is not known if similar patterns of sex difference attenuation are evident in gene expression.

Comparing sex effects and ASD effects ascertained from separate data sets, as in previous work (14,15), does not allow for these hypotheses to be evaluated directly. Instead, comparison of relative expression levels in male and female cases and controls from the same data generation effort are required. Additionally, since studies comparing ASD- and sex-differential expression in

brain were published, available genomic data sets of human brain tissue have expanded, ASD-dysregulated transcriptomic signatures (such as upregulation of glial cell markers) have been refined, and the number of ASD-associated risk genes has grown. Thus, an expanded and updated investigation of ASD- and sex-differential patterns in the human brain is warranted to identify specific genes and their associated functions that are affected by both sex and ASD in the human brain. These genes may point to the mechanistic underpinnings of male vulnerability or female protection for ASD.

Utilizing bulk RNA-seq data from cortical tissue from post-mortem donors of all sex and diagnosis combinations (female cases and controls, male cases and controls), here we test for genes with expression impacted by sex and ASD that may be involved in female-protective and male-vulnerability mechanisms. We performed transcriptome-wide analyses to address the following specific questions: 1) Are neurotypical sex-differential gene expression patterns maintained, attenuated, exaggerated, or reversed in ASD cases? 2) If not consistent, are changes in sex-differential gene expression in the ASD brain driven by expression changes primarily in one sex or both? 3) Which genes follow expression patterns suggestive of male-vulnerability to, or female-protection against, ASD? 4) Do male-vulnerability and female-protective genes relate to specific cellular or molecular functions or pathways?

We find neurotypical sex-differential gene expression patterns to be attenuated in across three cortical lobes of the brain in ASD cases. Further, we identify three general patterns of attenuation of sex-differential gene expression, sex-equalized (where changes are driven by both sexes), male-shifted (changes driven by male cases), and female-shifted (changes driven by female cases) expression in ASD cases. Then, we establish sets of genes in each cortical lobe with expression patterns suggestive of male-vulnerability to and female-protection against ASD, with the most pronounced signature of both existing in the parietal cortex. Furthermore, we determined female-protective genes to be enriched for markers of neuronal cells as well as known ASD risk variants and male-vulnerability genes to overlap with markers of glial and neuroimmune

cells at the blood-brain-barrier (BBB). Future studies of our identified female-protective and male-vulnerability genes may facilitate development of therapeutics that amplify protective or reduce vulnerability activities, which may address the needs of male and female individuals on the autism spectrum.

**Methods**

| Brain Region | N Donors | F CTL | M CTL | F ASD | M ASD |
|---|---|---|---|---|---|
| **Total** | 111 | 10 | 44 | 10 | 47 |
| Frontal | 104 | 10 | 41 | 10 | 43 |
| Temporal | 99 | 9 | 38 | 10 | 42 |
| Parietal | 81 | 5 | 28 | 7 | 41 |

**Table 1.** Unique donor information per region of the brain including breakdown by sex and diagnosis. ASD = Autism Spectrum Disorder; CTL = Controls; M = Males; F = Females.

**RNA-sequencing data.** RNA-seq data from a total of 111 unique human donors (44 M CTL, 47 M ASD, 10 F CTL,10 F ASD) aged 2-67.3 years were used, including tissue from multiple subregions of the frontal, temporal, and parietal lobe from the ASD UCLA project (8). Normalized, regressed expression with technical covariates removed were downloaded from https://github.com/dhglab/Broad-transcriptomic-dysregulation-across-the-cerebral-cortex-in-ASD/blob/master/main_datasets/A_GeneLevel/Gene_NormalizedExpression_TechnicalCovariatesRemoved.RData and boxplots of normalized expression per individual sample are found in **Supplemental Figure 1**. Pre-processing steps are detailed on https://github.com/dhglab/Broad-transcriptomic-dysregulation-across-the-cerebral-cortex-in-ASD/tree/6821cc55aaf17879a4e0d8eec454d98b2f87dd6a/code/01_RNAseqProcessing.

Processed data included 24,836 genes and a total of 676 samples from 111 donors. Sample and

donor demographics are shown in **Table 1** and **2**. Additional information on sample composition and processing steps can be found in Gandal et al. 2022 (8) and the corresponding github page (https://github.com/dhglab/Broad-transcriptomic-dysregulation-across-the-cerebral-cortex-in-ASD/).

| Brain Region | N Samples | F CTL | M CTL | F ASD | M ASD |
|---|---|---|---|---|---|
| **Total** | 676 | 45 | 249 | 69 | 313 |
| Frontal | 283 | 21 | 103 | 30 | 129 |
| BA9 | 121 | 10 | 48 | 13 | 50 |
| BA24 | 51 | 3 | 15 | 6 | 27 |
| BA4-6 | 59 | 4 | 24 | 4 | 27 |
| BA44-45 | 52 | 4 | 16 | 7 | 25 |
| Temporal | 210 | 12 | 78 | 23 | 97 |
| BA 41-42-22 | 107 | 8 | 38 | 13 | 48 |
| BA 20-37 | 57 | 2 | 24 | 7 | 24 |
| BA38 | 46 | 2 | 16 | 3 | 25 |
| Parietal | 183 | 12 | 68 | 16 | 87 |
| BA7 | 64 | 4 | 23 | 3 | 34 |
| BA39-40 | 63 | 4 | 25 | 7 | 27 |
| BA3-1-2-5 | 56 | 4 | 20 | 6 | 26 |

**Table 2.** Individual sample information per region of the brain including breakdown by sex and diagnosis. BA = Brodmann Area; ASD = Autism Spectrum Disorder; CTL = Controls; M = Males; F = Females.

**Differential expression analyses.** Differential expression analyses were performed separately for each cortical lobe using limma-voom (20) on normalized and covariate-adjusted $\log_2$(CPM+1) values from expressed genes (downloaded from github page). All differential expression models contained covariates of age (years), RNA-seq batch, RIN, and PMI. To account for non-independence of samples derived from the same donor, we used the limma::duplicateCorrelation (20) function to run a linear mixed effects model, with donor included as a random effect, in each differential expression analysis.

I. **Sex differential expression in ASD and CTL.** To examine sex differences, we first separated cases and controls in each lobe and then used differential expression models including sex as the main variable of interest with covariates as detailed above: ~Sex + RNA-seq batch + Age + RIN + PMI. We defined robust sex-differentially expressed genes (sex-DEGs) as genes with Benjamini-Hochberg false discovery rate (FDR) ≤0.1. Of note, the sets of ASD and CTL samples are well-matched in the overall number of samples as well as the number of male and female samples (see **Table 1, 2**).

II. **ASD differential expression in males and females.** Here, we first separated samples by sex (males, females) in each lobe and then used differential expression models including diagnosis as the main variable of interest with covariates as detailed above: ~Diagnosis + RNA-seq batch + Age + RIN + PMI. We utilized $\log_2$FC estimates from these comparisons to classify sex-DEGs, below.

III. **Sex-by-disorder interaction differential expression.** To test for sex-by-disorder status expression differences, we performed a differential expression analysis using an interaction term (~Diagnosis:Sex) as the main variable of interest: ~Diagnosis:Sex +

Diagnosis + Sex + RNA-seq batch + Age + RIN + PMI. Sex-by-disorder differentially expressed genes were those that met a threshold of FDR ≤0.1.

IV. **Risk group differential expression and identification of sex-differential risk pattern DEGs.** Here, we aimed to identify genes that follow two specific expression patterns suggestive of sex-differential risk expression: 1) “Male-vulnerability” genes, with increasing expression pattern by group of F CTL < M CTL < F ASD < M ASD, 2) “Female-protection” genes, with decreasing expression pattern by group of F CTL > M CTL > F ASD > M ASD. In order to detect genes that show these specific patterns, we assigned each sample a numeric risk score based on their sex-by-diagnosis risk group. Specifically, F CTLs were given a score of 1, being controls and the less prevalent sex in ASD, M CTLs were given a score of 2, being controls and the more prevalent sex in ASD, F ASDs were assigned to a score of 3, being cases but the less prevalent sex, and M ASDs were assigned a score of 4, being cases and the more prevalent sex. We then utilized this numeric risk score as the main variable of interest in differential expression models in each cortical lobe: ~RiskGroup + RNA-seq batch + Age + RIN + PMI. Genes that met a threshold of FDR ≤0.05 for differential expression by risk group were retained and further filtered by median and mean expression in each risk group to define specific sets of genes as:

1) Male-vulnerability genes, with both the mean and median expression pattern of F CTL < M CTL < F ASD < M ASD.
2) Female-protection genes, with both the mean and median expression pattern of F CTL > M CTL > F ASD > M ASD.

This step was done to filter out genes where the main effect of disorder status drives the significance of the risk group association and to retain genes that that follow our hypothesized risk patterns of interest by sex and diagnosis.

**Comparison and classification of sex-DEGs.** To assess the magnitude and direction of sex-DEGs in CTL vs. ASD, we used a Z-test to compare the regression coefficients for the union of sex-DEGs from CTL and ASD sex-differential analyses. Formula:

$$Z = \frac{\beta_{CTL} - \beta_{ASD}}{\sqrt{SE_{CTL}^2 + SE_{ASD}^2}}$$

We then leveraged these results to categorize CTL-specific sex-DEGs into several groups based on the following criteria:

1. “Female-shifted” genes, for which the sex difference in ASD is attenuated by a shift in females’ expression toward control male levels: Sex-DE FDR ≤0.1 in CTLs only, sex effect comparison in ASD vs. CTL $P < 0.05$ (Z-test), ASD-DE |log2(FC)| in females > ASD-DE |log2(FC)| in males, and absolute FC magnitude in females > 1.2.
    a. Expression up pattern, showing expression that rises in ASD to similar levels as male CTLs.
    b. Expression down pattern, showing expression that falls in ASD to similar levels as male CTLs.
2. “Male-shifted” genes, for which the sex difference in ASD is attenuated by a shift in males’ expression toward control female levels: Sex-DE FDR ≤0.1 in CTLs only, sex effect comparison in ASD vs. CTL $P < 0.05$ (Z-test), ASD-DE |log2(FC)| in females < ASD-DE |log2(FC)| in males, and FC magnitude in males > 1.2.
    a. Expression up pattern, showing expression that rises in ASD to similar levels as female CTLs.
    b. Expression down pattern, showing expression that falls in ASD to similar levels as female CTLs.

3. “Sex-equalized” genes, for which the attenuated sex difference in ASD is not accounted for by a strong shift in expression in one sex (no statistically significant differential direction of effect towards male or female CTL expression): Sex-DE FDR ≤0.1 in CTLs only, sex effect comparison in ASD vs. CTL $P < 0.05$ (Z-test), and absolute FC magnitude > 1.2 in either males and females from sex-stratified disorder differential expression.

**Functional annotation and enrichment tests.** To evaluate whether any sex-DEGs or “sex-differential risk pattern” DEGs represent differential function of specific cell types or biological processes, we compared DEGs to cell type marker and biological processes gene sets (**Table 3**). Neural cell type marker genes were selected from published lists by Lake et al. 2016 (21), derived from single-nuclei RNA-seq post-mortem human cortical tissue. We used gene lists of markers for neurons, excitatory neurons, inhibitory neurons, oligodendrocytes, astrocytes, microglia, and endothelial cells. The background gene set used was all expressed genes reported in Lake et al. 2016.

Additionally, we compared DEGs to specific BBB cell type marker genes from a single-nuclei RNA-seq study of post-mortem human neural vascular and cortical tissue (frontal cortex and hippocampus) (22). From this publication, we utilized gene lists for BBB-associated neurons, oligodendrocytes, oligodendrocyte progenitor cells (OPC), astrocytes, microglia/macrophage, brain endothelial cells (BEC), ependymal cells, pericytes, T cells, fibroblasts, and smooth muscle cells (**Table 3**). The background gene set used was all expressed genes reported in Yang et al. 2022 (22). We also tested for overlap with BBB-associated functions by comparing DEGs to curated Gene Ontology lists, published in Saverimuttu 2021 (23), specific for BBB transport and BBB integrity (**Table 3**).

To investigate the relationship with DNA variants associated with ASD risk, we compared DEGs to four sets of ASD risk-associated genes derived from recent publications (**Table 4**). These include three exome-sequencing studies identifying rare variant-implicated ASD risk genes:

Satterstrom et al. 2020 (3), Fu et al. 2022 (4), and Zhou et al. 2022 (5). Additionally, we considered one set of common variant-associated ASD risk genes identified by a genome-wide association study (24). The background gene set for rare-variant gene sets was autosomal, protein-coding genes from Gencode v21 and for the common variant ASD gene set, it was autosomal, protein-coding genes released from NCBI 37.3 in MAGMA (25).

| Gene List | Number of Genes | Publication |
|---|---|---|
| Neurons | 182 | Lake et al.(21) |
| Excitatory neurons | 113 | |
| Inhibitory neurons | 30 | |
| Oligodendrocytes | 142 | |
| Astrocytes | 175 | |
| Microglia | 183 | |
| Endothelial cells | 171 | |
| BBB neurons | 183 | Yang et al.(22) |
| BBB oligodendrocytes | 209 | |
| BBB OPC | 172 | |
| BBB astrocytes | 166 | |
| BBB microglia/macrophage | 188 | |
| Brain endothelial cells | 204 | |
| Ependymal cells | 210 | |
| Pericytes | 131 | |
| T cells | 142 | |
| Fibroblasts | 183 | |
| Smooth muscle cells | 77 | |
| BBB transport | 80 | Saverimuttu et al. (23) |
| BBB integrity | 22 | |

**Table 3.** Gene lists of cell type markers and functions used for functional annotation and enrichment analyses. Number of genes per each gene list and source publication are listed. BBB = Blood brain barrier. OPC = oligodendrocyte progenitor cells.

In order to explore potential connections to other neuropsychiatric disorders and phenotype risks beyond ASD, we conducted an analysis of the overlap between differentially expressed genes and disease risk-associated genes previously identified in various studies (**Table 4**). These include genes implicated by rare variants of large effect such as Developmental Delay (DD) (26), Epilepsy and Developmental Delay (EPDD) (27); as well as those implicated by genome-wide association studies of common variation: Educational Attainment (EA) (28), Attention Deficit/Hyperactivity Disorder (ADHD) (29), schizophrenia (SCZ) (30), major depressive

disorder (MDD) (31), multiple sclerosis (MS) (32), Parkinson's disease (PD) (33), and Alzheimer's disease (AD) (34). Protein-coding genes from Gencode v21 were set as the background for this analysis (35).

| Gene List | Method | Number of Genes | Publication |
|---|---|---|---|
| ASD | Exome-seq | 97 | Satterstrom et al.(3) |
| ASD | Exome-seq | 72 | Fu et al. (4) |
| ASD | Exome-seq | 60 | Zhou et al.(5) |
| ASD | GWAS | 16 | Grove et al.(24) |
| Developmental delay (DD) | Exome-seq | 91 | McRae et al.(26) |
| EPDD | Exome-seq | 32 | Heyne et al.(27) |
| Educational Attainment (EA) | GWAS | 963 | Lee et al.(28) |
| ADHD | GWAS | 10 | Demontis et al.(29) |
| Schizophrenia (SCZ) | GWAS | 259 | Ripke et al.(30) |
| MDD | GWAS | 30 | Wray et al.(31) |
| Multiple sclerosis (MS) | GWAS | 80 | Beecham et al.(32) |
| Parkinson's disease (PD) | GWAS | 37 | Chang et al.(33) |
| Alzheimer's disease (AD) | GWAS | 39 | Lambert et al.(34) |

**Table 4.** Gene lists of risk genes for neuropsychiatric disorders and phenotypes used for functional annotation and enrichment. Number of genes per each gene list and source publication and method of discovery are listed. Rare variants are those identified from exome-sequencing studies and common variants are those identified from genome-wide associated studies. ASD = Autism spectrum disorder; EPDD = Epilepsy and developmental delay; ADHD = Attention deficit/hyperactivity disorder; MDD = Major depressive disorder; GWAS = genome-wide association studies.

To confirm that our sets of sex-differential risk pattern DEGs were in agreement with previously defined ASD-associated modules from the same data sets here (7,8), we utilized gene lists belonging to ASD-impacted co-expression modules that were defined from the same data sets used in this study (7,8). We used genes identified in 14 ASD-upregulated modules (3 from Parikshak et al. (7); 11 from Gandal et al. (8)) and 10 ASD-downregulated modules (3 from Parikshak et al. (7); 7 from Gandal et al. (8)) The background gene set employed was all 16,092 expressed genes published in Parikshak et al. (7) and all 24,479 genes reported in Gandal et al. (8), respectively.

Given the relatively small number of attenuated sex-DEGs of interest, statistical tests for enrichment were only done on sets of sex-differential risk-pattern associated genes. Enrichments of DEGs in each category described above (cell type markers, functional pathways, ASD-

dysregulated modules, ASD risk genes, neuropsychiatric trait genes) were evaluated by two-sided Fisher's exact tests, taking into account a common background gene set of the intersection of all expressed genes in Gandal data set and all background genes from the comparison set. Sets of robust (FDR ≤0.05) male-vulnerability genes and female-protective genes in each cortical lobe (frontal, temporal, parietal) were tested separately against each gene set of interest. We separately applied a Bonferroni correction for multiple testing in each analysis, accounting for 2 risk-pattern gene sets (male-vulnerability, female-protective) and either 7 cell type marker sets (14 tests), 11 BBB cell type marker sets (22 tests), 2 BBB-associated function gene sets (4 tests), 6 ASD-dysregulated modules (Parikshak et al.; 12 tests), 18 ASD-dysregulated modules (Gandal et al.; 36 tests), each ASD risk gene set (2 tests for each list of ASD risk genes), or 9 neuropsychiatric disease gene sets (18 tests for 9 gene sets).

Additionally, functional enrichments were assessed with the gProfiler2 R package (version 0.2.1) for Gene Ontology (GO), KEGG pathways, Reactome, WikiPathways, TRANSFAC, miRTarBase, Human Protein Atlas, CORUM, and HPO (36). gProfileR used a significance threshold ≤ 0.05 for g:SCS (Set Counts and Sizes) method of multiple testing correction, an algorithm developed by gProfiler to specifically account for hierarchal clustering of GO terms by their specificity (36).

**Estimation of cell type composition.** To generate cell type signatures for the cortex, we utilized single-nuclei gene expression data from the middle temporal gyrus of adult epilepsy patients (N=8), published in Darmanis et al. (37). Gene-level counts from tissue samples were averaged across each cell type cluster: neurons, astrocytes, oligodendrocytes, microglia, and endothelial cells. Subsequently, averaged gene counts were then transformed to counts per million (CPM) and genes with >1 CPM in at least one of the cell types were retained to generate the final cell signature matrix. Estimations of cell type composition per sample were performed using the deconvolution method of dtangle (38) (version 2.0.9), with log2(CPM+1) expression as the input

(cite). Estimated cell type proportions from samples of each sex-diagnosis group (4 total: F CTL, M CTL, F ASD, M ASD) were calculated and tested for overall differences in cell type proportions using Kruskal-Wallis test (Formula: Cell type ~ Group). Subsequently, the Dunn test was used to compare differences between groups and identify sources of any group differences. Significant differences in cell type composition were defined as a P value ≤ 0.05 for a cell type.

**Results**

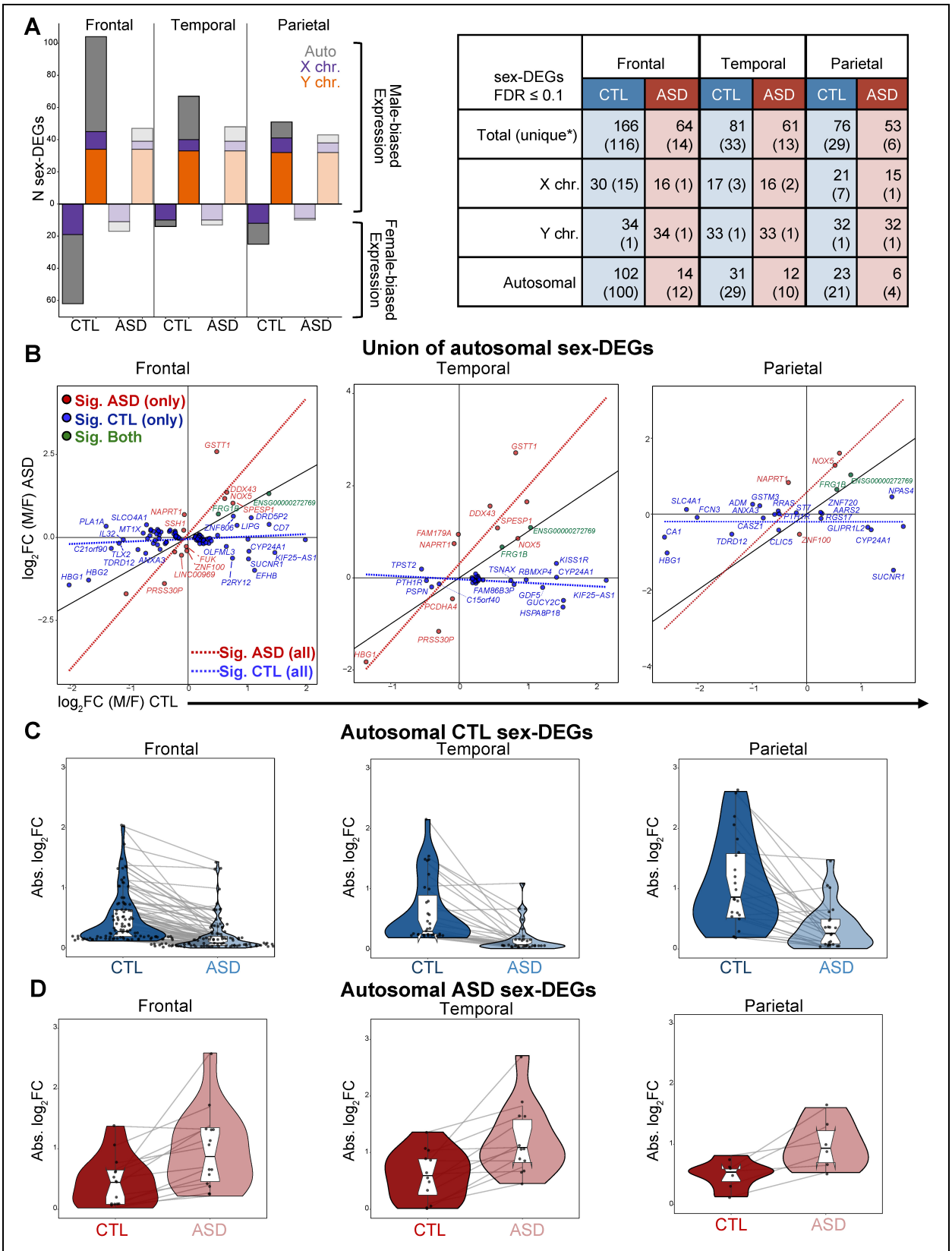
**Sex-differential expression is attenuated in the ASD cortex.** To characterize sex-disorder gene expression patterns in the ASD brain, we first sought to examine if sex-differential gene expression is different in the context of the ASD brain. Thus, we quantified and compared sex-differential gene expression in controls and cases separately from three different cortical lobes from de-identified human donors. We applied the same model to cases and controls separately to test for genes differentially expressed in males and females. We then assessed sex-DEGs identified in the control and ASD brain (FDR ≤0.1) in each cortical lobe.

Despite comparable numbers of male and female donors and samples in the ASD and control groups, we identified a greater number of sex-DEGs in controls than ASD in all three cortical lobes assayed, with the largest disparity in the frontal cortex (**Fig 1A, Table 5**). Many Y and X chromosome genes reached significance for sex-differential expression in ASD and controls in all lobes, while a relatively greater number of autosomal genes reached significance only in controls (**Fig 1A, Table 5**).

As expected, the direction and magnitude of sex-differential expression of *XIST*/*TSIX* and Y chr genes remained consistent between controls and cases as evidenced by highly correlated $\log_2$FC (fold change) (**Table 6, Supp Fig 2**), as did sex-DEGs on the X chromosome, reflecting consistent effects of X inactivation in both groups (**Table 6, Supp Fig 3**). However, for autosomal sex-DEGs detected in controls, regardless of sex-DE significance in ASD, sex effects ($\log_2$FC) are poorly correlated between ASD and controls in all tested cortical lobes (e.g. Pearson r = 0.24,

frontal cortex; **Fig 1B, Table 6, Supp Fig 5**), indicating specificity of these effects in controls. Though a smaller group of genes, the sex effects of autosomal sex-DEGs detected in ASD are more strongly correlated between the groups (e.g. Pearson r = 0.82, frontal cortex; **Fig 1B, Table 6, Supp Fig 5**), indicating greater consistency of these effects between groups.

This attenuation of apparent sex-differential expression in ASD may be attributed to either a reduction in the difference in gene expression level between ASD males and females, or to an increase in gene expression variance in ASD. We tested these two possibilities by comparing $log_2$FC magnitude and variance for sex-DEGs in ASD and CTL (**Fig 1C & D, Supp Fig 4)**. Comparing the magnitude of autosomal sex-DEG fold change per gene finds that indeed, autosomal sex-DEGs from controls show significantly greater sex effects in controls (e.g. frontal cortex, p = 3.13E-26, one-sided paired Wilcoxon test; **Fig 1C**, **Table 7**), while autosomal sex-DEGs from cases do not show significantly greater effects in ASD (e.g. frontal cortex, p = 1.00, one-sided paired Wilcoxon test; **Fig 1D**, **Table 7**). However, comparing the variance in estimated sex effects for autosomal sex-DEGs revealed no significant differences in either CTL sex-DEGs or ASD sex-DEGs (**Supp Fig 4, Table 8**). Thus, our findings suggest that attenuation of sex-differential gene expression in the autism brain is not due to increased variance of sex effects, but rather a reduction in the difference in gene expression levels between ASD males and females.




| sex-DEGs FDR ≤ 0.1 | Frontal | | Temporal | | Parietal | |
|---|---|---|---|---|---|---|
| | CTL | ASD | CTL | ASD | CTL | ASD |
| Total (unique*) | 166 (116) | 64 (14) | 81 (33) | 61 (13) | 76 (29) | 53 (6) |
| X chr. | 30 (15) | 16 (1) | 17 (3) | 16 (2) | 21 (7) | 15 (1) |
| Y chr. | 34 (1) | 34 (1) | 33 (1) | 33 (1) | 32 (1) | 32 (1) |
| Autosomal | 102 (100) | 14 (12) | 31 (29) | 12 (10) | 23 (21) | 6 (4) |

**Fig 1:** Attenuation of sex-differential expression in the ASD cortex. (A) Bar plot and table of number of sex-DEGs (FDR ≤ 0.1) in cases (lighter bars) and controls (darker bars) in each

cortical lobe. Number inside paratheses indicates number of sex-DEGs unique to controls (not significant in cases) or cases (not significant in controls). (B) Scatter plot of $log_2$ fold changes from sex-differential expression analysis separate in controls (x axis) and cases (y axis) for the union of autosomal sex-DEGs at FDR $\leq$ 0.1 in each cortical lobe. Point color denotes significance in only cases (red), only controls (blue), or in both cases and controls (green). Dotted red linear regression line includes genes significant in ASD (red points) and significant in both (green points). Dotted blue linear regression line includes genes significant in controls (blue points) and significant in both (green points). See **Table 5** for linear regression statistics. Black line indicates slope of 1. (C) Violin plots of absolute $log_2$ fold change in autosomal sex-DEGs detected in controls, regardless of their significance in cases. Each dot inside the violin plots indicates the absolute $log_2$ fold change for a specific gene in controls or cases. Light gray lines connect the absolute $log_2$ fold change values for a specific gene in controls and cases. Black line inside violin plot denotes median value. (D) Violin plots of absolute $log_2$ fold change in autosomal sex-DEGs detected in cases, regardless of their significance in controls. Each dot inside the violin plots indicates the absolute $log_2$ fold change for a specific gene in controls or cases. Light gray lines connect the absolute $log_2$ fold change values for a specific gene in controls and cases. Black line inside violin plot denotes median value. ASD = autism spectrum disorder cases; CTL = controls; sex-DEGs = sex-differentially expressed genes; Auto = autosomal genes; X chr. = X chromosome genes; Y chr. = Y chromosome genes; Sig. = significant at FDR $\leq$ 0.1; Abs. $log_2$FC = absolute value of $log_2$ fold change (male/female).

**Sex-shifted expression patterns in ASD-attenuated sex-DEGs are evident and associated with BBB cell types and neuroimmune functioning.** Sex-differential gene expression in ASD may be attenuated due to female-specific shifts in expression toward male levels, male-specific shifts in expression toward female levels, or expression shifts in both sexes that reach a similar endpoint. For sex-DEGs meeting significance only in controls in each cortical lobe, we selected genes with significantly greater sex effects in CTL versus ASD (Z-test, Methods) and categorized these genes into three groups based on the magnitude of expression change for each sex between CTL and ASD (Methods): female-shifted, male-shifted, or sex-equalized, representing whether in ASD cases, the level of expression is similar to male controls, female controls, or in between, respectively (**Fig 2A**). Across the three lobes, in accordance with the number of CTL-specific sex-DEGs, we detect the highest number of female- and male-shifted sex-DEGs in the frontal lobe (**Fig 2B**, **Table 9** (detailed lists found in **Supp. Tables 1-3**)). We detect minimal overlap of genes between lobes for both gene sets, with only 2 genes (*TPST2* and *KIF25-AS1*) shared in the frontal and temporal lobe and 1 gene (*SUCNR1*) in both frontal and parietal lobes for “female-

shifted" genes and only 1 "male-shifted" gene shared between all three lobes (*CYP24A1)* (**Fig 2B & 2C**). *CYP24A1* encodes a mitochondrial enzyme, 24-hydroxylase, that mediates the amount of vitamin D throughout the body (39), but has yet to be studied within the context of the sex differences and ASD. Sex-specific expression shifts in ASD can result from either increased or decreased expression in ASD cases relative to sex-matched controls. By sorting sex-shifted genes based on the direction of change in ASD, we find that female-shifted genes are more likely to have reduced expression in ASD females in frontal cortex (19/25 genes, p=0.014; two-sided binomial test), while male-shifted genes show no skew in the direction of change. This pattern potentially reflects preferential down-regulation, perhaps due to transcriptional repression, of sex-DEGs in ASD females.

Functional annotation and gene set enrichment analyses did not identify significant enrichments of sex-shifted gene sets for any one functional category, likely due to the small number of genes tested (**Table 9**), though we note that several female-shifted and male-shifted genes in the frontal and parietal lobe overlap with various gene sets related to BBB cell types and/or neuroimmune functioning (**Table 10**). We observe a common theme of sex-shifted genes that annotate to neuroimmune and BBB cell types, particularly female-shifted genes that overlap with endothelial cell marker genes (*TGM2, PLA1A, SLCO4A1, SDPR, IL32, C1orf64, AFF3, ANXA3, PTH1R,* and *PCDH11X*), and male-shifted expression of genes related to glial-immune functioning (*IL4R, SLC16A1,* and *P2RY12*).

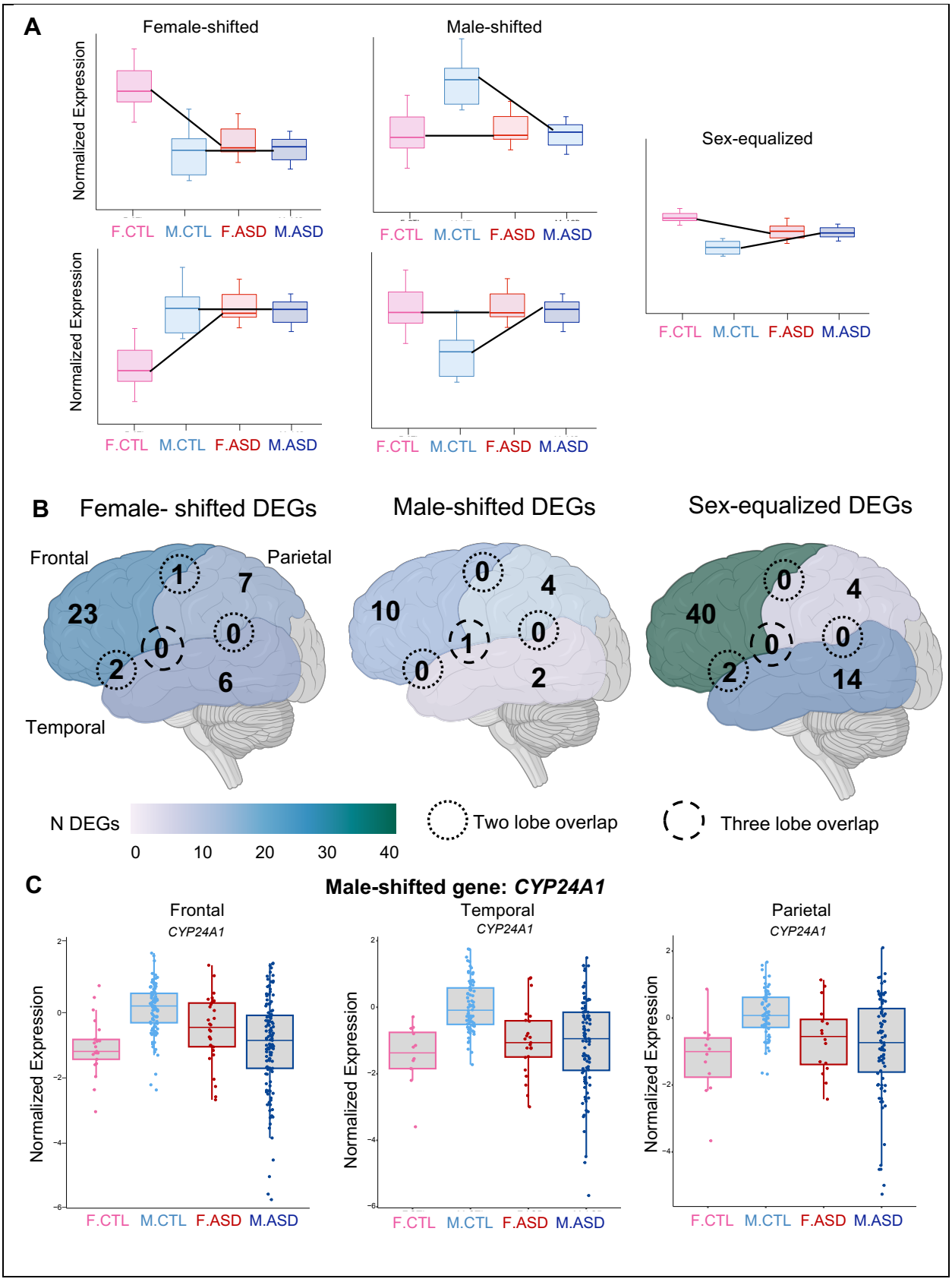

A
Female-shifted
Male-shifted
Sex-equalized
Normalized Expression
F.CTL M.CTL F.ASD M.ASD
B
Female- shifted DEGs
Male-shifted DEGs
Sex-equalized DEGs
Frontal
Parietal
Temporal
1
7
23
0
0
2
6
0
4
10
1
0
0
2
0
4
40
0
0
2
14
N DEGs
0 10 20 30 40
Two lobe overlap
Three lobe overlap
C
Male-shifted gene: CYP24A1
Frontal
Temporal
Parietal
CYP24A1
Normalized Expression
F.CTL M.CTL F.ASD M.ASD

Fig 2: Patterns of sex-shifted or sex-equalized expression in ASD-attenuated sex-DEGs are evident in the cortex. (A) Boxplot examples of patterns of attenuation of sex-differential expression (Methods). Female-shifted expression (left column) can either be represented by a shift down (top) or a shift up in expression (bottom) in female ASD cases. Male-shifted expression (middle column) can either be represented by a shift down (top) or a shift up in expression (bottom) in male ASD cases. (B) Number of ASD-attenuated sex-DEGs in either female-shifted (left brain), male-shifted (middle brain), or sex-equalized (right brain) in each cortical lobe with overlaps between lobes represented by dashed circles. Color of cortical lobe indicates number of genes on scale shown below. Created with BioRender.com. (C) Boxplots of normalized expression of male-shifted gene *CYP24A1* detected in all three cortical lobes. Each dot represents the normalized expression value for an individual sample. Color denotes sample group (female controls = pink, male controls = light blue; female ASD cases = red; male ASD cases = dark blue). ASD = autism spectrum disorder cases; CTL = controls; DEGs = differentially expressed genes; F = female; M = male.

**Risk-associated gene expression patterns are evident in the brain.** We hypothesize that genes participating in sex-differential risk mechanisms for ASD are likely to show 1) baseline sex-differential expression levels in controls, and 2) altered expression in ASD, indicating their involvement in ASD etiology or pathobiology. Given that males are more frequently diagnosed with ASD, we further hypothesize that control males' expression of genes involved in sex-differential risk mechanisms will be closer to levels seen in ASD as compared with control females' expression (i.e. expression level in control males is intermediate between control females and ASD). Here, we defined genes that follow two specific expression patterns indicative of potential roles in sex-differential ASD biology: 1) female-protective genes, showing highest expression in female controls and progressive reduction in expression to male controls, then female cases, and lowest expression in male cases (**Fig 3A)**; and 2) male-vulnerability genes (**Fig 3B)**, exhibiting highest expression levels in male cases and a stepwise reduction in expression to female cases, then male controls, and lowest expression in female controls (Methods). Higher expression of female-protective genes may be beneficial and may serve to buffer females against the impact of ASD risk exposures. Higher expression of male-vulnerability genes may be harmful and may serve to exacerbate or compound the impact of ASD risk exposures.

In all cortical lobes tested, we detected a high number of both male-vulnerability and female-protective genes; parietal lobe yielded the largest number of sex-differential risk-

associated DEGs for both categories (Methods), followed by temporal lobe, and frontal lobe showed the fewest risk-associated DEGs (**Fig 3C & 3D)**. This pattern corresponds to our observation of the largest number of ASD-attenuated sex-DEGs in the frontal lobe as compared with other lobes, as our definition of sex-differential risk-associated genes requires observation of sex effects in ASD. We find 11 male-vulnerability DEGs (10 autosomal: *HGF, TNPO1, ELP4, SLC1A4, RPN2, IL1B, FOXJ1, BTG1, NADK2, UBE2E1;* 1 X chr: *NXT2*), and 5 female-protective DEGs (5 autosomal: *MAP2K7, CORO2B, PEX5L, FAM171A1, SPNS2*), that are shared between all three cortical regions (**Fig 3C & 3D**).

**Female-protective genes enriched for neuronal cell markers and ASD risk genes; male-vulnerability genes enriched for glial and BBB cell markers.** We observe that female-protective DEGs from temporal and parietal cortices are enriched for neuronal cell type markers, including both excitatory and inhibitory neurons (21); these enrichments are more pronounced for parietal cortex, likely due to the larger number of sex-differential risk-associated genes in this lobe (**Fig 3E, Table 11**). Female-protective DEGs in all three cortical lobes were at least nominally enriched for BBB-associated oligodendrocyte marker genes (22) (**Fig 3E, Table 12**). However, this enrichment was not present when testing a set of oligodendrocyte markers derived from general cortex tissue (**Fig 3E, Table 11**, (21)). We note a similar trend for microglial markers in parietal cortex for female-protective DEGs, where significant enrichment is present for microglial markers from general cortex tissue, but not for microglial/macrophage markers from tissue at the BBB (**Fig 3E, Tables 11-12,** (21–23)).

In contrast to neuronal enrichment for female-protective genes, male-vulnerability DEGs from temporal and parietal cortex are enriched for glial-type and endothelial cell markers, including nominal enrichment for cortical microglial and endothelial cell markers (**Fig 3F, Tables 13-14,** (21)). For parietal male-vulnerability DEGs, we observe additional enrichment for markers of cortical oligodendrocytes, cortical and BBB-associated astrocytes, and T cells (**Fig 3F, Tables**

**13-14,** (22)). Male-vulnerability DEGs from the frontal lobe are also enriched for BBB-associated fibroblast cell markers (**Fig 3F, Table 14,** (22)). Given enrichments for cell types involved in BBB function and processes, we then compared male-vulnerability DEGs to sets of genes associated with BBB functions of integrity and transport (23) and found nominal enrichment for BBB integrity and transport (**Fig 3F, Table 15-16**) in parietal lobe male-vulnerability DEGs. This pattern is in agreement with previous studies reporting an upregulation of genes associated with glial and neuroimmune functions in the ASD cortex (7–9,40,41) and in the adult male brain (14,42). Furthermore, evidence from animal and cell culture models point to the potential involvement of the BBB in ASD pathogenesis, particularly in males (43–48) and suggests that, as a group, the expression of genes related to neuroimmune/ BBB functions and cell types may be involved in male-skewed pathology or risk mechanisms for ASD.

Additionally, we assessed the relationship of sex-differential risk-associated DEGs to ASD and neuropsychiatric disorder risk genes defined by studies evaluating rare (exome-sequencing) (3–5) or common variants (GWAS) (24). Female-protective DEGs in the parietal cortex were consistently enriched for rare-variant implicated ASD genes (3–5), across gene lists from different publications tested (**Fig 3E, Table 17, Supp. Fig 6**), and genes associated with DD and EPDD risk **(Fig 3E, Table 18**). Temporal cortex female-protective DEGs are also nominally enriched for two sets of rare-variant implicated ASD genes **(Fig 3E, Table 17)**. Interestingly, there is minimal overlap between female-protective DEGs in the temporal and parietal cortex driving such enrichments, only *KCNB1* is significant in both cortical areas. Instead, it is largely different subsets of rare-variant-implicated ASD risk genes that overlap with female-protective DEGs from the temporal and parietal cortex (**Fig 3G**). We do not find any enrichment nor any overlapping protective or vulnerability genes with common variant-associated ASD risk genes identified from GWAS (**Fig 3E, Table 19**). We do not observe enrichments of male-vulnerability genes for ASD risk genes, common or rare variant-implicated, suggesting that male-vulnerability genes may be acting downstream from, or in parallel with, mutation-carrying risk genes (**Fig 3F, Table 19**).

However, we do find nominal enrichment for Alzheimer's Disease risk genes (34) in male-vulnerability genes from the parietal cortex **(Fig 3F, Table 20**), which is driven by an overlap of three genes (*PVRL2, APOE, APOC1*) associated with microglial function.

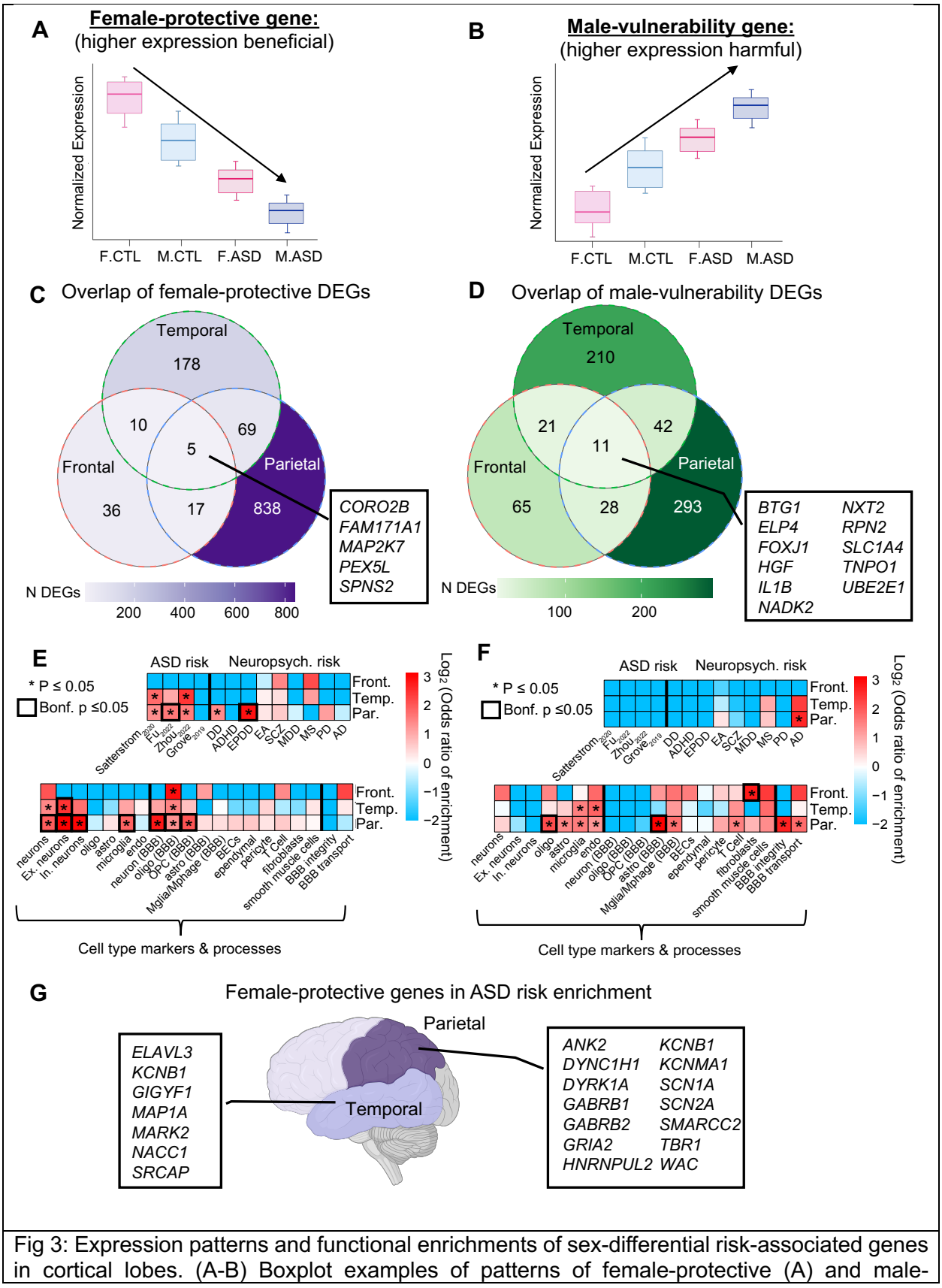



Fig 3: Expression patterns and functional enrichments of sex-differential risk-associated genes in cortical lobes. (A-B) Boxplot examples of patterns of female-protective (A) and male-

vulnerability (B) expression (Methods). (C) Venn diagram of number of female-protective DEGs (FDR < 0.05) detected in frontal, temporal, and parietal cortices and overlaps. Color of circle indicates number of genes on scale shown below. DEGs detected in all three lobes are listed in the side box. (D) Venn diagram of number of male-vulnerability genes (FDR < 0.05) detected in frontal, temporal, and parietal cortices and overlaps. Color of circle indicates number of genes on scale shown below. DEGs detected in all three lobes are listed in the side box. (E-F) Patterns of female-protective DEG (E) and male-vulnerability DEG (F) enrichments for 4 ASD risk gene sets (3–5,24), 9 neuropsychiatric risk sets(26–34) , 7 cortical cell type markers (21), 11 BBB-associated cell type markers (22), and 2 BBB function gene sets (23) using two-sided Fisher's exact test. (G) Female-protective DEGs by cortical lobe that overlap with ASD risk gene sets (3–5) listed in boxes. Created with BioRender.com. ASD = autism spectrum disorder cases; CTL = controls; DEGs = differentially expressed genes; F = female; M = male; DD = developmental delay; ADHD = attention deficit hyperactivity disorder; EPDD = epilepsy and developmental delay; EA = educational attainment; SCZ = schizophrenia; MDD = major depressive disorder; MS = multiple sclerosis; PD = Parkinson's disease; AD = Alzheimer's disease; Ex. Neurons = excitatory neurons; In. neurons = inhibitory neurons; Oligo = oligodendrocytes; Astro = astrocytes; Endo = endothelial cells; BBB = blood brain barrier; OPC = oligodendrocyte progenitor cell; Mglia/ Mphage = microglia/ macrophage; BECs = brain endothelial cells; Front. = frontal lobe; Temp. = temporal lobe; Par. = parietal lobe.

**Estimated cell type proportions do not differ significantly between groups.** Enrichments for cell type markers could either reflect an increased expression of said marker genes or a difference in cell type composition in sample groups. Towards this end, we employed dtangle (Methods) to estimate proportions of five cortical cell types in bulk tissue samples and tested for differences between all four groups to further examine biological sources that may be contributing to sex-differential ASD risk patterns. However, we find no significant differences between and within sample groups of any cell type tested in any cortical lobe (**Supp. Fig 7**). Thus, enrichments for neuronal markers in female-protective genes and astrocyte and oligodendrocyte markers in male-vulnerability genes appear not to be driven by large differences in relative proportions of cell populations, but may result from increased expression of certain marker genes or from smaller shifts in cellular composition that are not evident from deconvolution estimates.

**Disorder-by-sex interaction effects on differential expression are minimal.** Differential expression analyses to identify genes with an ASD-by-sex interaction effect yielded only one gene in the frontal lobe (*EFHB*) and one gene in the temporal lobe (*TPST2*) that passed an FDR

threshold of 0.1 (**Supp. Fig 8**); no genes in the parietal lobe met this threshold. This low rate of discovery is likely a consequence of small sample size, particularly due to low numbers of female donors, though we note that the small number of genes with significant interaction effects mirrors patterns seen in schizophrenia brain gene expression reported in Hoffman et al (49) from a much larger sample size (437 controls, 341 cases) finding zero significant genes, indicating that disorder-by-sex interaction effects on neural gene expression may be minimal in several contexts.

**Discussion**

ASD exhibits a profound sex disparity in prevalence, as evidenced by the consistently male-skewed diagnosis rate, indicating potential mechanisms that confer risk to males or protection to females. However, previous work characterizing transcriptomic patterns in ASD has primarily focused on differences between cases and controls and not directly examined the additional variable of sex. Thus, the mechanistic interactions of sex and ASD neurobiology, as reflected in gene expression, have remained largely unknown and warrant investigation to advance our understanding of the strong sex bias in ASD. Towards this end, we investigated differences in patterns of sex-differential gene expression and risk-associated expression signatures in the human cortex at the transcriptomic level in ASD.

We observe an attenuation of sex-differential gene expression in the ASD cortex, as evidenced by a smaller number of genes reaching significance for sex-differential expression, and a reduction in the magnitude of sex differences seen in controls. Attenuation of sex differences in the context of ASD has been hypothesized (50), and results from neuroimaging studies are consistent with this possibility (50). Whereas the “extreme male brain theory” of ASD may predict that individuals with ASD exhibit a hyper-masculinization of the brain (16), the story from neuroimaging is more complex. Such studies do observe that anatomical (auditory cortex, cerebellum (17,18)) and connectivity patterns (frontal-temporal network (19)) in the brains of females with ASD show signs of masculinization, but also that somato-motor network connectivity

(19) and patterns of hypo-connectivity in the thalamus and parietal cortex (19) in males with ASD are more akin to those seen in typical female controls. Here, we find genes with transcriptomic signatures representative of both male-shifted and female-shifted phenotypes in the autism brain, suggesting that such patterns are evident at the transcriptome level in addition to neural connectivity and functional level. We do detect a slightly larger number of female-shifted genes, in agreement with the proposed “extreme male brain theory”, but we also find evidence of several male-shifted genes, which is more in line with a pattern of sex-specific changes in the ASD brain.

On the whole, the attenuation of sex-differential expression in ASD could be due to reduced sexual differentiation of the cortex, and this effect may be particularly evident in the frontal cortex. Prior work comparing gene expression patterns between multiple cortical lobes have observed patterns suggesting reduced regional differentiation in ASD cortex compared with controls (“attenuated cortical patterning”, (6–8)). Our results indicate that cortical regions are also less transcriptionally distinct between sexes in ASD, which could suggest a more general phenomenon whereby ASD cortex is less responsive to differentiation signals from multiple contexts. However, we caution that the number of female donors available for this study is small, and that diagnostic and ascertainment biases may contribute to the attenuation of sex differences that we observe. Specifically, females who receive an ASD diagnosis and who are ascertained for post-mortem tissue donation may be those who are most phenotypically similar to ASD males, which may extend to gene expression patterns in the brain.

We further utilized this data set including cases and controls of both sexes to identify genes that follow expression patterns consistent with broadly defined mechanisms of female protection and male vulnerability. We find numerous genes displaying patterns suggestive of female protection or male vulnerability in all three cortical lobes, with the greatest contribution from the parietal cortex. Male-vulnerability genes from temporal and parietal lobes are enriched for markers of glial cell types in cortex and at the BBB, as well as common functional themes related to BBB cell types and processes. Our results align with previous research showing an

overlap of male-biased, ASD-upregulated gene expression related to neuroimmune functioning and glial cell types (14,15). We further enhance this by identifying genes with specific patterns of male vulnerability to ASD, previously only hypothesized, and refine functional enrichment patterns to specific BBB cell types and pathways, rather than previously reported general glial functioning (14,15). However, due to the nature of samples from post-mortem brains, at this time we cannot differentiate if such male-vulnerability signatures are involved in ASD onset or are the result of a lifetime of ASD. Future work teasing apart the temporal trajectories and mechanistic roles of the genes we prioritize here will be important.

We also detect sets of genes with a potential female-protective expression signature to be broadly enriched for neuronal cell type markers and processes. This is in agreement with previous work reporting female-biased enrichment for neuronal cell markers that were also down-regulated in a separate cohort of ASD brains (14,15). Additionally, we report detection of female-skewed expression of ASD-implicated risk genes, within the female-protective gene set. Previous studies have reported variable results with regard to sex-differential expression of ASD risk genes. Several prior assays of bulk human cortex do not find significant female-biased enrichment of ASD risk genes (14,15,51), while one study found ASD risk genes to have preferentially female-skewed expression in prenatal whole brain (52). More recent assessment of single nuclei data in developing human brain also finds enrichment of rare and common ASD risk variants in lineage-specific expression profiles elevated in neural cells from neurotypical female donors (53). We note that ASD risk genes classified as female-protective in this study do not meet significance for sex-differential expression independently in either cases or controls, but as defined (Methods), the mean and median expression of each of these genes is elevated in female cases and controls compared to their male counterparts. Together with findings from whole brain and in developmental lineages from single nuclei data, the patterns reported here suggest a model in which higher baseline expression of ASD risk genes in females may protect females by providing a transcriptional buffer against down-regulation resulting from loss-of-function mutations.

Finally, a striking pattern we noted in our analyses is the large number of sex-differential risk-associated genes in the parietal and temporal cortex, compared to the frontal cortex. Previous investigations of gene expression differences associated with have predominantly concentrated on the prefrontal cortex as the region of interest due to the relatively high expression and co-expression of ASD risk genes in prefrontal cortex (54,55), involvement of this cortical area in higher-order cognitive and social-processing functions that may be impaired in ASD, and evidence of structural and functional connectivity abnormalities in ASD from neuroimaging studies (56–58). However, our findings suggest that this focus may be too narrow. We identified a more substantial number of sex-differential risk mechanism-associated DEGs in the temporal and parietal lobes, indicating that such areas of the brain may be involved in etiological differences between sexes. Of note, our observation of the largest number of sex-differential risk pattern genes in the parietal cortex, as compared to the frontal and temporal, fits with the observation reported by Gandal et al. (8) in this same data set of an anterior-posterior gradient in ASD-associated gene expression differences, where ASD differences are greatest in posterior cortical regions. These insights collectively underscore the necessity for future research endeavors to broaden their scope beyond the prefrontal cortex. It is imperative that these studies include a wider array of cortical areas and perhaps even delve into sub-cortical regions to gain a more comprehensive understanding of the genetic underpinnings of ASD and sex-skewed disorders. This expanded approach could unveil new dimensions of the disorders and potentially lead to more effective interventions.

It is important to note that our analysis is limited by the small number of female ASD cases in the data set, as reflective of ASD's male-skewed prevalence. We also note that the control group was sex-matched to the ASD group and included samples from the same number of female donors, to best reduce the likelihood of confounding sex with case-control status. At present, to the best of our knowledge there is no available independent data set or one with additional ASD female samples. Additionally, we note that the small number of attenuated sex-DEGs that were

able to be categorized into either female-shifted or male-shifted and our lack of significant findings for sex-by-disorder interaction effects at the transcriptomic level are likely due to statistical power challenges stemming from the small number of female donors. In the future, more inclusive research is necessary, particularly with increased numbers of female human donors, in order to delineate mechanisms related to male-skewed ASD pathology and the female protective effect in ASD. While human studies are limited by available donor tissue, studies in animal models can be thoughtfully designed to facilitate well powered analyses of sex-matched samples, including investigation of the joint effects of sex and ASD-associated genetic risk variants, comparison between male and female variant carriers, within-sex comparisons between mutant and wild-type animals, and manipulation of sex chromosome and sex hormone exposures. Such experimental work, in parallel with data generation from human tissues, will be critical to advance our understanding of specific sex-differential risk or protective mechanisms.

Overall, our study contributes to bridging the gap in knowledge of how sex may come into play in ASD neurobiology its male-skewed prevalence at the transcriptomic level. We find evidence for attenuated sex-differential gene expression in the ASD brain, building on similar findings at the neural circuitry and morphological level. Furthermore, by detecting genes with specific expression patterns indicative of sex-differential ASD risk, we have identified sets of genes and associated functional pathways that may represent points of convergence between sex differences and ASD neurobiology that may be involved in female protection against and/or male vulnerability towards ASD. Our results also shed light on the need for further research utilizing greater numbers of female subjects and investigating areas of the brain outside the prefrontal cortex. In conclusion, the work presented here can contribute to the development of more effective diagnostic and treatment strategies that specifically address the unique needs of both male and female individuals on the autism spectrum. For example, the genes exhibiting female-protective expression patterns are indeed protective against ASD, then possible future therapeutics amplifying such activities could be beneficial. Relatedly, if male-vulnerability genes

are involved in pathogenesis of ASD, then development of therapeutics blocking or reducing such biological activities could also be helpful. Finally, the work here also sheds light on the broader question of why neurodevelopmental disorders, in general, exhibit patterns of sex-skewed prevalence.

| Cortical Lobe | Test | Independent Variable | Dependent Variable | N genes | Estimate | Std. Error | P-value | $R^2$ | Adj. $R^2$ |
|---|---|---|---|---|---|---|---|---|---|
| Frontal | All Chr- CTL DEGs | CTL $log_2FC$ | ASD $log_2FC$ | 166 | 0.941 | 0.016 | 2.75E-110 | 0.952 | 0.952 |
| | All Chr- ASD DEGs | ASD $log_2FC$ | CTL $log_2FC$ | 64 | 1.053 | 0.017 | 8.65E-58 | 0.984 | 0.984 |
| | X Chr- CTL DEGs | CTL $log_2FC$ | ASD $log_2FC$ | 30 | 1.098 | 0.086 | 6.36E-13 | 0.857 | 0.852 |
| | X Chr- ASD DEGs | ASD $log_2FC$ | CTL $log_2FC$ | 16 | 0.988 | 0.034 | 3.73E-13 | 0.985 | 0.983 |
| | Autosomal- CTL DEGs | CTL $log_2FC$ | ASD $log_2FC$ | 102 | 0.472 | 0.189 | 1.41E-02 | 0.059 | 0.049 |
| | Autosomal- ASD DEGs | ASD $log_2FC$ | CTL $log_2FC$ | 14 | 1.604 | 0.321 | 3.07E-04 | 0.676 | 0.649 |
| Temporal | All Chr- CTL DEGs | CTL $log_2FC$ | ASD $log_2FC$ | 81 | 0.882 | 0.017 | 7.53E-62 | 0.970 | 0.969 |
| | All Chr- ASD DEGs | ASD $log_2FC$ | CTL $log_2FC$ | 61 | 1.069 | 0.017 | 1.01E-55 | 0.985 | 0.985 |
| | X Chr- CTL DEGs | CTL $log_2FC$ | ASD $log_2FC$ | 17 | 1.152 | 0.062 | 2.65E-11 | 0.962 | 0.959 |
| | X Chr- ASD DEGs | ASD $log_2FC$ | CTL $log_2FC$ | 16 | 0.888 | 0.051 | 2.11E-10 | 0.959 | 0.956 |
| | Autosomal- CTL DEGs | CTL $log_2FC$ | ASD $log_2FC$ | 31 | -0.081 | 0.402 | 8.41E-01 | 0.001 | -0.033 |
| | Autosomal- ASD DEGs | ASD $log_2FC$ | CTL $log_2FC$ | 12 | 1.500 | 0.315 | 7.61E-04 | 0.694 | 0.664 |
| Parietal | All Chr- CTL DEGs | CTL $log_2FC$ | ASD $log_2FC$ | 76 | 1.044 | 0.028 | 5.82E-50 | 0.950 | 0.950 |
| | All Chr- ASD DEGs | ASD $log_2FC$ | CTL $log_2FC$ | 53 | 0.937 | 0.016 | 5.36E-48 | 0.985 | 0.984 |
| | X Chr- CTL DEGs | CTL $log_2FC$ | ASD $log_2FC$ | 21 | 1.405 | 0.095 | 1.74E-11 | 0.923 | 0.919 |
| | X Chr- ASD DEGs | ASD $log_2FC$ | CTL $log_2FC$ | 15 | 0.685 | 0.054 | 2.73E-08 | 0.930 | 0.924 |
| | Autosomal- CTL DEGs | CTL $log_2FC$ | ASD $log_2FC$ | 23 | 0.336 | 0.542 | 5.42E-01 | 0.018 | -0.029 |
| | Autosomal- ASD DEGs | ASD $log_2FC$ | CTL $log_2FC$ | 6 | 0.940 | 0.657 | 2.26E-01 | 0.339 | 0.173 |

**Table 5.** Results of linear regression tests of slope of $log_2FC$ for sex-DEGs detected in controls, regardless of significance level in ASD, and sex-DEGs detected in ASD cases regardless of significance level of controls. Sex-DEGs in all chromosomes, only X chromosome, and only autosomes were tested separately in each cortical lobe and are indicated above. ASD = autism spectrum disorder; CTL = control; DEGs = differentially-expressed genes; logFC = $log_2$ fold change (Male vs. Female); Std. Error = standard error.

| | Test | cor | statistic | p | conf.low | conf.high |
|---|---|---|---|---|---|---|
| **Frontal** | All Chr- CTL DEGs | 0.98 | 57.224 | 2.75E-110 | 0.967 | 0.982 |
| | All Chr- ASD DEGs | 0.99 | 62.684 | 8.65E-58 | 0.987 | 0.995 |
| | X Chr- CTL DEGs | 0.93 | 12.729 | 6.36E-13 | 0.847 | 0.965 |
| | X Chr- ASD DEGs | 0.99 | 28.763 | 3.73E-13 | 0.976 | 0.997 |
| | Autosomal- CTL DEGs | 0.24 | 2.499 | 0.0141 | 0.050 | 0.417 |
| | Autosomal- ASD DEGs | 0.82 | 5.004 | 3.07E-04 | 0.517 | 0.942 |
| **Temporal** | All Chr- CTL DEGs | 0.98 | 50.431 | 7.53E-62 | 0.976 | 0.990 |
| | All Chr- ASD DEGs | 0.99 | 62.762 | 1.01E-55 | 0.988 | 0.996 |
| | X Chr- CTL DEGs | 0.98 | 18.709 | 2.65E-11 | 0.943 | 0.993 |
| | X Chr- ASD DEGs | 0.98 | 17.451 | 2.11E-10 | 0.937 | 0.993 |
| | Autosomal- CTL DEGs | -0.038 | -0.202 | 0.841 | -0.387 | 0.321 |
| | Autosomal- ASD DEGs | 0.83 | 4.766 | 7.61E-04 | 0.497 | 0.952 |
| **Parietal** | All Chr- CTL DEGs | 0.97 | 37.587 | 5.82E-50 | 0.960 | 0.984 |
| | All Chr- ASD DEGs | 0.99 | 57.370 | 5.36E-48 | 0.987 | 0.996 |
| | X Chr- CTL DEGs | 0.96 | 14.732 | 1.74E-11 | 0.902 | 0.985 |
| | X Chr- ASD DEGs | 0.96 | 12.631 | 2.73E-08 | 0.888 | 0.989 |
| | Autosomal- CTL DEGs | 0.13 | 0.620 | 0.542 | -0.294 | 0.518 |
| | Autosomal- ASD DEGs | 0.58 | 1.431 | 0.226 | -0.435 | 0.946 |

**Table 6.** Results of Pearson correlation coefficient tests of $log_2$FC for sex-DEGs detected in controls, regardless of significance level in ASD, and sex-DEGs detected in ASD cases regardless of significance level of controls. Sex-DEGs in all chromosomes, only X chromosome, and only autosomes in each lobe were tested separately and are indicated above. ASD = autism spectrum disorder; CTL = control; DEGs = differentially-expressed genes; logFC = $log_2$ fold change (Male vs. Female); cor = correlation coefficient; p = p-value; conf.low = lower limit of 95% confidence interval; conf.high = upper limit of 95% confidence interval.

| | Test | P. value | Conf. low | Conf. high | Estimate |
|---|---|---|---|---|---|
| **Frontal** | All Chr- CTL DEGs | 1.94E-08 | 0.105 | Inf | 0.128 |
| | All Chr- ASD DEGs | 1.000 | -1.696 | Inf | -0.570 |
| | X Chr- CTL DEGs | 7.82E-03 | 0.027 | Inf | 0.066 |
| | X Chr- ASD DEGs | 0.837 | -0.102 | Inf | -0.029 |
| | Autosomal- CTL DEGs | 3.13E-26 | 0.155 | Inf | 0.199 |
| | Autosomal- ASD DEGs | 1.000 | -0.833 | Inf | -0.539 |
| **Temporal** | All Chr- CTL DEGs | 0.847 | -2.219 | Inf | -0.185 |
| | All Chr- ASD DEGs | 1.000 | -18.451 | Inf | -3.122 |
| | X Chr- CTL DEGs | 0.681 | -0.072 | Inf | -0.025 |
| | X Chr- ASD DEGs | 0.992 | -0.118 | Inf | -0.075 |
| | Autosomal- CTL DEGs | 3.26E-09 | 0.168 | Inf | 0.282 |
| | Autosomal- ASD DEGs | 1.000 | -1.156 | Inf | -0.785 |
| **Parietal** | All Chr- CTL DEGs | 1.70E-07 | 0.549 | Inf | 1.479 |
| | All Chr- ASD DEGs | 0.011 | 0.214 | Inf | 2.099 |
| | X Chr- CTL DEGs | 0.048 | 0.001 | Inf | 0.036 |
| | X Chr- ASD DEGs | 0.812 | -0.064 | Inf | -0.023 |
| | Autosomal- CTL DEGs | 5.13E-06 | 0.435 | Inf | 0.782 |
| | Autosomal- ASD DEGs | 1 | -1.021 | Inf | -0.552 |

**Table 7.** Results of one-sided (greater) paired Wilcoxon test of absolute fold change magnitude for sex-DEGs detected in controls, regardless of significance level in ASD, and sex-DEGs detected in ASD cases regardless of significance level of controls. Sex-DEGs in all chromosomes, only X chromosome, and only autosomes in each lobe were tested separately and are indicated above. ASD = autism spectrum disorder; CTL = control; DEGs = differentially-expressed genes; conf.low = lower limit of 95% confidence interval; conf.high = upper limit of 95% confidence interval.

| | Test | P. value | Conf. low | Conf. high | Estimate |
|---|---|---|---|---|---|
| **Frontal** | All Chr- CTL DEGs | 1.000 | -2.297 | Inf | -1.625 |
| | All Chr- ASD DEGs | 0.999 | -1.805 | Inf | -0.864 |
| | X Chr- CTL DEGs | 1.000 | -1.158 | Inf | -0.615 |
| | X Chr- ASD DEGs | 1.000 | -0.452 | Inf | -0.305 |
| | Autosomal- CTL DEGs | 1.000 | -2.759 | Inf | -1.957 |
| | Autosomal- ASD DEGs | 0.979 | -1.668 | Inf | -0.805 |
| **Temporal** | All Chr- CTL DEGs | 1.000 | -2.144 | Inf | -1.581 |
| | All Chr- ASD DEGs | 1.000 | -2.118 | Inf | -1.468 |
| | X Chr- CTL DEGs | 1.000 | -0.488 | Inf | -0.312 |
| | X Chr- ASD DEGs | 0.988 | -0.369 | Inf | -0.218 |
| | Autosomal- CTL DEGs | 1.000 | -1.711 | Inf | -0.782 |
| | Autosomal- ASD DEGs | 0.339 | -1.199 | Inf | 0.339 |
| **Parietal** | All Chr- CTL DEGs | 1.000 | -4.195 | Inf | -2.948 |
| | All Chr- ASD DEGs | 1.000 | -2.811 | Inf | -2.045 |
| | X Chr- CTL DEGs | 1.000 | -1.188 | Inf | -0.451 |
| | X Chr- ASD DEGs | 0.998 | -0.353 | Inf | -0.246 |
| | Autosomal- CTL DEGs | 1.000 | -11.063 | Inf | -8.814 |
| | Autosomal- ASD DEGs | 0.922 | -4.622 | Inf | -2.163 |

**Table 8.** Results of one-sided (greater) paired Wilcoxon test of variance of $\log_2$ fold change for sex-DEGs detected in controls, regardless of significance level in ASD, and sex-DEGs detected in ASD cases regardless of significance level of controls. Sex-DEGs in all chromosomes, only X chromosome, and only autosomes in each lobe were tested separately and are indicated above. ASD = autism spectrum disorder; CTL = control; DEGs = differentially-expressed genes; conf.low = lower limit of 95% confidence interval; conf.high = upper limit of 95% confidence interval.

| | Female-shifted | | Male-shifted | | Sex-equalized |
|---|---|---|---|---|---|
| | Exp. Up | Exp. Down | Exp. Up | Exp. Down | |
| **Frontal (Total)** | 7 | 19 | 5 | 6 | 42 |
| Autosomal | 7 | 19 | 4 | 5 | 41 |
| X chromosome | 0 | 0 | 1 | 1 | 1 |
| **Temporal (Total)** | 7 | 1 | 0 | 3 | 16 |
| Autosomal | 7 | 1 | 0 | 3 | 14 |
| X chr | 0 | 0 | 0 | 0 | 2 |
| **Parietal (Total)** | 3 | 5 | 4 | 1 | 4 |
| Autosomal | 2 | 5 | 4 | 1 | 4 |
| X chr | 1 | 0 | 0 | 0 | 0 |

**Table 9.** Counts of ASD-attenuated sex-DEGs by lobe and chromosome category that met criteria for expression patterns of female-shifted with either expression up in female cases or expression down in female cases, male-shifted with either expression up in male cases or expression down in male cases, or sex-equalized (Methods). Exp. = expression.

| Gene Name | Category | Annotations | Cortical Lobe |
|---|---|---|---|
| *IL32* | Female-shifted | Cortical endothelial cells | Frontal |
| *SLCO4A1* | | BECs | |
| *AFF3* | | BECs | |
| *PLA1A* | | BECs | |
| *SDPR* | | Cortical endothelial cells | |
| *C1orf64* | | Cortical endothelial cells | |
| *TGM2* | | BECs | |
| *IL4R* | Male-shifted | BECs | |
| *SLC16A1* | | Astros; BBB transport | |
| *P2RY12* | | Microglia | |
| *ANXA3* | Female-shifted | BECs | Parietal |
| *PTH1R* | | BBB pericytes | |
| *PCDH11X* | | Microglia | |

**Table 10**. ASD-attenuated sex-DEGs annotated to blood brain barrier (BBB) and neuroimmune cell types. BECs = brain endothelial cells; Astros = astrocytes.

| Test Set | Ref Set | N in Test | N in Ref | N Overlap | P-value | Odds Ratio | CI. Low | CI. High | Adj. P-value |
|---|---|---|---|---|---|---|---|---|---|
| **Frontal** | Astros | 68 | 175 | 0 | 1.000 | 0.000 | 0.000 | 7.801 | 1.000 |
| | Endo | 68 | 171 | 0 | 1.000 | 0.000 | 0.000 | 7.987 | 1.000 |
| | Microglia | 68 | 183 | 0 | 1.000 | 0.000 | 0.000 | 7.452 | 1.000 |
| | Neurons | 68 | 182 | 2 | 9.11E-02 | 4.079 | 0.480 | 15.540 | 1.000 |
| | Ex. Neuro | 68 | 113 | 0 | 1.000 | 0.000 | 0.000 | 12.182 | 1.000 |
| | In. Neuro | 68 | 30 | 0 | 1.000 | 0.000 | 0.000 | 48.134 | 1.000 |
| | Oligo | 68 | 142 | 0 | 1.000 | 0.000 | 0.000 | 9.649 | 1.000 |
| **Temporal** | Astros | 261 | 175 | 1 | 1.000 | 0.531 | 0.013 | 3.027 | 1.000 |
| | Endo | 261 | 171 | 2 | 0.705 | 1.099 | 0.131 | 4.071 | 1.000 |
| | Microglia | 261 | 183 | 4 | 0.132 | 2.090 | 0.559 | 5.510 | 1.000 |
| | Neurons | 261 | 182 | 5 | 4.59E-02 | 2.653 | 0.844 | 6.388 | 0.642 |
| | Ex. Neuro | 261 | 113 | 6 | 1.35E-03 | 5.302 | 1.886 | 12.080 | 1.90E-02 |
| | In. Neuro | 261 | 30 | 1 | 0.275 | 3.208 | 0.078 | 19.501 | 1.000 |
| | Oligo | 261 | 142 | 0 | 0.411 | 0.000 | 0.000 | 2.445 | 1.000 |
| **Parietal** | Astros | 924 | 175 | 8 | 0.547 | 1.223 | 0.518 | 2.477 | 1.000 |
| | Endo | 924 | 171 | 5 | 0.690 | 0.767 | 0.245 | 1.832 | 1.000 |
| | Microglia | 924 | 183 | 22 | 1.72E-06 | 3.544 | 2.150 | 5.584 | 2.41E-05 |
| | Neurons | 924 | 182 | 23 | 4.14E-07 | 3.756 | 2.302 | 5.873 | 5.79E-06 |
| | Ex. Neuro | 924 | 113 | 27 | 9.96E-15 | 8.213 | 5.095 | 12.856 | 1.39E-13 |
| | In. Neuro | 924 | 30 | 7 | 1.01E-04 | 7.810 | 2.822 | 18.865 | 1.42E-03 |
| | Oligo | 924 | 142 | 4 | 0.823 | 0.738 | 0.198 | 1.939 | 1.000 |

**Table 11**. Results of Fishers Exact test for cortical cell-type marker genes in female-protective DEGs (Methods). Astros = astrocytes; endo = endothelial cells; Ex. neurons = excitatory neurons; In. neurons = inhibitory neurons; oligo = oligodendrocytes; CI = confidence interval; Adj. P-value = Bonferroni- adjusted p-value.

| Test Set | Ref Set | N in Test | N in Ref | N Overlap | P-value | Odds Ratio | CI. Low | CI. High | Adj. P-value |
|---|---|---|---|---|---|---|---|---|---|
| Frontal | Astrocyte | 68 | 166 | 1 | 0.371 | 2.193 | 0.054 | 12.816 | 1 |
| | BECs | 68 | 204 | 0 | 1.000 | 0.000 | 0.000 | 6.673 | 1 |
| | Ependymal | 68 | 210 | 0 | 1.000 | 0.000 | 0.000 | 6.480 | 1 |
| | Fibroblasts | 68 | 183 | 0 | 1.000 | 0.000 | 0.000 | 7.452 | 1 |
| | Macrophage/microglia | 68 | 188 | 0 | 1.000 | 0.000 | 0.000 | 7.252 | 1 |
| | Neuron | 68 | 183 | 0 | 1.000 | 0.000 | 0.000 | 7.452 | 1 |
| | Oligo | 68 | 209 | 4 | 0.003 | 7.379 | 1.933 | 20.107 | 6.04E-02 |
| | OPC | 68 | 172 | 0 | 1.000 | 0.000 | 0.000 | 7.940 | 1 |
| | Pericyte | 68 | 131 | 0 | 1.000 | 0.000 | 0.000 | 10.476 | 1 |
| | Smooth muscle cells | 68 | 77 | 0 | 1.000 | 0.000 | 0.000 | 18.033 | 1 |
| | T Cell | 68 | 142 | 1 | 0.327 | 2.569 | 0.064 | 15.047 | 1 |
| Temporal | Astrocyte | 261 | 166 | 0 | 0.429 | 0.000 | 0.000 | 2.085 | 1 |
| | BECs | 261 | 204 | 1 | 0.729 | 0.455 | 0.011 | 2.586 | 1 |
| | Ependymal | 261 | 210 | 1 | 0.731 | 0.442 | 0.011 | 2.511 | 1 |
| | Fibroblasts | 261 | 183 | 1 | 1.000 | 0.508 | 0.013 | 2.891 | 1 |
| | Macrophage/microglia | 261 | 188 | 2 | 1.000 | 0.998 | 0.119 | 3.692 | 1 |
| | Neuron | 261 | 183 | 4 | 0.132 | 2.090 | 0.559 | 5.510 | 1 |
| | Oligo | 261 | 209 | 6 | 0.025 | 2.783 | 1.000 | 6.248 | 0.550 |
| | OPC | 261 | 172 | 3 | 0.435 | 1.655 | 0.336 | 4.973 | 1 |
| | Pericyte | 261 | 131 | 2 | 0.408 | 1.442 | 0.172 | 5.368 | 1 |
| | Smooth muscle cells | 261 | 77 | 1 | 0.563 | 1.222 | 0.030 | 7.075 | 1 |
| | T Cell | 261 | 142 | 1 | 1.000 | 0.657 | 0.016 | 3.752 | 1 |
| Parietal | Astrocyte | 924 | 166 | 8 | 0.414 | 1.293 | 0.547 | 2.622 | 1 |
| | BECs | 924 | 204 | 11 | 0.199 | 1.458 | 0.713 | 2.681 | 1 |
| | Ependymal | 924 | 210 | 12 | 0.143 | 1.552 | 0.786 | 2.786 | 1 |
| | Fibroblasts | 924 | 183 | 8 | 0.562 | 1.167 | 0.494 | 2.360 | 1 |
| | Macrophage/microglia | 924 | 188 | 9 | 0.439 | 1.284 | 0.576 | 2.504 | 1 |
| | Neuron | 924 | 183 | 37 | 4.27E-17 | 6.687 | 4.502 | 9.719 | 9.39E-16 |
| | Oligo | 924 | 209 | 18 | 1.32E-03 | 2.430 | 1.403 | 3.967 | 2.91E-02 |
| | OPC | 924 | 172 | 23 | 1.48E-07 | 4.010 | 2.453 | 6.284 | 3.25E-06 |
| | Pericyte | 924 | 131 | 6 | 0.641 | 1.225 | 0.440 | 2.753 | 1 |
| | Smooth muscle cells | 924 | 77 | 3 | 0.767 | 1.034 | 0.208 | 3.150 | 1 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | T Cell | 924 | 142 | 8 | 0.262 | 1.526 | 0.644 | 3.108 | 1 |

**Table 12.** Results of Fishers Exact Test for BBB-associated cell type markers (Yang et al. 2022) in female-protective DEGs (Methods). BBB = blood brain barrier; BECs = brain endothelial cells; oligo = oligodendrocytes; OPC = oligodendrocyte progenitor cells; CI = confidence interval; Adj. P-value = Bonferroni- adjusted p-value.

| Test Set | Ref Set | N in Test | N in Ref | N Overlap | P-value | Odds Ratio | CI. Low | CI. High | Adj. P-value |
|---|---|---|---|---|---|---|---|---|---|
| Frontal | Astros | 125 | 175 | 0 | 1.000 | 0.000 | 0.000 | 4.182 | 1 |
| | Endo | 125 | 171 | 3 | 5.74E-02 | 3.539 | 0.713 | 10.766 | 0.804 |
| | Microglia | 125 | 183 | 1 | 0.610 | 1.071 | 0.027 | 6.154 | 1 |
| | Neurons | 125 | 182 | 3 | 6.66E-02 | 3.321 | 0.669 | 10.093 | 0.933 |
| | Ex. Neuro | 125 | 113 | 0 | 1.000 | 0.000 | 0.000 | 6.530 | 1 |
| | In. Neuro | 125 | 30 | 0 | 1.000 | 0.000 | 0.000 | 25.816 | 1 |
| | Oligo | 125 | 142 | 2 | 0.164 | 2.812 | 0.334 | 10.566 | 1 |
| Temporal | Astros | 283 | 175 | 3 | 0.459 | 1.496 | 0.304 | 4.493 | 1 |
| | Endo | 283 | 171 | 7 | 3.85E-03 | 3.716 | 1.457 | 7.934 | 5.39E-02 |
| | Microglia | 283 | 183 | 6 | 1.99E-02 | 2.939 | 1.055 | 6.607 | 0.279 |
| | Neurons | 283 | 182 | 2 | 1.000 | 0.950 | 0.114 | 3.513 | 1 |
| | Ex. Neuro | 283 | 113 | 0 | 0.644 | 0.000 | 0.000 | 2.842 | 1 |
| | In. Neuro | 283 | 30 | 0 | 1.000 | 0.000 | 0.000 | 11.244 | 1 |
| | Oligo | 283 | 142 | 2 | 0.681 | 1.223 | 0.146 | 4.542 | 1 |
| Parietal | Astros | 373 | 175 | 7 | 1.81E-02 | 2.725 | 1.071 | 5.797 | 0.253 |
| | Endo | 373 | 171 | 6 | 4.75E-02 | 2.372 | 0.853 | 5.323 | 0.665 |
| | Microglia | 373 | 183 | 8 | 7.20E-03 | 2.997 | 1.264 | 6.099 | 1.01E-01 |
| | Neurons | 373 | 182 | 4 | 0.362 | 1.457 | 0.391 | 3.829 | 1 |
| | Ex. Neuro | 373 | 113 | 1 | 1.000 | 0.576 | 0.014 | 3.295 | 1 |
| | In. Neuro | 373 | 30 | 0 | 1.000 | 0.000 | 0.000 | 8.486 | 1 |
| | Oligo | 373 | 142 | 9 | 3.39E-04 | 4.456 | 1.978 | 8.811 | 4.75E-03 |

**Table 13**. Results of Fishers Exact test for cortical cell-type marker genes in male-vulnerability DEGs (Methods). Astros = astrocytes; endo = endothelial cells; Ex. neurons = excitatory neurons; In. neurons = inhibitory neurons; oligo = oligodendrocytes; CI = confidence interval; Adj. P-value = Bonferroni- adjusted p-value.

| Test Set | Ref Set | N in Test | N in Ref | N Overlap | P-value | Odds Ratio | CI. Low | CI. High | Adj. P-value |
|---|---|---|---|---|---|---|---|---|---|
| Frontal | Astrocyte | 125 | 166 | 2 | 0.208 | 2.398 | 0.285 | 8.986 | 1 |
| | BECs | 125 | 204 | 3 | 8.69E-02 | 2.955 | 0.596 | 8.964 | 1 |
| | Ependymal | 125 | 210 | 1 | 1.000 | 0.932 | 0.023 | 5.344 | 1 |
| | Fibroblasts | 125 | 183 | 6 | 3.61E-04 | 6.884 | 2.445 | 15.734 | 7.95E-03 |
| | Macrophage/microglia | 125 | 188 | 3 | 7.19E-02 | 3.212 | 0.648 | 9.758 | 1 |
| | Neuron | 125 | 183 | 0 | 1.000 | 0.000 | 0.000 | 3.996 | 1 |
| | Oligo | 125 | 209 | 0 | 0.631 | 0.000 | 0.000 | 3.491 | 1 |
| | OPC | 125 | 172 | 0 | 1.000 | 0.000 | 0.000 | 4.256 | 1 |
| | Pericyte | 125 | 131 | 1 | 0.490 | 1.503 | 0.037 | 8.675 | 1 |
| | Smooth muscle cells | 125 | 77 | 2 | 5.91E-02 | 5.264 | 0.619 | 20.086 | 1 |
| | T Cell | 125 | 142 | 1 | 0.518 | 1.385 | 0.035 | 7.985 | 1 |
| Temporal | Astrocyte | 283 | 166 | 1 | 1.000 | 0.516 | 0.013 | 2.941 | 1 |
| | BECs | 283 | 204 | 2 | 1.000 | 0.845 | 0.101 | 3.122 | 1 |
| | Ependymal | 283 | 210 | 1 | 0.738 | 0.407 | 0.010 | 2.311 | 1 |
| | Fibroblasts | 283 | 183 | 0 | 0.281 | 0.000 | 0.000 | 1.739 | 1 |
| | Macrophage/microglia | 283 | 188 | 5 | 6.78E-02 | 2.360 | 0.751 | 5.674 | 1 |
| | Neuron | 283 | 183 | 0 | 0.281 | 0.000 | 0.000 | 1.739 | 1 |
| | Oligo | 283 | 209 | 0 | 0.183 | 0.000 | 0.000 | 1.519 | 1 |
| | OPC | 283 | 172 | 0 | 0.273 | 0.000 | 0.000 | 1.852 | 1 |
| | Pericyte | 283 | 131 | 1 | 1.000 | 0.656 | 0.016 | 3.752 | 1 |
| | Smooth muscle cells | 283 | 77 | 3 | 5.99E-02 | 3.492 | 0.700 | 10.711 | 1 |
| | T Cell | 283 | 142 | 4 | 8.30E-02 | 2.499 | 0.667 | 6.617 | 1 |
| Parietal | Astrocyte | 373 | 166 | 19 | 1.06E-11 | 8.744 | 5.060 | 14.353 | 2.32E-10 |
| | BECs | 373 | 204 | 3 | 1.000 | 0.964 | 0.196 | 2.881 | 1 |
| | Ependymal | 373 | 210 | 3 | 1.000 | 0.936 | 0.191 | 2.796 | 1 |
| | Fibroblasts | 373 | 183 | 5 | 0.208 | 1.826 | 0.582 | 4.381 | 1 |
| | Macrophage/microglia | 373 | 188 | 9 | 2.46E-03 | 3.305 | 1.475 | 6.484 | 5.42E-02 |
| | Neuron | 373 | 183 | 0 | 0.122 | 0.000 | 0.000 | 1.313 | 1 |
| | Oligo | 373 | 209 | 0 | 8.15E-02 | 0.000 | 0.000 | 1.147 | 1 |
| | OPC | 373 | 172 | 1 | 0.528 | 0.376 | 0.009 | 2.138 | 1 |
| | Pericyte | 373 | 131 | 4 | 0.140 | 2.047 | 0.546 | 5.414 | 1 |
| | Smooth muscle cells | 373 | 77 | 3 | 0.113 | 2.633 | 0.528 | 8.059 | 1 |
| | T Cell | 373 | 142 | 6 | 2.19E-02 | 2.881 | 1.032 | 6.497 | 0.481 |

**Table 14.** Results of Fishers Exact Test for BBB-associated cell type markers (Yang et al. 2022) in male-vulnerability DEGs (Methods). BBB = blood brain barrier; BECs = brain endothelial cells; oligo = oligodendrocytes; OPC = oligodendrocyte progenitor cells; CI = confidence interval; Adj. P-value = Bonferroni- adjusted p-value.

| Test Set | Ref Set | N in Test | N in Ref | N Overlap | P-value | Odds Ratio | CI. Low | CI. High | Adj. P-value |
|---|---|---|---|---|---|---|---|---|---|
| **Frontal** | BBB integrity | 125 | 22 | 0 | 1.000 | 0.000 | 0.000 | 36.008 | 1.000 |
| | BBB transport | 125 | 80 | 2 | 0.063 | 5.060 | 0.596 | 19.286 | 0.253 |
| **Temporal** | BBB integrity | 283 | 22 | 0 | 1.000 | 0.000 | 0.000 | 15.684 | 1.000 |
| | BBB transport | 283 | 80 | 1 | 0.606 | 1.083 | 0.027 | 6.259 | 1.000 |
| **Parietal** | BBB integrity | 373 | 22 | 2 | 0.044 | 6.491 | 0.733 | 26.897 | 0.175 |
| | BBB transport | 373 | 80 | 4 | 0.034 | 3.427 | 0.906 | 9.214 | 0.136 |

**Table 15.** Results of Fishers Exact Test for BBB-associated functions (Saverimuttu 2021) in male-vulnerability DEGs (Methods).

| Test Set | Ref Set | N in Test | N in Ref | N Overlap | P-value | Odds Ratio | CI. Low | CI. High | Adj. P-value |
|---|---|---|---|---|---|---|---|---|---|
| **Frontal** | BBB integrity | 68 | 22 | 0 | 1.000 | 0.000 | 0.000 | 67.070 | 1.000 |
| | BBB transport | 68 | 80 | 1 | 0.200 | 4.596 | 0.113 | 27.265 | 0.799 |
| **Temporal** | BBB integrity | 261 | 22 | 0 | 1.000 | 0.000 | 0.000 | 17.030 | 1.000 |
| | BBB transport | 261 | 80 | 1 | 0.576 | 1.175 | 0.029 | 6.798 | 1.000 |
| **Parietal** | BBB integrity | 924 | 22 | 0 | 1.000 | 0.000 | 0.000 | 4.658 | 1.000 |
| | BBB transport | 924 | 80 | 2 | 0.771 | 0.653 | 0.078 | 2.446 | 1.000 |

**Table 16.** Results of Fishers Exact Test for BBB-associated functions (Saverimuttu 2021) in female-protective DEGs (Methods).

| Test Set | Ref Set | N in Test | N in Ref | N Overlap | P-value | Odds Ratio | CI. Low | CI. High | Adj. P-value |
|---|---|---|---|---|---|---|---|---|---|
| **Frontal** | Satterstrom 2020 | 63 | 96 | 0 | 1 | 0 | 0 | 12.206 | 1.000 |
| | Fu 2022 | 54 | 71 | 0 | 1 | 0 | 0 | 15.831 | 1.000 |
| | Zhou 2022 | 55 | 59 | 0 | 1 | 0 | 0 | 19.931 | 1.000 |
| | Grove 2019 | 54 | 12 | 0 | 1 | 0 | 0 | 109.948 | 1.000 |
| **Temporal** | Satterstrom 2020 | 250 | 96 | 4 | 0.037 | 3.337 | 0.883 | 8.947 | 0.073 |
| | Fu 2022 | 222 | 71 | 2 | 0.267 | 2.021 | 0.238 | 7.669 | 1.000 |
| | Zhou 2022 | 233 | 59 | 4 | 0.009 | 5.166 | 1.349 | 14.173 | 0.057 |
| | Grove 2019 | 225 | 12 | 0 | 1 | 0 | 0 | 25.495 | 1.000 |
| **Parietal** | Satterstrom 2020 | 872 | 96 | 10 | 0.012 | 2.464 | 1.137 | 4.776 | 0.023 |
| | Fu 2022 | 812 | 71 | 10 | 0.004 | 3.017 | 1.373 | 5.961 | 0.021 |
| | Zhou 2022 | 857 | 59 | 8 | 0.011 | 2.892 | 1.181 | 6.164 | 0.064 |
| | Grove 2019 | 815 | 12 | 0 | 1 | 0 | 0 | 6.745 | 1.000 |

**Table 17.** Results of Fishers Exact Test for gene sets of rare or common variants implicated in ASD risk in female-protective DEGs (Methods).

| Test Set | Ref Set | N in Test | N in Ref | N Overlap | P-value | Odds Ratio | CI. Low | CI. High | Adj. P-value |
|---|---|---|---|---|---|---|---|---|---|
| Frontal | ADHD | 63 | 9 | 0 | 1.000 | 0.000 | 0.000 | 157.700 | 1 |
| | AD | 63 | 30 | 0 | 1.000 | 0.000 | 0.000 | 40.812 | 1 |
| | DD | 63 | 91 | 0 | 1.000 | 0.000 | 0.000 | 12.884 | 1 |
| | EA | 63 | 836 | 2 | 1.000 | 0.721 | 0.085 | 2.725 | 1 |
| | EPDD | 63 | 32 | 0 | 1.000 | 0.000 | 0.000 | 38.133 | 1 |
| | MDD | 63 | 28 | 0 | 1.000 | 0.000 | 0.000 | 43.926 | 1 |
| | MS | 63 | 72 | 1 | 0.211 | 4.337 | 0.107 | 25.835 | 1 |
| | PD | 63 | 32 | 0 | 1.000 | 0.000 | 0.000 | 38.133 | 1 |
| | SCZ | 63 | 238 | 2 | 0.183 | 2.629 | 0.310 | 10.015 | 1 |
| Temporal | ADHD | 250 | 9 | 0 | 1.000 | 0.000 | 0.000 | 38.669 | 1 |
| | AD | 250 | 30 | 0 | 1.000 | 0.000 | 0.000 | 9.986 | 1 |
| | DD | 250 | 91 | 0 | 0.635 | 0.000 | 0.000 | 3.148 | 1 |
| | EA | 250 | 836 | 12 | 0.641 | 1.111 | 0.564 | 1.987 | 1 |
| | EPDD | 250 | 32 | 0 | 1.000 | 0.000 | 0.000 | 9.324 | 1 |
| | MDD | 250 | 28 | 0 | 1.000 | 0.000 | 0.000 | 10.749 | 1 |
| | MS | 250 | 72 | 2 | 0.241 | 2.178 | 0.257 | 8.249 | 1 |
| | PD | 250 | 32 | 0 | 1.000 | 0.000 | 0.000 | 9.324 | 1 |
| | SCZ | 250 | 238 | 4 | 0.556 | 1.302 | 0.349 | 3.422 | 1 |
| Parietal | ADHD | 872 | 9 | 0 | 1.000 | 0.000 | 0.000 | 10.674 | 1 |
| | AD | 872 | 30 | 1 | 1.000 | 0.725 | 0.018 | 4.386 | 1 |
| | DD | 872 | 91 | 10 | 0.008 | 2.617 | 1.205 | 5.087 | 0.163 |
| | EA | 872 | 836 | 45 | 0.233 | 1.208 | 0.867 | 1.647 | 1 |
| | EPDD | 872 | 32 | 8 | 6.90E-05 | 7.070 | 2.738 | 16.329 | 1.38E-03 |
| | MDD | 872 | 28 | 1 | 1.000 | 0.779 | 0.019 | 4.740 | 1 |
| | MS | 872 | 72 | 0 | 0.079 | 0.000 | 0.000 | 1.105 | 1 |
| | PD | 872 | 32 | 3 | 0.176 | 2.181 | 0.424 | 7.060 | 1 |
| | SCZ | 872 | 238 | 16 | 0.114 | 1.527 | 0.854 | 2.549 | 1 |

**Table 18.** Results of Fishers Exact Test for gene sets implicated in neuropsychiatric disorders and phenotypes in female-protective DEGs (Methods). DD = developmental delay; ADHD = attention deficit hyperactivity disorder; EPDD = epilepsy and developmental delay; EA = educational attainment; SCZ = schizophrenia; MDD = major depressive disorder; MS = multiple sclerosis; PD = Parkinson's disease; AD = Alzheimer's disease.

| Test Set | Ref Set | N in Test | N in Ref | N Overlap | P-value | Odds Ratio | CI. Low | CI. High | Adj. P-value |
|---|---|---|---|---|---|---|---|---|---|
| **Frontal** | Satterstrom 2020 | 113 | 96 | 0 | 1.000 | 0 | 0 | 6.699 | 1 |
| | Fu 2022 | 115 | 59 | 0 | 1.000 | 0 | 0 | 9.339 | 1 |
| | Zhou 2022 | 106 | 71 | 0 | 1.000 | 0 | 0 | 7.911 | 1 |
| | Grove 2019 | 106 | 12 | 0 | 1.000 | 0 | 0 | 54.835 | 1 |
| **Temporal** | Satterstrom 2020 | 265 | 96 | 0 | 0.645 | 0 | 0 | 2.808 | 1 |
| | Fu 2022 | 246 | 59 | 0 | 1.000 | 0 | 0 | 4.295 | 1 |
| | Zhou 2022 | 231 | 71 | 0 | 0.629 | 0 | 0 | 3.567 | 1 |
| | Grove 2019 | 231 | 12 | 0 | 1.000 | 0 | 0 | 24.818 | 1 |
| **Parietal** | Satterstrom 2020 | 341 | 96 | 0 | 0.421 | 0 | 0 | 2.170 | 1 |
| | Fu 2022 | 329 | 59 | 0 | 0.633 | 0 | 0 | 3.190 | 1 |
| | Zhou 2022 | 311 | 71 | 0 | 0.407 | 0 | 0 | 2.631 | 1 |
| | Grove 2019 | 312 | 12 | 0 | 1.000 | 0 | 0 | 18.251 | 1 |

**Table 19.** Results of Fishers Exact Test for gene sets of rare or common variants implicated in ASD risk in male-vulnerability DEGs (Methods).

| Test Set | Ref Set | N in Test | N in Ref | N Overlap | P-value | Odds Ratio | CI. Low | CI. High | Adj. P-value |
|---|---|---|---|---|---|---|---|---|---|
| Frontal | ADHD | 113 | 9 | 0 | 1.000 | 0.000 | 0.000 | 86.724 | 1.000 |
| | AD | 113 | 30 | 0 | 1.000 | 0.000 | 0.000 | 22.434 | 1.000 |
| | DD | 113 | 91 | 0 | 1.000 | 0.000 | 0.000 | 7.077 | 1.000 |
| | EA | 113 | 836 | 2 | 0.245 | 0.395 | 0.047 | 1.465 | 1.000 |
| | EPDD | 113 | 32 | 0 | 1.000 | 0.000 | 0.000 | 20.945 | 1.000 |
| | MDD | 113 | 28 | 0 | 1.000 | 0.000 | 0.000 | 24.142 | 1.000 |
| | MS | 113 | 72 | 0 | 1.000 | 0.000 | 0.000 | 9.001 | 1.000 |
| | PD | 113 | 32 | 0 | 1.000 | 0.000 | 0.000 | 20.945 | 1.000 |
| | SCZ | 113 | 238 | 0 | 0.409 | 0.000 | 0.000 | 2.652 | 1.000 |
| Temporal | ADHD | 265 | 9 | 0 | 1.000 | 0.000 | 0.000 | 36.390 | 1.000 |
| | AD | 265 | 30 | 2 | 0.064 | 5.140 | 0.590 | 20.566 | 1.000 |
| | DD | 265 | 91 | 0 | 0.640 | 0.000 | 0.000 | 2.966 | 1.000 |
| | EA | 265 | 836 | 8 | 0.362 | 0.682 | 0.290 | 1.370 | 1.000 |
| | EPDD | 265 | 32 | 0 | 1.000 | 0.000 | 0.000 | 8.786 | 1.000 |
| | MDD | 265 | 28 | 0 | 1.000 | 0.000 | 0.000 | 10.128 | 1.000 |
| | MS | 265 | 72 | 2 | 0.261 | 2.052 | 0.242 | 7.768 | 1.000 |
| | PD | 265 | 32 | 0 | 1.000 | 0.000 | 0.000 | 8.786 | 1.000 |
| | SCZ | 265 | 238 | 1 | 0.389 | 0.299 | 0.008 | 1.698 | 1.000 |
| Parietal | ADHD | 341 | 9 | 0 | 1.000 | 0.000 | 0.000 | 28.164 | 1.000 |
| | AD | 341 | 30 | 3 | 0.016 | 6.198 | 1.198 | 20.288 | 0.315 |
| | DD | 341 | 91 | 0 | 0.415 | 0.000 | 0.000 | 2.293 | 1.000 |
| | EA | 341 | 836 | 18 | 0.419 | 1.231 | 0.717 | 1.989 | 1.000 |
| | EPDD | 341 | 32 | 0 | 1.000 | 0.000 | 0.000 | 6.790 | 1.000 |
| | MDD | 341 | 28 | 0 | 1.000 | 0.000 | 0.000 | 7.825 | 1.000 |
| | MS | 341 | 72 | 2 | 0.366 | 1.586 | 0.188 | 5.990 | 1.000 |
| | PD | 341 | 32 | 0 | 1.000 | 0.000 | 0.000 | 6.790 | 1.000 |
| | SCZ | 341 | 238 | 2 | 0.451 | 0.466 | 0.056 | 1.716 | 1.000 |

**Table 20.** Results of Fishers Exact Test for gene sets implicated in neuropsychiatric disorders and phenotypes in male-vulnerability DEGs (Methods). DD = developmental delay; ADHD = attention deficit hyperactivity disorder; EPDD = epilepsy and developmental delay; EA = educational attainment; SCZ = schizophrenia; MDD = major depressive disorder; MS = multiple sclerosis; PD = Parkinson's disease; AD = Alzheimer's disease.

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## SUMMARY, LIMITATIONS, AND FUTURE DIRECTIONS

### Summary

Autism spectrum disorder (ASD) was first described and characterized by Leo Kanner in 1943 (1). Throughout the last 80 years of research on this condition, a consistent finding has been the male preponderance in prevalence, with a current ~4:1 male to female ratio (2,3), suggestive of a prominent role for sex-differential biology in the risk and presentation of ASD (**Fig 1**). Additionally, research has identified different levels of biology, such as alterations in neural circuitry and gene expression, contribute to the ASD phenotype, but how sex plays into these neurobiological features of ASD has been understudied. It has been hypothesized that ASD may develop from multiple “hits” involving some combination of genetics, sex (intrinsic factors), and environmental triggers (extrinsic factors) and possibly at specific developmental stages, leading to the imbalance of males and females in ASD prevalence (**Fig. 1**) (4–9). Given this complexity and unknown mechanistic roles, sex differences may contribute at any level, impacting the additive hit to result in development of ASD (**Fig. 2**). Toward this end, studying gene expression, and specifically utilizing transcriptomic approaches that facilitate comparisons across data sets, allows for the discovery of biological processes that may overlap or influence biological “hits” leading to ASD development, such as sex effects.

Here, we employed such transcriptomic approaches to investigate sex-differences in gene expression in the brain during prenatal cortical development and changes within the ASD cortex. First, we defined robust sex-differential transcriptomic signatures in the human cortex during a specific timepoint in prenatal development by leveraging two large, independent RNA-sequencing studies available, which previously had not been fully addressed in the field. This study determined that sex-differential gene expression in human prenatal cortical tissue is mostly confined to sex chromosome genes, except for a few autosomal genes encoding neuronal proteins, and does not overlap with ASD risk variants, suggesting that sex-differential biology may

intersect with ASD neurobiology in other brain regions, developmental stages, or mechanisms. Then, in the postnatal cortex, we established sex-differential gene expression patterns and changes within the context of ASD brain and found attenuation of sex effects in the ASD brain that primarily occurs in autosomal genes. We also identified genes in the postnatal brain that show specific expression patterns reflective of sex-differential risk mechanisms and biological functions, processes, and pathways associated with these patterns. These genes demonstrated that transcriptomic signatures reflecting female protection overlap with neuronal cell markers and ASD risk genes and signatures representing male-vulnerability are related to neuroimmune and blood-brain-barrier (BBB) functions. Additionally, this study demonstrated that the most pronounced signatures of female protection or male vulnerability occur in cortical regions posterior to the frontal cortex. Overall, through comparison and identification of patterns of gene expression affected by variables of sex and/or ASD, we have highlighted genes and functional expression patterns important in human cortical development in males and females which also may be implicated within sex-differential aspects of ASD neurobiology and prevalence, providing avenues for future research into addressing precise mechanisms that may contribute to ASD's profound sex bias.

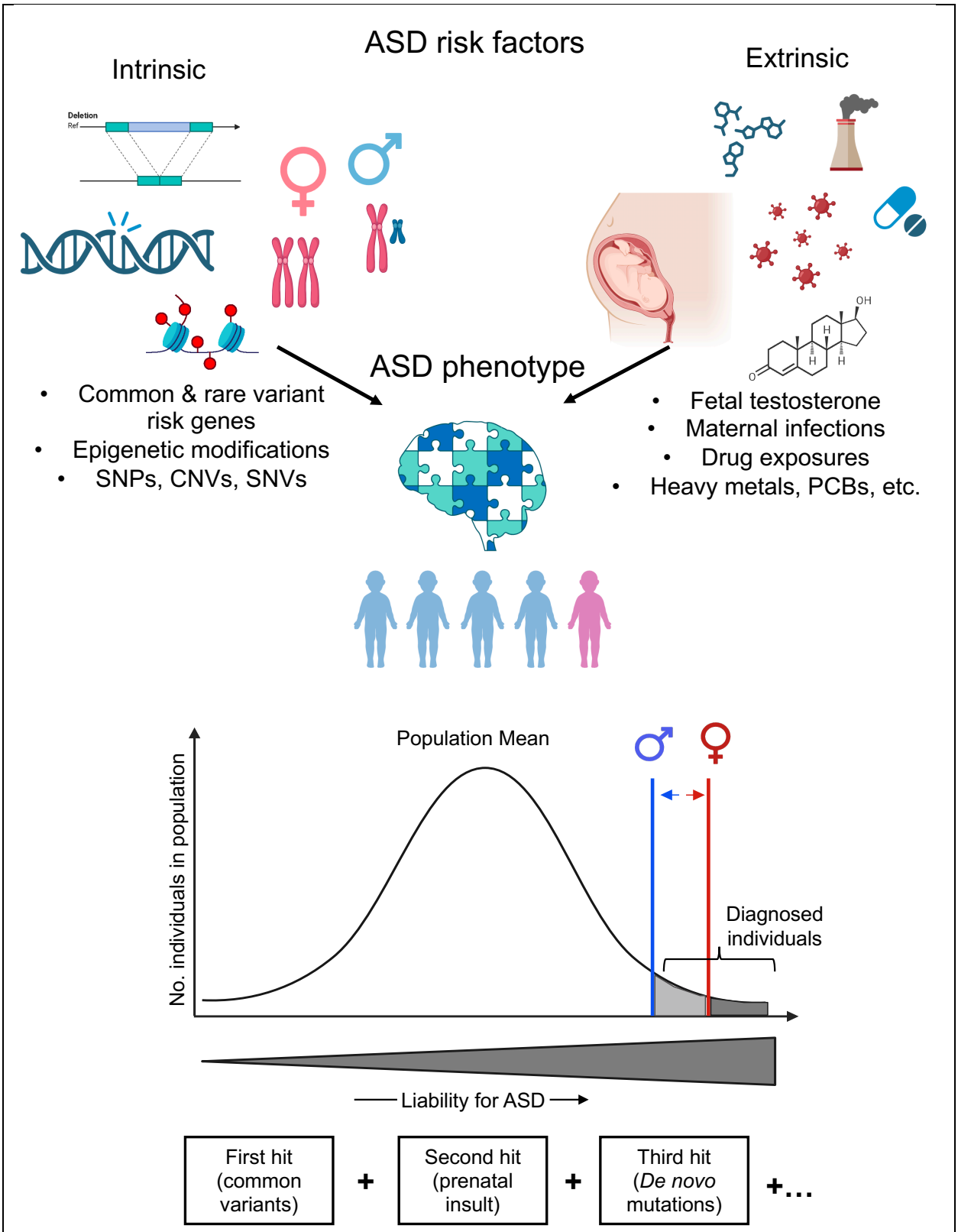


**Fig 1:** Summary of known ASD risk factors and multi-hit hypothesis leading to ASD phenotype. SNPs = single-nucleotide polymorphisms; CNVs = copy number variants; SNVs = single nucleotide variants; PCBs = polychlorinated biphenyls. Created with BioRender.com.

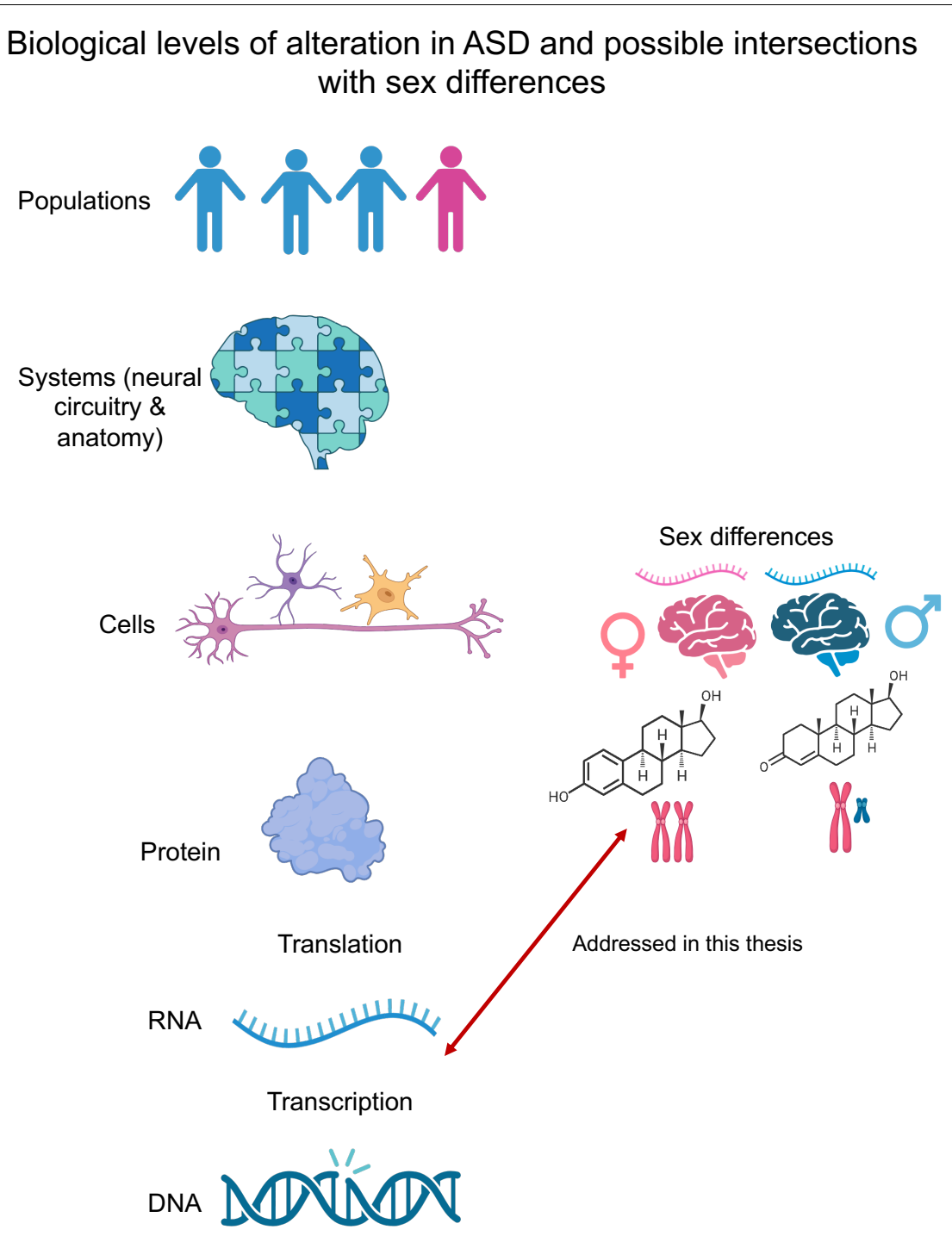


**Fig 2:** Biological levels of alterations in ASD and possible intersections with sex differences. Created with BioRender.com.

**Sex effects on gene expression as related to ASD phenotype and genetic risk**

In both studies, and consistent with previous literature (10–12), we detected strong sex-differential expression in the sex chromosomes and more subtle effects in autosomal genes in the cortex and during the developmental timepoints examined here (prenatal 14-21 post-conception weeks (PCW) and postnatal). Even though effects of sex on gene expression are minimal, our second study supports that even these subtle differences between male and female expression are attenuated in ASD. However, a caveat of our study, and the field in general, is that females with an ASD diagnosis, and therefore those who contribute to human tissue studies, may be more likely to have a similar phenotype as ASD males. This phenotypic similarity could result in limited detection of sex differences in currently available tissue samples. That said, a similar phenomenon has been demonstrated in several neuroimaging studies, with evidence for reduction of neurotypical sex differences in measures of total white matter volume, regional gray matter volume, and neural connectivity in ASD subjects (13–18). Here, we demonstrate that such a pattern is evident at the transcriptomic level in cortical areas. Similarly, from an overlapping set of samples, Parikshak et al.(19) and Gandal et al.(20) reported a pattern of “attenuated cortical patterning” of gene expression in ASD cortical tissue compared to control tissue. In other words, cortical regions of the brain, i.e. frontal lobe vs. temporal lobe, are less transcriptionally distinct in the ASD brain compared to neurotypical brains. Building on this, our results indicate that cortical regions are also less transcriptionally distinct between sexes in ASD. Together, this could possibly reflect a dysregulation of cortical development to respond to various differentiation cues, whether that be a sex-specific cue or region-specific biological signal. Future research utilizing animal models could test these possibilities by ablating expression of genes during development and analyze resulting changes in neural gene expression, cortical patterning, and behavioral phenotypes. Possibly, there exists a “master regulator”, such as a transcription factor, that has altered activity or expression during development, leading to a decrease in differentiation of the cortex.

In terms of direct effects of sex on mutation-causing ASD genes, such as ASD risk genes being sex-differentially expressed/regulated, in both studies here and parallel with previous reports (21,22), we can specifically conclude that the current set of ASD risk genes, identified from exome-sequencing (23–25), are not preferentially sex-differentially expressed in the human prenatal nor postnatal cortex tissue during age ranges studied here (**Fig 3**). These results contribute to the field by concretely demonstrating that currently known mutation-causing ASD risk genes are not sex-differentially regulated themselves. However, it is important to acknowledge certain limitations and considerations regarding currently known ASD risk variants. First, the present list of ASD risk genes from exome sequencing are those most amenable to discovery due to their relatively large damaging effect (23–26), and currently only account for 20-30% of cases (27). Considering the majority of ASD cases are idiopathic and do not express any known ASD risk variants, it is hypothesized ASD is most likely a polygenic disorder (23,28,29), possibly resulting from a combination of genes with individually smaller effects on ASD risk, but thus far only a small set of common variants and rare inherited variants, both of which are less damaging, have been discovered (25,30). As our knowledge of genetic variants contributing to ASD expands, testing for sex-differential regulation of such genes will need to be revisited, as we may find evidence of this in ASD risk genes that have not yet been identified.

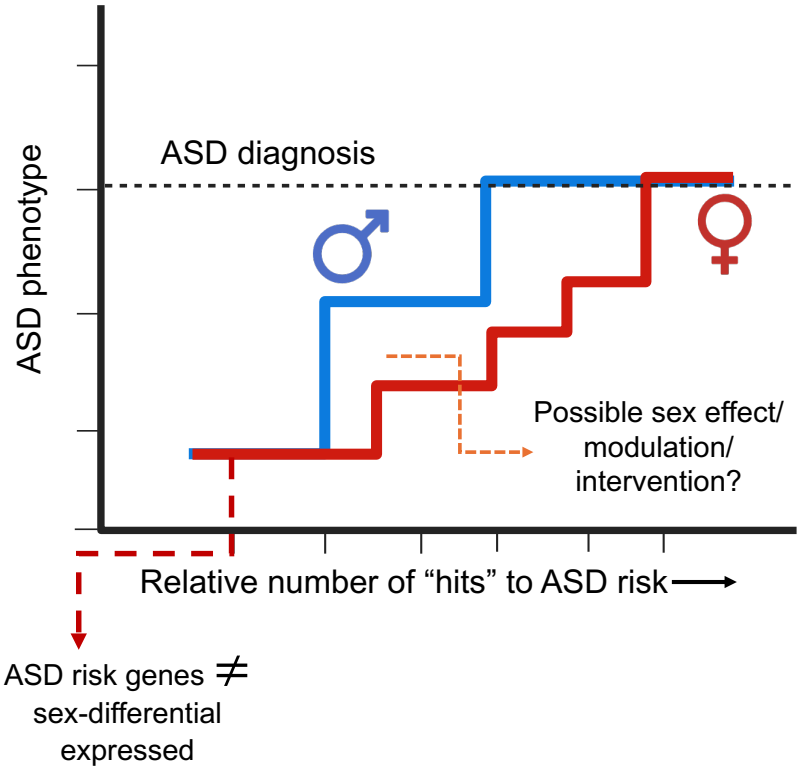


**Fig 3:** Summary of multi-hit hypothesis and intersection with sex-differential effects to reach ASD diagnosis. Created with BioRender.com.

Additionally, at present, ASD risk genes from exome sequencing are entirely autosomal (23–25,31). This is largely due to challenges in statistical power for risk gene discovery on the X chromosome in a male-skewed condition like ASD, in which the majority of cases have only 1 X chromosome that is maternally inherited, while *de novo* variants driving gene-based association results primarily arise on paternal chromosomes. This is a major limitation of the field as the X chromosome contains multiple genes that are involved in neurodevelopment and mutations of such genes lead to neurodevelopmental disorders (NDD) (32–36). Furthermore, evidence from my thesis work, as well as other studies, demonstrates consistent sex-differential expression of X chromosome genes, particularly those that may escape X inactivation, and suggest the possibility of sex-differential influences on human neurodevelopment and autism etiology at these loci (32,35–40). Moreover, work by Turner et al. (33) found sex-specific risk effects of X chromosome variants contributing to NDDs, which overlap with sex-differentially expressed genes

consistently detected here. Thus, it remains possible, and highly likely, that future work may identify X chromosome genes involved in ASD risk that show different expression levels in males and females. Possibly, female-biased expression of X chromosome genes that escape X inactivation, resulting in higher baseline expression in females, may be involved in the female protective effect to other autism risk factors (35,40,41). For example, if an X chromosome ASD risk gene escapes X inactivation and is expressed at a higher level in females than males, then mutations in this gene in males could be more detrimental compared to females with a “back-up” level of normal expression. Overall, at present, we do not find evidence of sex-differential transcriptional regulation of currently known ASD risk genes, but as these gaps in research have been filled in and the list of ASD risk genes expands, this will need to be re-evaluated.

Besides sex chromosomes, sex differences in brain function and development are also impacted by effects of circulating sex hormones and may be involved in ASD etiology (42–46). Indeed, hormonal expression patterns and fetal exposure to elevated levels of testosterone and oestrogens have been linked to development of ASD phenotypes later in life in human studies (43–45,47). However, RNA-seq data sets utilized in this thesis do not include any measurement of hormone levels nor pubertal status for the postnatal samples. Evaluation of genes involved in sex hormone biology (48,49), including estrogen receptors *ESR1*, *ESR2*, androgen receptor (*AR*), and aromatase (*CYP19A1*) shows no sex- or ASD-differential expression or age effects in either data set, suggesting that these genes are not reliable proxies for sex hormone exposure in cortex. Thus, the influence of sex hormones cannot be accounted for nor addressed in this thesis work. However, current knowledge of transcriptomic signatures regulated and/or influenced by levels of sex hormones in the human brain is minimal (10,50–52) and animal studies have largely utilized non-brain tissue for characterizing sex hormone effects (further reviewed in (10,51)). Recent work in mice finds neonatal hormone surges act on *ESR1*-expressing cells in the bed nucleus of stria terminalis, aiding in establishment of neural circuitry, and the genome of these cells continues to be responsive later in life to hormonal alterations (49). Although such work was conducted in only

a specific area of the murine brain, it demonstrates the careful experiments that could be executed to delineate mechanisms of sex hormone influence on the transcriptome in other cell populations and/or tissue in the brain. As future research fills in these gaps, examining differences in expression levels between males and females, and within the context of the human ASD brain, or the brain of ASD models, will be important.

**Limitations and future directions: Cell types, brain regions, development (Fig 4)**

Our analyses and the current understanding of sex-differential biology and its role in sex-skewed disorders, particularly at the transcriptomic level, may be limited by the methodology employed thus far in the field. Specifically, our analyses and previous work utilizing bulk RNA-sequencing consistently report minimal detection of sex-differential gene expression. Although we can see profound transcriptional differences in ASD v. CTL in bulk cortical tissue (19,20,53), our work demonstrates and builds on previous research that strong sex effects are not seen at the whole tissue level and could possibly be “washed out” in bulk RNA sequencing. Although we do find more sex-differentially expressed genes in the meta-analysis, it is possible that sex effects may be more pronounced, or are evident in a larger number of genes, at the level of individual cell populations that we are unable to detect with bulk tissue data. In other words, differences between male and female gene expression could be more evident, or readily detectable, in specific cell populations, as opposed to the mixed population of cells in bulk brain tissue. At present, only a few studies have employed single-nuclei RNA sequencing methods to study differential expression in certain cell populations during prenatal cortical development, but the majority have focused on the effects of developmental age, rather than sex (54–57). Promisingly, Velmeshev and colleagues (58) reported detection of hundreds of sex-specific gene expression events in certain neural cell populations during developmental time, providing groundwork that transcriptional differences between males and female are reflected in specific neuronal types, including subplate neurons and L6 excitatory neurons. Furthermore, they found evidence of

female-specific, upregulated expression of candidate ASD genes (SFARI high confidence), including transcription factors *NR4A2* and *MEF2C*, in female neuronal subplate cells, suggesting that an intersection of sex differences and ASD-associated biology may be reflected in transcriptomic signatures of certain cell types (58). For example, if such risk genes are expressed at a higher baseline level in female neurons, as compared to neurons in the male brain, it is possible that this female-skewed expression could protect against haploinsufficiency in females. These initial findings highlight and motivate the need to expand to single cell sequencing if we want to define clear mechanisms, such as transcriptional regulation, of how female-skewed neuronal expression of ASD risk genes results in protection against ASD etiology, as well as identify other potential developmental timepoints of overlap between sex differences and ASD at the transcriptomic level, that could reflect mechanisms contributing to strong male-biased prevalence in ASD.

In this thesis work, we concentrated on sex-differential expression and the connection to ASD transcriptomic signatures in cortex tissue, primarily the prefrontal area due to previous work establishing pronounced expression of ASD rare variant genes in this brain region (24,59,60) and the broader implication of prefrontal involvement in higher cognitive processes that may be affected in ASD. Our findings revealed limited expression differences between sexes and overlap with known ASD-implicated genes and pathways in the prefrontal cortex, consistent with previous work examining sex-differential expression in this area of the brain during adulthood (21,22). However, in our second study, we detected a large number of genes with sex-differential risk patterns, potentially reflecting an intersection of sex differences and ASD neurobiology, in the parietal cortex, suggesting that areas outside of the prefrontal cortex may have more pronounced or different signatures of sex and ASD effects. Additionally, Gandal and colleagues (20) report an anterior-posterior gradient across the cortex of increasing differences in ASD and control

transcriptomes, with the largest disparities in the occipital cortex, further emphasizing the relevance of studying brain regions beyond just the prefrontal lobe.

Furthermore, sex-specific differences have been observed in subcortical regions of the brain, including hypothalamus, amygdala, nucleus accumbens, and bed nucleus of stria terminalis in studies of adult human brain tissue (12,61–65) and animal studies (10,51,66–68). For example, in a recent study by Fitzgerald and colleagues (69), a large amount of sex-differentially expressed genes were found in the nucleus accumbens in rat brain, and female-upregulated genes in astrocytes, neurons, and oligodendrocytes were enriched for GWAS signals of psychiatric disorders. However, sex-differential and sex-and-ASD-impacted gene expression in human subcortical areas of the brain, and during developmental stages, are largely understudied and should be a focus for future research endeavors on identifying areas of the brain and mechanisms involved in sex-skewed ASD pathobiology. For example, it is possible that sex differences are more evident in subcortical regions, and this sex-differential input from subcortical structures may impact specific patterns of overall cortical development (70,71), thereby increasing or decreasing the sensitivity of males vs. females to ASD risk. Evidence of alterations in functional connectivity between cortical and subcortical structures, correlated with diagnosis and/or sex, has been found in several neuroimaging studies (17,72–76), but results across the field have been inconsistent thus far (76–79). Nevertheless, these findings underline the importance of addressing the role of subcortical structures in ASD etiology and their role in sex-differential risk.

Developmental timing will also be an important factor to study in future research of sex-differential ASD risk. It has been hypothesized that etiological mechanisms of autism may involve multistage disruption during brain development (80,81). We studied prenatal ages 14-21 PCW as results from prior work demonstrate peak cortical expression of ASD risk genes during this period of prenatal neurodevelopment (24,59,60,82), which also happens to coincide with presumed initial onset of sex hormone circulation (reviewed in (10,51)). Through our robust meta-analysis of the developing prefrontal tissue, we are able to confidently rule out the existence of strong sex-

differential expression, outside of sex chromosome genes, and of the currently known autism risk variants during 14-21 PCW. However, the human brain during 3rd trimester, perinatal period, and puberty, important developmental stages that coincide with sex hormone surges (reviewed in (10,51)), is largely understudied, partly due to the paucity of post-mortem brain tissue available for research during those times. Moreover, it is possible that sex differences in expression may be transient during development, and we do not see them with the current, rather broad, age ranges in our study and other work. Evidence of this possibility comes from a recent study by Velmeshev and colleagues (58), in which sex-specific expression patterns briefly emerge in excitatory and inhibitory neuronal genes, such as *MN1* and *KLHL5*, during 3rd trimester and perinatal period; these stages were not included in our studies due to insufficient sample availability. This promising result highlights the need to further investigate sex differences during these understudied timepoints of human neural development, and with larger sample sizes, in order to further determine how sex effects during development may interact with ASD etiology.

Finally, it is crucial to acknowledge the context of ASD at the diagnostic level that may contribute to the preponderance of males in ASD prevalence. Current knowledge of ASD symptoms and diagnostic criteria is largely based on research involving male subjects (83,84), which could make recognition of ASD in females more difficult and result in a decreased tendency to diagnose ASD in females (2,71). Furthermore, several studies have indicated variations in behavioral phenotypes and age of diagnosis between males and females with ASD(81,85–87), suggesting the disorder may present differently in females and points towards a limited understanding regarding the disorder's manifestation in females. For example, in our second study, our identified sets of female-protective genes and male-vulnerability genes may be more applicable to our current diagnostic understanding of ASD, which is largely based on male-predominant studies. It is possible that transcriptomic signatures of ASD in females could be slightly different than those in males based on phenotypic differences in disorder manifestation. Thus, across disciplines of ASD research, more female-balanced studies are necessary (further

reviewed in (88)) if our goal is to identify and understand mechanisms involved in protection of females against ASD development, which could lead to future therapeutic targets mimicking such effects.

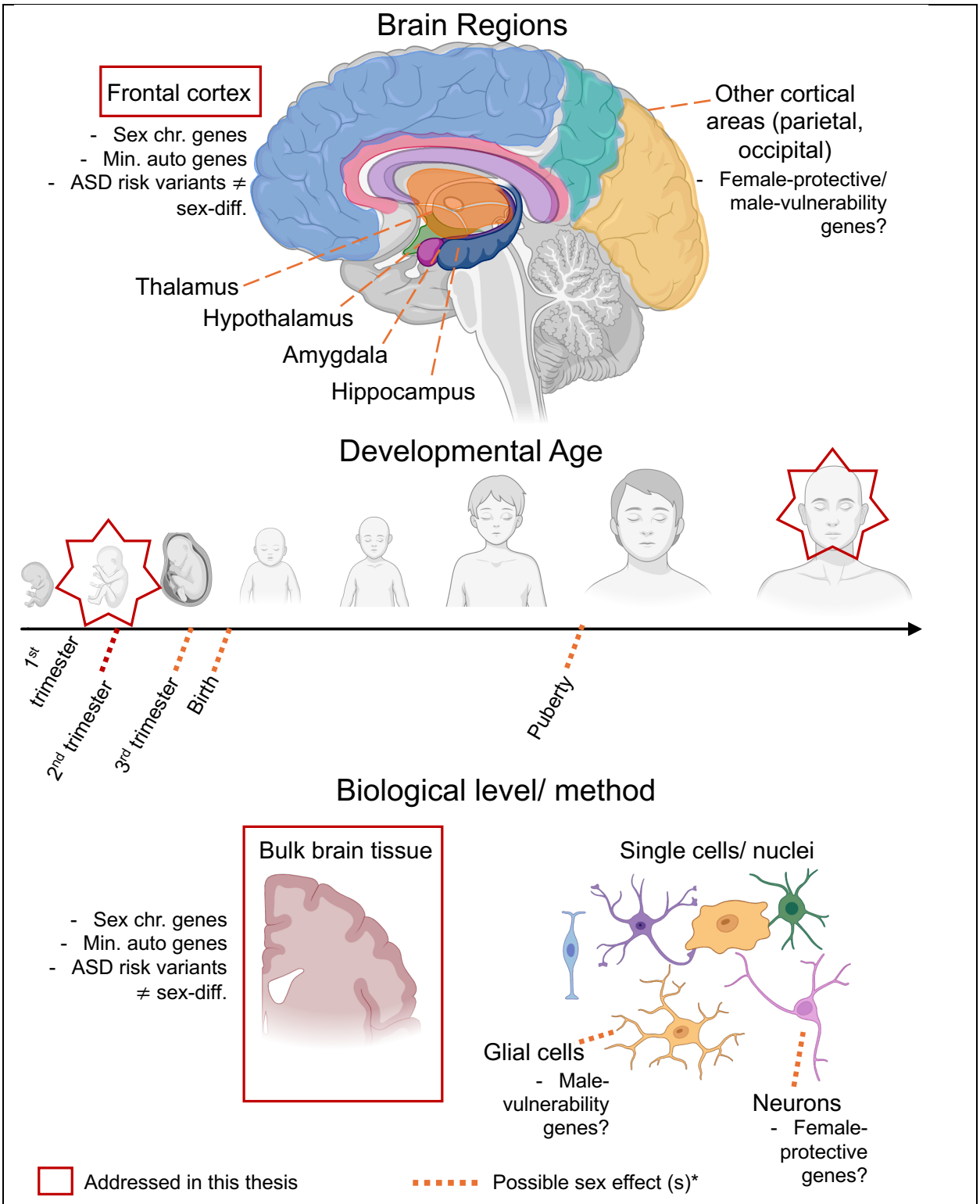


**Fig 4:** Summary of areas of intersection of sex-differential and ASD-associated neurobiology addressed in this thesis (red boxes/shapes) and areas of future investigation where possible sex effects may be seen (orange dotted lines). Created with BioRender.com.

**Conclusion**

In essence, this dissertation work contributes to bridging the gap in knowledge of how biological sex comes into play in neurodevelopment and ASD. First, by identifying robust patterns of sex-differential expression in the developing prenatal cortex and changes in sex-differential gene expression in the ASD brain, this work has created a valuable resource that catalogues transcriptomic signatures to be utilized for future research in genomics, neural development and disorders, among other areas. Furthermore, our robust analysis of sex-differential gene expression during prenatal cortical development concretely demonstrates that known ASD risk variants are not expressed at different levels in males and females, at least during 14-21 PCW in the cortex, which is important for motivating the field forward to look at other areas of the brain, stages in development, and with finer resolution (single nuclei sequencing). Rather, our work in the second study demonstrates that expression of some ASD risk genes, associated with neuronal functioning, show a specific pattern reflective of female-biased protection from ASD and suggest potential targets that may be beneficial by mimicking these expression patterns. Furthermore, the identification of sets of genes and functional expression patterns impacted by both sex and ASD in the brain, with specific patterns representative of sex-differential risk profile in ASD, has provided crucial insights into the complex interaction between sex and ASD at the molecular level, including neuronal genes are female-skewed expression pattern that suggests a protective mechanism, possibly against other “hits” involved in ASD risk, and male-skewed expression of neuroimmune and glial cell type markers that point to possible vulnerability to other “hits” of ASD risk in males. These findings not only contribute to unraveling the long-standing puzzle of profound male prevalence in ASD, but also enhances our comprehension of biological differences between sexes at a fundamental level.

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## APPENDIX A: SUPPLEMENTAL MATERIALS FOR CHAPTER I

### Sex-differential gene expression in developing human cortex and its intersection with autism risk pathways

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**Supplemental Materials and Methods**

**Prenatal BrainVar data set**. RNA-sequencing data from the BrainVar project were used (1). As detailed in the originating publication, for the BrainVar source data, STAR was used to map RNA-seq reads to the GRCh38 human reference genome, and HTSeq used to calculate gene-level read counts based on Gencode v21 annotation. Data from tissue samples from the dorsolateral prefrontal cortex (DLPFC) from subjects aged 14 to 21 post-conception weeks (PCW) **(Table S1)** were retained for the present analysis, an age range that corresponds to prenatal sex hormone production and influence of sex chromosomes on neurodevelopment. Normalization of library sizes was performed by calculating scaling factors with edgeR (version 3.38.4) (2). Gene-level read counts were transformed to counts per million mapped reads (CPM; using edgeR:cpm function) and then non-expressed genes that did not meet the criteria of expression level of 1 CPM or greater in more than 50% of all samples from either sex were removed. After gene filtering and outlier removal, 15,649 genes and 87 samples (41 males, 46 females) were retained for analysis.

**Prenatal UCLA data set.** RNA-sequencing data from Walker et al. (2019) were used (3). As detailed in the originating publication, RNA-seq data were evaluated by FastQC and lanes with low-quality data were removed. Remaining RNA-seq reads were then aligned by STAR to the GRCH37.p13 human reference genome. Samtools was used to merge BAM files from different sequencing lanes, and HTSeq was used to calculate gene-level read counts based on Gencode v19 annotation. These gene-level read count data were acquired from the Geschwind lab at UCLA. Data from whole cortical tissue samples from subjects aged 14-21 PCW **(Table S2)** were retained as to allow for comparison with BrainVar. Normalization of library sizes, CPM transformation, and gene expression filtering were performed as described above for BrainVar. After gene filtering and outlier removal, 16,193 genes and 186 samples (101 males, 85 females)

were retained for analysis. Gencode v19 was used for gene annotation, as used in original publication on this data set (3).

**Cell type deconvolution**

**Estimation of cell type composition.** To generate prenatal cell type signatures for the prefrontal cortex, single-cell gene expression data from prenatal human brain tissue (age range: 17-25 gestational weeks) were downloaded from https://dev-brain-regions.cells.ucsc.edu (4). Gene counts from neocortex tissue samples were averaged across cells belonging to each cell cluster: excitatory neuronal, dividing, radial glial, endothelial, vascular, intermediate progenitor cells (IPC), interneuron, astrocyte, microglia, and oligodendrocyte. In accordance with the protocol published in Sutton et al. (5), gene-level counts were transformed to transcripts per million (TPM) and a minimum expression threshold was set at >1 TPM in at least one of the cell types to generate the final signature matrix.

Cell type composition per sample was estimated separately for BrainVar and UCLA data using the deconvolution method of dtangle (version 2.0.9), with $\log_2$(CPM+1) (edgeR cpm transformation) expression as the input (6). Cell type proportion estimates from female and male samples were calculated and tested for sex differences within each data set using the two-sided Wilcoxon test. Sex-differential cell type composition was defined by a $P$ value ≤ 0.05 for a cell type.

**Estimation of cell type-specific expression.** To investigate cell-type specific sex-differential expression signatures, we utilized the bMIND (v0.3.3) package in R (7). The inputs for bMIND are estimated proportions of 10 cell types from dtangle (version 2.0.9), bulk gene expression in the form of log2(CPM +1), and covariates (sex, age, batch, RIN, PMI and all surrogate variables (SVs)). Cell-type-specific differential expression analysis was performed separately for each cell type using limma voom (version 3.52.4) and the bMIND estimated cell type-specific gene expression.

**Differential expression analysis of individual datasets.** For all analyses, differential expression analysis was performed using limma voom (version 3.52.4) (2,8) on $\log_2$(CPM +1) (edgeR cpm transformation) from expressed genes. For both data sets, we applied a differential expression model that included sex as the main variable of interest and covariates age (post-conception weeks), RNA-seq batch, RIN, PMI, estimated intermediate progenitor cell (IPC) proportion (to adjust for variability in cortical dissection depth between samples and data sets), and all surrogate variables (SVs) identified by SVAseq with default num.sv option “be” (version 3.44.0 (9)) in addition to the listed covariates (11 SVs for BrainVar, 16 SVs for UCLA). Additionally, we applied the variancePartition tool (version 1.33.0) (10) to assess the contribution of each variable included in the model to individual genes’ expression variance, and to overall gene expression variance **(Tables S18-21)** and plotted the percent variance explained by each variable in the form of violin plots **(Fig. S7, S8)**. For comparison, we also estimated expression variance for a model without SVs, and calculated the median variance explained (R^2), AIC, and BIC values for the model both including and excluding SVs. The median R^2 for the model including SVs is 0.843 versus 0.173 for the model without SVs. The median AIC and BIC values for the SV-included model are -17.1 (AIC) and 42.1 (BIC) versus 113.3 (AIC) and 145.3 (BIC) for the model without SVs, supporting the use of SVs in these analyses. Correlation between variables included in the differential expression models are shown in **Fig. S9** (BrainVar) and **Fig. S10** (UCLA).

**Meta-analysis of BrainVar and UCLA data sets.** To ensure the similarity of the two data sets**,** we first estimated the fraction of the number of differentially-expressed genes identified in one data set that were also associated with the other data set (“pi1”) using the ‘propTrueNull’ function from limma (11–13). Differential expression results from the two individual datasets were subsequently combined for meta-analysis. For both differential expression models (full model, baseline model), we utilized the MetaRNASeq (14) package to perform inverse-normal p-value

combination analysis to increase statistical power. Results from meta-analysis were then used to define robust sex-differentially expressed genes (sex-DEGs) as those genes with a Benjamini-Hochberg false discovery rate (FDR) ≤ 0.1. Additionally, to facilitate comparison across models and with ASD-associated sex-differential expression patterns reported previously from pre- and postnatal human brain (15), we further considered an extended set of the top 200 sex-DEGs as ranked by ascending *P* value (meta-analysis), with the same direction of fold change in both data sets.

**Power calculation for differential expression.** The power to observe differential expression depends on the averaged gene expression differences, the variance of gene expression, and the sample sizes for two conditions (here, males and females). Following Smyth, 2004 (16), we model this problem using the two-sample test and the corresponding t-statistics. The degree of freedom is provided by the limma::eBayes() function, which is df.brainvar = 67.39886 and df.ucla = 169.1104. The power for the two datasets with two confidence level alpha = 0.05 and alpha = 0.1, minimum absolute t-statistics for power = 0.8, and histograms of the power for all autosomal genes and significant sex-DEGs (FDR ≤ 0.1) are shown in **Fig S11**.

**X chromosome gene classification.** X inactivation is a process intended to equalize expression dosage of X chromosome genes in males and females, though X inactivation “escape” or extension of X inactivation into the X-Y homologous pseudoautosomal regions (PAR) can lead to sex-differential expression. We defined four categories of X chromosome genes, according to their chromosomal location or sex-differential expression in human adult brain from GTEx (17): 1) PAR, 2) X Inactivation Escape (XIE)-high probability, with female/male logFC > 0 and *P* value ≤ 0.05 in GTEx brain, 3) XIE-low probability, with female/male logFC > 0 but *P* value > 0.05, and 4) X-other, including any remaining genes classified as “variable XIE” (17) that had female/male

logFC > 0 and $P > 0.05$ and/or male-biased expression (F/M logFC < 0); the female-biased *XIST* transcript is considered separately **(Table S5)**.

**Functional annotation and enrichment testing**

**Gene sets of interest.** To investigate whether ASD risk genes show evidence of sex-differential expression in human prenatal cortex, we compared sex-DEGs to four sets of ASD risk-associated genes derived from recent publications, including three exome-sequencing studies identifying rare variant-implicated ASD risk genes: Satterstrom et al. (97 genes (18)), Fu et al. (72 genes (11)), and Zhou et al. (60 genes(19)); and one set of common variant-associated ASD risk genes identified by a genome-wide association study (16 genes) (20) **(Table S3)**. The background gene set for rare-variant gene sets was autosomal, protein-coding genes from Gencode v21, as used in the previous publication of BrainVar (1) and for the common variant ASD gene set was autosomal, protein-coding genes released from NCBI 37.3 in MAGMA (21).

To investigate overlap with ASD-affected neurobiology, beyond risk variant-carrying risk genes, gene lists belonging to co-expression modules with altered expression in ASD (22,23) were used for gene set enrichment analyses. From a study of cortical regions from all four cortical lobes by Gandal et al. (23), genes in 11 ASD-upregulated modules of GeneM15 (240 genes), M8 (253 genes), M32 (65 genes), M2 (737 genes), M21 (110 genes), M7 (253 genes), M12 (170 genes), M9 (243 genes), M34 (55 genes), M33 (61 genes), and M27 (94 genes) and genes in 7 ASD-downregulated modules (GeneM14 (160 genes), M19 (168 genes), M3 (691 genes), M5 (398 genes), M23 (102 genes), M25 (102 genes), M24 (102 genes)) were used **(Table S3**). The background gene set used was all 24,479 expressed genes reported in Gandal et al. (23).

Comparison of ASD-associated co-expression modules from a previous study of frontal and temporal cortex tissue from overlapping donors by Parikshak et al. (22) to cortical sex-differential expression from the BrainSpan data set found evidence for enrichment of prenatal, male-biased DEGs in ASD-upregulated modules, M19 and M9 (Kissel & Werling, Biol Psych

2022). Here, we sought to determine whether this pattern could be replicated in larger prenatal data sets. From Parikshak et al. (22), genes in the ASD up-regulated modules of ctx.m9 (522 genes), ctx.m19 (273 genes), and ctx.m20 (346 genes) and genes in the ASD down-regulated modules of ctx.m4 (245 genes), ctx.m10 (257 genes), and ctx.m16 (283 genes) were used (**Table S3**). The background gene set used was all 16,092 expressed genes reported in Parikshak et al. (22).

To assess the overlap with neuropsychiatric disease/phenotype risk, we compared sex-DEGs to disease risk-associated genes as previously defined (1), including genes implicated by studies of rare variants of large effect: Developmental Delay (DD; 91 genes) (24), Epilepsy and Developmental Delay (EPDD; 32 genes) (25); and genes implicated by studies of common variation (genome-wide association studies): Educational Attainment (EA; 963 genes) (26), Attention Deficit/Hyperactivity Disorder (ADHD; 10 genes) (27), schizophrenia (SCZ; 259 genes) (28), major depressive disorder (MDD; 30 genes) (29), multiple sclerosis (MS; 80 genes) (30), Parkinson's disease (PD; 37 genes) (31), and Alzheimer's disease (AD; 39 genes) (32) **(Table S3)**. The background gene set for this analysis was protein-coding genes from Gencode v21, as used in the previous publication of BrainVar (1).

To assess whether sex-DEGs represent sex-differential function of specific cell types or biological processes, we compared sex-DEGs to cell type marker gene sets and databases of biological functions. Prenatal cell type marker genes were selected as the top 200 expressed genes per cell type, using the find_markers function in dtangle with n markers set as 200, from a single-cell RNA-seq study of fetal neocortex tissue (4). From this data set, we tested the top 200 genes in neurons, dividing cells, radial glia, interneurons, oligodendrocytes, intermediate progenitor cells (IPC), astrocytes, microglia, endothelial cells, and vascular cells **(Table S3)**. The background gene set used was all 29,805 expressed genes reported in the data downloaded from https://dev-brain-regions.cells.ucsc.edu (4).

**Enrichment testing approaches.** Enrichments of sex-differential genes in each category described above (ASD risk genes, ASD-dysregulated modules, neuropsychiatric trait genes, cell type markers) were evaluated by two-sided Fisher's exact tests, taking into account a common background gene set for each (intersection between all expressed genes in the BrainVar and UCLA data sets) and all background genes from the comparison set. Robust (FDR ≤ 0.1) male-biased genes and female-biased genes were tested separately against each gene set of interest. We separately applied a Bonferroni correction for multiple testing in each analysis, accounting for 2 sex-differential gene sets (male-biased, female-biased) and either 18 ASD modules (Gandal et al.; 36 tests for 18 modules), 6 ASD modules (Parikshak et al.; 12 tests for 6 modules), 9 disease gene sets (18 tests for 9 disease risk gene sets), or 10 cell type marker sets (20 tests for 10 cell types). ASD risk gene sets were considered independently (2 tests for each ASD risk gene set).

We also investigated enrichments for these gene sets of interest using a threshold-free approach, Gene Set Enrichment Analysis (GSEA, version 4.3.2) (33). We used results from the sex-differential meta-analysis to rank genes from most male-biased to most female-biased by multiplying each gene's meta-analysis p-value by the sign of its effect. The effect sign for genes with consistent effect directions in both BrainVar and UCLA was determined by the sign of the gene's log2FC (positive for male-biased genes, negative for female-biased genes). For genes with disparate effect directions, we used the data set-specific variance of each gene to calculate a weighted sum of log2FC values from BrainVar and UCLA. The sign of this joint log2FC was applied to determine gene ranking as either male-biased or female-biased. We then applied the GSEA Preranked tool to test the gene sets described above for skewed distribution across the sex-differentially expressed genes.

**Functional annotation.** We also applied the gProfiler2 R package (version 0.2.1) to assess functional enrichments for Gene Ontology (GO), KEGG pathways, Reactome, WikiPathways, TRANSFAC, miRTarBase, Human Protein Atlas, CORUM, and HPO (34). gProfileR used a significance threshold ≤ 0.05 for g:SCS (Set Counts and Sizes) method of multiple testing

correction, an algorithm developed by gProfiler to specifically account for hierarchal clustering of GO terms by their specificity (34).

**Co-expression network analysis**

**WGCNA network construction and module identification.** We applied Weighted Gene Co-Expression Network Analysis (WGCNA, version 1.71) (35) to identify sex-specific modules in the prenatal human cortex. First, samples were separated by sex and filtered for quality control using the WGCNA function goodSamples. Hierarchical sample clustering was plotted to visually detect outlier samples. Then, cutreeStatic function was applied with an appropriate cutHeight of 180 to remove outlier samples. Additionally, we applied the goodGenes function in the WGCNA package to filter out genes with low expression and/or zero variance (35). After these pre-processing steps in BrainVar, 39 male samples and 46 female samples expressing 23,327 genes were retained for co-expression analysis. In the UCLA data set, 101 male samples and 85 female samples expressing 16,531 genes were retained for co-expression analysis. The soft power cutoff threshold for WGCNA was then selected based on a mean connectivity (mean.k) less than 50 and scale-free topology (SFT.R.sq) greater than 0.8.

To further reduce any outlier bias, we applied the blockwiseConsensusModules function across 100 subsampled iterations (66% of sample size) to generate co-expression networks separately for male and female samples. Within this function, we performed WGCNA using a signed network with additional parameters of a minimum module size of 200, a network calibration of ‘single quantile’ of 0.95, a consensus quantile of 0.2, pamStage = FALSE, deep split of 2, a merge cut height of 0.1, and the respective soft power threshold. Subsequently, we generated the network dendrogram using the average linkage hierarchal clustering of the consensus topological overlap dissimilarity matrix (1-TOM) with a tree cut height set to 0.999 (**Fig. S1, S5**). Modules were then summarized by their module eigengene (ME) value and genes were assigned a kME value using the WGCNA function of signedKME. Additional merging of modules was not needed

as we did not detect any modules with high correlations (module eigengene correlation value ≥ 0.9). From this analysis, in BrainVar we detected 14 co-expression modules in females and 11 co-expression modules in males (**Fig. S1**) and in the UCLA data set we detected 20 modules in females and 21 in males (**Fig. S5**).

**Co-expression comparison between sexes in BrainVar and UCLA.** To assess whether any of the modules detected were sex-specific, we compared female and male modules using module preservation analysis in the WGCNA R package (35,36). Expression data belonging to modules from one sex were set as the reference network and expression data from the other sex were used as the test set. Specifically, we applied the modulePreservation function with 200 permutations of the expression data to calculate module preservation statistics. Sex-specific modules were defined as those with a composite Z-summary score of less than 10, which indicates poor preservation in one sex of a module's co-expression network structure identified in the other sex (35–37).

In BrainVar, we also assessed module similarity across sexes by directly comparing the gene content of male- and female-identified modules, and by multidimensional scaling (MDS) on per-sample module eigengene values for all module gene sets. We grouped together similar modules using the following steps: 1) We applied hierarchical clustering to a matrix indicating module membership by gene and merged together pairs of modules, one from each sex, that clustered into adjacent, clustered leaves of the resulting dendrogram (**Fig. S2**). 2) Unpaired modules for which >50% of their genes overlapped with a module in an existing group were added to that group. 3) Unpaired modules that clustered in immediate proximity to any module in a group in MDS space were added to that group (e.g. mod6.F and mod13.F, which show 73% and 46% gene content overlap with mod18.M, respectively) (**Fig. 3A, Fig. S2)**. Remaining modules were left ungrouped and considered as possibly sex-differential. Sex-merged and remaining sex-specific modules were then functionally annotated for further interpretation.

**WGCNA functional enrichment.** Merged and sex-specific co-expression modules were functionally annotated using a two-sided Fisher's exact test to compare to gene sets of interest described above (cell type markers, ASD-associated co-expression modules, ASD and neuropsychiatric disorder risk genes), extended sets of male-biased and female-biased sex-DEGs identified here, and for functional enrichments using gProfiler with a background gene set of all annotated genes reported in Ensembl GRCh38.p13 (34,38).

**sLED.** To identify individual genes contributing most strongly to co-expression differences between sexes in the two data sets, we applied a sparse leading eigengene driven (sLED) test to expression data of all genes included in WGCNA (39–41). sLED evaluates the difference matrix between the correlation matrices derived from gene expression in males and females and then identifies the genes driving differences in correlation network structure (39). We first removed genes that belonged to the grey WGCNA modules (unassigned genes) from both the male and female-specific analyses (intersection) and retained 14,611 genes (BrainVar) and 13,218 genes (UCLA) for sLED input. Parameters for sLED were set as adj.beta = 0 (for correlation matrices), rho = 1000, sumabs.seq of 0.2 (as recommended for bulk RNA-sequencing data), and 1000 permutations. To identify genes driving significant differences in correlation network structure, we then selected genes with leverage > 0 and subset them into 2 categories: 1) primary genes, which cumulatively account for 90% of the leverage; and 2) secondary genes, which account for the remaining 10%. To validate module preservation observations, we then compared primary and secondary high leverage genes to all sex-specific WGCNA modules and ran further functional annotation using gProfiler as above.

**Age-by-sex differential expression analysis**

During prenatal development of the cortex, age is a powerful driver of differences in gene expression (1), and developmental processes may interact with sex-differential factors. To identify genes affected by both age and sex in the prenatal cortex, we performed differential expression analysis separately in male and female samples, with age as the main variable of interest. Initial gene filtering was performed as described above and SVA was run on filtered CPM data, including age and sex as the variables of interest, with IPC proportion and RNA-sequencing batch as adjustment variables. Samples were then separated by sex and a differential expression model was run using age (post-conception weeks) as the main variable of interest with IPC proportion, RNA-seq batch, RIN, PMI, and all SVs (BrainVar: N=11; UCLA: N=16) as covariates. Robust age-impacted differential genes (age-DEGs) were defined as those genes with FDR $\leq$ 0.1. We then evaluated the correlation of age $\log_2$FC (equivalent to beta coefficient for age) in the union of male and female age-DEGs. We additionally investigated if any genes exhibited a combined interaction effect of sex by prenatal age. Here, we used both male and female samples and performed differential expression using an interaction term (~Sex:Age) as the main variable of interest with sex, age, IPC proportion, RNA-seq batch, RIN, PMI, and all SVs (BrainVar: N=11; UCLA: N=16) as covariates. A threshold of FDR $\leq$ 0.1 was used to detect sex-age DEGs with a significant interaction effect of age and sex.

**Supplemental Results**

**Sex-differential expression in the prenatal human cortex.** Prior to conducting a meta-analysis, we compared sex-DEGs detected between BrainVar and UCLA data sets. The union of sex-DEGs between the two data sets contains 102 genes at FDR ≤ 0.1, with 49 genes (28 X chr, 19 Y chr, 2 autosomal) that are significant in both UCLA and BrainVar. The two autosomal sex-DEGs that replicate are protein-coding gene *CCDC158* and lincRNA *CECR7*. In terms of functional enrichment, common terms from BrainVar and UCLA sex-DEGs include HPO terms for 'Y-linked inheritance' for male-biased sex-DEGs and 'X-linked inheritance' for female-biased sex-DEGs. Female-biased sex-DEGs are also enriched for 'estrogen metabolism' (WP:WP697), and 'sulfuric ester hydrolase activity' (GO: 0008484) in both data sets, which are driven by 3 X chromosome genes, ARSD and STS in both BrainVar and UCLA, and ARSL in UCLA only.

**Cell type-specific sex-differential expression estimates.** Sex-differential expression analysis of cell type-specific gene expression estimates from bMIND (Methods) identifies a variable number of sex-DEGs per cell type in both data sets (**Tables S9, S10**), with more abundant cell types like neurons, IPCs, and vascular cells tending to show a greater number of sex-DEGs at FDR≤0.1. We identify fewer sex-DEGs in less abundant astrocytes, microglia, oligodendrocytes, and interneurons. Like for bulk tissue measurements, these sex-DEGs are found predominantly on the sex chromosomes. Cell type sex-DEGs overlap substantially with sex-DEGs from bulk tissue, but this analysis also identifies genes that are uniquely sex-differential within particular cell types, highlighting the utility of this approach for additional discovery.

**Age-by-sex differential expression analysis.** In our sex-separated age differential expression analysis, we noted a substantially larger number of age-DEGs detected in the UCLA data set compared to those detected in BrainVar (**Tables S6, S7**). Hypothesizing that this may be due increased statistical power in the UCLA data set compared to BrainVar, we downsampled the

UCLA data to a randomly selected 87 samples to match BrainVar. We identify only 7,350 age-DEGs at FDR $\leq 0.1$ in this downsampled analysis, compared to 10,409 age-DEGs in the full UCLA data set. This is still greater than the 2,804 age-DEGs seen in BrainVar, however, suggesting that statistical power is not the only contributor. Another possibility is that since the UCLA data set contains whole cortical tissue, rather than just cortical plate like BrainVar, more age-related changes may be evident when gene expression is included from cell types in deeper layers (e.g. radial glia, intermediate progenitor cells).

**Functional annotation and gene set enrichment for sex-specific co-expression modules.** Putatively sex-differential co-expression modules include modules defined in females: $mod22_F$, $mod16_F$, $mod12_F$, $mod11_F$ and module $mod20_M$, defined in males. $Mod22_F$ and $mod16_F$ are both enriched for markers of radial glia, oligodendrocytes, and astrocytes (**Fig. 3C, Table S14**), as well as GO terms related to anatomical development (**Fig. 3D, Table S14**). Genes in $mod16_F$ are also enriched for almost every cell type in the prenatal brain, with the exception of microglia and dividing cells (**Fig. 3C, Table S14**). $Mod12_F$ annotates to GO terms related to general cell processes and metabolism (**Fig. 3C**), and $mod11_F$ is enriched for GO terms related to translation and peptide processing (**Fig. 3D, Table S15**).

**Supplemental Figures**

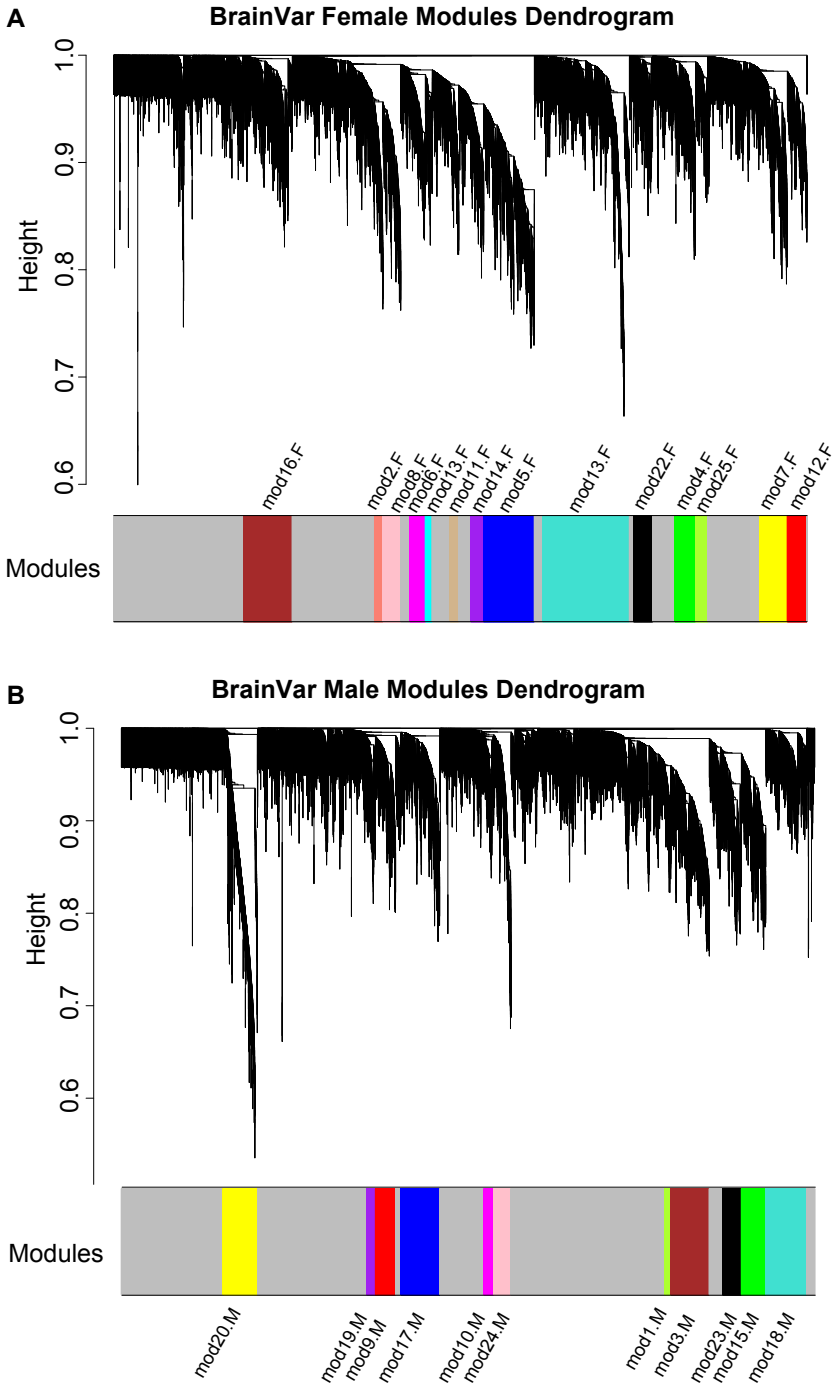


Figure S1. WGCNA modules detected in BrainVar in males and females. (A-B) Dendrograms of cortically-expressed genes assigned to WGCNA modules in females (A) and males (B).

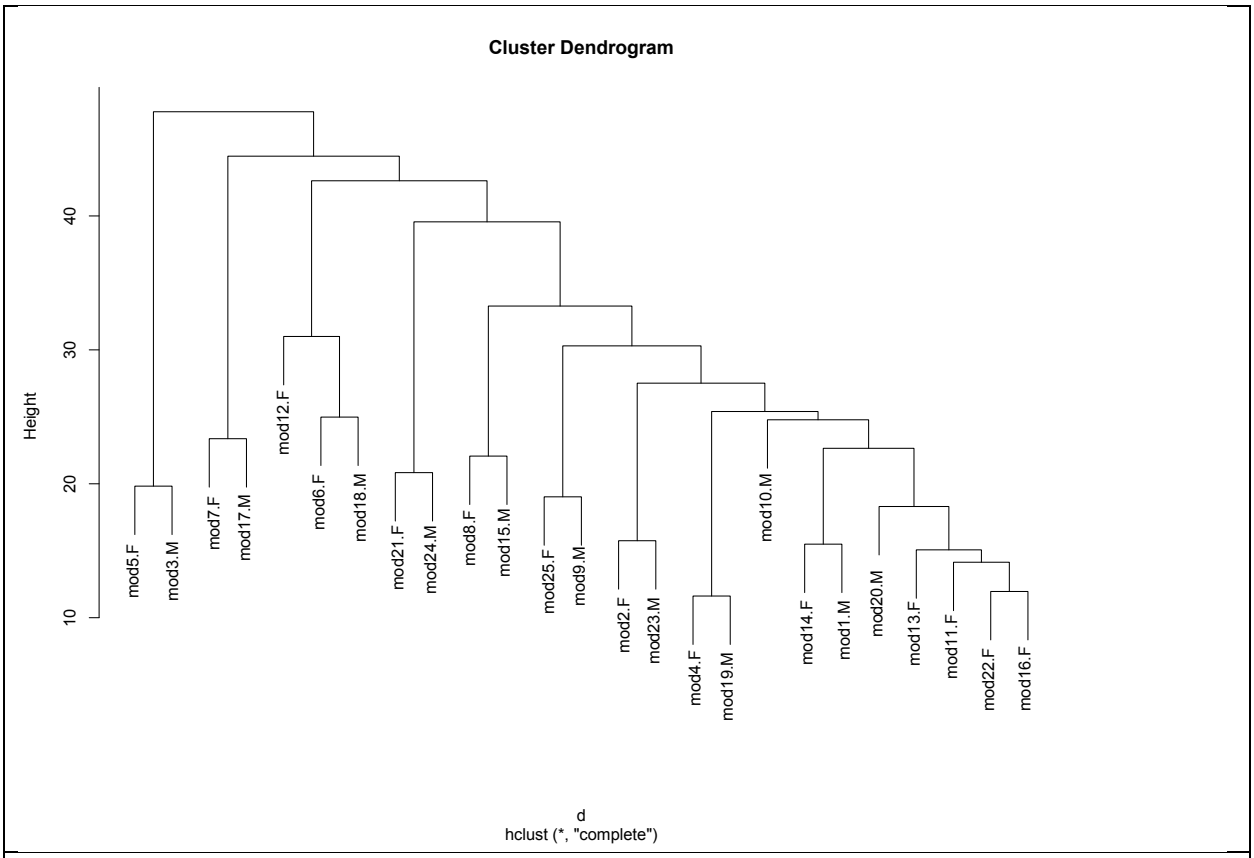


Figure S2. WGCNA module relationships between sexes. Cluster dendrogram of similarity in gene content between modules detected in males and females by hierarchical clustering.

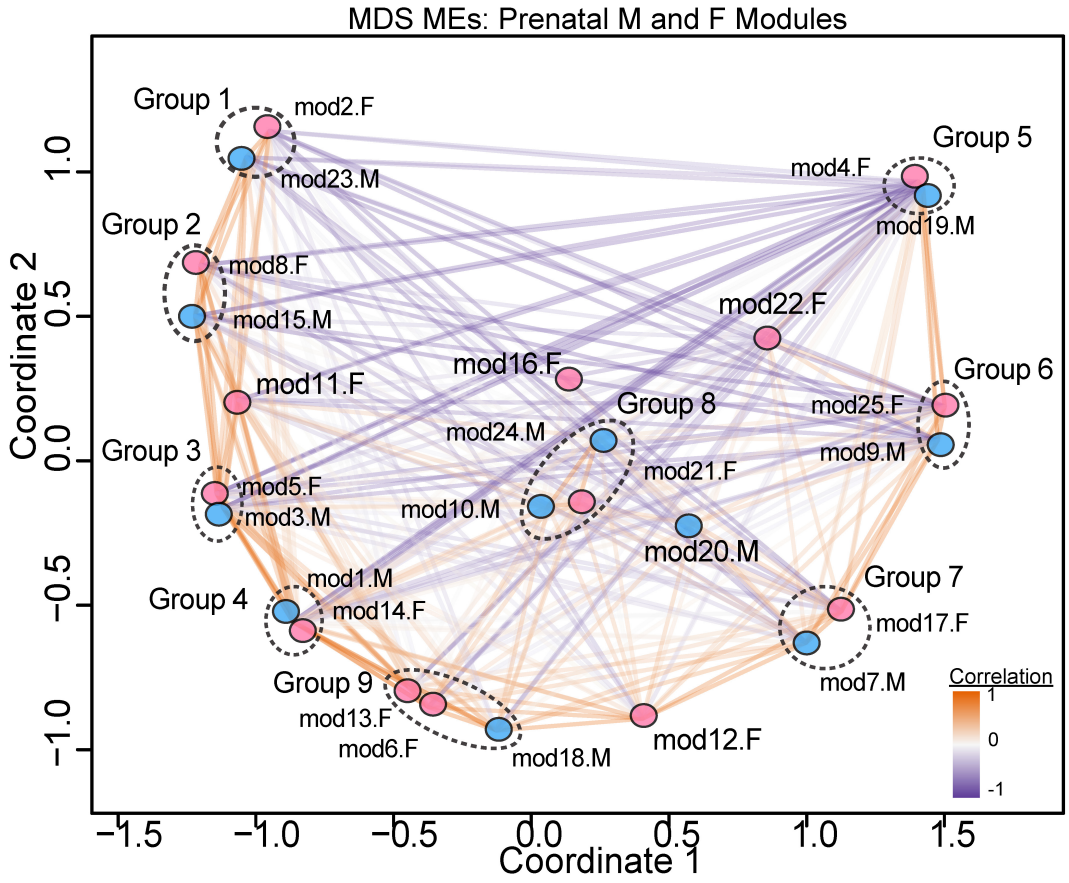


Figure S3. Multidimensional scaling plot of module eigengenes of grouped and ungrouped modules. Module colors and letter after number denote male (blue, M) or female (pink, F) WGCNA. Line color indicates the direction and degree of correlation between module eigengenes.

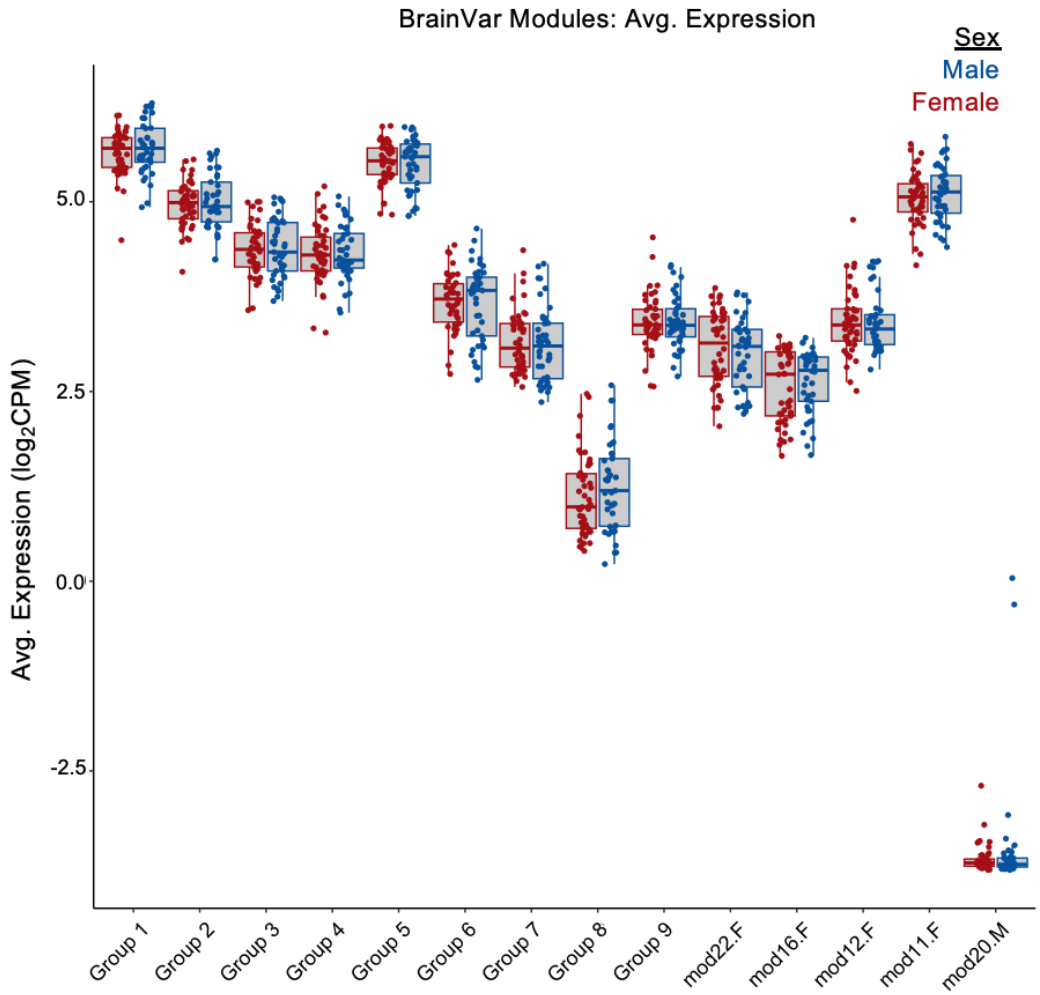


Figure S4. Average expression of BrainVar modules and groups. Each dot represents the average expression of genes in a specific module for an individual sample.

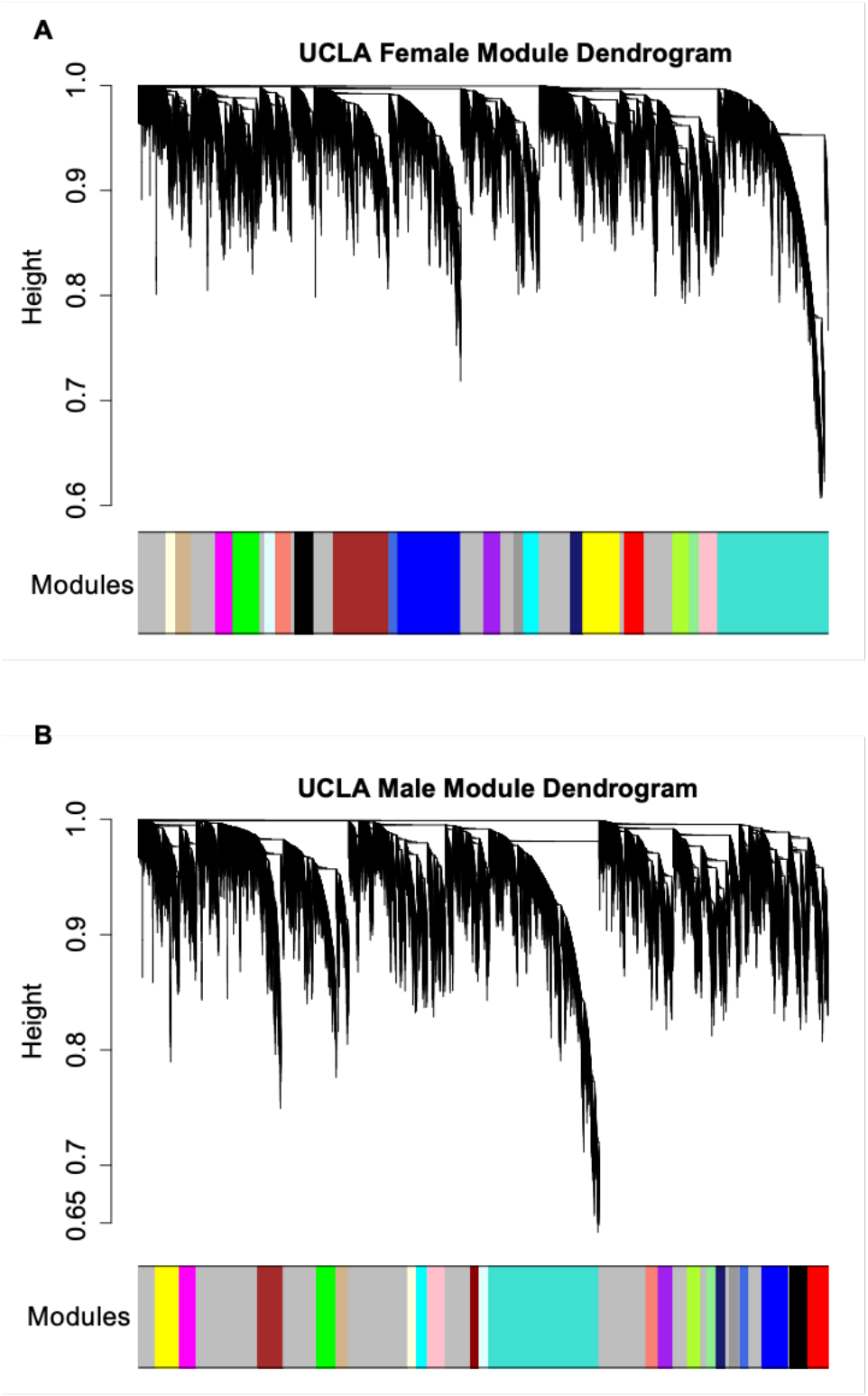


Figure S5. WGCNA modules detected in UCLA data set in males and females. (A-B) Dendrograms of cortically-expressed genes assigned to WGCNA modules in females (A) and males (B).

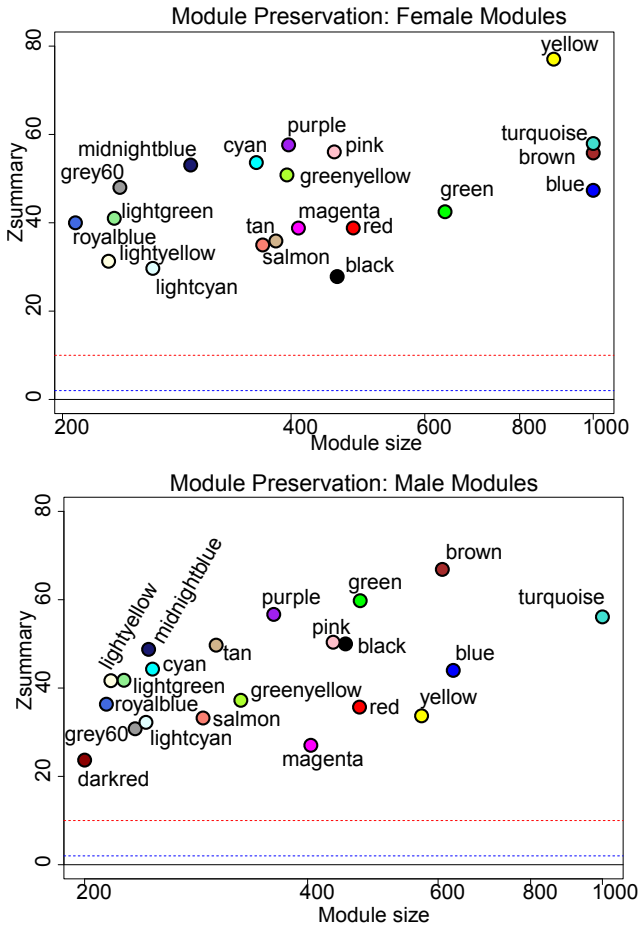


Figure S6. Module preservation between prenatal females and males in UCLA data set. (A) Module preservation in males for modules identified in females. (B) Module preservation in females for modules identified in males.

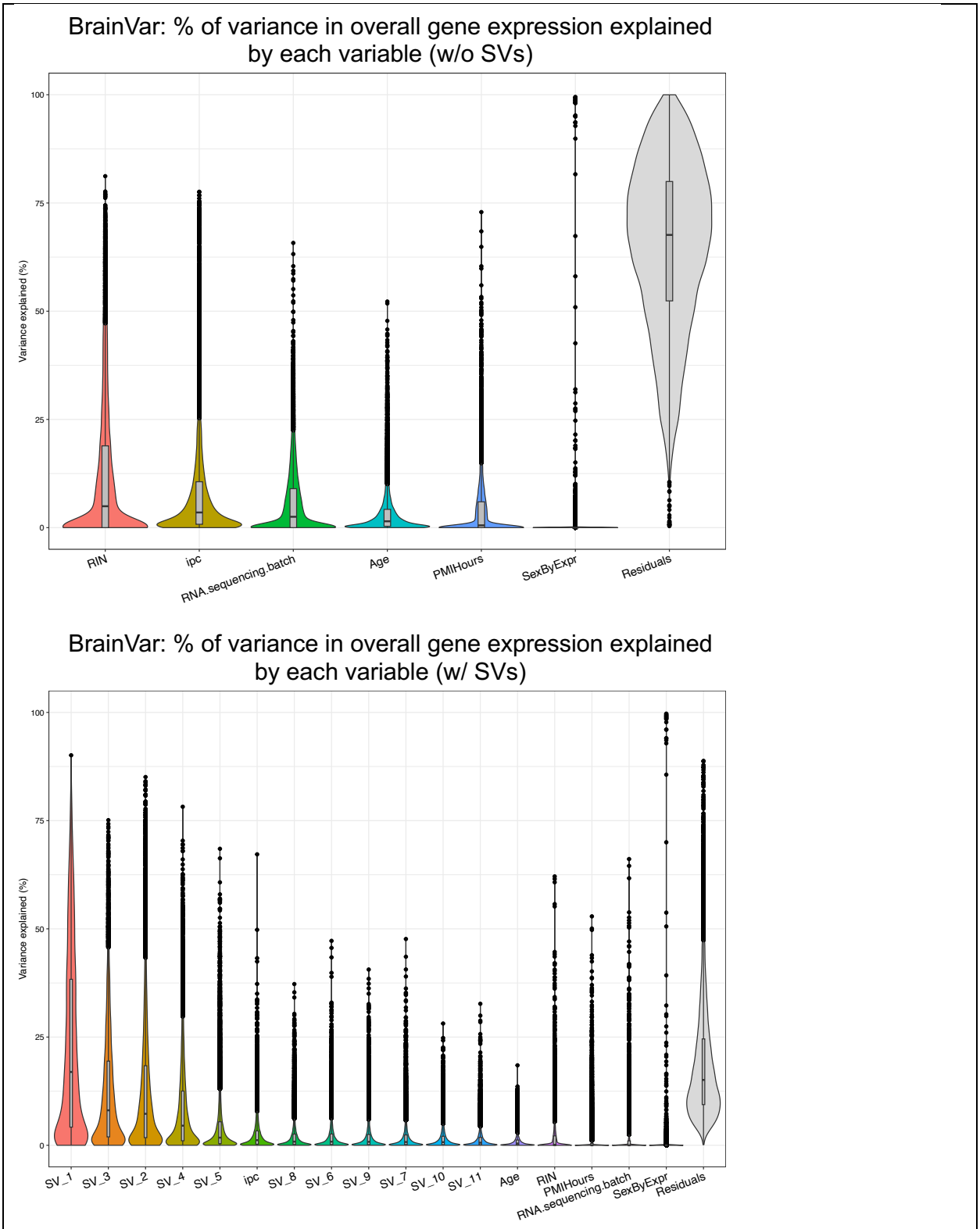


Figure S7. Percent Variance in overall gene explained by each variable from BrainVar baseline model without surrogate variables (top panel) and BrainVar main model with surrogate variables (bottom panel).

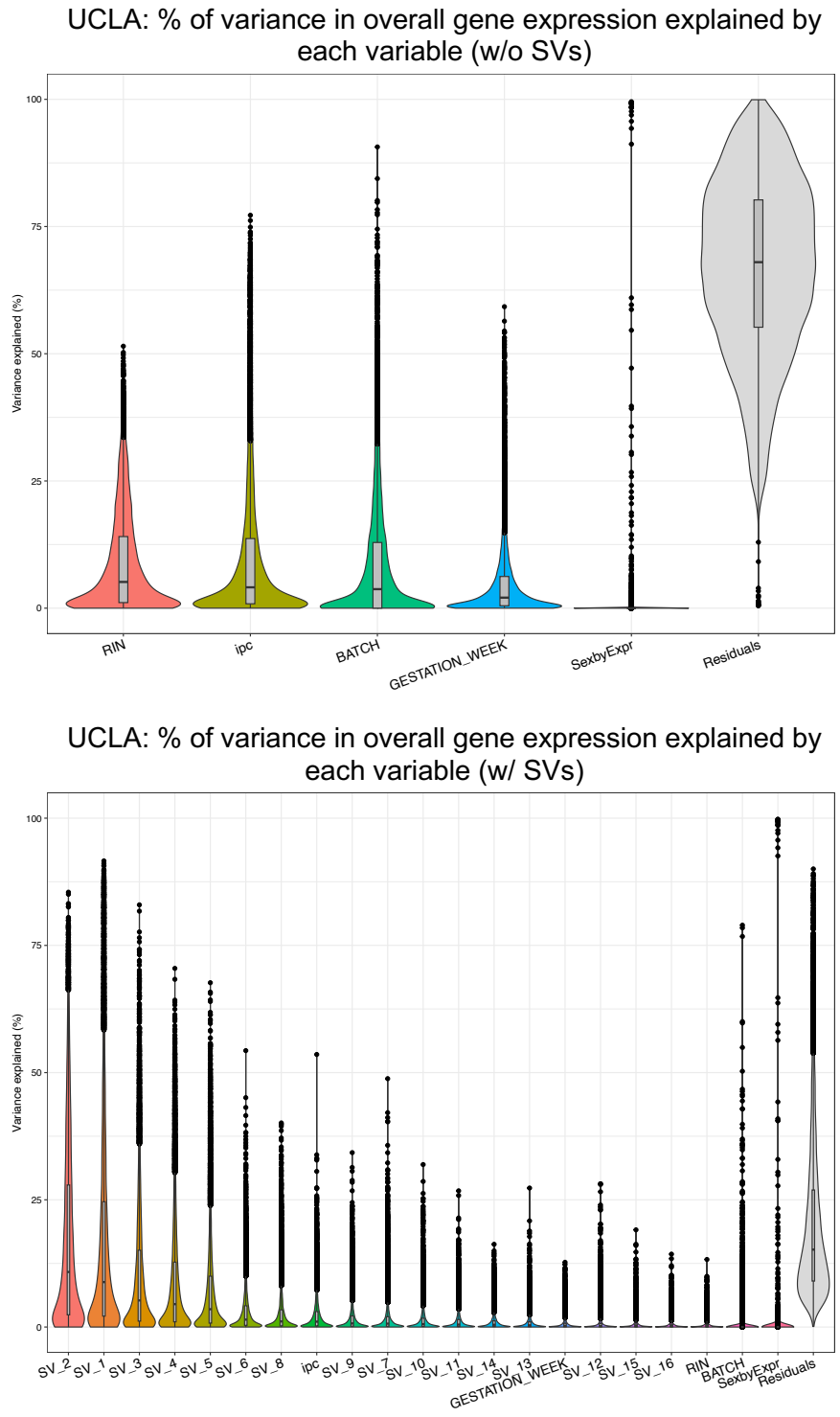


Figure S8. Percent Variance in overall gene explained by each variable from UCLA baseline model without surrogate variables (top panel) and UCLA main model with surrogate variables (bottom panel).

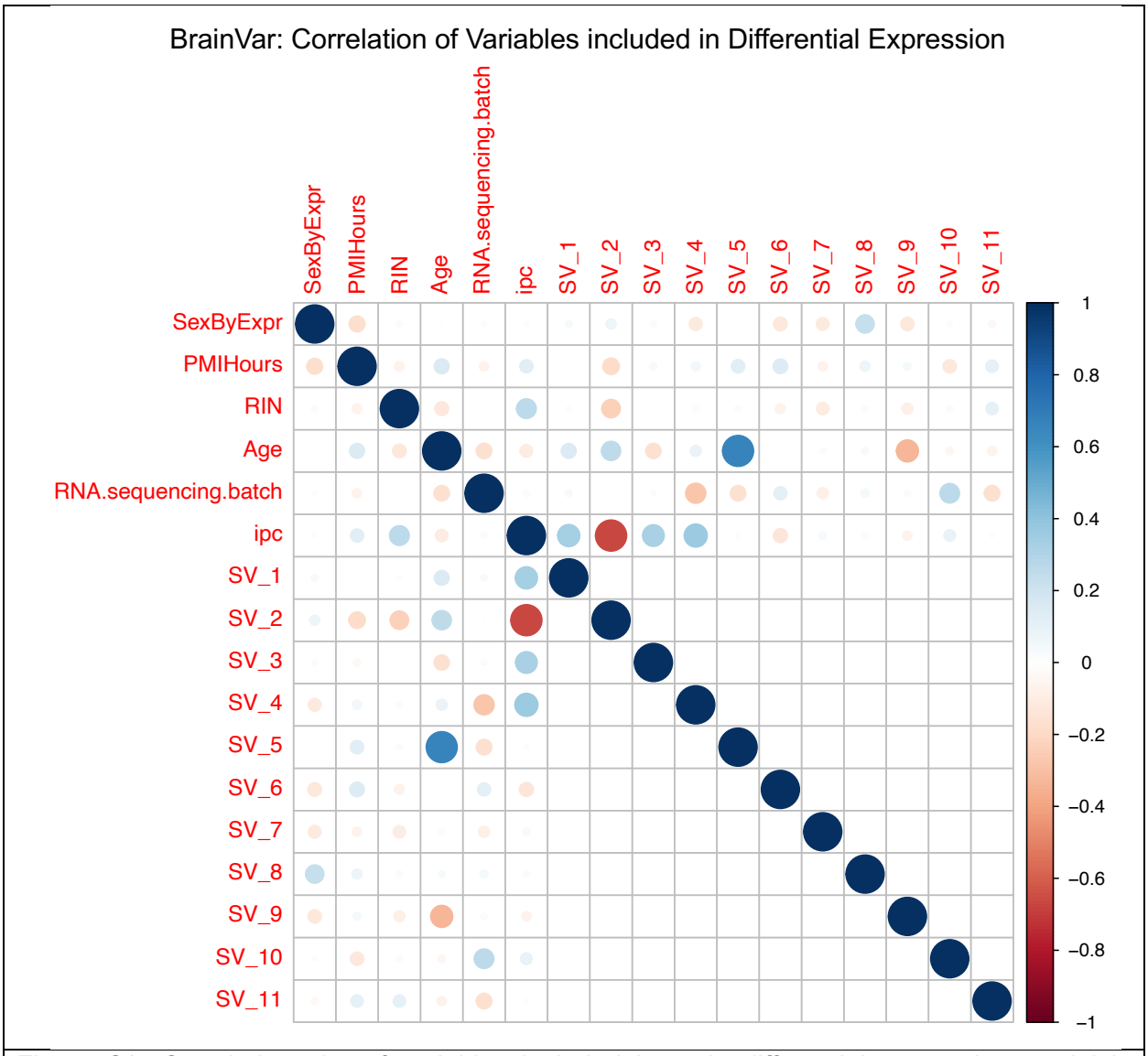


Figure S9. Correlation plot of variables included in main differential expression model in BrainVar.

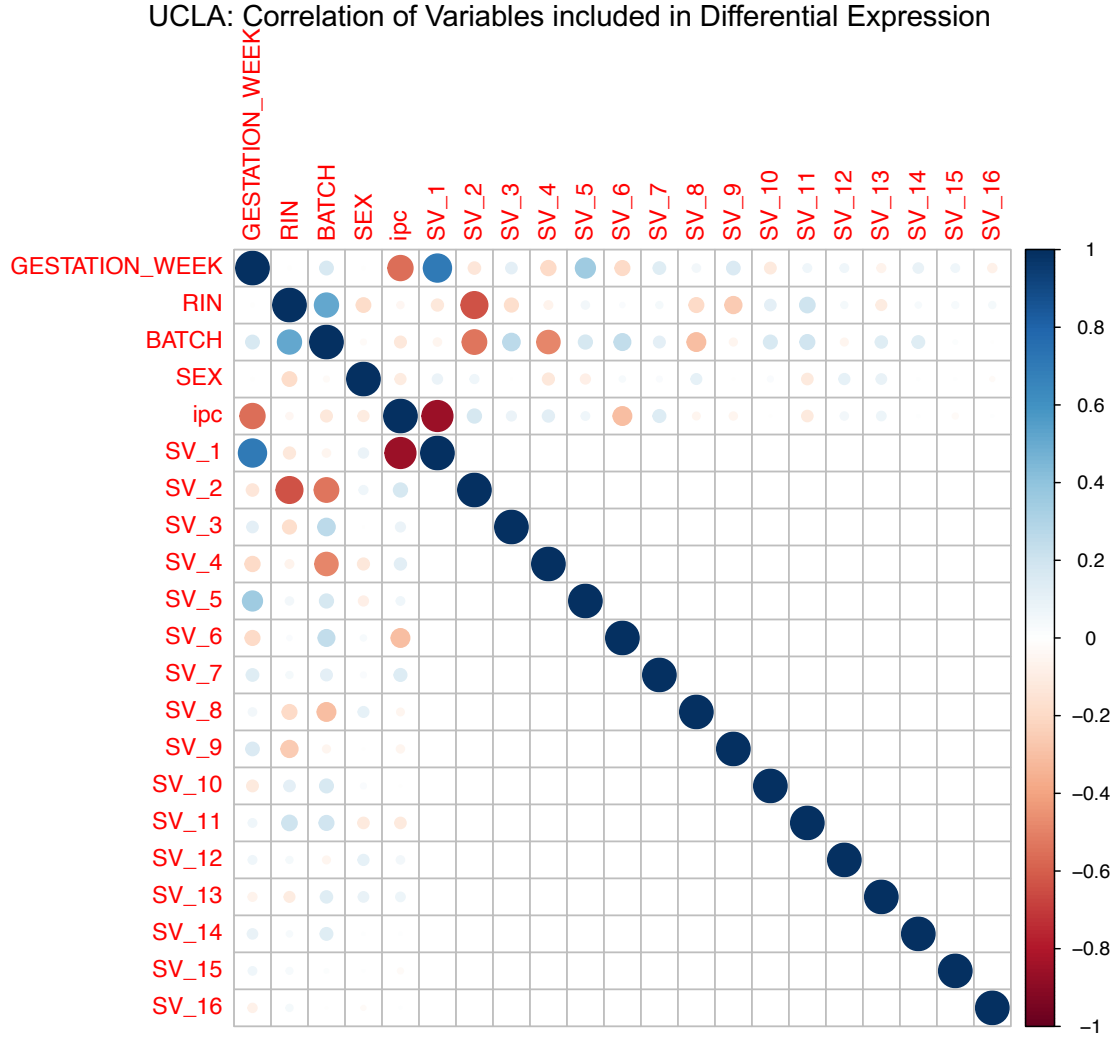


Figure S10. Correlation plot of variables included in main differential expression model in UCLA.

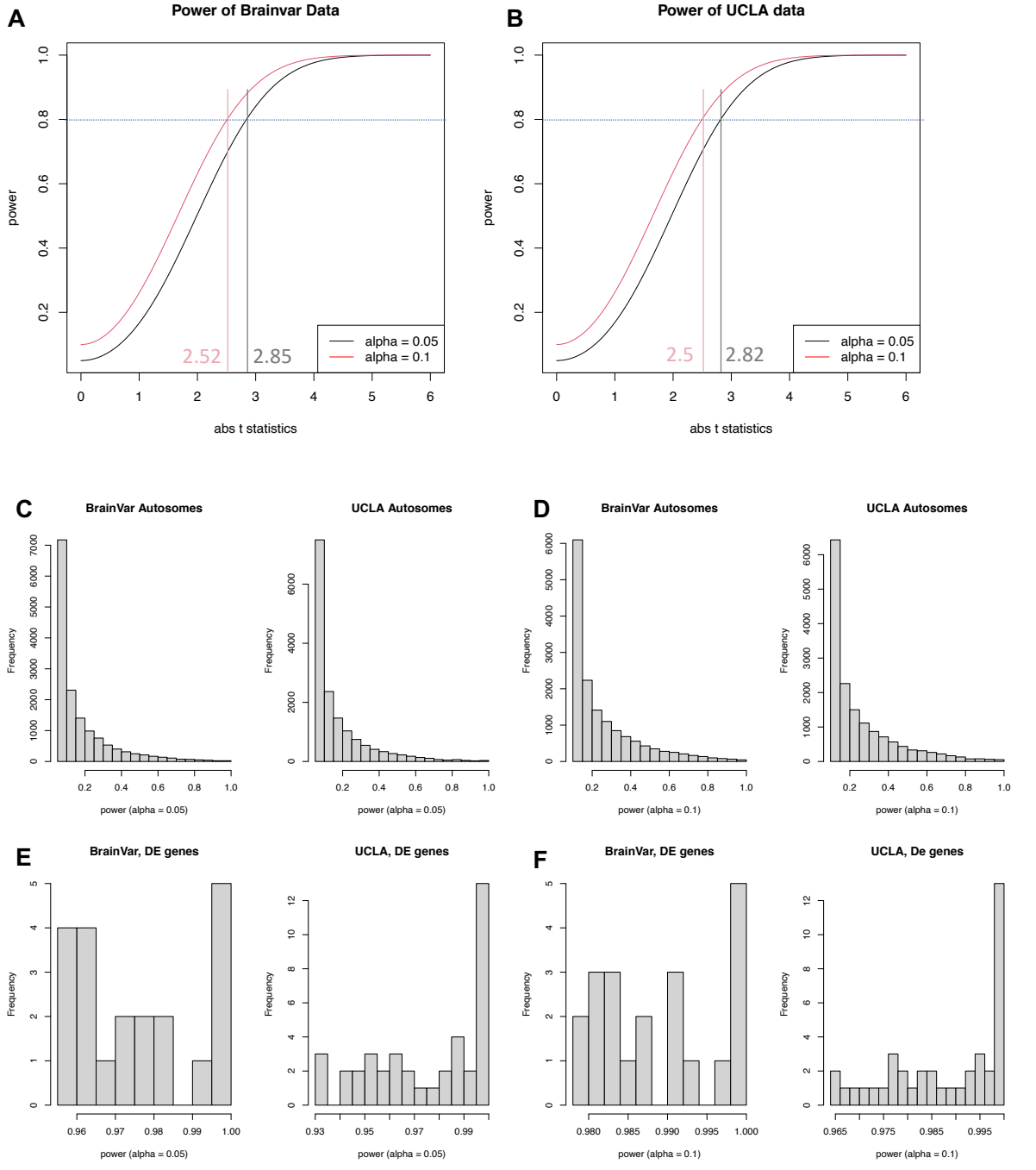


Figure S11. Power analysis for sex-differential expression. The power curves for (A) BrainVar and (B) UCLA as calculated for a two-sample test using degrees of freedom from limma:eBayes() with two confidence levels, alpha = 0.05 (gray) and alpha = 0.1 (pink). The minimum absolute t-statistics for power = 0.8 are noted on the plot. Histograms of the power for all autosomal genes at (C) alpha = 0.05 and (D) alpha = 0.1, and for the significant sex-DEGs (FDR < 0.1) at (E) alpha = 0.05 and (F) alpha = 0.1 (panels C-F: left, BrainVar; right, UCLA).

**List of Supplemental Tables (available upon request)**

**Supp. Table 1:** Meta-data for BrainVar RNA-seq data set.

**Supp. Table 2:** Meta-data for UCLA RNA-seq data set.

**Supp. Table 3:** Gene sets used for functional annotation with Fisher's Exact Test.

**Supp. Table 4:** Differential expression results from each data set and meta-analysis results.

**Supp. Table 5:** X chromosome gene categorization from GTeX brain tissue study by Tukiainen et al. 2017.

**Supp. Table 6:** Combined differential expression results from sex-separated age differential expression analysis, and age-by-sex interaction, in BrainVar.

**Supp. Table 7:** Combined differential expression results from sex-separated age differential expression analysis, and age-by-sex interaction, in UCLA.

**Supp. Table 8:** Results from Fisher's Exact Tests (FET) on sex-biased genes detected from meta-analysis of differential expression results

**Supp Table 9:** Results from cell type-specific sex-differential expression as estimated by bMIND, BrainVar

**Supp Table 10:** Results from cell type-specific sex-differential expression as estimated by bMIND, UCLA

**Supp Table 11:** Results from GSEA on transcriptome-wide sex-differential expression as determined by meta-analysis

**Supp. Table 12:** List of module genes, kME values, ME values per sample from sex-separated WGCNA (before grouping) in BrainVar data set.

**Supp. Table 13:** Lists of Ensembl IDs for each BrainVar WGCNA module or group.

**Supp. Table 14:** Results from Fisher's Exact Tests (FET) from BrainVar WGCNA modules and groups.

**Supp. Table 15:** gProfiler results from BrainVar WGCNA modules and groups.

**Supp. Table 16:** Lists of genes and results from sLED analysis on BrainVar RNA-seq.

**Supp. Table 17:** Lists of genes and results from sLED analysis on UCLA RNA-seq.

**Supp. Table 18:** Results from variance partition analysis with percent variance explained per gene per variable in model including SVs for differential expression in BrainVar RNA-seq data set.

**Supp. Table 19:** Results from variance partition analysis with percent variance explained per gene per variable in model without SVs for differential expression in BrainVar RNA-seq data set.

**Supp. Table 20:** Results from variance partition analysis with percent variance explained per gene per variable in model including SVs for differential expression in UCLA RNA-seq data set.

**Supp. Table 21:** Results from variance partition analysis with percent variance explained per gene per variable in model without SVs for differential expression in UCLA RNA-seq data set.

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## APPENDIX B: SUPPLEMENTAL MATERIALS FOR CHAPTER II

### Sex-differential gene expression in the ASD brain and relationship to risk-associated mechanisms

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## Supplemental Figures

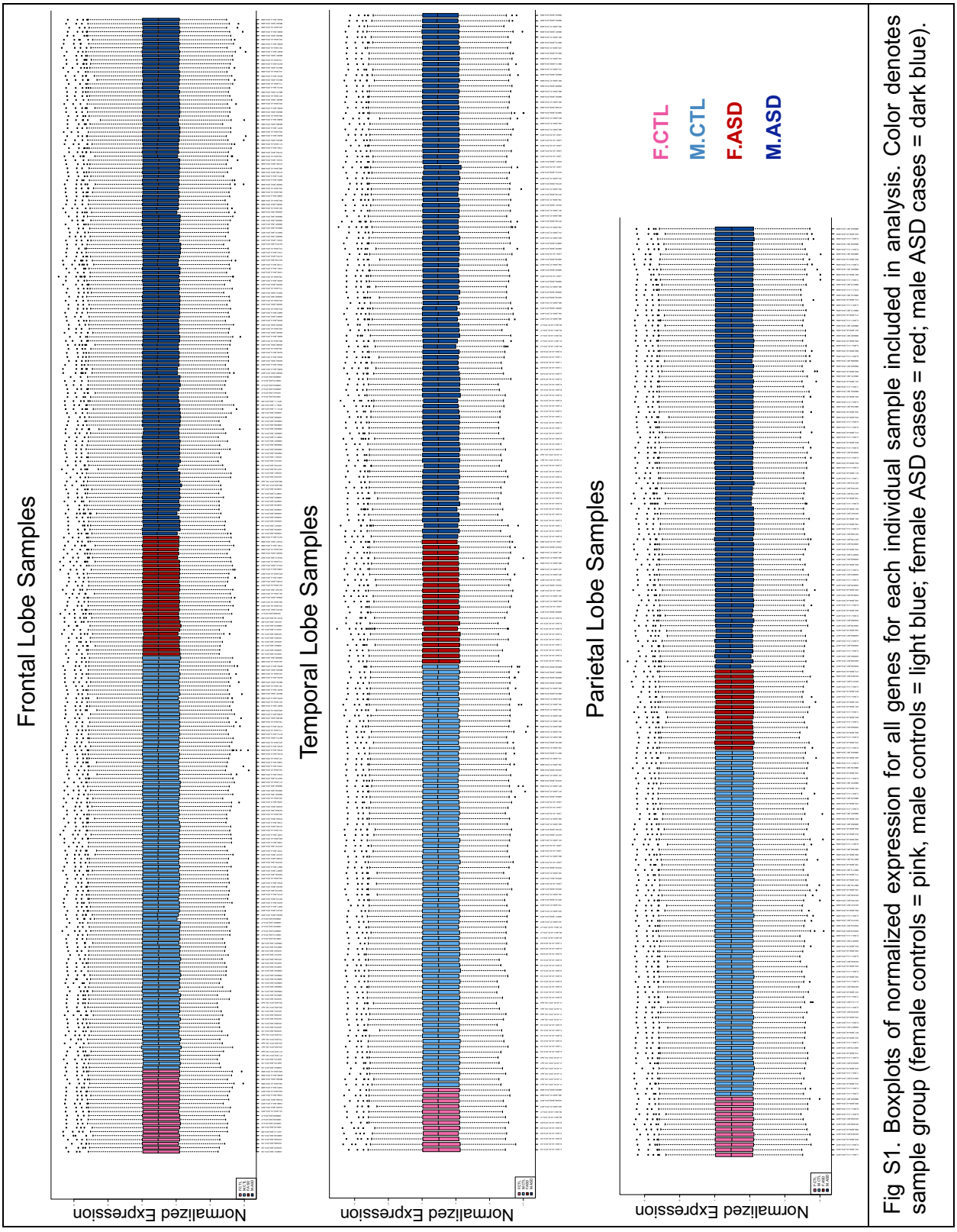



Fig S1. Boxplots of normalized expression for all genes for each individual sample included in analysis. Color denotes sample group (female controls = pink, male controls = light blue; female ASD cases = red; male ASD cases = dark blue).

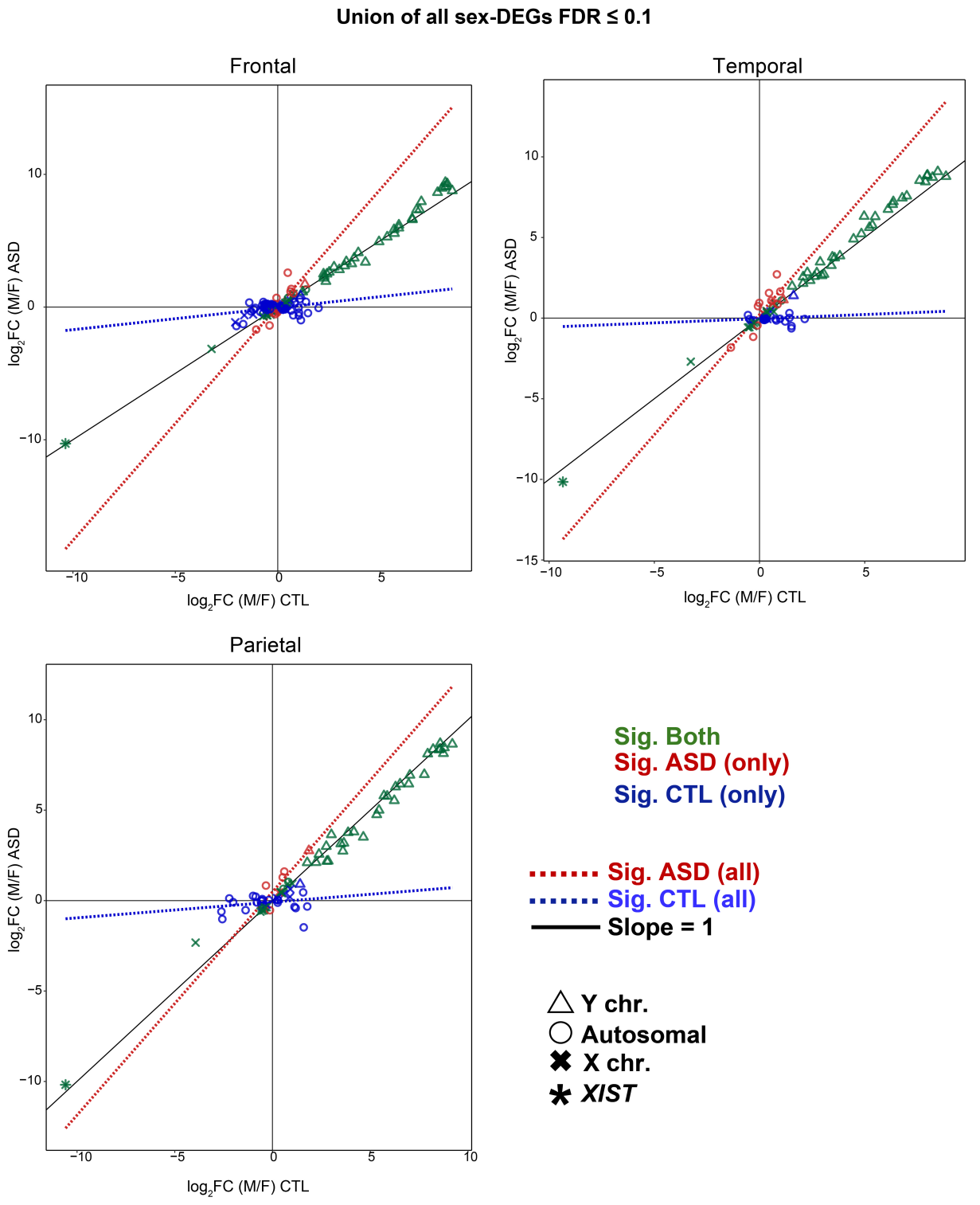



Fig S2. Scatter plot of $\log_2$ fold changes from sex-differential expression analysis separate in controls (x axis) and cases (y axis) for the union of all sex-DEGs at FDR ≤ 0.1 in each cortical lobe. Point color denotes significance in only cases (red), only controls (blue), or in both cases

and controls (green). Point shape indicates chromosome of gene. Dotted red linear regression line includes genes significant in ASD (red points) and significant in both (green points). Dotted blue linear regression line includes genes significant in controls (blue points) and significant in both (green points). See **Table 5** for linear regression statistics. Black line indicates slope of 1.

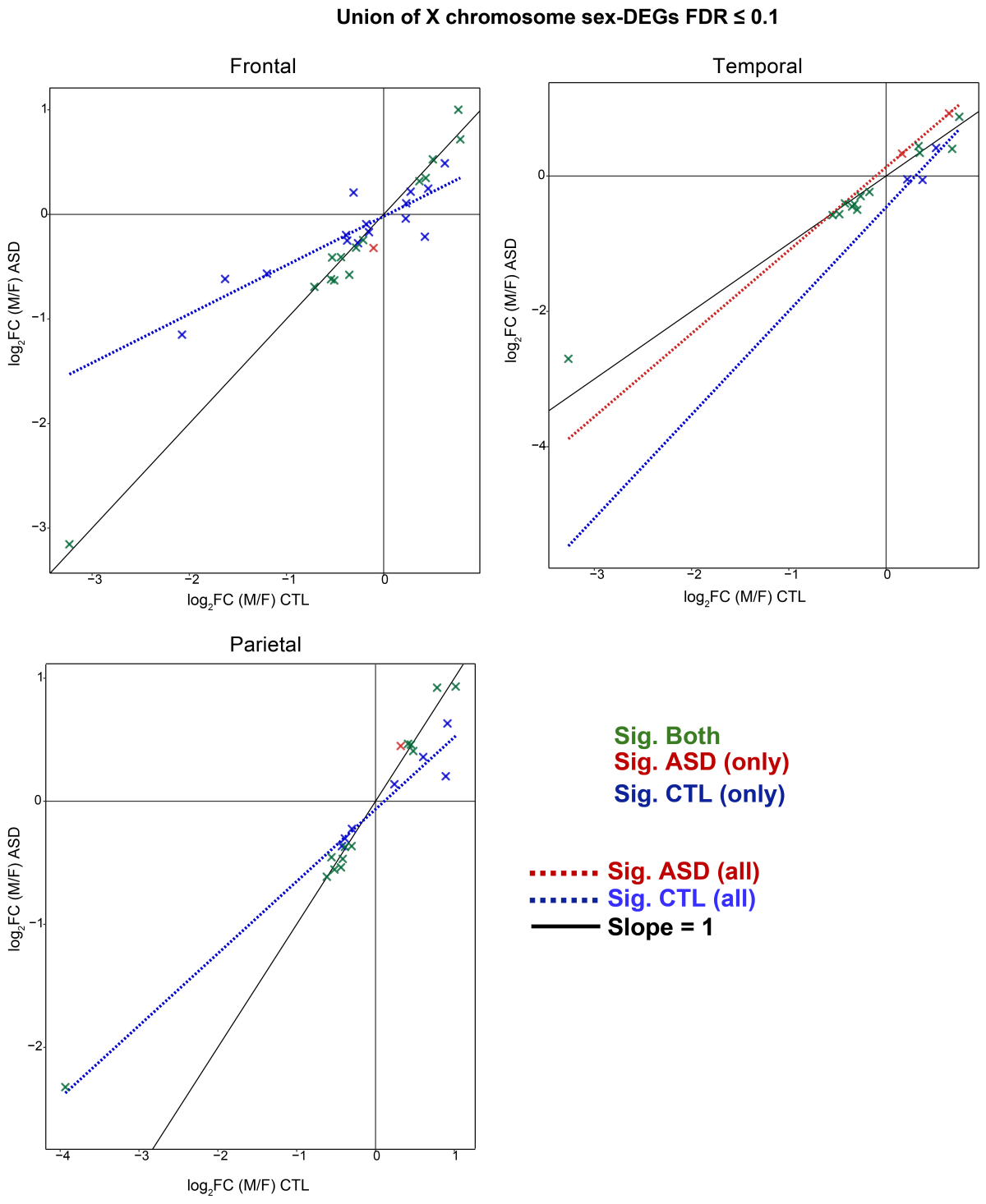



Fig S3. Scatter plot of $\log_2$ fold changes from sex-differential expression analysis separate in controls (x axis) and cases (y axis) for the union of X chromosome sex-DEGs at FDR $\leq 0.1$ in each cortical lobe. Point color denotes significance in only cases (red), only controls (blue), or in both cases and controls (green). Dotted red linear regression line includes genes significant

in ASD (red points) and significant in both (green points). Dotted blue linear regression line includes genes significant in controls (blue points) and significant in both (green points). See **Table 5** for linear regression statistics. Black line indicates slope of 1.

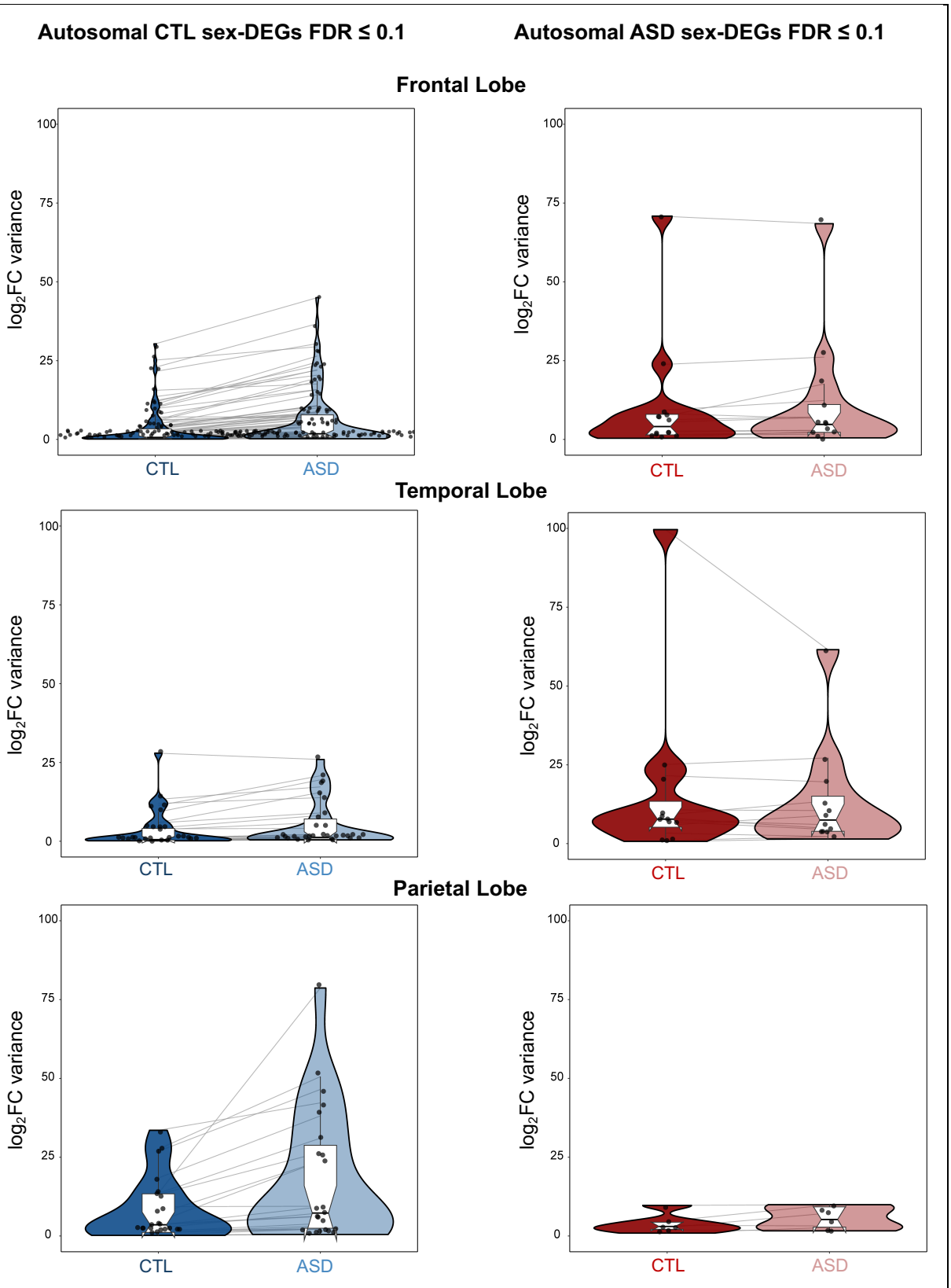



Fig S4. Violin plots of $\log_2$ fold change variance in autosomal sex-DEGs detected in controls, regardless of their significance in cases (left column) and autosomal sex-DEGs detected in

cases, regardless of their significance in controls (right column). Each dot inside the violin plots indicates the value of $\log_2$ fold change variance for a specific gene in controls or cases. Light gray lines connect the $\log_2$ fold change variance values for a specific gene in controls and cases. Black line inside violin plot denotes median value.

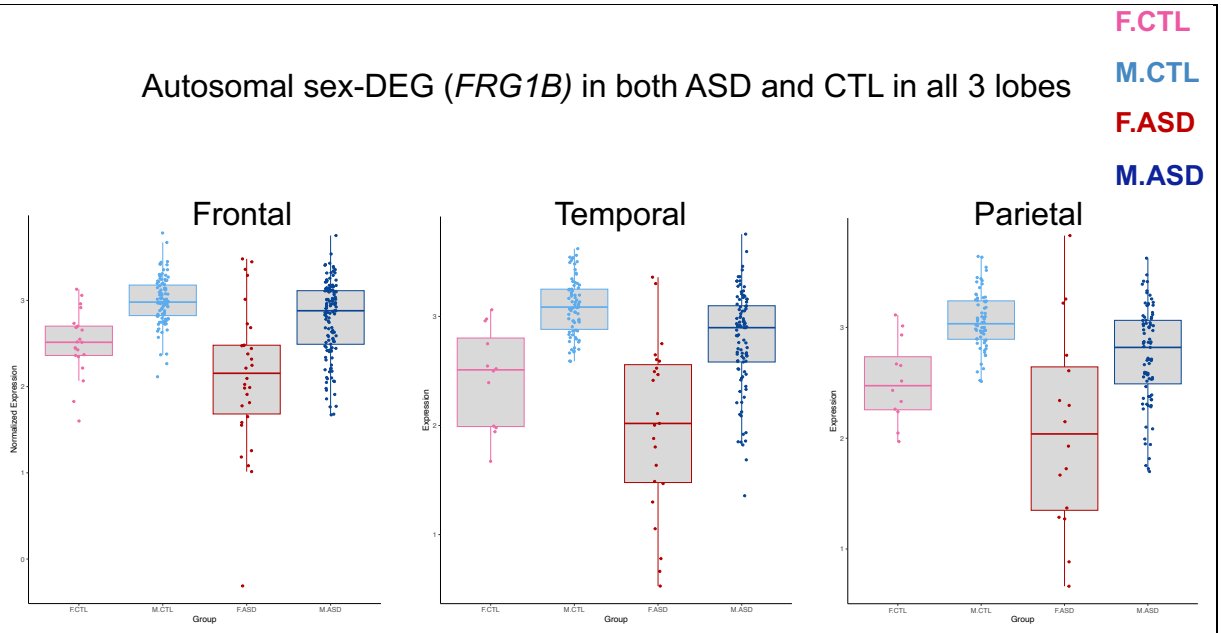
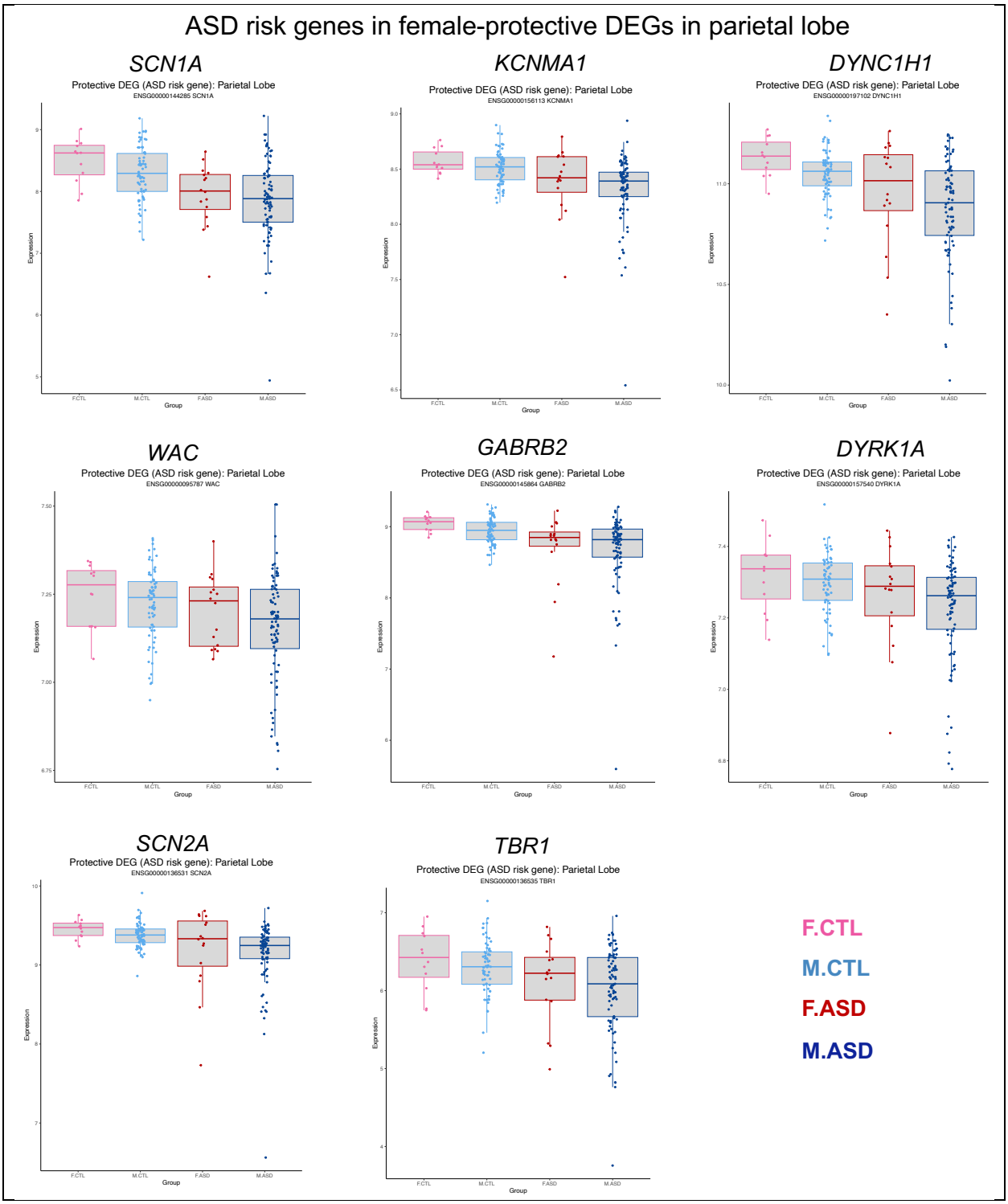



Fig S5. Boxplots of normalized expression of autosomal sex-DEG *FRG1B* significant in both cases and controls in all three cortical lobes. Each dot represents the normalized expression value for an individual sample. Color denotes sample group (female controls = pink, male controls = light blue; female ASD cases = red; male ASD cases = dark blue).


ASD risk genes in female-protective DEGs in parietal lobe
SCN1A
Protective DEG (ASD risk gene): Parietal Lobe
ENSG00000144285 SCN1A
KCNMA1
Protective DEG (ASD risk gene): Parietal Lobe
ENSG00000156113 KCNMA1
DYNC1H1
Protective DEG (ASD risk gene): Parietal Lobe
ENSG00000197102 DYNC1H1
WAC
Protective DEG (ASD risk gene): Parietal Lobe
ENSG00000095787 WAC
GABRB2
Protective DEG (ASD risk gene): Parietal Lobe
ENSG00000145864 GABRB2
DYRK1A
Protective DEG (ASD risk gene): Parietal Lobe
ENSG00000157540 DYRK1A
SCN2A
Protective DEG (ASD risk gene): Parietal Lobe
ENSG00000136531 SCN2A
TBR1
Protective DEG (ASD risk gene): Parietal Lobe
ENSG00000136535 TBR1
Expression
Group
F.CTL
M.CTL
F.ASD
M.ASD

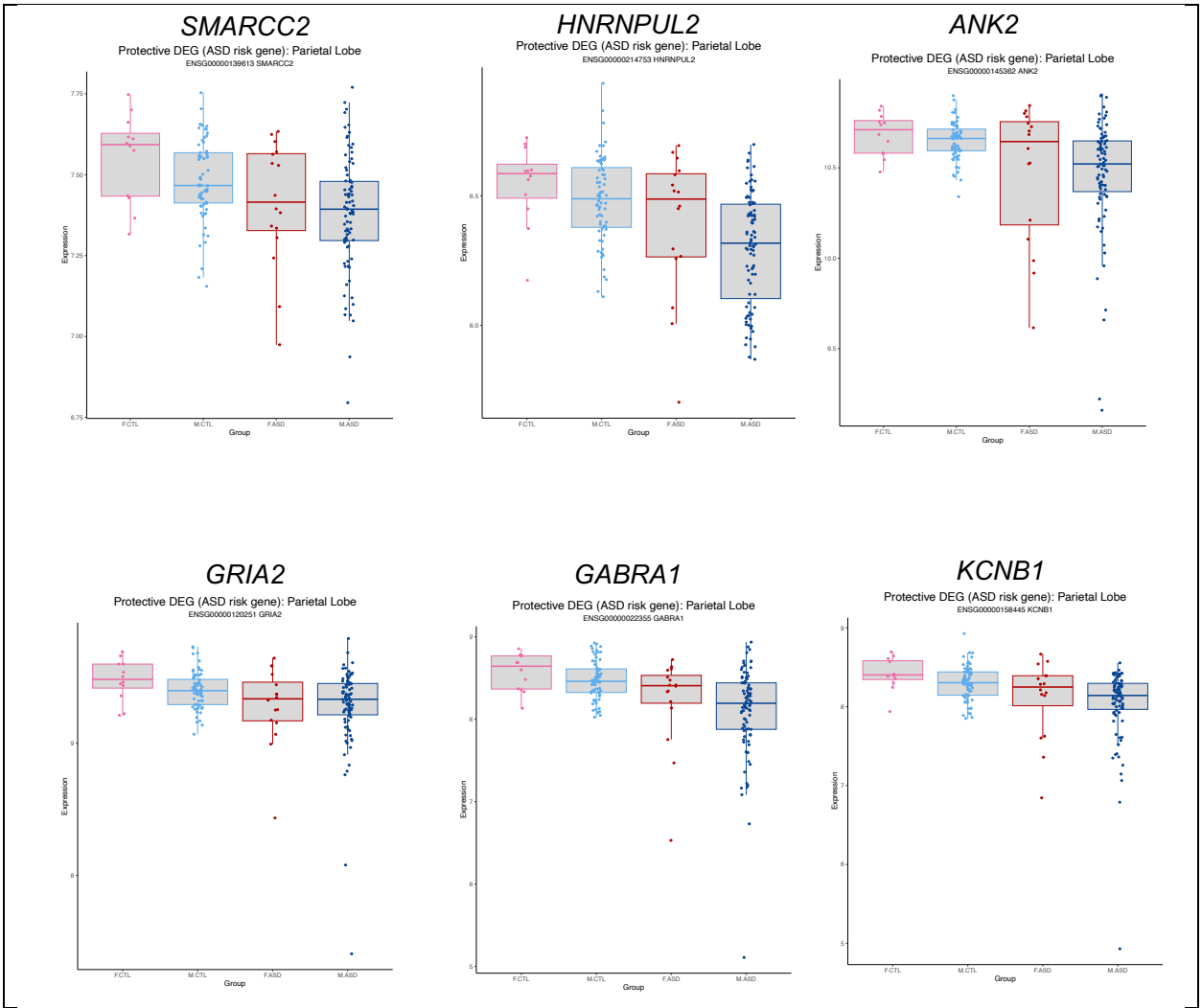



Fig S6. Boxplots of normalized expression of female-protective DEGs in the parietal lobe that overlap with ASD risk genes (1–3). Each dot represents the normalized expression value for an individual sample. Color denotes sample group (female controls = pink, male controls = light blue; female ASD cases = red; male ASD cases = dark blue).

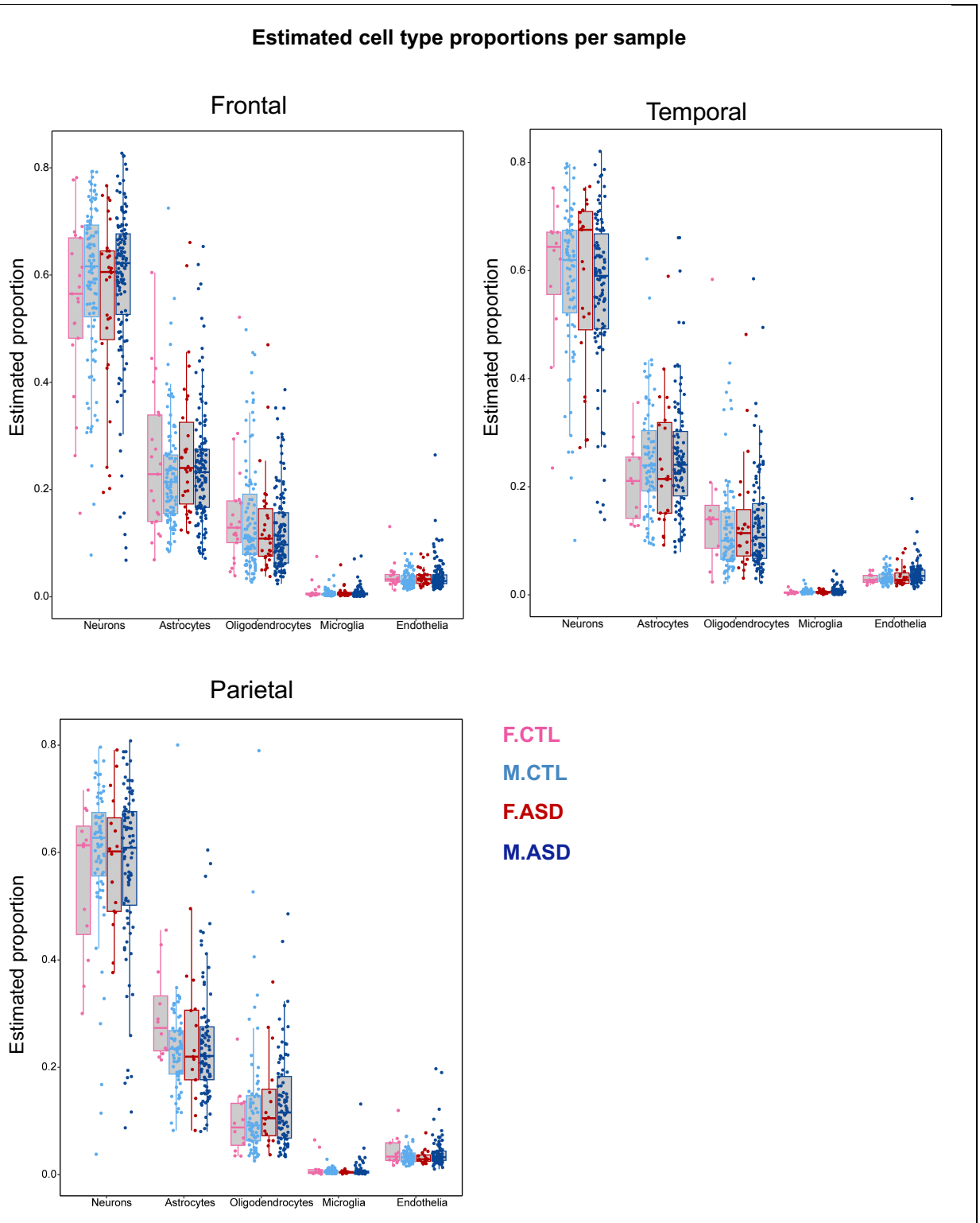



Fig S7. Estimated proportions of five cell types in female controls (pink), male controls (light blue), female ASD cases (red), and male cases (dark blue) in each cortical lobe. Each dot represents a specific cell type estimate for an individual sample.

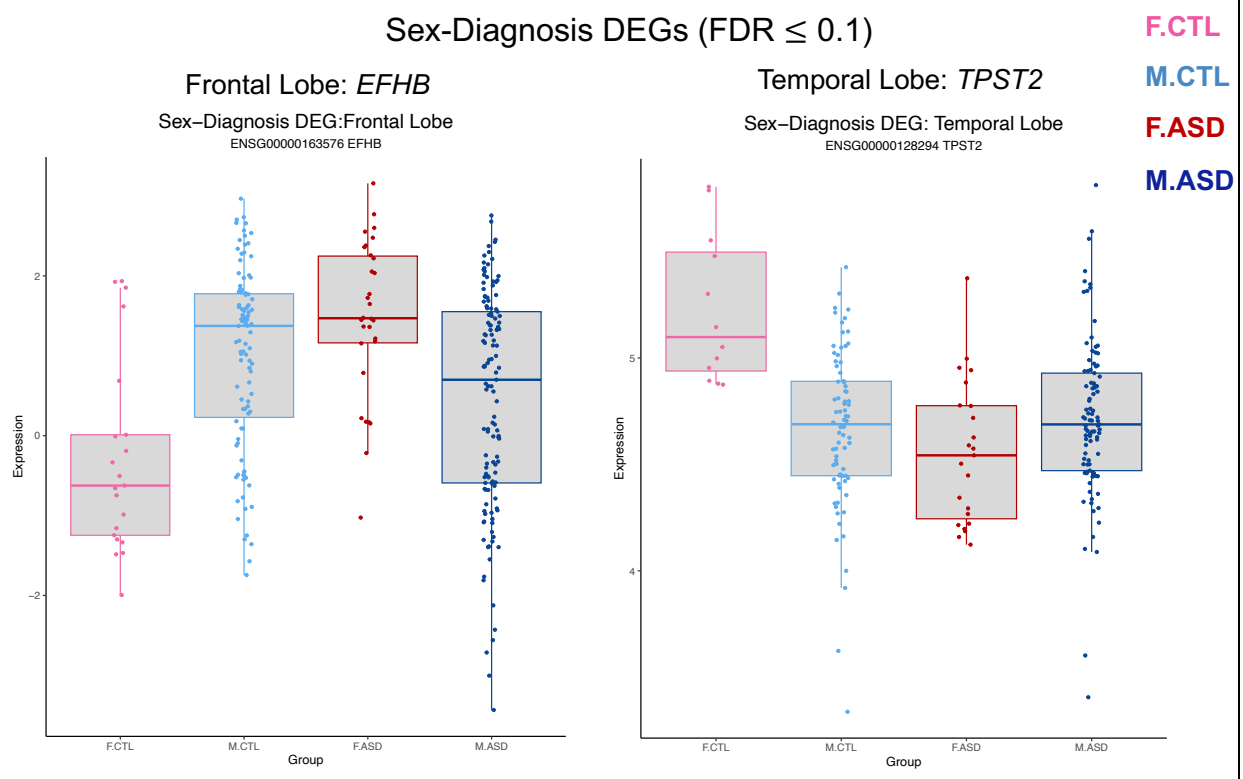



Fig S8. Boxplots of normalized expression of genes significant at FDR ≤ 0.1 from sex-by-diagnosis interaction differential expression analysis in each cortical lobe (none significant in parietal lobe). Each dot represents the normalized expression value for an individual sample. Color denotes sample group (female controls = pink, male controls = light blue; female ASD cases = red; male ASD cases = dark blue).

**Supplemental Tables**

| Ensembl ID | Gene Name | Chromosome | Category |
|---|---|---|---|
| ENSG00000001630 | *CYP51A1* | 7 | Sex-equalized |
| ENSG00000013561 | *RNF14* | 5 | |
| ENSG00000060762 | *MPC1* | 6 | |
| ENSG00000067064 | *IDI1* | 10 | |
| ENSG00000068024 | *HDAC4* | 2 | |
| ENSG00000083937 | *CHMP2B* | 3 | |
| ENSG00000099622 | *CIRBP* | 19 | |
| ENSG00000104671 | *DCTN6* | 8 | |
| ENSG00000108561 | *C1QBP* | 17 | |
| ENSG00000110987 | *BCL7A* | 12 | |
| ENSG00000114942 | *EEF1B2* | 2 | |
| ENSG00000116918 | *TSNAX* | 1 | |
| ENSG00000120837 | *NFYB* | 12 | |
| ENSG00000123836 | *PFKFB2* | 1 | |
| ENSG00000124733 | *MEA1* | 6 | |
| ENSG00000127125 | *PPCS* | 1 | |
| ENSG00000141759 | *TXNL4A* | 18 | |
| ENSG00000144589 | *STK11IP* | 2 | |
| ENSG00000145293 | *ENOPH1* | 4 | |
| ENSG00000147454 | *SLC25A37* | 8 | |
| ENSG00000150787 | *PTS* | 11 | |
| ENSG00000154803 | *FLCN* | 17 | |
| ENSG00000155016 | *CYP2U1* | 4 | |
| ENSG00000155959 | *VBP1* | X | |
| ENSG00000160752 | *FDPS* | 1 | |
| ENSG00000164258 | *NDUFS4* | 5 | |
| ENSG00000165264 | *NDUFB6* | 9 | |
| ENSG00000167074 | *TEF* | 22 | |
| ENSG00000172366 | *FAM195A* | 16 | |
| ENSG00000172775 | *FAM192A* | 16 | |
| ENSG00000182093 | *WRB* | 21 | |
| ENSG00000185414 | *MRPL30* | 2 | |
| ENSG00000186010 | *NDUFA13* | 19 | |
| ENSG00000186480 | *INSIG1* | 7 | |
| ENSG00000189403 | *HMGB1* | 13 | |
| ENSG00000196792 | *STRN3* | 14 | |
| ENSG00000197375 | *SLC22A5* | 5 | |
| ENSG00000198839 | *ZNF277* | 7 | |
| ENSG00000203705 | *TATDN3* | 1 | |
| ENSG00000205323 | *SARNP* | 12 | |
| ENSG00000214253 | *FIS1* | 7 | |
| ENSG00000241258 | *CRCP* | 7 | |
| ENSG00000008517 | *IL32* | 16 | Female-shifted |
| ENSG00000100342 | *APOL1* | 22 | |
| ENSG00000101187 | *SLCO4A1* | 20 | |
| ENSG00000105227 | *PRX* | 19 | |

| | | | |
|---|---|---|---|
| ENSG00000108551 | *RASD1* | 17 | |
| ENSG00000109906 | *ZBTB16* | 11 | |
| ENSG00000115297 | *TLX2* | 2 | |
| ENSG00000116774 | *OLFML3* | 1 | |
| ENSG00000122359 | *ANXA11* | 10 | |
| ENSG00000126368 | *NR1D1* | 17 | |
| ENSG00000128253 | *RFPL2* | 22 | |
| ENSG00000128294 | *TPST2* | 22 | |
| ENSG00000144182 | *LIPT1* | 2 | |
| ENSG00000144218 | *AFF3* | 2 | |
| ENSG00000144837 | *PLA1A* | 3 | |
| ENSG00000147799 | *ARHGAP39* | 8 | |
| ENSG00000163576 | *EFHB* | 3 | |
| ENSG00000168497 | *SDPR* | 2 | |
| ENSG00000173762 | *CD7* | 17 | |
| ENSG00000183888 | *C1orf64* | 1 | |
| ENSG00000198829 | *SUCNR1* | 3 | |
| ENSG00000198959 | *TGM2* | 20 | |
| ENSG00000205056 | NA | 12 | |
| ENSG00000229921 | *KIF25-AS1* | 6 | |
| ENSG00000235453 | *TOPORS-AS1* | 9 | |
| ENSG00000239556 | NA | 7 | |
| ENSG00000019186 | *CYP24A1* | 20 | |
| ENSG00000077238 | *IL4R* | 16 | |
| ENSG00000102317 | *RBM3* | X | |
| ENSG00000112972 | *HMGCS1* | 5 | |
| ENSG00000120437 | *ACAT2* | 6 | |
| ENSG00000126767 | *ELK1* | X | Male-shifted |
| ENSG00000155380 | *SLC16A1* | 1 | |
| ENSG00000169313 | *P2RY12* | 3 | |
| ENSG00000182912 | *C21orf90* | 21 | |
| ENSG00000187193 | *MT1X* | 16 | |
| ENSG00000197142 | *ACSL5* | 10 | |

**Supplemental Table 1.** ASD-attenuated sex-DEGs in the frontal lobe that met criteria (Methods) for sex-equalized, female-shifted, or male-shifted expression pattern.

| Ensembl ID | Gene Name | Chromosome | Category |
|---|---|---|---|
| ENSG00000001630 | *CYP51A1* | 7 | Sex-equalized |
| ENSG00000034693 | *PEX3* | 6 | |
| ENSG00000077458 | *FAM76B* | 11 | |
| ENSG00000083937 | *CHMP2B* | 3 | |
| ENSG00000087470 | *DNM1L* | 12 | |
| ENSG00000103769 | *RAB11A* | 15 | |
| ENSG00000111237 | *VPS29* | 12 | |
| ENSG00000115464 | *USP34* | 2 | |
| ENSG00000116874 | *WARS2* | 1 | |
| ENSG00000120805 | *ARL1* | 12 | |
| ENSG00000124333 | *VAMP7* | X | |
| ENSG00000160801 | *PTH1R* | 3 | |
| ENSG00000173275 | *ZNF449* | X | |
| ENSG00000174842 | *GLMN* | 1 | |
| ENSG00000180228 | *PRKRA* | 2 | |
| ENSG00000273035 | NA | 2 | |
| ENSG00000070019 | *GUCY2C* | 12 | Female-shifted |
| ENSG00000116014 | *KISS1R* | 19 | |
| ENSG00000125965 | *GDF5* | 20 | |
| ENSG00000128294 | *TPST2* | 22 | |
| ENSG00000173295 | *FAM86B3P* | 8 | |
| ENSG00000229921 | *KIF25-AS1* | 6 | |
| ENSG00000232953 | *HSPA8P18* | 3 | |
| ENSG00000249465 | *RBMXP4* | 4 | |
| ENSG00000019186 | *CYP24A1* | 20 | Male-shifted |
| ENSG00000116918 | *TSNAX* | 1 | |
| ENSG00000180773 | *SLC36A4* | 11 | |

**Supplemental Table 2.** ASD-attenuated sex-DEGs in the temporal lobe that met criteria (Methods) for sex-equalized, female-shifted, or male-shifted expression pattern.

| Ensembl ID | Gene Name | Chromosome | Category |
|---|---|---|---|
| ENSG00000004866 | *ST7* | 7 | Sex-equalized |
| ENSG00000091844 | *RGS17* | 6 | |
| ENSG00000124608 | *AARS2* | 6 | |
| ENSG00000197302 | *ZNF720* | 16 | |
| ENSG00000102290 | *PCDH11X* | X | Female- shifted |
| ENSG00000126458 | *RRAS* | 19 | |
| ENSG00000134202 | *GSTM3* | 1 | |
| ENSG00000138772 | *ANXA3* | 4 | |
| ENSG00000160801 | *PTH1R* | 3 | |
| ENSG00000180481 | *GLIPR1L2* | 12 | |
| ENSG00000198829 | *SUCNR1* | 3 | |
| ENSG00000248103 | NA | 5 | |
| ENSG00000004939 | *SLC4A1* | 17 | Male-shifted |
| ENSG00000019186 | *CYP24A1* | 20 | |
| ENSG00000133742 | *CA1* | 8 | |
| ENSG00000142748 | *FCN3* | 1 | |
| ENSG00000148926 | *ADM* | 11 | |

**Supplemental Table 3.** ASD-attenuated sex-DEGs in the parietal lobe that met criteria (Methods) for sex-equalized, female-shifted, or male-shifted expression pattern.

**References**

1. Satterstrom FK, Kosmicki JA, Wang J, Breen MS, De Rubeis S, An J-Y, *et al.* (2020): Large-scale exome sequencing study implicates both developmental and functional changes in the neurobiology of autism. *Cell*, vol. 180. pp 568-584. e23.

2. Zhou X, Feliciano P, Shu C, Wang T, Astrovskaya I, Hall JB, *et al.* (2022): Integrating de novo and inherited variants in 42,607 autism cases identifies mutations in new moderate-risk genes. *Nature Genetics* 54: 1305–1319.

3. Fu JM, Satterstrom FK, Peng M, Brand H, Collins RL, Dong S, *et al.* (2022): Rare coding variation provides insight into the genetic architecture and phenotypic context of autism. *Nature Genetics* 54: 1320–1331.

## APPENDIX C: PEER REVIEWED PUBLICATIONS

The following is a list of peer reviewed publications and presentations I have authored during my time in the Neuroscience Training Program at University of Wisconsin-Madison. Although these papers and presentations are not all directly related to my thesis in terms of content, each one has played a valuable role in my development as a scientist and is an indication of my breadth of study and contributions to science. The contributions I have made to these works have all been important in expanding my skills and knowledge that have been vital in my academic and scientific career.

I also include these works as an additional appreciation for my colleagues and the collaborations I have had during my time at UW. To everyone involved in these works, I thank you for involving me in your research efforts!

- Lee

Publications:

Shen, M.; Sirois, CL; Guo, Y.; Li, M.; Dong, Q; Méndez-Albelo, NM; Gao, Y; Khullar, S; **Kissel, L.**; et al. *Species-specific FMRP regulation of RACK1 is critical for prenatal cortical development*. Neuron, 2023. S0896-6273(23)00702-X.

Rayasam, A.; Kijak, JA; **Kissel, L**; Choi, YH.; Kim, T.; Hsu, M.; Joshi, D.; Laaker, CJ.; Cismaru, P.; Lindstedt, A.; Kovacs, K.; Vemuganti, R.; Chiu, SY.; Priyathilaka, TT.; Sandor, M.; Fabry, Z. *CXCL13 expressed on inflamed cerebral blood vessels recruit IL-21 producing TFH cells to damage neurons following stroke*. J Neuroinflammation, 2022. 19(1):125.

Rayasam, A; Hsu, M; Kijak, J; **Kissel, L**; Hernandez, G.; Sandor, M.; Fabry, Z. *Immune responses in stroke: how the immune system contributes to damage and healing after stroke and how this knowledge could be translated to better cures.* Immunology, 2018. 154(3):363-376.

Presentations:

**Kissel, L.**; Pochareddy, S.; An, JY.; Roeder, K.; Sestan, N.; Sanders, SJ.; Werling, DM. Sex-differentially expressed genes in the developing and ASD cortex implicate glial function in sex-differential ASD risk. Presented at the 2023 INSAR Annual Meeting, Stockholm, Sweden, May 3-6, 2023.

**Kissel, L.**; Pochareddy, S.; An, JY.; Roeder, K.; Sestan, N.; Sanders, SJ.; Werling, DM. Defining patterns of sex-differential expression in the human cortex during prenatal development and the intersections with Autism Spectrum Disorder. Presented at the 2022 SfN Annual Meeting, San Diego, CA, United States, November 12-16, 2022.

**Kissel, L.**; Rayasam, A.; Choi, Y.; Kijak, J.; Hsu, M.; Kovacs, K.; Sandor, M.; Fabry, Z. T follicular helper cell recruitment to the ischemic brain. Presented at the 2019 AAI Annual Conference, San Diego, CA, United States, May 9-12, 2019.