ARTICLE

The Link Between Autism and Sex-Related Neuroanatomy, and Associated Cognition and Gene Expression

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Objective: The male preponderance in prevalence of autism is among the most pronounced sex ratios across neurodevelopmental conditions. The authors sought to elucidate the relationship between autism and typical sex-differential neuroanatomy, cognition, and related gene expression.

Methods: Using a novel deep learning framework trained to predict biological sex based on T_1-weighted structural brain images, the authors compared sex prediction model performance across neurotypical and autistic males and females. Multiple large-scale data sets comprising T_1-weighted MRI data were employed at four stages of the analysis pipeline: 1) pretraining, with the UK Biobank sample (>10,000 individuals); 2) transfer learning and validation, with the ABIDE data sets (1,412 individuals, 5–56 years of age); 3) test and discovery, with the EU-AIMS/AIMS-2-TRIALS LEAP data set (681 individuals, 6–30 years of age); and 4) specificity, with the NeuroIMAGE and ADHD200 data sets (887 individuals, 7–26 years of age).

Results: Across both ABIDE and LEAP, features positively predictive of neurotypical males were on average significantly more predictive of autistic males (ABIDE: Cohen's d=0.48; LEAP: Cohen's d=1.34). Features positively predictive of neurotypical females were on average significantly less predictive of autistic females (ABIDE: Cohen's d=1.25; LEAP: Cohen's d=1.29). These differences in sex prediction accuracy in autism were not observed in individuals with ADHD. In autistic females, the male-shifted neurophenotype was further associated with poorer social sensitivity and emotional face processing while also associated with gene expression patterns of midgestational cell types.

Conclusions: The results demonstrate an increased resemblance in both autistic male and female individuals' neuroanatomy with male-characteristic patterns associated with typically sex-differential social cognitive features and related gene expression patterns. The findings hold promise for future research aimed at refining the quest for biological mechanisms underpinning the etiology of autism.

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Autism spectrum conditions (henceforth autism) are a set of neurodevelopmental conditions diagnosed four to five times more often in males than in females (1). This male preponderance in prevalence has been attributed partially to an underrecognition of autistic females (2). Even after taking this into account, studies report a male:female ratio of 3:1 (3), implying involvement of neurobiological mechanisms in the sex-biased prevalence of autism. This raises the critical question of how quantitative biological factors related to typical sexual differentiation are involved in the neurobiology of autism. Identifying such mechanisms related to biological sex will broaden our understanding of the sex-differential etiology of autism.

Studies of biological sex differences in the autistic neural phenotype have gained considerable attention in recent years (4). However, how typical sexual differentiation is associated with the neuroanatomy of autistic males and females remains unclear (5–7). Initial efforts show that autistic females tend to exhibit a brain phenotype resembling that of neurotypical males (6,8–11), while findings are more mixed in autistic males (12). It thus remains to be established how a shift toward "neurotypical maleness"

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differentially informs neurobiological underpinnings in autistic males and females.

Given the neurodevelopmental origins of autism (13), the underlying biological mechanisms are likely to act early ontogenetically. The prenatal period is a critical developmental window in which genetic, hormonal, and neuroimmune organizational factors have long-lasting effects on sexual differentiation and pathways implicated in neurodevelopmental conditions such as autism (14). Different etiological models have been put forward explaining the sex-differential autism likelihood (15–17), for example, the female protective effect (18–20) and increased steroidogenic activity in prenatal neurodevelopment (15, 16, 21, 22). Thus, a key question is how proposed explanations translate into neurobiological cascades from genome to neural and behavioral phenotype. Specifically, we need to better understand how sex-differential neural phenotypes are linked to cognition and genomic mechanisms, and whether they can be traced back to the prenatal developmental period and specific pathways. Recent work shows that studying transcriptional patterns that spatially overlap with brain structural changes provides a mechanistic lens into the physiology of neurodevelopmental conditions ("the transcriptional vulnerability model" [23, 24]). Brain structure is particularly relevant in the developmental biology of neurodevelopmental conditions such as autism (25, 26) and can be considered an intermediate phenotype between genetics and behavior (27). Specifically, autism has been associated with spatially selective alterations in neuroanatomy that have been linked to both autistic behavioral features (28) and genes relevant to these features (29–31).

Motivated by the need to address previous shortcomings, such as small sample sizes (especially of females) and lack of independent replication, tests of methodological robustness (32), and careful accounting for confounding variables (e.g., brain volume [7]), our aim in this study was to comprehensively establish whether the neuroanatomy in male and female autistic people shows, on average, a shift toward (and beyond) the typical male neuroanatomy, and whether this is associated with autism-associated and typically sex-differential cognition and related gene expression. In line with etiological models and evidence suggesting that typical male neurobiology is associated with a higher likelihood of autism (14, 16, 19, 21), we derived three hypotheses, using a T_1-weighted structural brain imaging–based sex prediction classifier pretrained in neurotypical males and females to predict male sex: 1) among autistic individuals, females will be less accurately classified than males; 2) among females, autistic individuals will be less accurately classified than neurotypical individuals; and 3) among males, autistic individuals will be more accurately classified than neurotypical individuals. We further examined whether male-shifted neuroanatomy is specific to autism; neurodevelopmentally different across age; associated with specific brain regions and clinical features; and linked to gene expression associated with autism, prenatal development, or sex differences.

To test our hypotheses, we employed a novel deep learning framework leveraging several large-scale neuroimaging data sets with proportionally large numbers of autistic females: the UK Biobank (33), comprising over 10,000 individuals; the Autism Brain Imaging Data Exchange (ABIDE [34, 35]); and the EU-AIMS/AIMS-2-TRIALS Longitudinal European Autism Project (LEAP [36, 37], the largest European multicenter initiative aimed at identifying biomarkers in autism). Based on T_1-weighted structural brain images, we first established a sex prediction classifier with high accuracy in the UK Biobank, next transferred and validated it in ABIDE, and then tested it in LEAP to assess sex classification accuracies in autistic males and females. To establish the specificity of our results to autism, we then applied the sex prediction model to an independent sample of individuals with attention deficit hyperactivity disorder (ADHD)—another male-biased neurodevelopmental condition.

We next employed a novel framework to translate global prediction accuracies into spatially specific sex predictions at the level of the brain to identify the most implicated brain regions and associated cognitive features. Finally, to understand associated biological processes, we investigated the enrichment of genes highly expressed in regions associated with sex-differential prediction with a range of (prenatal) genes associated with autism or sex. We tested the hypotheses that genes expressed in male-shifted brain regions are enriched for prenatal genes, autism-associated genes including common genetic variants (38), rare genetic variants (39, 40) and transcriptionally dysregulated genes (41–43), and/or genes differentially expressed by sex or gonadal steroids (44).

METHODS

Samples

Pretraining. We used the preprocessed and quality-controlled first release of UK Biobank data (33, 45), comprising 14,503 individuals (7,584 neurotypical females, 6,919 neurotypical males; ages 44–80 years) to pretrain the convolutional neural networks based on individuals' T_1-weighted structural brain images (46) (see the online supplement).

Validation/transfer. We selected a sample from the publicly available ABIDE I and II data sets (34, 35). The selection process (see the online supplement) resulted in a total of 1,412 individuals (115 autistic females, 526 autistic males, 239 neurotypical females, 532 neurotypical males; ages 5–56 years) (see information and Table S1 in the online supplement; numbers were balanced across sex and diagnosis at all stages of model training, transfer, and validation).

Testing. We included participants from the LEAP cohort (36, 37). After selection procedures (see the online supplement), the final sample consisted of 681 individuals (286 autistic males, 109 autistic females, 188 neurotypical males, 98 neurotypical females; ages 6–30 years) (Table 1; see also Figure S1A in the online supplement; numbers were balanced across sex and diagnosis when testing the model). For a list of clinical and cognitive measures included in analyses, see the online supplement.

TABLE 1. LEAP sample characterization[a]

Variable	Autistic Males (N=286)		Autistic Females (N=109)		Neurotypical Males (N=188)		Neurotypical Females (N=98)		Post Hoc
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Age (years)	17.13	5.49	16.92	6.27	17.22	5.82	17.02	5.98	ASC=NT
IQ									
Full-scale IQ	98.85	19.35	97.19	19.85	104.79	17.16	105.14	19.80	ASC<NT
Verbal IQ	97.39	19.24	97.75	19.85	103.74	18.20	105.34	20.94	ASC<NT
Performance IQ	100.39	21.17	96.62	21.06	105.05	19.08	103.82	19.76	ASC<NT
Autism Diagnostic Interview									
Social	17.16	6.62	15.25	6.88					M>F
Communication	13.71	5.78	12.43	5.20					M>F
Restricted, repetitive behavior	4.59	2.67	3.72	2.48					M>F
Autism Diagnostic Observation Schedule									
CSS	5.75	2.80	4.63	2.55					M>F
Social affect CSS	6.36	2.61	5.50	2.55					M>F
Restricted, repetitive behavior CSS	4.97	2.73	4.10	2.58					M>F
	N	%	N	%	N	%	N	%	
ADHD	120	42	39	36	13	7	11	11	

[a] ADHD=attention deficit hyperactivity disorder; CSS=calibrated severity score.

Specificity. To assess specificity of results, we selected an independent sample of individuals with ADHD based on the publicly available ADHD200 (47) and the local NeuroIMAGE (48) samples (324 males with ADHD, 130 females with ADHD, 225 neurotypical males, 208 neurotypical females; ages 7–26 years; see information and Table S2 and Figure S1B in the online supplement).

Sex Predictions

The detailed image parameters and steps describing image preprocessing, model training, and testing are outlined in the supplemental information and Table S3 in the online supplement. To summarize, we first pretrained the simple fully convolutional neural network (SFCN) model in UK Biobank for sex classification (i.e., yielding a probability of male sex) using the brain-extracted, bias-corrected, and linearly registered (12 degrees of freedom) T_1-weighted images as input (Figure S2 in the online supplement). Next, we applied the pretrained models to ABIDE data via transfer learning (49, 50). For this, we randomly resampled the ABIDE cohort 100 times for training and validation using balanced numbers of individuals in each diagnostic (autism, neurotypical) and sex (male, female) group across all sites (see Figure S1C and S1E and Table S4 in the online supplement). We then applied the 100 fine-tuned models to every individual in LEAP, again using sex- and diagnostic-group-balanced numbers. To summarize prediction results, we computed 1) individual-level ensemble prediction probabilities for every individual based on the median value of the 100 prediction probabilities, which provides an ensemble measure of predictive confidence, and 2) group-level ensemble prediction proportions (i.e., the true positive rate [correctly classified autistic males] and the true negative rate [correctly classified autistic females], which provides an ensemble measure of predictive accuracy, hereafter referred to as "sex prediction accuracy") for each sex/diagnostic group (autistic male, autistic female, neurotypical male, neurotypical female). For this, all 100 individual-level prediction probabilities were compared against a sex classification threshold, which was defined by the median prediction probability across neurotypical males and females (since we were interested in sex-specific accuracy differences in autism that differed from the neurotypical baseline reference). This implies that the accuracy of the group-level ensemble sex prediction of female and male individuals was the same within the neurotypical group (i.e., the true positive and true negative rates were the same). Finally, group differences in these group-level sex prediction accuracies were computed with one-sample t tests and associated Cohen's d.

To identify potential age effects, we further checked the sex prediction accuracies as a function of age. For this, we generated four sliding age bins, each spanning 10 years (5–15 years, N=144; 10–20 years, N=204; 15–25 years, N=192; and 20–30 years, N=120), and computed the median sex prediction accuracy for each bin and sex/diagnostic group. To test functional relevance of sex prediction accuracies, we investigated the associations with autism-related, clinical, and sex-differential cognitive features (see the online supplement). The same procedure was also applied to the ADHD sample (see Figure S1D and S1F and Table S4 in the online supplement) and to autistic individuals with and without co-occurring ADHD. Finally, we ran several sensitivity analyses to control for model choice, total brain volume, and age (see the online supplement).

Interpreting Sex Predictions at the Brain Level

Usually, deep learning models can be considered black boxes that do not provide any information on which brain features

are most important for classification. Thus, to identify the most predictive and biologically meaningful features that drive our prediction at the brain level, we employed a novel model interrogation approach—region-aligned prediction (RAP) (51)—in the LEAP sample. This method generates spatially resolved estimates of sex prediction accuracy and predicts labels at the brain region level (see the online supplement). Specifically, it aligns the intermediate feature maps (layer "mp4," corresponding to the 4th layer of the SFCN; see Figure S2 in the online supplement) across all individuals in the data set (including both the training and validation sets) and extracts the feature matrix at one spatial location at a time for each individual. These feature encodings are then used in an L2-regularized multiple regression to predict sex at every spatial location for every individual. Given that the original convolutional neural network was trained 100 times, this resulted in spatially specific sex prediction maps where every spatial location received 100 predictions (one per model) for each individual. We used the median value as the ensemble prediction for each spatial location, resulting in one RAP imaging map per person. Here, each spatial location in each person's RAP imaging map consists of probabilistic values between 0 and 1 (where 0 means "least likely male" and 1 means "most likely male"). Next, we used these individual RAP imaging maps to compute group-level RAP imaging t-maps (autistic males vs. neurotypical males; autistic females vs. neurotypical females; neurotypical males vs. neurotypical females) to discover the spatial group differences related to the sex predictions. Here, positive t statistics imply a higher male probability in both autistic males and autistic females. Finally, we also applied a quartile split on the prediction probabilities in autistic males and autistic females to also compare highly misclassified and highly correctly classified individuals on their spatial sex predictions.

Cognitive Decoding

We investigated two specific RAP imaging t-maps (autistic females vs. neurotypical females and autistic males vs. neurotypical males) focusing on regions where autistic males and autistic females showed higher male regional predictive probabilities (i.e., positive t values). For this, we used the Neurosynth Image Decoder to visualize the top 100 terms most strongly associated with the two RAP imaging t-maps. To identify regions with the highest male regional predictive probabilities, the RAP imaging t-maps were cluster corrected (voxel-level $Z=2.3$, $p=0.05$). Significant regions were then used as masks to extract the average RAP values for each autistic male or female. These region-specific RAP prediction values were then submitted to general linear models to test the association with different cognitive measures. These were picked post hoc based on the cognitive domains implicated by the cognitive decoding analyses (see the online supplement). Results were false discovery rate (FDR) corrected.

Gene Expression Decoding and Enrichment Analyses

To establish genomic associations, we next isolated genes with expression patterns highly similar to the topology present in the RAP imaging t-maps (autistic females vs. neurotypical females and autistic males vs. neurotypical males, with neurotypical females vs. neurotypical males as reference) using the gene expression decoding functionality integrated in Neurosynth (52) and NeuroVault (53). This approach has been used in several autism-related studies (29, 30, 44, 54–56). It uses the six donor brains from the Allen Human Brain gene expression atlas (57) and statistically tests the similarity between spatial gene expression patterns of all 20,787 protein decoding genes and our input maps. For this, an approximate random-effects analysis calculates the slope of best linear fit for each donor. Next, a one-sample t test is performed on the beta estimates to test how consistent the relations between the gene expression and imaging map values are. This way, we identified genes whose spatial expression patterns were highly similar to the evaluated maps, and this consistently across the six donor brains. In the resulting list of genes, only those with expression enriched in brain regions predictive of male phenotypes were retained, that is, genes with positive t statistics resulting from the similarity analysis between positive RAP imaging t-maps (i.e., higher male probability) and high gene expression in these areas (i.e., FDR $p<0.05$; see the online supplement).

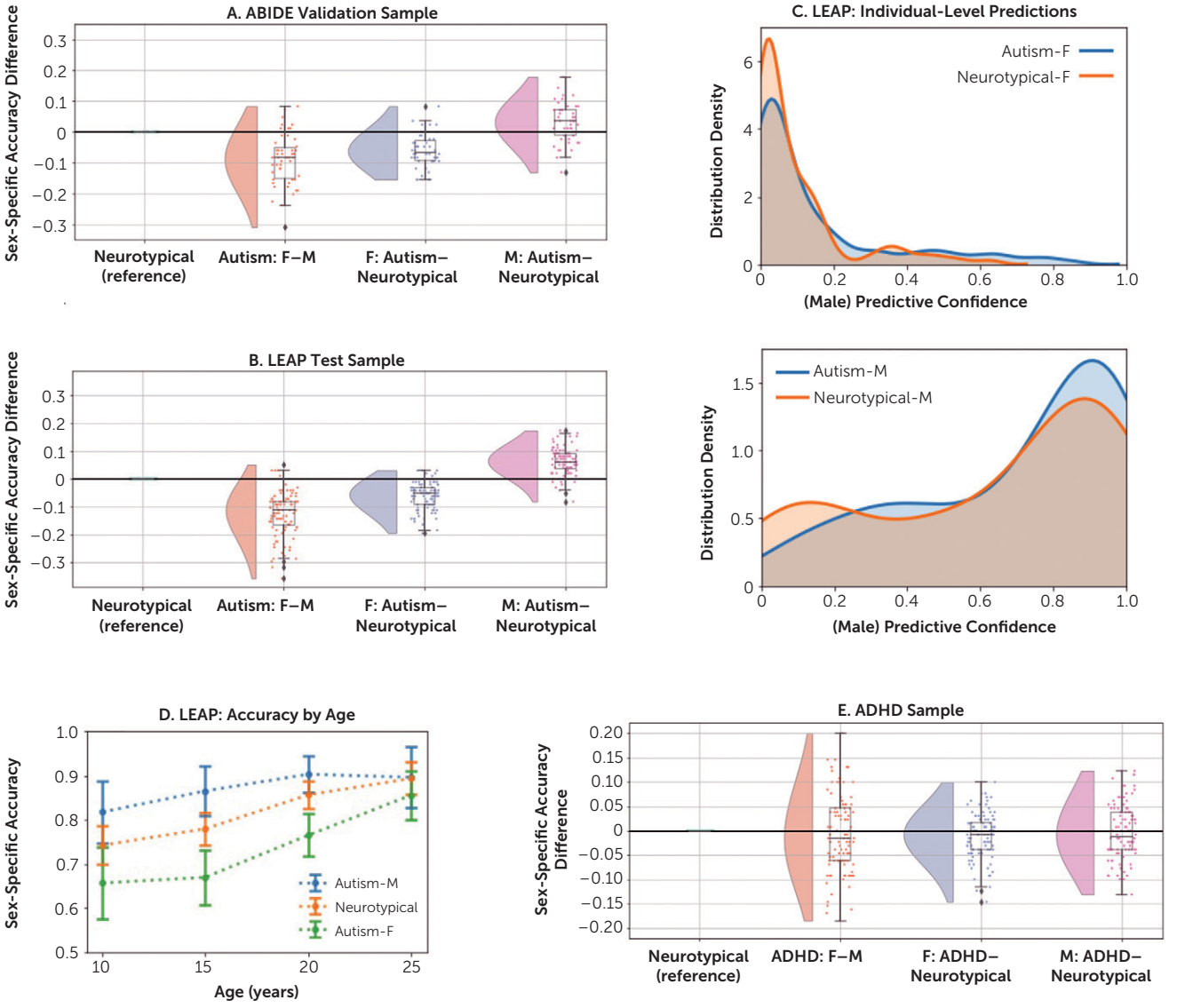
Next, we tested the overlap (i.e., enrichment) of our gene lists with different classes of genes that act prenatally and/or are relevant in the context of autism and sexual differentiation. These included 1) autism-associated genes, including common genetic variants (38), rare genetic variants (39, 40), and transcriptionally dysregulated genes (41–43); 2) genes from prenatal cell types (58); and 3) sex-differentially regulated genes such as genes with sex-differential expression from prenatal brain samples (59, 60) and sex-differentially regulated genes by dihydrotestosterone (DHT) (44, 54, 61) from embryonic neural stem cells, and estrogen (62) (see the online supplement). Analyses examining the enrichment between two lists of genes were implemented using hypergeometric tests based on the *sum(dhyper)* function in R (44) (see custom code at https://github.com/mvlombardo/utils/blob/master/genelistOverlap.R). The background set size for enrichment analyses was set to the number of protein decoding genes considered in the Neurosynth Gene Expression Decoding analyses (i.e., 20,787). To avoid biasing our findings toward genes expressed in brain (29, 30), we conducted another enrichment analysis using a more conservative list of 16,906 background genes based on real estimates of genes expressed in cortical tissue (63). Enrichment analyses yielded enrichment odds ratios, hypergeometric p values, and FDR q values. Only genes surviving FDR $p<0.05$ were considered.

RESULTS

Sex Prediction Accuracies in Autism

In ABIDE, at the group level, the proportion of correctly classified autistic females (72%) was on average 9% lower than that of autistic males (82%) (hypothesis 1: Cohen's

FIGURE 1. Comparison of sex prediction model performance across diagnostic and sex groups[a]



[a] We compared sex prediction accuracy differences between autistic males and females, autistic females and neurotypical females, and autistic males and neurotypical males in both the ABIDE validation sample (panel A) and the LEAP test sample (panel B). Each dot represents one model out of 100 models in total. Negative values mean that the model performs worse in the first sex/diagnostic group. Panel C shows the distribution of individual-level sex prediction accuracies (i.e., the predictive confidence of being classified as male) across autistic females and neurotypical females in the upper panel and across autistic males and neurotypical males in the lower panel in the LEAP sample. In panel D, we generated four sliding age bins, each spanning 10 years (5–15 years, N=144; 10–20 years, N=204; 15–25 years, N=192; 20–30 years, N=120), and compared the sex prediction accuracies as a function of increasing age. Panel E shows a comparison of sex prediction accuracy differences between males and females with attention deficit hyperactivity disorder (ADHD), females with ADHD and neurotypical females, and males with ADHD and neurotypical males in the combined ADHD200 and NeuroIMAGE sample. F=female; M=male.

d=1.19, p=1.1e−20) and 7% lower than that of neurotypical individuals (79%) (hypothesis 2: d=1.25, p=3.9e−22). The proportion of correctly classified autistic males was 3% higher than that of neurotypical individuals (hypothesis 3: d=0.48, p=1.0e−5) (Figure 1A). These results replicated when testing the 100 ABIDE fine-tuned models in LEAP (Table 2). Specifically, for the same group comparisons, we found that at the group level the proportion of correctly classified autistic females (74%) was on average 12% lower than that of autistic males (86%) (hypothesis 1: d=1.59, p=5.5e−29) and 6% lower than that of neurotypical individuals (80%) (hypothesis 2: d=1.29, p=1.2e−22). The proportion of correctly classified autistic males was 6% higher than that of neurotypical individuals (hypothesis 3: d=1.34, p=1.1e−23) (Figure 1B). These results were also reflected in differences in individual-level predictive confidence in LEAP (Figure 1C): after ranking the individual-level ensemble sex prediction probabilities, we compared the top 10% values using a Wilcoxon rank sum test. Autistic females showed lower predictive confidence (75%) than neurotypical individuals (84%) (W=2.69, p=7.1e−03), while autistic males showed higher predictive confidence (89%) than

neurotypical individuals (84%; W=2.08, p=3.8e−02). Effect sizes of sex differences in sex prediction accuracies between autistic and neurotypical individuals were largest in younger individuals, and sex prediction accuracies improved toward adulthood across all groups (Figure 1D). Finally, autistic females showed an association between poorer performance on a mentalizing test with typical sex-differential performance (the "Reading the Mind in the Eyes Test" [64]) and higher individual-level male predictive confidence (t=−2.36, p=0.01, q=0.04; see information and Figure S3A in the online supplement).

In the ADHD sample, sex prediction accuracies did not differ across the groups (Figure 1E, Table 2; see also information and Figure S4 and Table S5 in the online supplement). Also, in LEAP, the patterns remained the same when autistic individuals were stratified according to co-occurring ADHD (see Figure S5 and Table S6 in the online supplement). All sets of control analyses confirmed these observed patterns (see Figure S6 and Table S7 in the online supplement).

TABLE 2. Sex-specific prediction accuracies across the autism and ADHD samples[a]

Cohort and Group	Sex-Specific Accuracy	
	Mean	SD
ABIDE		
Neurotypical	0.789	0.030
Autistic females	0.724	0.046
Autistic males	0.818	0.050
LEAP		
Neurotypical	0.800	0.024
Autistic females	0.737	0.053
Autistic males	0.863	0.041
ADHD		
Neurotypical	0.733	0.046
ADHD females	0.722	0.069
ADHD males	0.730	0.064

[a] ADHD=attention deficit hyperactivity disorder.

Region-Specific Sex Predictions

The most differentiating regions between neurotypical males and neurotypical females were in posterior auditory cortex and frontal-temporal/-occipital white matter pathways (Table S8 in the online supplement and Figure 2A). Similarly, the regions with the highest shifts toward maleness in autistic females were also in posterior auditory cortex regions, cerebellum, and occipital fusiform gyrus (gray matter) and corpus callosum and frontal-temporal/-occipital white matter pathways (Figure 2B; see also Table S9 in the online supplement). In autistic males, regions most similar to neurotypical males were mostly in subcortical regions (amygdala, pallidum, thalamus), cerebellum, frontal medial cortex, and supplementary motor cortex (gray matter) and in superior fronto-occipital fasciculus (white matter) (Figure 2C; see also Table S10 in the online supplement). The neurotypical males–versus–neurotypical females RAP imaging t-map was correlated with the autistic females–versus–neurotypical females RAP imaging t-maps at r=0.38, whereas it was correlated with the autistic males–versus–neurotypical males RAP imaging t-maps at r=0.19, further highlighting the similarity between autistic females and neurotypical males. For differences across highly misclassified and correctly classified autistic females and autistic males, see the information and Figure S7 in the online supplement.

Cognitive Decoding

The most common cognitive terms associated with male-shifted regions in autistic females were primarily related to face perception, visual processing, and speech (Figure 2D), whereas in autistic males, they were primarily related to motor and reward processing (Figure 2E). Based on these results, we tested the association between the region-of-interest-specific RAP-based sex prediction values and cognitive measures in LEAP associated with 1) face processing and communication in autistic females and 2) motor and reward processing in autistic males (see the online supplement for specific measures). While we found no relationships in autistic males, in autistic females there was a significant association between predicted maleness and lower accuracy on the Karolinska Directed Emotional Faces task (t=−2.6, p=0.01, q=0.02) (see Figure S3B in the online supplement).

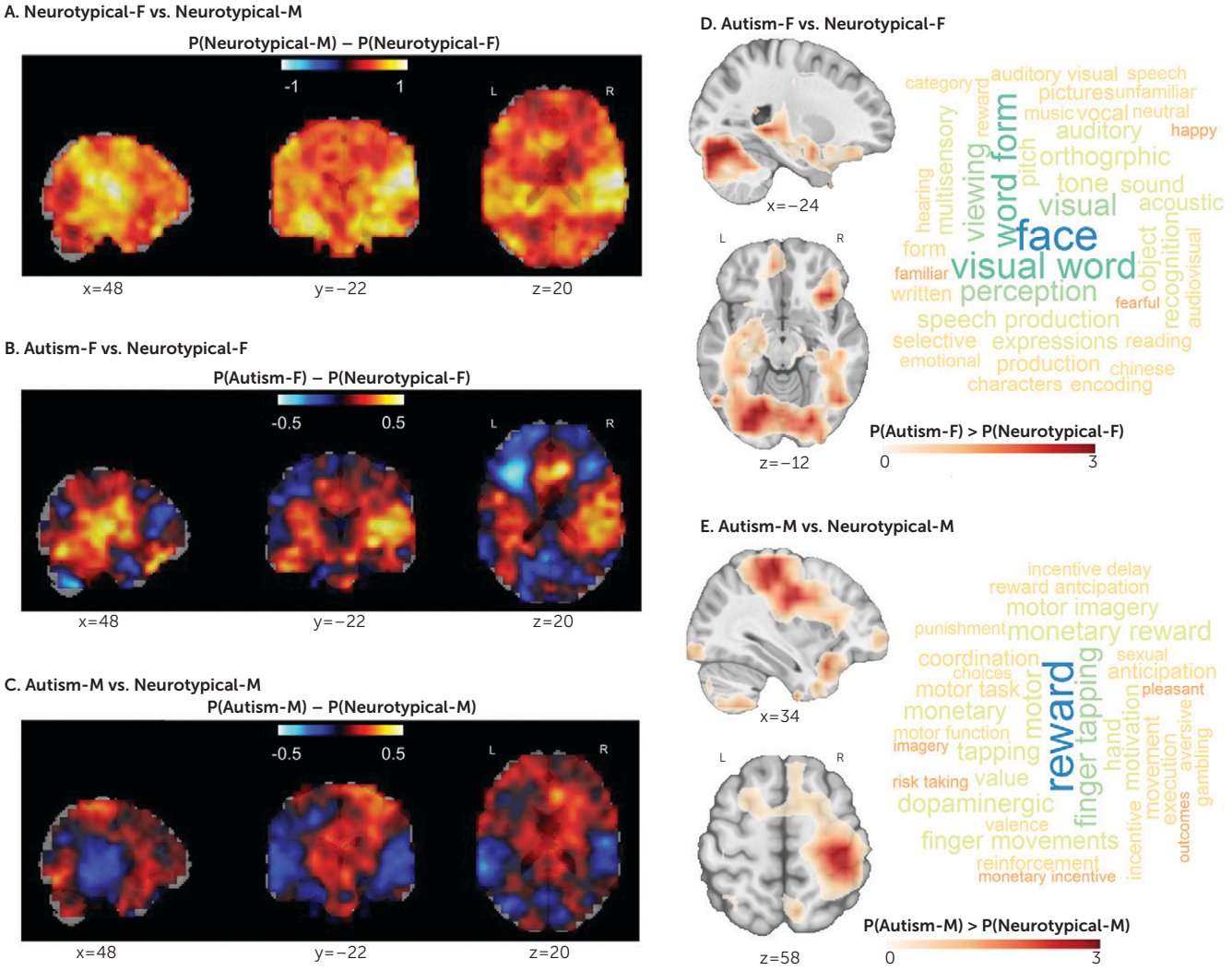
Gene Expression Decoding and Enrichment Analysis

Across both autistic females and autistic males, genes that correlated with male-shifted regions showed significant enrichment for a set of transcriptionally upregulated genes associated with autism (Figure 3B, leftmost and middle panels). In contrast, genes associated with sex-differential regions between neurotypical males and neurotypical females showed significant enrichment for transcriptionally downregulated autism-associated genes (Figure 3B, rightmost panel). Genes correlated with male-shifted regions in autistic females were significantly enriched for prenatal excitatory neuronal cell types (58) (Figure 3A, middle panel) and with autosomal female differentially expressed genes, X-chromosomal male differentially expressed genes, and genes downregulated by estrogen (Figure 3C, middle panel). Strikingly, transcriptional patterns similar to sex-differential regions in neurotypical males and neurotypical females were similar to male-shifted regions in autistic females (Figure 3A–C, middle and rightmost panels). On the other hand, male-shifted regions in autistic males were significantly enriched for prenatal microglial, progenitor, and radial glial cell types (58) (Figure 3A, leftmost panel) and genes upregulated by DHT and estrogen (Figure 3C, leftmost panel). These results remained largely unchanged when we used a more restrictive background total of 16,906 genes (63) (expressed in cortical tissue) (see Figure S8 in the online supplement) (29, 30).

DISCUSSION

Our study demonstrates the overlap of neuroanatomical features characteristic of neurotypical males with those of autistic individuals. This pattern was specific to autism and

FIGURE 2. Spatial representation of the sex predictions, in region-aligned prediction (RAP) maps[a]

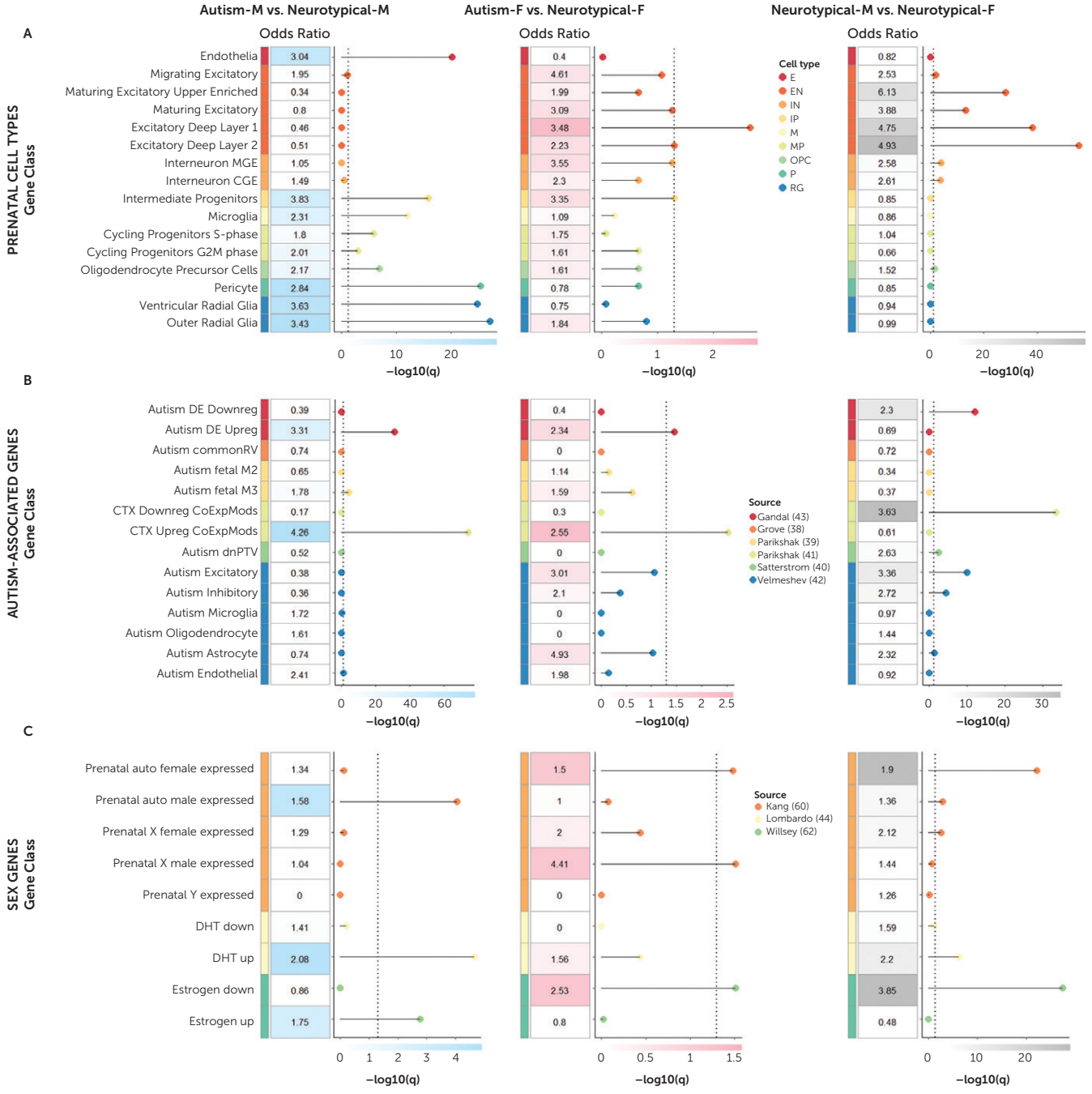


[a] Brain maps in panels A–C depict Cohen's d maps associated with the different RAP imaging t-maps (spatial representation of the sex predictions) across neurotypical females versus neurotypical males, autistic females versus neurotypical females, and autistic males versus neurotypical males. The color map encodes the average maleness probability (P; yellow/positive values=higher probability of being classified as male; blue/negative values=higher probability of being classified as female). Panel D shows regions where autistic females showed higher male prediction probabilities than neurotypical females, and panel E regions where autistic males showed higher male prediction probabilities than neurotypical males. To explore the cognitive domains implicated in these (panel D and E), we used the Neurosynth Image Decoder (http://neurosynth.org/decode/) to visualize the top 100 terms most strongly associated with the two RAP imaging t-maps showing correlations with the imaging maps between r values of 0.2 and 0.06. Results showed that the most common cognitive terms associated with the female RAP t-maps were primarily related to face perception, visual processing, and speech in autistic females, and to motor and reward processing in autistic males. F=female; M=male; P=probability; P(Neurotypical-F)=probability of neurotypical females being classified as males; P(Neurotypical-M)=probability of neurotypical males being classified as males; P(Autism-F)=probability of autistic females being classified as males; P(Autism-M)=probability of autistic males being classified as males.

was not observed in ADHD, pointing to possible different underlying biology in different neurodevelopmental conditions, despite both having a male-predominant prevalence. Overall, autistic females constituted the more critical test case for our hypotheses, likely because masculinizing sex-differentiation effects are less likely to reach a ceiling in females. Consistent with this, we observed an association between greater shifts toward neuroanatomical maleness and cognitive functional difficulties in social sensitivity and emotional face processing in autistic females. Genes relevant to brain regions predictive of male sex in autistic females were highly similar to those differentiating neurotypical males from neurotypical females, primarily comprising prenatal cell types (excitatory neurons) and upregulated autism-associated genes. The findings provide key insights into potential neurobiological and genomic underpinnings associated with male-biased autism prevalence.

We emphasize that the majority of autistic females were correctly classified by sex, and we cannot derive individual-level predictions of autism given the overlapping distributions across neurotypical and autistic individuals. This highlights the large heterogeneity inherent to autism and the importance of identifying biologically meaningful subgroups (65, 66). Among these, at least one subgroup is characterized

FIGURE 3. Gene set enrichment analyses[a]



[a] The figure shows odds ratios at a false discovery rate–corrected $p<0.05$ resulting from the gene set enrichment analyses for RAP imaging t-maps (autistic males vs. neurotypical males; autistic females vs. neurotypical females; neurotypical males vs. neurotypical females) and associated gene lists with different classes of genes acting prenatally and relevant in the context of autism and sexual differentiation. Panel A lists genes from prenatal cell types (58): endothelia (E), excitatory neurons (EN; migrating excitatory, maturing excitatory upper enriched, maturing excitatory, excitatory deep layer 1, excitatory deep layer 2), interneurons (IN; interneuron MGE and interneuron CGE), intermediate progenitors (IP), microglia (M), mitotic progenitors (MP; cycling progenitors S-phase and cycling progenitors G2M phase), oligodendrocyte precursors (OPC), pericytes (P), and radial glia (RG; ventricular radial glia and outer radial glia). Panel B lists autism-associated genes, including common genetic variants (Autism commonRV) (38), de novo mutations (fetal gene coexpression modules [Autism fetal M2, Autism fetal M3] [39]; 102 rare, de novo protein truncating genes [Autism dnPTV]) (40), and transcriptionally dysregulated genes (differentially expressed downregulated [Autism DE Downreg], differentially expressed upregulated [Autism DE Upreg] (43); cortical downregulated coexpression modules [CTX Downreg CoExpMods], cortical upregulated coexpression modules [CTX Upreg CoExpMods] [41]; Autism Excitatory, Autism Inhibitory, Autism Microglia, Autism Oligodendrocyte, Autism Astrocyte, Autism Endothelial [42]). Panel C lists sex-differentially expressed genes acting prenatally, such as genes differentially regulated by dihydrotestosterone (DHT) (44, 54, 61), estrogen (62), and (autosomal and X-/Y-chromosome linked) sex-differential gene array expression data from prenatal samples (59, 60). F=female; M=male.

by multivariate features in brain structure that are more similar to those of neurotypical males. These multivariate results of male-shifted whole-brain patterns add to previous mass-univariate neuroimaging studies that identify shifts toward the neurotypical male profile in autistic females across both brain structure (6, 8, 67) and function (10, 11, 32). A male-like profile in autistic individuals has previously been reported in specific aspects of cognitive style (68,69). Our study extends this observation to multivariate, male-shifted characteristics in brain structure. Importantly, the findings are in line with the notion that there is no strict sexual dimorphism in human neuroanatomy (70, 71), but brains exhibit a "multimorphic" mosaic of male-like and female-like features (72, 73) that can reliably distinguish males from females with above-chance to high accuracy (72, 74, 75). This means that not all autistic individuals have an "extreme male brain," but multivariate patterns characteristic of neurotypical males are on average more common in a subset of autistic individuals who are overall shifted toward the male neurophenotype. We previously reported such functional brain mosaicism in autistic males who exhibited both shifts toward male- and female-like network connectivity depending on the neural circuit involved (12). We thus emphasize the need for future studies to also examine the shifts toward a female-like neurophenotype in autism (76).

One striking finding is the similarity of neuroanatomical and associated transcriptional patterns between male-shifted regions in autistic females and regions differentiating neurotypical males from neurotypical females. Regions most strongly differentiating neurotypical males from neurotypical females overlapped with well-established sex-differential regions in posterior auditory and visual regions and hippocampus (77, 78). These visual, face, and language processing areas were also among those that least accurately predicted female sex in autistic females and have previously been associated with male-shifted patterns in autistic females (6, 32, 79) as well as with atypical brain structure in autism (80, 81). Higher predicted maleness was also associated with cognitive difficulties in autistic females such as poorer mentalizing and emotional face processing, pointing to the clinical relevance of a male-shifted neurophenotype in autistic females (16, 68). Furthermore, both sex-differential and the cell-type-specific expression patterns overlapping with male-shifted regions in autistic females and with regions differentiating neurotypical males from neurotypical females were highly similar to each other. These multilevel findings jointly suggest that cellular mechanisms mediating neurotypical brain sexual differentiation likely play a role in the etiology of autism, especially in females.

In autistic males, on the other hand, the male-shifted regions were particularly relevant to reward processing and motor functions. The former has been implicated in recent work showing striatal hypoactivation during reward processing in autism (82) and male-specific atypicality in reward learning in an animal model of autism (83). Also, somatomotor networks have frequently been found to be atypical in autism (55, 84–86), and bilateral volumetric increases in somatosensory, motor, and premotor cortex have been associated with fetal testosterone exposure (87).

Particularly, the influence of prenatal masculinization via androgens has been suggested to play a mechanistic role in the etiology of autism (15, 16, 61, 76, 88, 89). Other steroid hormones, including estrogens, have also been found to be associated with autism-related outcomes, when cohorts were restricted to males (21, 22, 90). Also, neuroimaging research shows that gray matter volumes in posterior auditory regions (exhibiting male shifts in autistic females here) and in somatomotor areas (exhibiting male shifts in autistic males here) are associated with increased androgen sensitivity (87, 91).

We further observe that genes relevant to male-shifted regions in autistic males are enriched both for genetic markers of intermediate progenitor (IP) and radial glial (RG) cell types, as well as genes upregulated by DHT and estrogen. In line with this, it has been shown that DHT increases proliferation of IP and RG cell types (92). Also, differences in proliferative processes through symmetric cell division in IP and RG cell types lead to atypical cortical expansion of surface area in autism (56, 93, 94). These downstream consequences of DHT may impact excitatory neuronal signaling, due to expansion of surface area via increased numbers of cortical columns and neurons within each column. Furthermore, genes involved in excitatory postsynaptic potentials and autism-associated genes affecting excitatory neuronal lineages are dysregulated by DHT (44, 54), and consequently, excitation-inhibition imbalance is asymmetrically more affected in autistic males (54).

On the other hand, in autistic females, male-shifted regions are particularly enriched for later differentiated excitatory neurons and genes downregulated by estrogen, but not for genes upregulated by DHT (as in autistic males). This is in line with clinical findings indicating that autistic females may be characterized by a relative imbalance between androgens and estrogens, rather than high steroid levels across all pathways (95, 96). Conditions related to androgen/estrogen imbalances in females (e.g., polycystic ovary syndrome) have previously been linked to autism likelihood in both mothers and their children (97–100). Further, estrogen-regulated signaling can influence region-specific neurodevelopmental male shifts and affect neuronal excitation (101–103), but more studies are needed to demonstrate this in human-derived cell models. Thus, the enrichment effects in IP, RG, and DHT in autistic males and in excitatory neuronal cell types and estrogen in autistic females may be relevant to the overall idea that one key emergent phenomenon of brain masculinization is the effect it has on excitation-inhibition imbalance (104), with potentially different underlying sex-steroid-related mechanisms in autistic males and females (90).

Furthermore, microglial neuroimmunological processes may be major contributors to brain masculinization (14). Werling et al. (59) reported enrichment of male-biased microglial expression with autism-upregulated coexpression modules along with enrichment of female-biased synaptic

expression with autism-downregulated coexpression modules, corroborating that molecular downstream pathways regulating neurotypical male development interact with those of autism-associated genes. These observations are consistent with our discoveries that 1) genes expressed in regions overlapping with male-shifted regions in both autistic males and autistic females are enriched for upregulated autism-associated genes mapping onto inflammatory pathways (41) (likely underlying vulnerability mechanisms associated with males [105]), 2) regions differentiating neurotypical females from neurotypical males are enriched for autism-downregulated expression modules (likely underlying protective mechanisms associated with females [105]), 3) the male-shifted regions in autistic males are enriched for prenatal microglial cell types, and 4) downstream transcriptionally dysregulated pathways are more involved in sex-differential processes than they are in upstream genetic susceptibility mechanisms.

Our findings of reduced and superior sex prediction performance in autistic females and males, respectively, are most pronounced in childhood and decrease throughout development to young adulthood. In the age bin of 20–30 years, we do not observe classification differences across groups. Similarly, two other studies in adult samples with mean ages of 22 years (7) and 26 years (5) also showed no sex prediction accuracy differences between autistic and neurotypical adults. It is likely that these age-related patterns are influenced by dynamically unfolding interactions between sex-differential and neurodevelopmental process across the lifespan. Sex differentiation emerges in utero, and many on-average sex differences persist into adulthood, while others are transient and context dependent, meaning they become apparent at specific developmental stages depending on factors such as hormonal milieu and experiences (71). For example, sex steroid hormones are thought to initially act organizationally during perinatal development, and then activationally during puberty to influence the formation and expression of sex differences at different developmental windows (106). Clinically, the rate of pubertal differentiation was, for example, found to interact with the association of autistic traits with fetal testosterone levels in a longitudinal cohort (107). Also, different molecular mechanisms are highly region specific, and they are again expressed at different developmental periods (71). One example of an age-dependent neurophenotype in autism is early brain overgrowth, which is more likely observed in autistic children but not later in life (108, 109). Atypical age-related cortical development has also been demonstrated by deviations from the typical developmental trajectories both in cortical thinning in autistic children and adolescents in longitudinal studies (110–112) and in neuronal differentiation in in vitro models (113). Here, we identified associations with prenatal cell types that have been linked to atypical cortical expansion in autism (56) and likely link back to sex-specific excitation-inhibition imbalance across neurodevelopment. On top of these sex-related mechanisms, gendered experiences, gender role expectations, and gender socialization based on one's sex assigned at birth in a society may also influence neurobiology and postnatal brain development (2, 114). Considering this mechanistic complexity, future studies (especially longitudinal ones) need to unravel the observed spatiotemporal sex-differential profiles in autism and establish how experiential, environmental, genetic, and hormonal effects interact to differentially contribute to the observed patterns across different developmental windows (e.g., fetal/neonatal stage, childhood, puberty/adolescence, adulthood/reproductive age, and old-age/postmenopause). Finally, we cannot exclude nosological issues and ascertainment bias contributing to the observed age-related patterns. Autism diagnostic practices have been described as male biased (3). It is thus possible that females presenting with "typical/classical" (i.e., male-like) autistic features (which might be related to the observed neuroanatomical patterns) may be diagnosed earlier in life than females presenting with more nuanced phenotypes that are less well captured by current diagnostic practices (115), hence contributing to the age differences in classification performance. We have no available data on age at diagnosis in the present study; however, future studies should disentangle the effects of chronological age and timing of autism diagnosis.

Finally, it has been suggested that the male preponderance in neurodevelopmental conditions may be driven by similar genetic and neuroendocrinological mechanisms. Here, we show that autism is associated with a different sex-differential neuroanatomical profile in males and females than is ADHD. A proposed pathway through which sex-differential prevalence and presentation in ADHD is mediated may be atypical dopaminergic system function modulated by gonadal hormones (116, 117), such as an increase in the striatal dopamine receptor density in prepubertal development in males but not females (118). Future research needs to pinpoint which exact sex-related mechanisms shape differential neurophenotypes in different male-biased conditions.

Strengths and Limitations

Our analytical approach has multiple strengths. We employ four of the largest cohorts available for our populations of interest (neurotypical, autism, ADHD), thus ensuring both replicability and specificity of findings. We carefully address potential confounders, confirming that the results are not driven by differences in brain volume, age, and model choice. Our analyses do not rely on artificial features, but by employing a novel deep convolutional neural network model with superior performance (46), we take whole-brain anatomical data as predictive features. Nevertheless, there are several limitations. Even though our samples span wide age ranges and the results imply the likely involvement of neurodevelopmental mechanisms, only when investigating a longitudinal cohort will we be able to make causal inferences. Further, we did not address social, cultural, or experiential factors (114) that can influence sex-differential brain development (71), or gender identity, which might differ from sex assigned at birth, especially in autistic individuals (119). The

data sources employed in the current study did not gather information specifically distinguishing sex at birth and gender identity. As such, the potential for misspecification of participants' sex at birth exists, but the rate is anticipated to be low. Within this limitation, given that our models were trained to predict biological sex, we used the term sex-specific prediction accuracy. In addition, while utilizing a highly accurate approach for sex classification, the high level of nonlinearity intrinsic to the convolutional neural network model makes straightforward interpretations more difficult compared to simpler linear models. We address this issue by a novel approach for model introspection by means of region-aligned prediction. This approach has the advantage of being able to generate spatially resolved estimates of sex-specific accuracy prediction, but due to the convolutional structure of our model, it achieves this with lower spatial fidelity than the original input data. Yet, being able to spatially resolve the sex prediction accuracy enables the further association analyses with cognition and gene expression. We further note that our enrichment analyses are a correlational approach that cannot reveal the causal genetic mechanisms of our brain structural findings. However, this approach can highlight genes for further molecular analyses to establish mechanisms and directionality of findings (23). Also, we only tested a select number of genes for which there is a prior literature implicating them as robust markers of autism likelihood, prenatal development, and sex-differential biology. While we also acknowledge the methodological limitation of using adult brain expression data in association with prenatal cell types, our results provide important signals that neuroanatomical male shifts in autism are associated with genes that are predominantly expressed in prenatal development and may therefore affect neurodevelopmental processes. Also, the Allen Human Brain gene expression atlas is the most comprehensive and highly sampled gene expression atlas currently available—albeit one based on adult donors only. Once high-resolution, whole-brain, and age-specific gene expression maps become available, future studies should investigate gene expression profiles in the developing human brain. This will further elucidate the genes associated with autism-related sexual differentiation acting at different developmental windows.

CONCLUSIONS

Understanding the male preponderance in autism prevalence requires understanding biological processes involved in brain sexual differentiation (14, 16). Our results suggest that, on average, autistic individuals are more likely to show a biologically typical male expression of neuroanatomy. Specifically, in autistic females this shift is associated with typically sex-differential cognitive features and overlaps with sex-differential, steroid-regulated, and excitatory cell-type specific expression patterns. Identifying neuroanatomical, cognitive, and genetic interrelations at the intersection of sex-differential and autism-associated neurobiology will provide promising avenues for research into biological mechanisms underpinning the etiology of autism. Echoing recent calls for an increased inclusion of females in basic neuroscience research (120), we extend this to clinical neuroscience, where increased attention to females in neurodevelopmental research will help elucidate sex-differential likelihood factors as well as potential sex-related diagnosis and support that are informed by neurobiology.

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