

Beyond a Binary Classification of Sex: An Examination of Brain Sex Differentiation, Psychopathology, and Genotype

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Objective: Sex differences in the brain are traditionally treated as binary. We present new evidence that a continuous measure of sex differentiation of the brain can explain sex differences in psychopathology. The degree of sex-differentiated brain features (ie, features that are more common in one sex) may predispose individuals toward sex-biased psychopathology and may also be influenced by the genome. We hypothesized that individuals with a female-biased differentiation score would have greater female-biased psychopathology (internalizing symptoms, such as anxiety and depression), whereas individuals with a male-biased differentiation score would have greater male-biased psychopathology (externalizing symptoms, such as disruptive behaviors).

Method: Using the Philadelphia Neurodevelopmental Cohort database acquired from database of Genotypes and Phenotypes, we calculated the sex differentiation measure, a continuous data-driven calculation of each individual's degree of sex-differentiating features extracted from multimodal brain imaging data (magnetic resonance imaging [MRI] /diffusion MRI) from the imaged participants ($n = 866$, 407 female and 459 male).

Results: In male individuals, higher differentiation scores were correlated with higher levels of externalizing symptoms ($r = 0.119$, $p = .016$). The differentiation measure reached genome-wide association study significance ($p < 5 \times 10^{-8}$) in male individuals with single nucleotide polymorphisms Chromosome5:rs111161632:*RASGEF1C* and Chromosome19:rs75918199:*GEMIN7*, and in female individuals with Chromosome2:r-s78372132:*PARD3B* and Chromosome15:rs73442006:*HCN4*.

Conclusion: The sex differentiation measure provides an initial topography of quantifying male and female brain features. This demonstration that the sex of the human brain can be conceptualized on a continuum has implications for both the presentation of psychopathology and the relation of the brain with genetic variants that may be associated with brain differentiation.

Key words: sex, gender, differentiation, mosaic, genotype

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The human brain may reflect and be influenced by biological and environmental sex-specific differences during development, thus putting the brain on a male–female continuous spectrum.¹ Recent work suggests that the human brain may not be strictly male or strictly female; rather, it may be composed of both characteristically male and characteristically female regions.² Here, we propose that the human brain is not classifiable into strict biological binary male and female groups, and that the composition of sex differentiating traits in the brain is linked to the presentation of psychopathology. We also explore whether the composition of these traits is influenced by genetic differences. Sex-differentiating brain traits are brain features that are more common in one sex than in the

other. In the present study, we developed a measure to quantify sex differentiation of the brain using a definition based on a biological sex male–female classification. We then examined the relation between this measure of differentiation and sex-biased psychopathology. Sex differences in psychopathology have been widely reported.^{3,4} In addition, to identify whether genetic variants play a role in the composition of sex differentiating traits, we conducted a genome-wide association (GWA) analysis. Recent studies have shown that genetic effects for complex traits are sex specific, and are also found in genes located not only on the sex chromosomes but also on the autosomes.⁵ Therefore, we also examined whether we could detect genetic variants that are associated with brain sex differentiation.

Sex differences in psychopathology often emerge during childhood.⁶ At key peripubertal stages of development, girls and boys differentiate in their clinical presentation of psychopathology: whereas girls present more often with internalizing disorders (eg, depression or anxiety), boys present more often with externalizing disorders (eg, attention-deficit/hyperactivity disorder [ADHD], oppositional defiant disorder [ODD], or conduct disorder [CD]). These factor labels (internalizing, externalizing, thought disturbance) emerge as the most frequent factors in investigations of the structure of psychiatric disorders.^{7–11} These labels were chosen because participants with symptoms of depression or anxiety clustered into a latent variable, the internalizing factor, whereas participants with symptoms of disruptive behavioral disorders including ADHD, ODD, or CD clustered into an externalizing factor. In large cohorts of youth, sex differences have been documented separately in brain development, psychopathology, and cognition^{12–15}; however, few studies have related how the degree of sex differentiation in the brain is related to psychopathology.

We posit that the degree of sex differentiation in the brain is associated with sex differences in psychopathology, such that a preponderance of brain features seen more often in female individuals is associated with internalizing psychopathology, and a preponderance of brain features seen more often in male individuals is associated with externalizing psychopathology. That is, individuals with brains comprising more characteristically female traits may be more likely to present with internalizing psychopathology, whereas individuals with brains that comprise more characteristically male traits may be more likely to present with externalizing psychopathology. Although this hypothesis is well supported by the extant literature in which sex is treated as a binary variable,^{16–18} it has not been tested using sex as a continuous measure, in which a greater degree of features of one sex (ie high differentiation) affects sex-biased variance in psychopathology. A brain-derived continuous measure may better reflect sex-biased variance in psychopathology than a binary chromosomal assignment of XX or XY.

Genetic influences play an important role during brain development. The majority of brain measures show high heritability.¹⁹ Also, sex differences in the heritability of white matter have been found.²⁰ The next step is to identify genetic variants that are associated with the degree of sex differentiating traits in the brain.

In this study, we investigated the relations among neuroimaging data, psychopathology scores, and genomic data reported in the Philadelphia Neurodevelopmental Cohort (PNC). First, we generated a composite continuous brain sex differentiation measure of each participant's sexual differentiation, based on neuroimaging data (structural and diffusion

magnetic resonance imaging [MRI]), which reflects the relative degree of female and male features in an individual participant. Second, we explored whether the differentiation measure (1) is related to psychopathology that is known to be sex biased in childhood (ie, individuals with a female-scored brain will be more likely exhibit internalizing psychopathology features, whereas male-scored brains more likely externalizing psychopathology); and 2) is related to genomic variants, which we investigated with a GWA analysis.

METHOD

Participants

We downloaded the PNC database from the database of Genotypes and Phenotypes (dbGaP) after being approved for controlled access to individual-level data²¹ (N = 8,719; mean age = 13.76 ± 3.68 years, sex distribution = 4,498 females and 4,221 males).

Psychopathology Data: Variable Clustering and Reduction via Factor Analysis

Demographic, medical, and psychopathology histories were assessed using a structured computerized instrument, GOASSESS,²² which was developed from the Kiddie-Schedule for Affective Disorders and Schizophrenia.²³ In addition to standard demographic data, the psychopathology screener assesses symptom- and criterion-related assessments of mood, anxiety, disruptive behavioral, eating, psychotic, and substance use disorders. Both subject and collateral informant data were acquired for children and adolescents aged 11 to 17 years; for children under age 11, only collateral data were acquired, whereas for adolescents and young adults older than age 18, only subject report was acquired. Psychopathology data were extracted from 252 individual item-level responses to a semi-structured interview from dbGaP).²⁴ We conducted a factor analysis to dimensionalize the psychopathology data using R's Psych Package²⁵ (see Supplement 1: Factor Methods Details, available online). Factor analysis is useful to organize common processes underlying psychopathology²⁶ and has been previously conducted within this same data sample.²⁷

Five factors emerged that included symptoms in the following broad categories: (1) psychosis; (2) mania; (3) anxiety and depression; (4) disruptive behaviors (ADHD, ODD, and CD); and (5) fear (Figures S1 and S2, available online). Anxiety, depression, and fear were labeled as *internalizing* factors; disruptive behaviors were labeled *externalizing* factors; and psychosis and mania were labeled *thought disturbance* factors.

Structural and Diffusion Image Processing

We processed structural MRI MPRAGE data in BrainSuite (<http://brainsuite.org/>) using the cortical extraction

pipeline. For the brain surface extraction, each brain was individually examined to ensure a satisfactory cortical extraction. Participants with excessive motion, as defined by impaired image clarity or image artifacts, were dropped. For the bias field correction, we applied the iterative option to reduce potential image inhomogeneity. SVREG (<http://brainsuite.org/processing/svreg/>) was used to register data to the Brainsuite BCI-DNI_brain atlas (http://brainsuite.org/svreg_atlas_description/).

Diffusion tensor imaging (DTI) data were assessed for quality using DTIPrep (<https://www.nitrc.org/projects/dtiprep/>). DTIPrep is a program that is designed to address data quality problems that affect diffusion MRI, and a detailed description of the program is available in Oguz *et al.*²⁸ During processing of a participant's brain scan, DTIPrep removes individual diffusion-weighted volumes found to be affected by corrupting artifacts. If more than 80% of a participant's diffusion-weighted volumes were not removed, that subject was considered to have passed quality control (QC). If a participant's data passed QC, that individual's QC'd diffusion weighted volumes were then registered to the structural data using BDP (<http://brainsuite.org/processing/diffusion/>). BDP was used to correct for geometric distortions in diffusion images (registration-based distortion correction) and to co-register diffusion and anatomical images. BDP registrations were individually inspected to ensure a satisfactory registration. Axial and radial diffusivity were chosen to obtain a comprehensive assessment of diffusivity in both gray and white matter regions across the entire brain.²⁹ Finally, we extracted cortical thickness, area, volume, and axial and radial diffusivity values from the 95 regions of interest (ROIs) that were defined from the BCI-DNI atlas for each participant. Of these regions, 66 are labeled on the surface, and cortical thickness was also obtained. SVREG further subdivides some ROIs into gray and white matter based on T1 tissue intensity values. (Details for ROIs are available via http://brainsuite.org/svreg_atlas_description/.)

Estimation of Brain Sex Differentiation: Likelihood Ratio Approach

We estimated brain sexual differentiation based on adaptations of methods presented previously.² We used all brain variables and scored the analyses continuously to retain the overall characteristic sex differentiation of the brain. This allowed for an automated continuous data-driven calculation of each variable and each participant's degree of sex-differentiating features.

For each brain measurement (axial/radial diffusion, area, volume, thickness) available within the ROIs and using a total of 698 brain features, we estimated sexual

differentiation measures for each participant using the likelihood ratio of male and female distributions, as described below. For features influenced by brain volume (area, volume), we normalized with whole brain volume (total brain volume excluding cerebrospinal fluid). This is a significant methodological decision given that, on average, male and female individuals differ in brain volume. By making the decision to control for brain volume, common in research examining sex differences in brain imaging, we attempted to generate a measure of sex differences that is not directly related to volumetric differences. In this likelihood ratio approach, we separately estimated the male and female population distribution of each metric using the Gaussian kernel density estimate with bandwidth selected via Scott's rule.³⁰ (Code replicating this log likelihood computation and all experiments is available at <https://github.com/OwenPhillips/differentiation>.) We then calculated the differentiation measure of each participant for a particular brain feature within the ROI by taking the log likelihood ratio of the two estimated distributions. Thus, the differentiation measure for each brain feature within the ROI is a measure of the odds that the participant's data came from the distribution of male scores versus the distribution of female scores. At this stage, we implemented two rules for our approach. First, features that did not have an adequate level of difference between male and female individuals were dropped. Variables with nearly identical distributions in the male and female populations provide little basis for differentiation. To smoothly interpolate between including all variables and restricting our differentiation measure to only the most sensitive variables, we introduce a tunable cutoff value and include only those variables for which the male and female distributions differ by more than that cutoff value. We adopt the Hellinger Distance, a standard measure of distributional distance,³¹ as our dissimilarity measure so that non-differentiating features would be excluded. After eliminating all variables with a Hellinger Distance below 0.12, a total of 502 variables remained. This was done because when the two probability density functions were essentially overlapping, the differentiation measure for these individual features would essentially be zero. The divergence cutoff value is tunable where a lower cutoff would allow more variables to be included and a higher cutoff would further remove more variables. The Hellinger Distance of 0.12 was chosen by inspection of remaining overlap as a compromise between allowing the inclusion of all features and restricting the differentiation measure to the most sensitive features. The benefit of this approach is that it allows for the initial inclusion of all available data while automatically removing noncontributing features.³¹

Second, in order to remove bias from outliers in estimated distributions, we winsorized the differentiation measures to within 2 standard deviations. A value of 2 standard deviations was chosen because 95% of values were within this range, and thus we would minimize the contribution from outliers. Brain measures within each participant were then averaged across all their ROIs to create a single final mean sexual differentiation measure for each participant. This process was also done with the divergence value of each brain measure within each ROI to create a mean effect size for each ROI. The divergence value measures the degree of difference between the two distribution functions. These divergence values were then rank ordered and used to generate a whole-brain visualization of the differentiation measure on the brain (Figure 1). No statistical tests were performed to generate this representative image; rather, this represents an ordered ranking of how much the different brain regions vary between the sexes according to the “differentiation measure.” (The underlying code for the “differentiation score” calculation is available here at <https://github.com/OwenPhillips/differentiation>.)

Statistical Analysis

We first computed partial correlations, with age as a covariate, between the factor scores and sex to establish which factors were sex biased. Next, we computed partial correlations within sex, with age as a covariate, between the differentiation measure (a single measure of brain sex differentiation) and the factor scores that were sex biased (ie internalizing and externalizing factors).

Genetic Data Processing

All samples included in this study were genotyped on one of four Illumina arrays: the HumanHap550v1.1, HumanHap550v3.0, Human610_Quad1_B, and HumanOmniExpress-12v1.0. Genetic data processing steps were applied on the full sample with genotyping data ($N = 8,741$) (see Supplement 2: Genetic Processing, available online) for details. To examine biological consequences of the detected variants, we analyzed expression values from GTEx project,³² Encode roadmap methylation data from the Haploreg project,³³ and single cell expression data from adult and fetal brain using R and previously defined brain cell populations.³⁴

Genome-wide Association Study

Associations of single nucleotide polymorphisms (SNPs) and the differentiation measure was conducted using linear regression. Calculations were carried out with PLINK (–linear standard-beta –assoc qt-means) within male individuals only, within female individuals only, and as a

supplement within all subjects (Figures S3 and S4, available online). Participants of African descent and European descent were analyzed separately and then combined via PLINK's meta. Age and PCA principal components 1 to 10 (in order to control for variability in ethnicity) were included as covariates within male individuals and within female individuals separately. The genome-wide significance level was set at 5×10^{-8} . Manhattan and quantile–quantile (QQ) plots were generated with the R package qqman (<https://CRAN.R-project.org/package=qqman>).

To examine how much genetic variation explains, we estimated SNP-based observed heritability and correlation with psychiatric disorders using previously published GWAS available on LDhub, which provides an atlas of genetic correlations across complex human traits.³⁵ SNP-based observed heritability was calculated with LD score regression.³⁵

RESULTS

The number of participants with both structural and diffusion data was 883. Of these, 865 (aged 8–21 years, mean 14.31, $SD = 3.39$; 407 male individuals, aged 8–21 years, mean 14.54, $SD = 3.44$; and 459 female individuals, aged 8–21 years, mean 14.06, $SD = 3.32$) passed quality control. Figure 2 shows a histogram of the brain sex-differentiation measure.

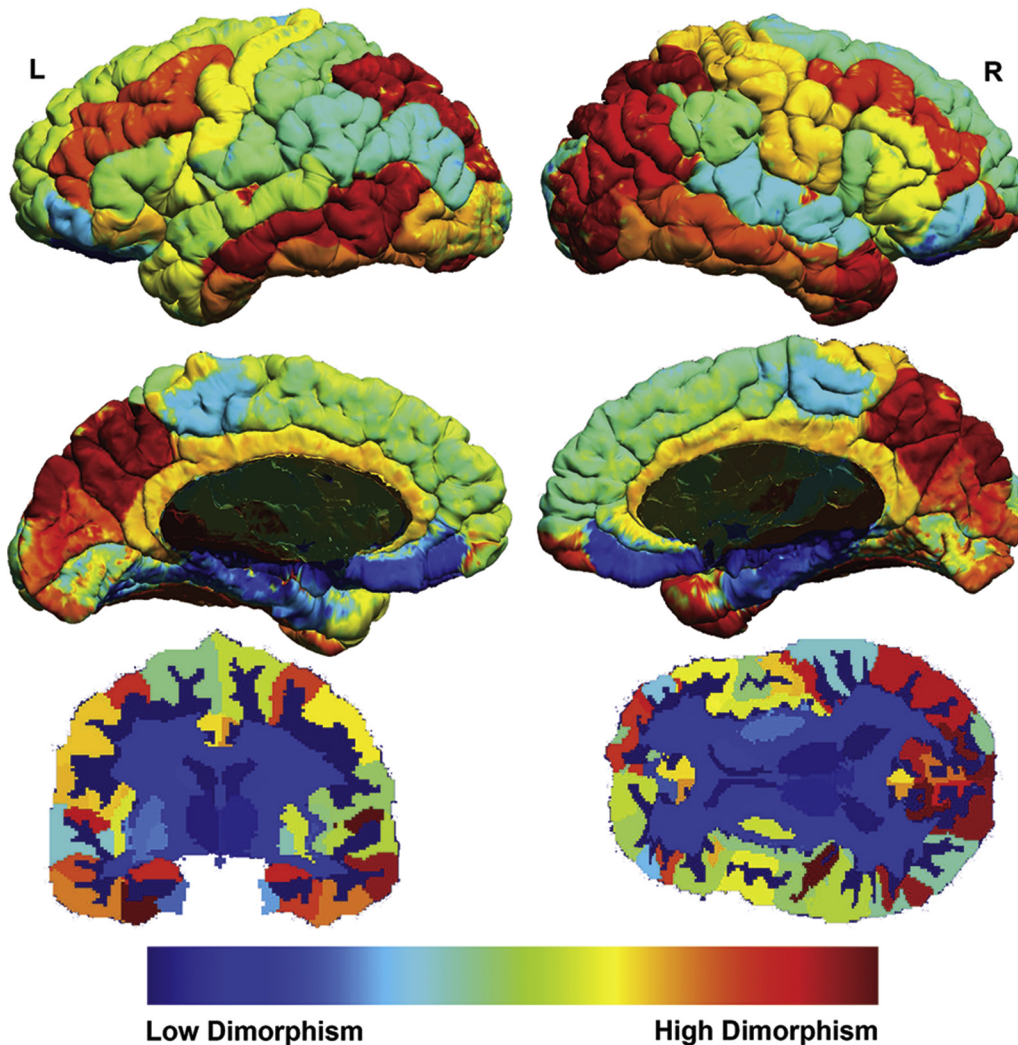
Factor Analysis

Additional details for the Factor Analysis are provided in the Supplementary Material, available online (see Table S1, Figures S1 and S2, available online). In brief, the factor analysis yielded the following results: thought disorder factors: factor 1: mean = 4.90, $SD = 5.90$; factor 2: mean = 3.18, $SD = 4.71$; internalizing factors (depression, anxiety): factor 3: mean = 5.42, $SD = 5.66$, factor 5: mean = 5.49, $SD = 5.1$; externalizing factor (disruptive behavior): factor 4: mean = 5.68, $SD = 5.00$.

Correlations

Relation Between Factor Scores and Sex. Partial correlations between sex and the externalizing factor for disruptive behavioral symptoms ($r = 0.125$, $p = .001$) and between sex and the internalizing factors for anxiety/depression symptoms ($r = -0.154$, $p = .001$) and for fear symptoms ($r = -0.217$, $p = .001$) were all significant. Partial correlations between sex and the thought disorder factors of psychosis-related symptoms ($r = 0.018$, $p = .60$) and mania-related symptoms ($r = -0.007$, $p = .827$) were not significant.

Relation Between the Differentiation Measure and Externalizing Symptoms. Partial correlations within male individuals were significant between the differentiation measure and the externalizing factor for disruptive behavioral symptoms ($r = 0.119$, $p = .016$). Partial correlations

FIGURE 1 Mapping the Brain's Sex Differentiation by Region

Note: Maps show the order ranked divergence value of mean sex differentiation within each brain region. Cool colors indicate low differentiation (the spread of the underlying variable for the two populations is low; that is, male and female brains tend to be similar in these areas). Hot colors indicate high differentiation (the spread of the underlying variable for the two population is high; that is, male and female brains tend to be different in these areas). Please note color figures are available online.

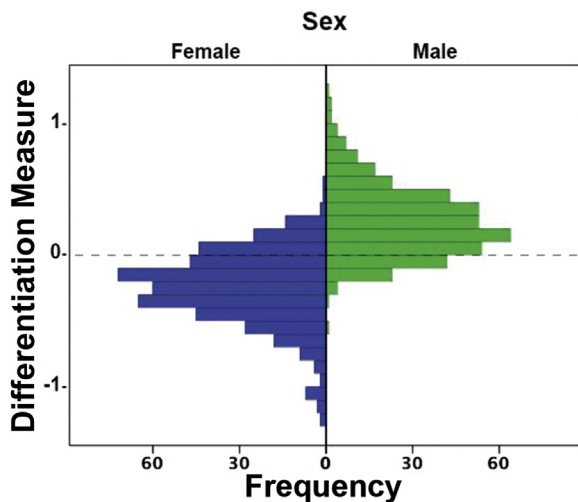
within female individuals were not significant ($r = 0.009$, $p = .854$).

Relation Between the Differentiation Measure and Internalizing Symptoms. Partial correlations within male individuals between the differentiation measure and the internalizing factors for anxiety/depression symptoms ($r = -0.026$, $p = .597$) and fear symptoms ($r = 0.049$, $p = .321$) were not significant, nor were they within female individuals for anxiety/depression symptoms ($r = 0.043$, $p = .354$) or fear symptoms ($r = -0.030$, $p = .516$).

Genome-wide Association Analysis. Of the 407 male and 459 female subjects who had MRI data that passed QC,

336 male and 396 female subjects also had GWA data from autosomal and the X chromosome (Figure 3), consisting of 3,543,016 imputed SNPs that passed our stringent QC.

Association Between the Differentiation Measure and Genome-wide SNPs Within Male Individuals. Two SNPs reached genome-wide significance (Figures 3 and 4, and see Figure S5, available online). First, SNP rs111161632 ($p = 4.032E-8$), from chromosome 5 located on RasGEF Domain Family Member 1C (RASGEF1C). RASGEF1C, is expressed in the Brain (Cerebellum (x9.8) and Cerebellar Hemisphere (x9.3) (GTExPortal <https://gtexportal.org>). RASGEF1C has been shown in both male and female hypothalamic neural-progenitor/stem cells³⁶ to be

FIGURE 2 Histogram of Likelihood Ratio Sex Differentiation Measure

Note: On the left of the histogram, the distribution of the differentiation measure (degree of sex-differentiating features) is shown for female individuals. Female individuals with a very negative differentiation measure have a high level of female-skewed brain features; female individuals with scores closer to zero have a greater mix of male- and female-biased features; and female individuals with very positive scores have a high level of male-biased features. On the right is the distribution of the differentiation measure for male individuals. Male individuals with a very positive differentiation measure have a high level of male-skewed brain features; male individuals with scores closer to zero have a greater mix of male- and female-biased features; and male individuals with very positive scores have a high level of male-biased features. Please note color figures are available online.

glucocorticoid regulated (see Figures S6 and S7, available online).

Second, SNP rs75918199 ($p = 4.82\text{E-}8$) from chromosome 19 located on the Gem Nuclear Organelle Associated Protein 7 (GEMIN7). GEMIN7 is a component of the core survival motor neuron protein (SMN) complex, which is required for pre-mRNA splicing in the nucleus and involved in neuron-specific functions, such as neurite outgrowth and axonal transport.³⁷ GEMIN7 is expressed in all tissues including the pituitary and neurons.^{38,39} (see Figures S8 and S9, available online). Furthermore, this SNP is located on the H3K4me1 binding site in neuronal tissues based on the Encode 15 state model (http://archive.broadinstitute.org/mammals/haploreg/detail_v4.1.php?query=&id=rs75918199).³³ The relation between SNPs in RAS-GEF1C and GEMIN7 and the externalizing factor score was not significant.

Association Between the Differentiation Measure and Genome-wide SNPs Within Female Individuals. Two SNPs reached GWA significance (Figures 3 and 4, and Figure S10, available online). First, SNP rs78372132 ($p = 1.64\text{E-}8$) (chromosome 2) is located on intron of Par-3 Family Cell Polarity Regulator Beta (PARD3B). The expression by

UniProt/SwissProt (<http://www.uniprot.org/>) shows intermediate levels in the brain, and the Allen brain Atlas shows widespread expression throughout the gray matter of the brain (see Figures S11 and S12, available online).^{40,41} Furthermore, this SNP is located on the H3K4me1 binding site in neuronal tissues based on the Encode 15 state model (http://archive.broadinstitute.org/mammals/haploreg/detail_v4.1.php?query=&id=rs78372132).

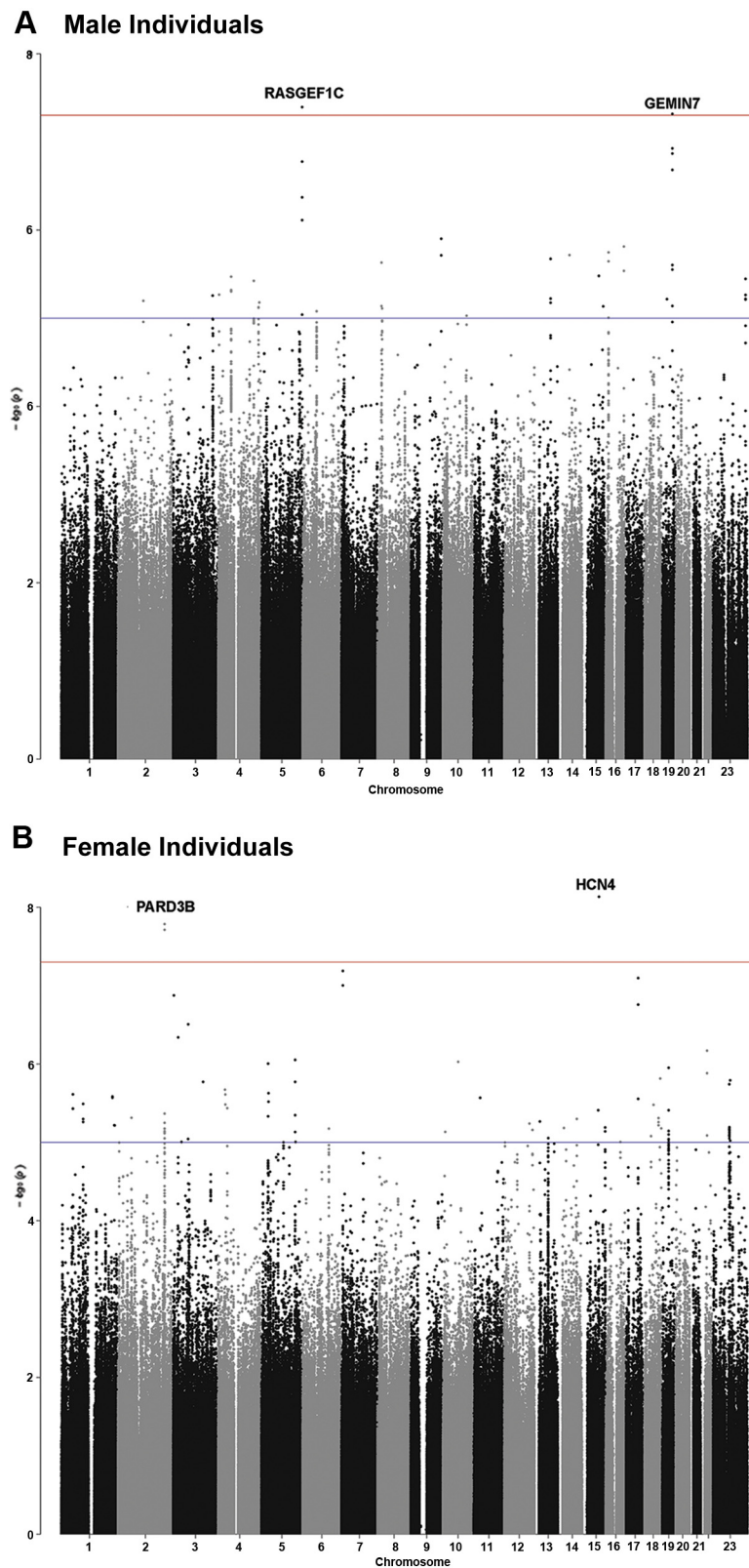
Second, SNP rs73442006 ($p = 7.34\text{E-}9$) from chromosome 15 is located on the Hyperpolarization Activated Cyclic Nucleotide Gated Potassium Channel 4 (HCN4). HCN4 encodes a member of the hyperpolarization-activated cyclic nucleotide-gated potassium channels, and HCN4 subunits may also play a physiological role in the developing hippocampus and may help control the rhythmic activation of pacemaker neurons during brain development.^{42,43} The protein differential expression in normal tissues indicates expression in the frontal cortex, and there is evidence for expression in subcortical regions of the brain (see Figures S13 and S14, available online).⁴⁴ Furthermore, this SNP is located on the H3K4me1 binding site in neuronal tissues based on the Encode 15 state model (http://archive.broadinstitute.org/mammals/haploreg/detail_v4.1.php?query=&id=rs73442006).

Observed Heritability

Although common variants did not explain heritability in brain sex differentiation in the total sample, there was a significant proportion of heritability in male individuals, specifically $h^2_{\text{snp}} = 0.015$ ($\text{SE} = 0.011$). We then examined whether genome-wide variants in brain sex differentiation were shared with psychiatric disorders using previously published GWA study data available on LDhub. In LDhub, a significant genetic correlation in male individuals was obtained between our differentiation score and schizophrenia ($p = .047$, $r = -0.28$, $\text{SE} = 0.14$). The lack of heritability estimate in the female individuals or our total PNC sample is likely reflected by lack of power due to relatively small N.

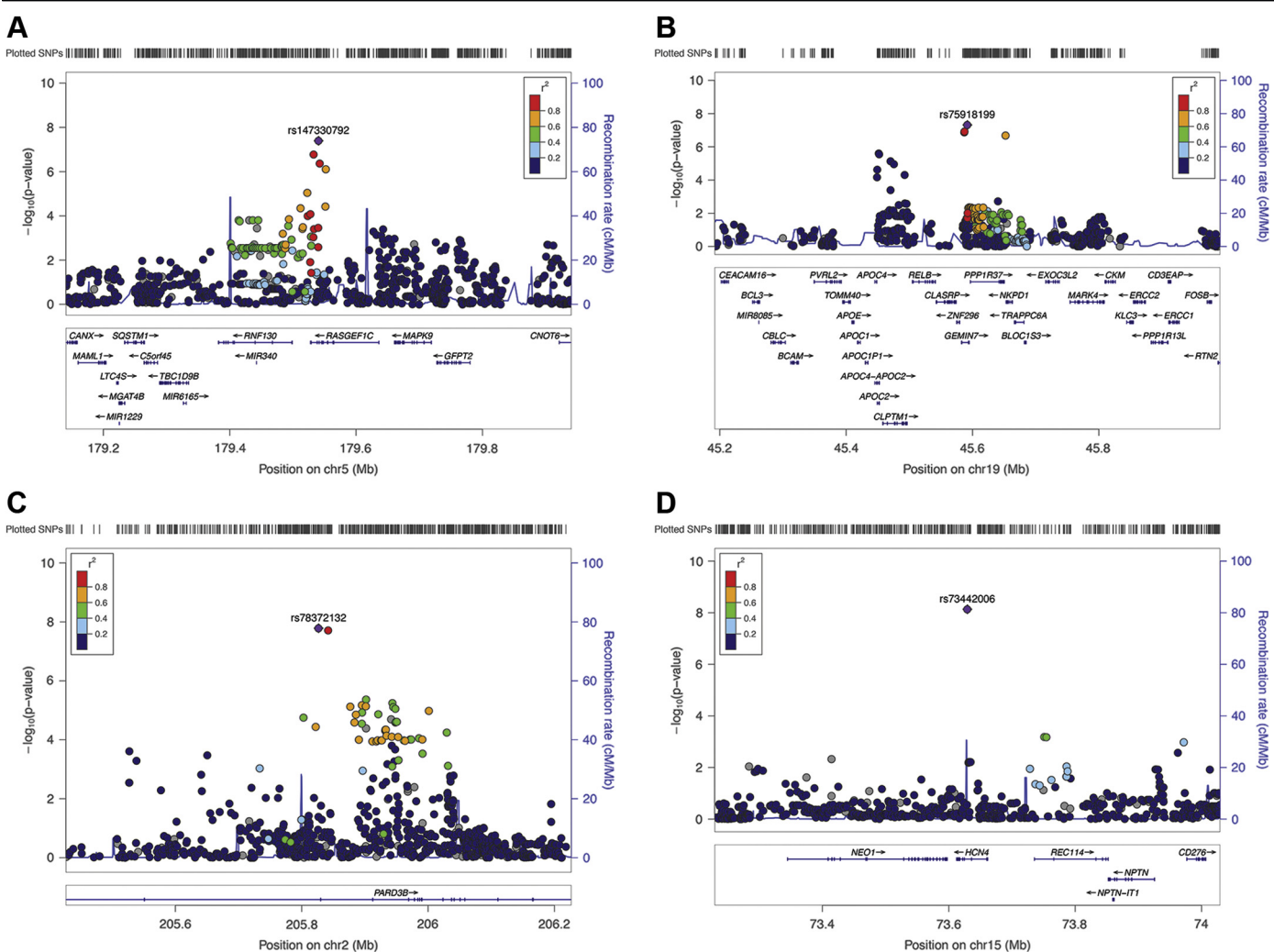
DISCUSSION

We developed a continuous measure to quantify the level of heterogeneity due to sex differences in the brain, and then applied this measure to explore how brain sex differences were related to differences in psychopathology and the genome in youth. Two main findings emerged from this investigation: (1) within male individuals, the brain sex differentiation measure was associated with an externalizing “disruptive behavior” factor; and (2) within both male and female individuals, variants in different genes were associated with the differentiation measure.

FIGURE 3 Genome-wide Association for the Sex Differentiation Measure

Note: (A) Manhattan plot of the meta-analyses for male individuals and the sex differentiation measure. (B) Manhattan plot of the meta-analyses for female individuals and the differentiation measure. Horizontal line indicates threshold for genome-wide significance ($p < 5 \times 10^{-8}$). Please note color figures are available online.

FIGURE 4 LocusZoom of Regional Hits for the Sex Differentiation Measure



Note: Regional plots of the top hit in the association results based on the meta-analysis. Blue lines indicate the recombination rate for the Han Chinese and Japanese populations in the 1000 Genome Project. The Y axis is $-\log_{10}(\text{p values})$ of the single-nucleotide polymorphisms (SNPs), and the X axis is chromosomal position (hg19). The linkage disequilibrium (r^2) between the top and the remaining SNPs is indicated by color. (a) RASGEF1C gene cluster; (b) GEMIN7; (c) PARD3B; (d) HCN4. Please note color figures are available online.

Our results partially support our hypothesis that the degree of sex-differentiated brain features is related to psychopathology, but sex biologically defined may be a stronger determinant of psychopathology. Numerous studies have found significant differences in brain structure between the sexes^{16,45–47}; however, recently Joel *et al.* analyzed four large imaging datasets and found that most brains are composed of unique mosaics of characteristics, with some characteristics more common in female individuals and others more common in male individuals.² Although the methodological approach taken is not without criticism,⁴⁸ other research supports the notion that sex as a simple categorical variable can be problematic in studies of the function and structure of the human brain.^{49–51} Elucidating the biological sex of the

brain is increasingly relevant to the field of psychiatry, in which specific recommendations have been proposed to incorporate sex as a variable in psychiatric research.⁵² In this study, we developed a single quantitative differentiation measure that seeks to capture the degree of sex bias of an individual brain. We correlated this measure with factor scores of psychopathology (internalizing factors: depression, anxiety, and fear; and an externalizing factor: disruptive behaviors) that are known to be affected by sex. We found that within male individuals, a differentiation measure indicative of a higher degree of male skewed features was significantly correlated with externalizing “disruptive behavior” symptoms. A recent study focused on autism that used a multivariate probabilistic classification to

compute the biological sex from cortical thickness suggested that male neuroanatomical characteristics carry a higher risk for autism.⁵³ Another study using the same dataset as the present work used a probability classification approach, and also found a link between classification for male or female brain sex and neurocognitive function across a number of behavioral domains.¹⁵ Although the methodological approaches taken and the behavior under investigation by our own and these recent studies vary, they are similar in that they all suggest that there is a link between the degree of sex-differentiating features in the brain and behavior. Our study adds novel findings that relate brain measures specifically to psychopathology in male individuals but not in female individuals, in whom the differentiation measure was not associated with increased internalizing symptoms. Thus, although a female brain is linked to internalizing symptoms, a more “female-type” brain does not appear to be associated with internalizing psychopathology. However, it is possible that there are sex-specific pathways leading to a similar psychopathology phenotype in male individuals and female individuals. Such pathways have been described previously in the context of psychiatric disorders.^{54,55} These pathways may in turn interact with the sexual differentiation of the brain. Future work will be needed to investigate this possibility.

We also found that the differentiation measure was associated with different genetic variants in male and female individuals. To our knowledge, this the first study to link the degree of sex-differentiating traits in the brain to the genome. Specifically, in male individuals, two SNPs were genome-wide significant. One of them was rs111161632, located in RAS-GEF1C, and the other was rs75918199, located in GEMIN7. In female individuals, two SNPs were genome-wide significant. One of them was rs78372132, located in PARD3B, and the other was rs73442006, located in HCN4. Overall, the association between the differentiation measure and the genes identified suggest that these genes may play a role in the natural variation of brain sex differentiation; however, these genes have not been previously linked to sex differences, and the findings should be replicated in a larger independent sample. Interestingly, all four significant SNPs were located on genes that are active in the brain, as evidenced both by expression and methylation data. For example, GEMIN7 is expressed in the pituitary, and HCN4 is expressed in the hippocampus; both are brain regions that contain important biochemical pathways that are critical for the expression and regulation of stress and have been shown to have sex-specific differences in stress response.^{56,57} Together, this suggests that variants in the identified genes may influence the degree of sex-differentiating traits in the brain.

Overall, a single in vivo measure of the brain's sex heterogeneity (ie, our differentiation measure) may capture more of the complexity of how different systems interact through the influence of sex compared to the classical binary measure, and we hope that this initial article can be a step toward understanding that complexity. However, given the complexity of the brain and the difficulty in obtaining and incorporating brain data from children and adolescents, a single measure is not completely explanatory and is limited by several methodological and conceptual considerations, which should be addressed in future work.

This research demonstrates that the relative degree of characteristically male or female features can be represented by a continuous brain differentiation measure; however, this single score is limited in determining precisely what drives the differentiation measure to be high or low. For example, an individual with an even mix of highly male and highly female features would have a similarly low differentiation measure as an individual with many features that were not characteristically male or female. Furthermore, with the single score, we cannot determine whether nonfocal brain regions and/or features are driving the effects that we have observed. Through the open source software that we have made freely available (<https://github.com/OwenPhillips/differentiation>), and the publicly accessible PNC data, these questions can be probed in future work.

The brain's plasticity is likely variable across the lifespan, which suggests that the differentiation measure is also variable across the lifespan. This is especially true in the developing brain, where developmental patterns can vary significantly; and beyond the strict effects of age, we did not have pubertal stage information or testosterone data available, which also affects development.⁵⁸ Previous research has also indicated that sex steroid hormones have an impact on sex-specific differentiation of the brain.¹ In future work, it will be important to control for the stage of pubertal development and consider contributions from other sociodemographic factors such as socioeconomic status, education level, and occupation for older youth.

Beyond the developmental impact on the differentiation measure, there are interactions between age/sex and the development of psychopathology⁵⁹ that were likely not captured in this analysis. In our analyses, we accounted for age by its use as a confounding variable; however, future research that seeks to identify how the brain's sex changes with age would be of significant interest. Similarly, in relation to the genome and the differentiation measure, we have provided evidence that the underlying genome is associated with the presentation of degree of sex-differentiated brain features; however, it is likely that societal and environmental influences also have an impact. Future work across a wider spectrum of

development and through the lifespan is needed. It is also important to emphasize that the brain sex differentiation measure is a measure of biological sex classification, not a gender sex classification.

The differentiation measure was characterized by incorporating multiple metrics from both structural and diffusion imaging. It is likely that incorporating more information, such as data from higher-resolution scanners, more precise regions of interest, other imaging modalities (functional MRI/spectroscopy), would increase the accuracy of the measure. Furthermore, within the participants' brain scans, there may be subtle variations in the amount of movement that could influence the assessment of the quantitative measures (eg, participants with more externalizing symptoms may experience more motion in the scanner, which in turn could influence the cortical thickness measurement).⁶⁰ This movement in turn could influence the differentiation measure. Future analyses of the PNC cohort may benefit from more granular assessments and matching of participant characteristics as related to motion within the scanner.

It is important to note that, overall, a conceptual choice was made whereby the brain sex-differentiation score was initially developed from a biological sex male–female classification; however, a different type of classification approach that does not adhere to an initial binary construct of biological sex may be more useful. Furthermore, we made another conceptual decision to calculate a single whole-brain differentiating measure using a multiregional approach; however, an approach focused on specific regions of interest may be more useful in identifying associations between psychopathology and genetics. Moreover, we made a methodological decision to remove “nondifferentiating” information from the calculation of the differentiating measure; however, what is or what is not differentiating may vary across development. In addition, the methodological decisions and the likelihood that the differentiation measure is influenced throughout development make it possible that both significant and nonsignificant associations with the differentiation measure vary across the lifespan. Overall, future studies that explore regional variation in differentiation across the lifespan would be helpful.

We should also emphasize that the relation between an individual's differentiation measure and psychopathology is not preclusive. For example, if an individual has a high differentiation score reflecting a high degree of characteristically male brain features, it does not necessarily mean that this individual will exhibit disruptive behavior. This is reflected in the statistical analysis in which the correlation between the measure of sex-differentiating brain features and externalizing symptoms in male individuals is

significant but weak ($r = 0.119$, $p = .016$). Therefore, this association is likely to be more useful for understanding population-level sex differences in the presentation of psychopathology, but is limited for understanding any particular individual's psychopathology. Furthermore, due to the exploratory nature of our analysis, we did not adjust our analyses for multiple comparisons to generate novel hypotheses in this nascent field.

Finally, although this study contains a large number of participants for an MRI study, it is comparatively small for a GWA study; this is further complicated by the fact that it is an ethnically diverse population. We made an effort to minimize this complication by including only the two largest ethnic groups (ie, those of European and African descent), while still maintaining the value of including a diverse group of participants. However, future studies including more participants would be beneficial in confirming the effects of the genome on the differentiation measure. Ultimately, this study has significant limitations, and before strong conclusions can be made resolving how the brain's sex “differentiation score” relates to psychopathology and the genome, this research should be replicated in a larger dataset with participants across a wider age range. The now in collection UK BioBank (<http://www.ukbiobank.ac.uk/>), or the The Lifespan Human Connectome Project Development (<https://www.humanconnectome.org/study/hcp-lifespan-development>), would be particularly appealing to deal with a number of the limitations mentioned, and to further investigate how the brain's sex is related to psychopathology, age effects, and the underlying genome.

In this article, we describe an initial attempt to move beyond the observation that the brain is a mosaic of male and female traits to quantifying the level of heterogeneity and its relation to sex-differentiated psychopathology. To do so, we developed an automated continuous data-driven calculation of each participant's degree of sex-differentiating brain features, which we called the “differentiation measure.” Although the differentiation measure that we developed has clear limitations, it provides an initial topography of male and female brain features, their associations with psychopathologies that are sex biased, and the underlying genetic influence on the presentation of the brain sex topography. Taken together, our research supports the formulation that the sex of the human brain can be conceptualized along a continuum rather than as binary. An individual's placement on this continuum can have important implications for the presentation of psychopathology. Furthermore, genetic variants can affect an individual's placement on this continuum.

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