Integrated Transcriptome Analyses Revealed Key Target Genes in Mouse Models of Autism

Weicheng Duan, Kang Wang, Yijie Duan, Xufeng Chu, Ruoyun Ma, Ping Hu, and Bo Xiong

Genetic mutations are the major pathogenic factor of Autism Spectrum Disorder (ASD). In recent years, more and more ASD risk genes have been revealed, among which there are a group of transcriptional regulators. Considering the similarity of the core clinical phenotypes, it is possible that these different factors may regulate the expression levels of certain key targets. Identification of these targets could facilitate the understanding of the etiology and developing of novel diagnostic and therapeutic methods. Therefore, we performed integrated transcriptome analyses of RNA-Seq and microarray data in multiple ASD mouse models and identified a number of common downstream genes in various brain regions, many of which are related to the structure and function of the synapse components or drug addiction. We then established protein–protein interaction networks of the overlapped targets and isolated the hub genes by 11 algorithms based on the topological structure of the networks, including *Sdc4*, *Vegfa*, and *Cp* in the Cortex-Adult subgroup, *Gria1* in the Cortex-Juvenile subgroup, and *Kdr*, *S1pr1*, *Ubc*, *Grm2*, *Grin2b*, *Nrxn1*, *Pdyn*, *Grin3a*, *Itgam*, *Grin2a*, *Gabra2*, and *Camk4* in the Hippocampus-Adult subgroup, many of which have been associated with ASD in previous studies. Finally, we cross compared our results with human brain transcriptional data sets and verified several key candidates, which may play important role in the pathology process of ASD, including *SDC4*, *CP*, *S1PR1*, *UBC*, *PDYN*, *GRIN2A*, *GABRA2*, and *CAMK4*. In summary, by integrated bioinformatics analysis, we have identified a series of potentially important molecules for future ASD research. ***Autism Res*** *2020, 13: 352–368.* © 2019 International Society for Autism Research, Wiley Periodicals, Inc.

Lay Summary: Abnormal transcriptional regulation accounts for a significant portion of Autism Spectrum Disorder. In this study, we performed transcriptome analyses of mouse models to identify common downstream targets of transcriptional regulators involved in ASD. We identified several recurrent target genes that are close related to the common pathological process of ASD, including *SDC4*, *CP*, *S1PR1*, *UBC*, *PDYN*, *GRM2*, *NRXN1*, *GRIN3A*, *ITGAM*, *GRIN2A*, *GABRA2*, and *CAMK4*. These results provide potentially important targets for understanding the molecular mechanism of ASD.

Keywords: autism; transcriptome; integrated analysis; gene regulation; PPI network; synaptic transmission

Introduction

Autism Spectrum Disorder (ASD), a neurodevelopmental disorder that impairs social skills, cognition, and behavior, occurs approximately 1 out of 59 children based on current diagnostic criteria (Centers for Disease Control and Prevention [CDC, 2018] www.cdc.gov/ncbddd/autism/data.html). According to the epidemiological investigation, the number of patients is rapidly increasing in recent years [WHO, 2017]. However, the etiology of ASD remains largely unknown, partially due to the extremely high genetic heterogeneity of the disease [Lai, Lombardo, & Baron-Cohen, 2014].

Based on the recent large-scale sequencing efforts using various high-throughput technologies, de novo mutations of many single genes have been highlighted as important pathogenic factors for ASD [Iossifov et al., 2014; Sanders et al., 2012; Stessman et al., 2017; Turner et al., 2017]. Interestingly, a significant number of ASD genes have been shown to play important roles in gene expression regulation, including transcriptional factors, chromatin remodeling factors and other types of epigenetic regulators, such as *CHD8*, *FMR1*, *MECP2*, and so on [Alonso-Gonzalez, Rodriguez-Fontenla, & Carracedo, 2018; De Rubeis et al., 2014]. Accordingly, numerous loss of function animal models were generated, especially the mouse models, to recapitulate the disease phenotypes and to explore the underlying molecular mechanisms [Araujo et al., 2017; Arbogast et al., 2018; Caubit et al., 2016; Celen et al., 2017; Cheng et al., 2018; Gabel et al., 2015; Harrington et al., 2016; Huang

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et al., 2018; Jung et al., 2018; Kong et al., 2014; McGill et al., 2018; Prilutsky et al., 2015; Provenzano et al., 2016; Raman et al., 2018; Scandaglia et al., 2017; Sgado et al., 2013; Suetterlin et al., 2018; Zhao, Goffin, Johnson, & Zhou, 2013]. With these models, RNA-Seq or microarray experiments have been performed in various brain regions to identify the downstream targets of the corresponding ASD genes. In each of these studies, hundreds to thousands of genes were identified as differentially expressed, either overexpressed or downregulated. However, it remains unclear, which of the targets are the major drivers of the ASD-related phenotypes. Since the core phenotypes caused by mutations in these transcription regulators are very similar, it is possible that a number of overlapped downstream genes may play important roles in disease pathology. Therefore, we hypothesized that the ASD genes that are involved in transcriptional regulation may affect nervous system development through some common targets or biological pathways. In this regard, we performed integrated bioinformatics analyses to identify the overlapped differentially expressed genes (DEGs) in specific brain regions of various ASD mouse models in order to provide insight into the mechanisms of how alteration of the transcriptional activities would lead to ASD.

Methods

Ethics Statement

The object of this study is mice, and the data sets related to mice were obtained from GEO and ArrayExpress database. The data of human patients were extracted from the published literature with known ethics approval. Since we performed bioinformatics analysis based on these data, additional ethics approval of ethics was not required.

Identification of Literature and Collection of Information

The literature search using PubMed was conducted independently by two investigators. The comprehensive search strategies included the Mesh term and Keywords: ("Autism," "autistic disorder," ASD, Kanner's syndrome), ("mice," "mouse"), ("knock out," "knock-out," deletion, mutation, homozygote, heterozygote), and ("transcriptome," "Gene Expression Profiles," "Gene Expression Profile," "Gene Expression Signatures") through April 03, 2019. All eligible studies were retrieved and examined carefully. Review articles and references of other relevant researches were used to find additional eligible studies. The inclusion criteria were as follows: (a) Mouse models were used; (b) ASD models were constructed by genome editing to generate homozygote or heterozygote mutants; (c) The targeted genes are known to be transcriptional regulators; (d) Animal models exhibit phenotypes related to ASD; (e) RNA-Seq or microarray experiments were performed in the specific brain regions including cerebellum, cortex, or hippocampus; (f) The database and project number of positioned transcriptome data were specified in the paper; (g) Transcriptome data were available in the corresponding database. The exclusion criteria were: (a) Non-mice studies, meta-analysis, comments, letters, or reviews; (b) The mutated gene was not transcriptional regulator; (c) The behavior or phenotypes of the models were not confirmed; (d) Expression profiles analysis only in specific cell type or the whole tissue. The following information on included studies was collected: first author, gene symbol, age of mice, analyzed brain region, test platform, database, and project number. With all above, the major clinical information related to these mutant genes was collected from OMIM database and several reviews [Carratala-Marco et al., 2018; D'Angelo, Moller, Alonso, & Koiffmann, 2015; Geets, Meuwissen, & Van Hul, 2019; Merner et al., 2016; Tucci, Ciaccio, Scuvera, Esposito, & Milani, 2016].

Identification of Overlapped DEGs

The processed microarrays gene expression profiles were obtained from the NCBI GEO database and the raw gene expression matrix of RNA-Seq was downloaded from ARCHS4 database [Lachmann et al., 2018]. The preprocessing and normalization of microarray expression profiles were carried out using the affy package of Bioconductor, with the following parameters: RMA (background correction), quantiles (normalization), pmonly (perfect match correction), and median polish (probe summarization). Data downloaded from the ARCHS4 database have already been mapped and normalized using the Kallisto aligner [Bray, Pimentel, Melsted, & Pachter, 2016], no further preprocessing was performed. The Linear Models for Microarray Data (Limma) package in bioconductor was applied to analyze the gene expression profiles from the processed microarray data and to identify the DEGs by comparing gene expression levels between the mutant and the control group. For the RNA-Seq data, Edge R package was used to identify DEGs. Only genes with absolute value of fold change ≥1.2 and P value <0.05 were regarded as significant DEGs. These DEGs in different studies were separately analyzed to avoid the heterogeneity. Based on the stage of animal models (Embryo, Juvenile: ≤6 weeks, Adult: >6 weeks) and encephalic regions examined, the DEGs of different data sets were divided into several subgroups to obtain the overlapped DEGs, including the Cerebellum-Adult subgroup, the Cortex-Juvenile subgroup, the Cortex-Adult subgroup, and the Hippocampus-Adult (Hip-Adult) subgroup. The DEGs overlapped in more than three data sets were selected and then used for further functional enrichment analyses and network analyses. The intersection of DEGs in each subgroup was visualized by the Venn diagram (data sets ≤3) or the Upset package (data sets ≥4) based on R 3.5.2.

Functional Analyses of DEGs

First, the ASD risk gene list was downloaded from the Simons Foundation and Autism Research Initiative (SFARI)

GENE database (https://gene.sfari.org/) to carry out the enrichment analyses by hypergeometric distribution test. Subsequently, the functional enrichment analyses of the DEGs, including Gene Ontology (GO) function analyses and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, were carried out using annotation, visualization, and integrated discovery tools in the Metascape database (metascape.org) [Zhou et al., 2018] the GO analyses, cellular component (CC), biological process (BP), and molecular function (MF) terms were analyzed, and *P*-value <0.001 and gene counts ≥3 were used as statistically significant cutoffs. For the KEGG pathways analyses, enriched pathways were identified according to the hypergeometric distribution with a *P*-value <0.01 and gene counts ≥3.

Network Analyses Among the DEGs

The protein products of the overlapped DEGs in different subgroups were analyzed using STRING database (a database of known and predicted protein interactions) to calculate the PPI network by importing the official gene symbol. A combined score of not less than 0.4 (medium confidence score) was considered as significant. Detailed data from STRING database were exported and then imported into the Cytoscape 3.7.1 software to visualize the PPI networks. The MCODE algorithm, one of the Cytoscape v3.7.1 plugins, was used to find highly interconnected or density modules in the networks with default parameters (node score cut-off = 0.2, degree cut-off = 2, k-core = 2, maximum depth set at 100). Subsequently, GO and KEGG pathways enrichment analyses were carried out in the identified modules to annotate these functions (hit genes ≥3, $P < 0.001$). In the PPI networks, the hub genes (proteins) were first selected according to the number of direct interactors of a node (more than 3), whose number would be the threshold of the other filter methods. Next, the hub genes were screened using 10 other algorithms of CytoHubba plugin, including MCC, DMNC, MNC, EPC, BottleNeck, EcCentricity, Closeness, Radiality, Betweenness, and Stress, then the results were cross compared to generate a final list of hub genes. Finally, the overlapped DEGs and the hub genes were validated by the results of another study based on student's *t*-test, which integrated multiple transcriptional data sets that containing over 2,000 brain samples from individuals with schizophrenia (SCZ), ASD, and controls [Gandal et al., 2018].

Results

Retrieval of ASD Mouse Model Studies

The process of the literature retrieval was displayed on the flowchart in Figure 1. A total of 113 studies were identified according to the result of the described retrieval strategy from the PubMed database. On the basis of our inclusion and exclusion criteria, 79 studies were first excluded for as meta-analyses, reviews, non-mice studies, non-transcriptome studies, or non-single gene researches. Then, 34 studies were selected for full-text review. However, 16 studies were further excluded, because one study lacked available transcriptome data, five focused on genes other than transcriptional regulators, and 10 studies focused on expression profiles analyses of specific cell type or the whole tissue. Finally, 18 studies were included in our analyses. The specific information about the included studies was exhibited in Table 1. The primary clinical symptoms and signs associated with included mutant genes were listed in Table 2, showing significantly overlapped phenotypes, such as autism features, head abnormity, facial dysmorphic features, intellectual disability (ID), and so on, indicating that certain biological processes may be commonly affected in the related disease models.

Identification of Overlapped DEGs Among Various ASD Mouse Models

To identify the commonly affected downstream genes in the selected ASD mouse models, we first subgrouped the studies based on the age of the mice and brain regions used for transcriptome experiments. As can be seen in Figure 2A,B, the Cerebellum-Adult subgroup contained three data sets, involving mutants in *Mecp2*, *Fmr1*, and *En2*. There were no overlapped upregulated DEGs and four overlapped downregulated DEGs in the Cerebellum-Adult subgroup, which were *Tspan18*, *Ncald*, *Nfatc2*, and *Chst15*. The numbers of overlapped DEGs vary in different combinations of the six studies, which were focused on the hippocampus region of adult mice (Fig. 2C,D). Since no overlapped DEGs present in all six studies, we set the threshold for DEG identification as overlapped in at least three data sets. Finally, 53 upregulated DEGs and 86 downregulated DEGs were isolated in the Hip-Adult subgroup. Similarly, in the Cortex-Adult subgroup, 83 upregulated DEGs and 62 downregulated DEGs were overlapped in more than three data sets, where five upregulated DEGs (*Adam33*, *mt-Nd2*, *mt-Nd6*, *Sult1a1*, and *Spp1*) and two downregulated DEGs (*Cntn5* and *Zfp608*) present in all data sets (Fig. 2E,F). As for the Cortex-Juvenile subgroup (Fig. 2G,H), 52 upregulated DEGs and 60 downregulated DEGs overlapped in more than three data sets, whereas only one upregulated DEGs (*Neurod1*) was the common targets in all studies. Next, the DEGs that were overlapped in at least three models in each subgroup were used for subsequent functional enrichment analyses and PPI network constructions.

Functional Annotation and Pathway Enrichment of the Overlapped DEGs

First, to evaluate the importance of the overlapped DEGs, we performed an ASD-related genes enrichment analysis

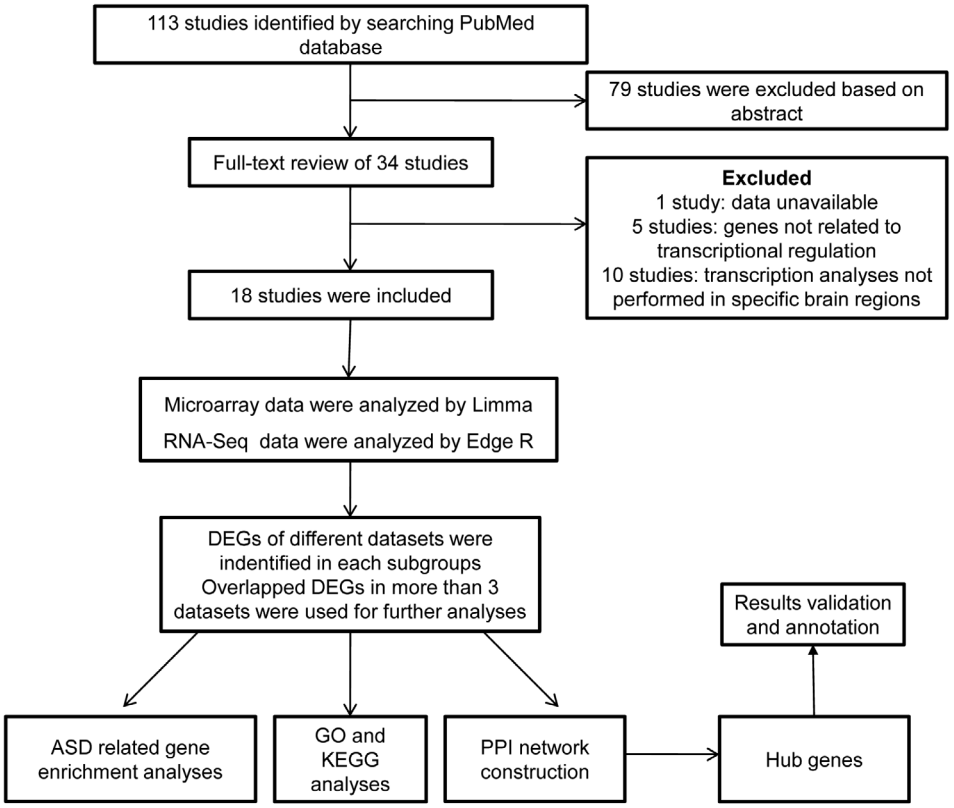

Figure 1. Flow chart of the overall process for the integrated transcriptome analyses.

based on a hypergeometric distribution test by comparing directly to the repository of the SFARI GENE database (Table 3). The ASD related genes were significantly enriched in the isolated DEGs set (Cortex-Adult: hit genes = 14, P = 4.37E-03. Cortex-Juvenile: hit genes = 14, P = 4.12E-04. Hip-Adult: hit genes = 11, P = 4.15E-02), indicating that our

Table 1. Characteristics of the Included Studies

Study	Gene	Genotype	Age	Brain region			Platform	Database	Data sets
				Cortex	Cerebellum	Hippocampus		GEO	GSE
Cheng Y	Mir137	Heterozygous Homozygote	P12	+			Illumina HiSeq 2000	GEO	GSE79661
Jung H	CHD8	Heterozygote	P25			+	Illumina's HiSeq 2000	GEO	GSE103377
Huang L	Upf3b	Homozygote	8-11W	+			Illumina HiSeq 4000	GEO	GSE99112
McGill BE	CTCF	Homozygote	3-6M			+	Agilent-026655	GEO	GSE108825
Suetterlin P	CHD8	Heterozygote	P5	+			Illumina HiSeq 4000		GSE81103
Arbogast T	Ktcd13	Homozygote Heterozygote	3W 15W	+		+	Illumina HiSeq2500	ArrayExpress	E-MTAB-7398
Raman AT	Mecp2	Heterozygote	8-9W		+		Illumina HiSeq 2000	GEO	GSE105045
Araujo DJ	Foxp1	Homozygote	P47	+		+	Illumina NextSeq 500	GEO	GSE97181
Celen C	Arid1b	Heterozygote	P78-P82			+	Illumina NextSeq 500	GEO	GSE92238
Scandaglia M	Kdm5c	Homozygote	2-6M			+	Illumina HiSeq 2500	GEO	GSE85874
Caubit X	THSZ3	Heterozygous	E18.5	+			Illumina HiSeq 2000	GEO	GSE85512
Harrington AJ	MEF2C	Homozygote	3W	+			Illumina HiSeq 2500	GEO	GSE87202
Provenzano G	En2	Homozygote	3-5M			+	Agilent-014868	GEO	GSE81501
Kimberly M. Huber	Fmr1	Homozygote	E17-18	+		+	Affymetrix Mouse Genome 430 2.0 Array	GEO	GSE71034
Gabel HW	Mecp2	Heterozygous	8W	+			Illumina HiSeq 2000	GEO	GSE67294
Kong SW	Fmr1	Homozygote	8-10W		+		Affymetrix Mouse Gene 1.0 ST Array	GEO	GSE40630
Sgadò P	En2	Homozygote	3-5M		+	+	Agilent-014868	GEO	GSE51612
Zhao YT	Mecp2	Heterozygous	P7 P90				Affymetrix Mouse Exon 1.0 ST Array	GEO GEO GEO	GSE42895

Table 2. Core Clinical Symptom of These Included Genes

Symptom	Mir137	CHD8 (OMIM)	UPF3B (OMIM)	CTCF (OMIM)	KCTD13 16p11.2	Mecp2 (OMIM)	Foxp1 (OMIM)	Arid1b (OMIM)	MEF2C (OMIM)	TSHZ3 (OMIM)	EN2	FMR1 (OMIM)
Head	Macrocephaly	Macrocephaly	Macrocephaly	Microcephaly	Macrocephaly	Microcephaly,	Macrocephaly		Microcephaly,			Macrocephaly
Dysmorphic features	Y	Y	Y	Y			Y	Y	Y	Y		Y
Ocular problems	Y			Y			Y	Y				
CHEST abnormity			Y									Y
Cardiac defect				Y								Y
Skeletal abnormity			Y		Y			Y		Y		Y
Hypotonia	Y	Y	Y	Y		Y	Y	Y	Y	Y		
Gastrointestinal problems		Y		Y		Y		Y		Y		
Autism features	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Intellectual disability	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Seizures		Y			Y	Y		Y	Y	Y	Y	Y
Motor Coordination problems					Y				Y		Y	
Speech delay	Y				Y		Y	Y	Y	Y	Y	
psychomotor development delay	Y			Y	Y	Y	Y	Y	Y	Y	Y	
Aggressive behavior	Y						Y					
Hyperactivity					Y		Y					Y

Abbreviation: Y, yes.

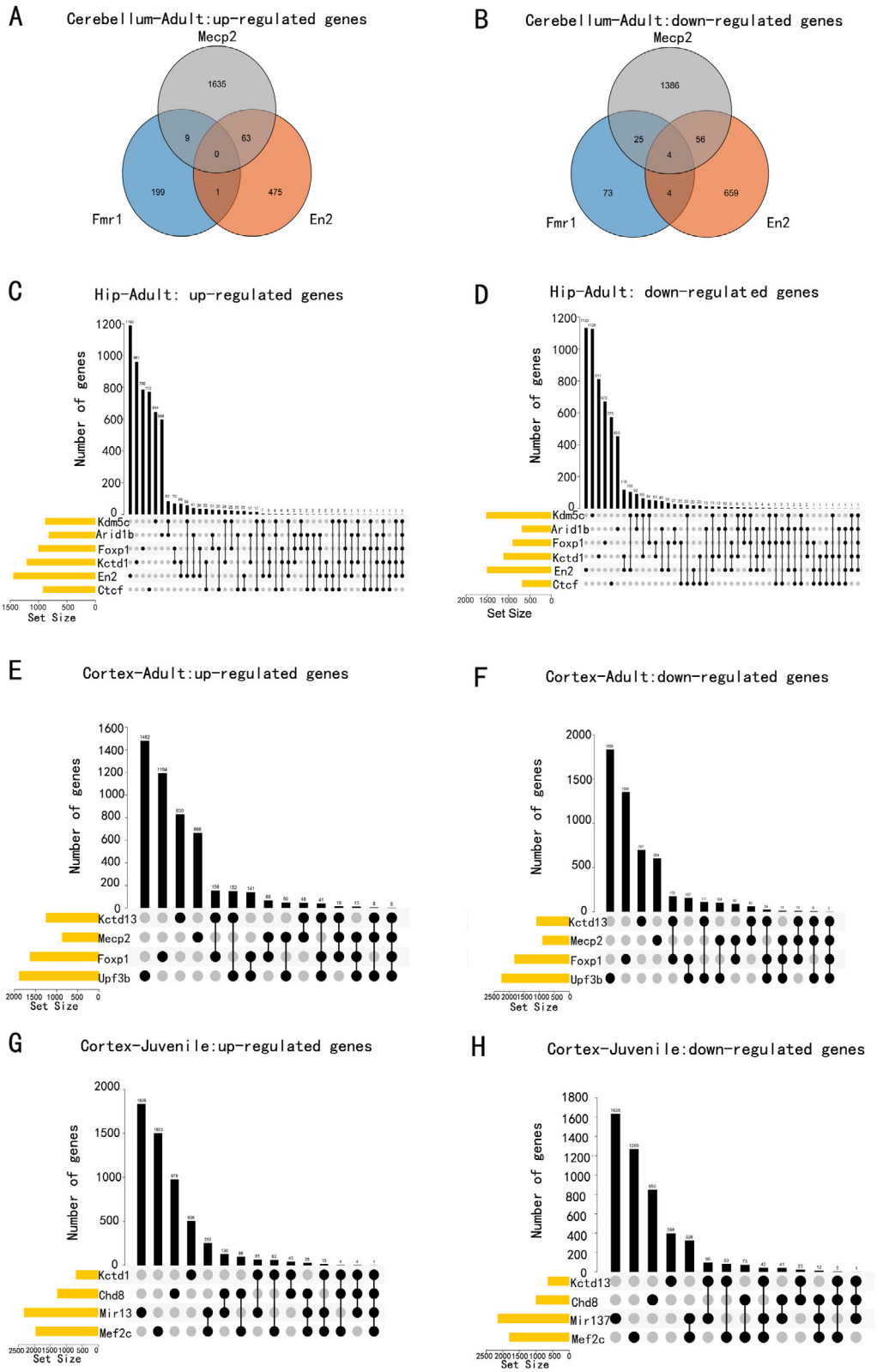

Figure 2. The intersection of the DEGs in different subgroups. (**A**, **B**) Venn diagram of the upregulated and downregulated DEGs in the Cerebellum-Adult subgroup. The overlapped areas in the diagram represent the common downstream genes among different ASD-related transcriptional regulators. **C**,–**H**: Upset diagrams of the upregulated and downregulated DEGs in the Hip-Adult subgroup, the Cortex-Adult subgroup, and the Cortex-Juvenile subgroup. The bars represent the number of common downstream genes of the regulators highlighted by dots and links below.

Table 3. ASD Risk Genes Enrichment Analysis

Data	SFARI genes	%Enrichment	*P* value	Gene symbol
Cortex-Adult	14	10.45	0.004368176	CNTN5, FAT1, EPHA6, GRIK4, SDK, CDH13, KMT2A, PCDH19, MET, CHD3, NEFL, GABRA1, MYO1E, EXT1
Cortex-Juvenile	14	13.33	0.0004117898	HTR2A, GRIA1, SYN2, SEMA5A, SDK1, CLSTN2, EPHA6, PLXNA4, ELAVL2, OGT, PCDH11X, CNTN4, CDH8, FOXP1
Hip-Adult	11	8.5	0.04150845	ATRNL1, AUTS2, GRIN2A, PCDH19, NBEA, GRIN2B, CAMK4, SYT17, NRXN1, NTNG1, CBLN1

analyses indeed highlighted genes that are relevant to the disease pathology.

To explore the specific functional features of the overlapped DEGs, we performed GO and KEGG enrichment analyses using Metascape. The GO terms were divided into MF, CC, and BP ontology, and only the most significant hierarchically cluster terms were exhibited (Fig. 3A–C). In addition, the results of the KEGG pathway enrichment analysis with the criteria $P < 0.01$ are displayed (Fig. 3D).

For the Cortex-Adult subgroup (Fig. 3A, Supplementary Table S1), the significant GO-MF terms were mainly ion gated channel activity and NADH dehydrogenase (ubiquinone) activity. The significant GO-CC terms were mainly GABA-ergic synapse, axon, and receptor complex. In addition, the significant GO-BP terms were mainly related to cell adhesion, cell morphogenesis, response to copper ion and exploration behavior. Moreover, KEGG analyses revealed that oxidative phosphorylation and nicotine addiction pathways were significantly enriched in the overlapped DEGs set. It is noteworthy that the terms related to synaptic components were significantly enriched, such as GABA-ergic synapse, axon, cell adhesion, and cell morphogenesis, indicating that the dysfunction of synapses could be one key pathological basis of ASD, which is a commonly accepted viewpoint in the field [Gokoolparsadh et al., 2016]. In addition, genes involved in NADH dehydrogenase (ubiquinone) activity were enriched in overlapped DEGs, in agreement with the previous finding that mitochondrial dysfunction is involved in ASD and intellectual disorder [Goh, Dong, Zhang, DiMauro, & Peterson, 2014; Fernandez et al., 2019].

In the Cortex-Juvenile subgroup (Fig. 3B, Supplementary Table S2), MF related to G-protein alpha-subunit binding, calcium ion binding, and glycosaminoglycan binding were markedly enriched. CC related to glutamatergic synapse and cell–cell junctions were significantly enriched. The enriched top three GO-BP terms were cell–cell adhesion via plasma-membrane adhesion molecules, response to ammonium ion and regulation of endothelial cell proliferation. For KEGG pathway analyses, the processes involved in gap junction, morphine addiction, and calcium signaling pathway were enriched. Similar to the results of the cortex-adult group, many of the overlapped DEGs play important roles in the structure and function of synapses. Moreover, some other enriched processes including neural circuits formation, intracellular calcium dysregulation and calcium-dependent pathways, have also been associated with ASD [Booker et al., 2018]. Therefore, misregulation of these overlapped DEGs and the related biological processes in the developing cortex may contribute to the pathology of ASD.

For the Hip-Adult subgroup (Fig. 3C, Supplementary Table S3), the enriched GO-MF terms were primarily associated with cell adhesion molecule binding. The significant GO-CC terms mainly included a synaptic membrane, glutamatergic synapse, axon, and dense core granule. In addition, the significant GO-BP terms mainly involved in neurotransmitter transport, apoptotic, postsynapse organization, cell adhesion, and neuron death. Moreover, the enriched pathways in KEGG analyses were primarily associated with drug addiction, neuroactive ligand–receptor interaction, glutamatergic synapse, cAMP signaling pathway, and long-term potentiation. The hippocampus has been widely reported to be involved in learning and memory, cognition and emotional control, which are critical for many forms of neurodevelopmental disorders [Drieu & Zugaro, 2019]. Interestingly, the enriched pathways, such as the cAMP signaling pathway, long-term potentiation, and glutamatergic synapse function, have been associated with abnormal hippocampus function in various ASD models [Booker et al., 2018; Shin et al., 2019; Zamarbide et al., 2019]. Therefore, these overlapped DEGs in the hippocampus region may play critical functions that are related to ASD phenotypes.

We next set out to examine what the common GO and KEGG terms were in all three subgroups (Fig. 3E,F). Four GO terms were significantly enriched in all three subgroups, which were synaptic membrane, intrinsic component of synaptic membrane, integral component of postsynaptic membrane, integral component of synaptic membrane, again indicating that broad synaptic dysfunction is an important feature of ASD. No KEGG pathway terms were enriched among all three subgroups, but three KEGG terms were shared between the Cortex-Juvenile subgroup and the Hip-Adult subgroup, covering Long-term potentiation, Glutamatergic synapse, and Amphetamine addiction. One KEGG term was in common between the Cortex-Adult subgroup and the Hip-Adult subgroup, which was Nicotine addiction. These results indicated that some processes and

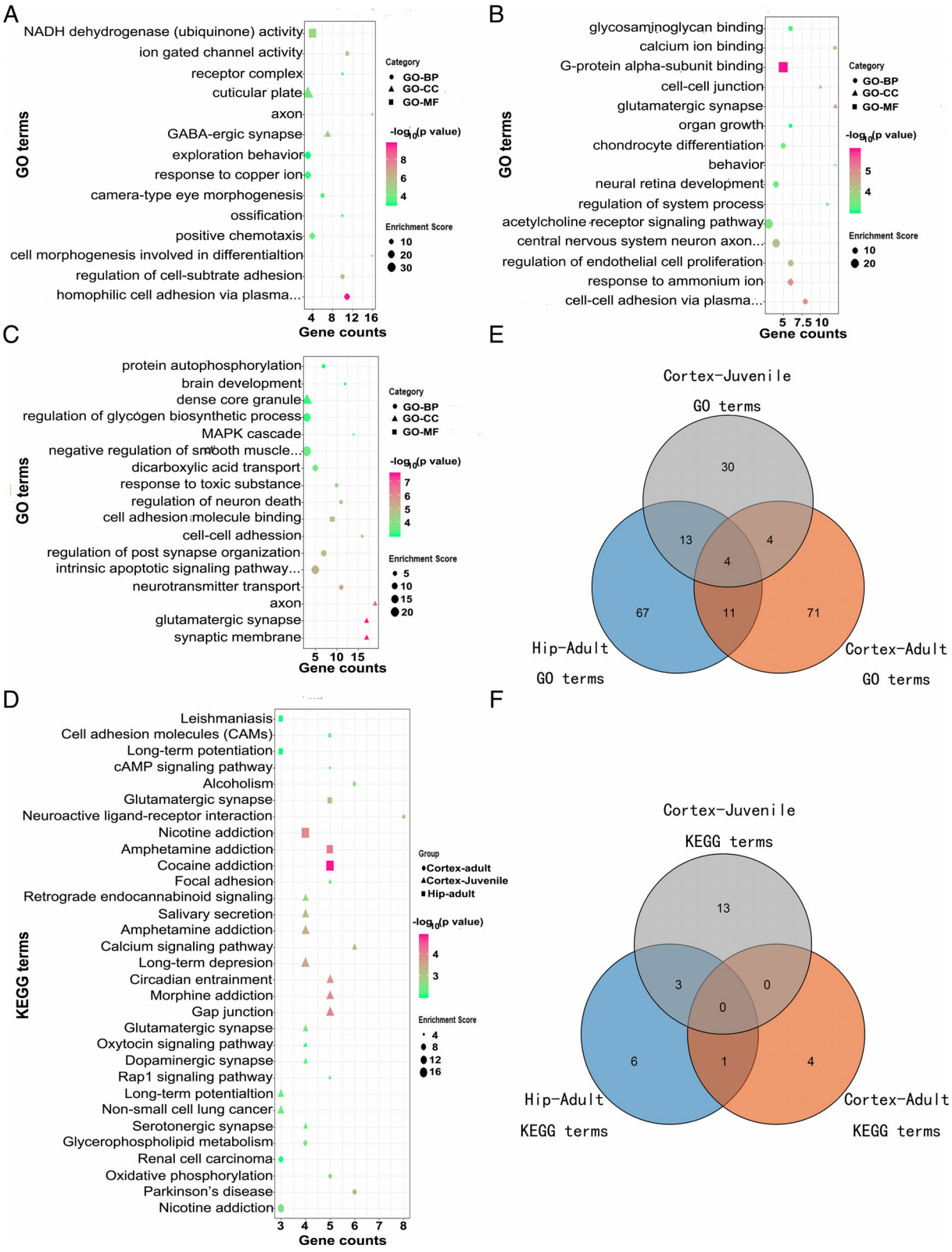

Figure 3. GO and KEGG pathway enrichment analyses of the overlapped DEGs. (**A**–**C**) Significantly enriched GO terms in the Cortex-Adult subgroup, the Cortex-Juvenile subgroup, and the Hip-Adult subgroup. (**D**) Significant KEGG pathways in the three subgroups. (**E**) The intersections of enriched GO terms. (**F**) The intersections of enriched KEGG pathways.

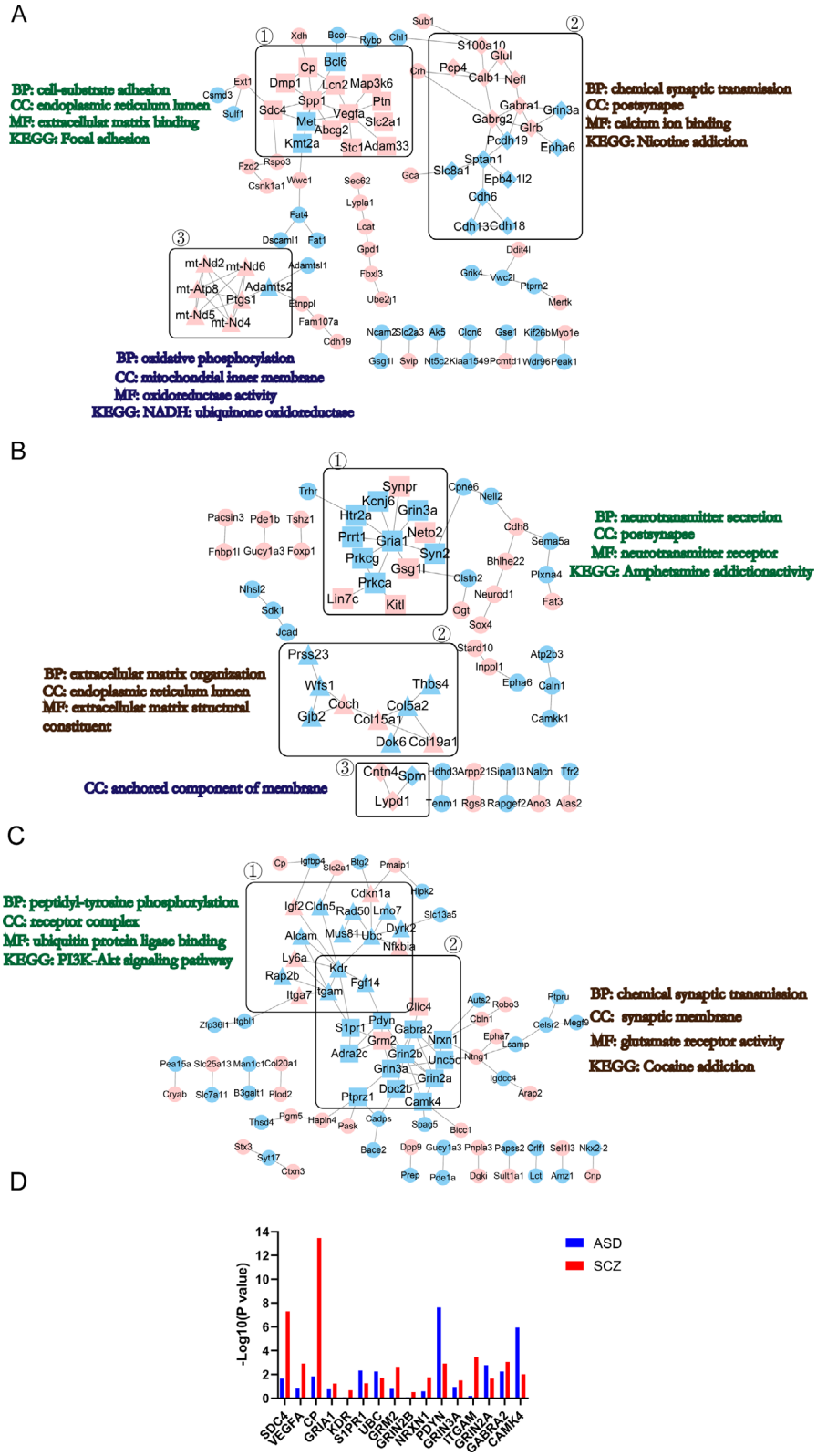

Figure 4. Protein–protein interaction (PPI) networks and functional modules among the overlapped DEGs. (**A**–**C**) PPI networks for the DEGs identified in the Cortex-Adult subgroup, the Cortex-Juvenile subgroup, and the Hip-Adult subgroup. The proteins (genes) in the rectangles represent the functionally related sub-modules in the overall networks based on the topological structures. The most significant annotations of the modules are displayed next to the rectangles. (**D**) The statistical results of the expression levels of the identified hub genes in ASD and schizophrenia (SCZ) patients based on human brain RNA-Seq data sets. Statistics were performed using student's *t*-tests.

Table 4. GO and KEGG Pathways Enrichment Analysis in the Modules of Networks

Group	Module	Category	Description	log *P*
Cortex-Adult	1	GO-BP	Regulation of cell–substrate adhesion	−6.82719
			Maternal placenta development	−5.90218
			Regulation of cellular response to stress	−5.46799
		GO-CC	Extracellular matrix	−2.41077
			Endoplasmic reticulum lumen	−3.075
		GO-MF	Extracellular matrix binding	−5.25538
			Integrin binding	−4.16016
			Receptor ligand activity	−3.75308
		KEGG	Focal adhesion	−3.63283
			PI3K-Akt signaling pathway	−2.94774
	2	GO-BP	Adherens junction organization	−5.59068
			Regulation of postsynaptic membrane potential	−5.60297
			Chemical synaptic transmission	−5.23818
		GO-CC	GABA-ergic synapse	−8.84966
			Postsynapse	−6.96702
			Catenin complex	−5.98056
		GO-MF	Calcium ion binding	−9.53532
			Transmitter-gated ion channel activity involved in Regulation of postsynaptic membrane potential	−7.53201
			Neurotransmitter receptor activity involved in regulation of postsynaptic membrane potential	−7.42159
		KEGG	Nicotine addiction	−5.55065
			GABAergic synapse	−4.51412
			Neuroactive ligand-receptor interaction	−4.44008
	3	GO-BP	Oxidative phosphorylation	−9.82045
			Mitochondrial electron transport	−9.07716
			NADH dehydrogenase complex assembly	−8.80735
		GO-CC	Respiratory chain	−8.01851
			Inner mitochondrial membrane complex	−7.4918
			Mitochondrial inner membrane	−7.24186
		GO-MF	NADH dehydrogenase activity	−9.39692
			Oxidoreductase activity	−8.92211
		KEGG	Oxidative phosphorylation	−10.0104
			Parkinson's disease	−9.8664
Cortex-Juvenile	1	GO-BP	Neurotransmitter secretion	−7.69881
			Signal release from synapse	−7.68605
			Glutamate receptor signaling pathway	−6.71657
		GO-CC	Postsynapse	−11.5388
			Asymmetric synapse	−9.8661
			Neuron to neuron synapse	−9.67944
		GO-MF	Ligand-gated cation channel activity	−4.65541
			Neurotransmitter receptor activity	−4.51475
		KEGG	Amphetamine addiction	−7.39501
			Circadian entrainment	−6.78808
			Glutamatergic synapse	−6.48749
	2	GO-BP	Sensory perception of sound	−4.77023
			Extracellular matrix organization	−3.55353
		GO-CC	Endoplasmic reticulum lumen	−6.79636
			Collagen-containing extracellular matrix	−6.24135
		GO-MF	Extracellular matrix structural constituent conferring tensile strength	−6.42196
	3	GO-CC	Anchored component of membrane	−6.45774
Hip-Adult	1	GO-BP	Peptidyl-tyrosine phosphorylation	−4.22617
			Toll-like receptor signaling pathway	−3.99533
			DNA biosynthetic process	−3.63926
		GO-CC	Receptor complex	−3.66532
			Plasma membrane receptor complex	−3.12908
			Membrane raft	−3.04289
		GO-MF	Ubiquitin protein ligase binding	−3.15923
		KEGG	PI3K-Akt signaling pathway	−4.32214
			Cell adhesion molecules	−4.03899

(Continues)

Table 4. Continued

Group	Module	Category	Description	log *P*
			Rap1 signaling pathway	−3.56417
	2	GO-BP	Chemical synaptic transmission	−9.52989
			Anterograde trans-synaptic signaling	−9.52989
			Trans-synaptic signaling	−9.48672
		GO-CC	Synaptic membrane	−8.01839
			Presynapse	−7.64382
			NMDA selective glutamate receptor complex	−7.32244
		GO-MF	Glutamate receptor activity	−8.5353
			NMDA glutamate receptor activity	−7.9951
			Transmitter-gated ion channel activity	−7.06839
		KEGG	Cocaine addiction	−9.7719
			Neuroactive ligand-receptor interaction	−9.35963
			Amphetamine addiction	−9.0376

pathways may be important in ASD pathology in multiple brain regions.

PPI Network Construction and Hub Genes Screening

To construct the PPI network of the overlapped DEGs in different subgroups, a minimum interaction score of 0.4 was used as the cutoff value (STRING database). The generated PPI networks were then mapped using Cytoscape for visualization and further analyses. First, based on the topological structure of these networks, the MCODE algorithm with the parameters mentioned above identified three modules in the Cortex-Adult and the Cortex-Juvenile subgroups and two modules in the Hip-Adult subgroup (Fig. 4A–C). For each module, we performed GO and KEGG enrichment analysis to predict the most related functions of the interaction network (Table 4). The module 1 of the Cortex-Adult network was associated with cell–substrate adhesion, cellular response to stress, and PI3K-Akt signaling pathway (Fig. 4A). The module 2 of the Cortex-Adult network was involved in synaptic function, especially for the GABAergic synapses (Fig. 4A). The module 3 of the Cortex-Adult network mainly participated in the regulation of mitochondrial function (Fig. 4A). The functions of module 1 and module 2 have been clearly linked to the pathomechanism of ASD [Gokoolparsadh et al., 2016]. For the module 3, mitochondrial dysfunction has recently been reported to cause cortical under-connectivity and cognitive impairment in neurodevelopmental disorders [Fernandez et al., 2019]. In the Cortex-Juvenile network (Fig. 4B), the module 1 was significantly associated with the process of neurotransmitter secretion, particularly in glutamatergic synapses. The module 2 and the module 3 were mainly involved in the extracellular matrix organization and anchored component of membrane, respectively, which may be involved in ASD by influencing the microenvironment or structure of the neurons [Nguyen, Mahida, Smith-Hicks, & Campeau, 2018; Olde et al., 2017]. For the Hip-Adult network (Fig. 4C), peptidyltyrosine phosphorylation, PI3K-Akt signaling pathway, Cell adhesion molecules, and Rap1 signaling pathway were significantly enriched in the module 1, whereas chemical synaptic transmission and drug addiction were enriched in the module 2. These pathways are critical for neuronal development and function and have been frequently linked to pathophysiological activities related to ASD. These results indicate that the calculated modules are potentially important for ASD studies.

The hub genes of the networks, which interact with multiple other genes, may play critical roles in the related

Table 5. Final Hub Genes and Its Interactors

Data	Hub gene	Interaction
Cortex-Adult	Sdc4	Cp, Dmp1, Lcn2, Vegfa, Abcg2, Met, Sdc4, Kmt2a, Xdh,
	Vegfa	Lcn2, Map3k6, Ptn, Slc2a1, Adam33, Stc1, Abcg2, Met, Spp1
	Cp	S100a10, Glul, Nefl, Gabrg2, Crh, Pcp4
Cortex-Juvenile	Gria1[a]	Kcnj6, Synpr, Grin3a, Neto2, Syn2, Gsg1l, Prkca, Prkcg, Prrt1, Htr2a
Hip-Adult	Kdr	Ubc, Igf2, Cldn5, Itgam, Alcam, Ly6a, S1pr1, Fgf14
	Grin2b[a]	Gabra2, Pdyn, Grm2, Grin3a, Doc2b, Camk4, Grin2a, Nrxn
	Ubc	Cdkn1a, Lmo7, Dyrk2, Nfkbia, Kdr, Mus81, Rad50
	Grin2a[a]	Nrxn1, Gabra2, Grm2, Grin2b, Grin3a, Doc2b, Camk4
	Itgam	Kdr, Alcam, Ly6a, S1pr1, Itga7, Rap2b,
	Pdyn	Fgf14, S1pr1, Adra2c, Grm2, Grin2b, Gabra2
	Grm2	Pdyn, S1pr1, Adra2c, Grin3a, Grin2a, Grin2b
	Gabra2[a]	Unc5c, Grin2a, Grin2b, Grin3a, Pdyn, Clic4
	Grin3a	Grm2, Gabra2, Unc5c, Grin2b, Grin2a, Ptprz1
	S1pr1	Kdr, Itgam, Adra2c, Grm2, Pdyn
	Camk4[a]	Grin2a, Grin2b, Spag5, Bicc1
	Nrxn1[a]	Auts2, Cbin1, Ntng1, Grin2a, Grin2b

[a]Reported ASD risk genes.

Table 6. Relationship Between Significant Hub Genes and Mental/Behavior Disease

Gene	Disease/phenotype	Association type	Database	PMID (disease)
SDC4	Neural tube closure	/	GO_REF:0000107	/
CP	Dementia	Biomarker	CTD_human	25490030
			HPO	12572682
	Autistic disorder	Biomarker	CTD_human	15363659
			BEFREE	12363196
	Schizophrenia	Biomarker	CTD_human	16842975
	Depressive disorder	Biomarker	HPO	
	Language delay	Biomarker	HPO	
S1PR1	Bipolar disorder	Biomarker	PSYGENET	24387768
			BEFREE	
	Impaired cognition	GeneticVariation	BEFREE	17437622
UBC	Lewy body disease	AlteredExpression	BEFREE	16380264
	Bipolar disorder	GeneticVariation	BEFREE	9433566
PDYN	Drug abuse	PosttranslationalModification	PSYGENET	28336495
		GeneticVariation	CTD_human	26502829
		Biomarker	BEFREE	16529859
		AlteredExpression		24816773
	Depressive disorder	Biomarker	PSYGENET BEFREE	23293137
		AlteredExpression		24231353
	Schizophrenia	GeneticVariation	PSYGENET	15301734
		Biomarker	BEFREE	12207142
	Amnestic state	Therapeutic	CTD_human	7768285
	Intellectual disability	Biomarker	GENOMICS_ENGLAND	
GRM2	Schizophrenia	PosttranslationalModification	BEFREE	23149219
		AlteredExpression Biomarker	LHGDN	18853337
			CTD_human	18923069
	Drug abuse	Biomarker	PSYGENET	23407939
			BEFREE	20211215
NRXN1	Autistic disorder	Biomarker	RGD	25420124
		GeneticVariation	BEFREE	26563496
		AlteredExpression		22504536
	Intellectual disability	Biomarker	CTD_human	28191889
		GeneticVariation	BEFREE	24832020
	Schizophrenia	GeneticVariation	BEFREE	26563496
		AlteredExpression	CTD_human	21424692
		Biomarker	LHGDN	17989066
	Speech delay	GeneticVariation	BEFREE	22617343
			CTD_human	20157312
	Bipolar disorder	Biomarker	BEFREE	21915259
			PSYGENET	20162629
	Drug abuse	AlteredExpression	GWASCAT BEFREE	20162629
		GeneticVariation		23942779
		Biomarker		20468056
GRIN3A	Schizophrenia	GeneticVariation	BEFREE	26257337
		Biomarker	PSYGENET	23237318
		AlteredExpression		15474907
	Bipolar disorder	AlteredExpression	BEFREE	15474907
		Biomarker	PSYGENET	
	Nicotine dependence	Biomarker	BEFREE	20084518
ITGAM	Bipolar disorder	AlteredExpression	BEFREE	19488045
		Biomarker	PSYGENET	
	Schizophrenia	AlteredExpression	BEFREE	23566496
		Biomarker	PSYGENET	
GRIN2A	Epilepsy, mental retardation, speech dyspraxia, autosomal dominant	GeneticVariation	UNIPROT	27864847
		Biomarker	CLINVAR	28492532
			CTD_human	
	Schizophrenia	GeneticVariation	GWASCAT	28540026
		Biomarker	BEFREE	27021555
			PSYGENET	22833210
	Alcoholic intoxication	GeneticVariation	BEFREE	26289945
		Biomarker	PSYGENET	24397780
	Depressive disorder	Biomarker	BEFREE	23557693

(Continues)

Table 6. Continued

Gene	Disease/phenotype	Association type	Database	PMID (disease)
		PosttranslationalModification	PSYGENET	19834457
	Autistic disorder	GeneticVariation	CTD_human	15830322
		Biomarker	BEFREE	
	Intellectual disability	Biomarker	BEFREE	20890276
				20384727
GABRA2	Drug abuse	GeneticVariation	BEFREE	20133874
		Biomarker	PSYGENET	24557088
			CTD_human	22253714
	Anxiety disorders	GeneticVariation	CTD_human	18313124
		Biomarker	BEFREE	16874763
	Autistic disorder	Biomarker	CTD_human	18821008
CAMK4	Cocaine dependence	GeneticVariation	PSYGENET	19001277
		Biomarker	CTD_human	
	Alcoholic intoxication	Biomarker	BEFREE	28734942
			PSYGENET	18606955
	Autism spectrum disorders	AlteredExpression	BEFREE	24442360

processes. Therefore, we performed a primary search in each subgroup using the degree method to isolate the hub genes. To validate the results, we then cross compared the hub genes generated using the MCC, DMNC, MNC, EPC, BottleNeck, EcCentricity, Closeness, Radiality, Betweenness, and Stress algorithms. The most significant hub genes were *Sdc4*, *Vegfa*, and *Cp* in the Cortex-Adult subgroup, *Gria1* in the Cortex-Juvenile subgroup, and *Kdr*, *S1pr1*, *Ubc*, *Grm2*, *Grin2b*, *Nrxn1*, *Pdyn*, *Grin3a*, *Itgam*, *Grin2a*, *Gabra2*, and *Camk4* in the Hip-Adult subgroup. Interestingly, some of these hub genes have been implicated in various nervous system functions and ASD (Table 5). We further annotated the function of these hub genes using the DisGenet database (http://disgenet.org/) to explore their roles in mental/behavior disorders (Table 6). The results indicated that these hub genes were involved in drug abuse, bipolar disorder, depressive disorder, SCZ, and autistic disorder, which are a group of closely related disorders that may share some similar pathomechanisms with ASD.

Finally, to evaluate whether the regulation networks related to ASD models could be conserved in human, we examined the expression levels of the hub genes with the data generated in an integrated transcriptional study using data sets of 2,000 high-quality postmortem brain samples of individuals with SCZ or ASD [Gandal et al., 2018]. The significance of the alteration of expression levels of the hub genes based on student's *t*-test were calculated (Fig. 4D). The data indicated that the expression levels of *SDC4*, *CP*, *S1PR1*, *UBC*, *PDYN*, *GRIN2A*, *GABRA2*, and *CAMK4* are significantly different in ASD patients compared to controls (*SDC4*: $P = 0.022713077$, *CP*: $P = 0.014066147$, *S1PR1*: $P = 0.004851289$, *UBC*: $P = 0.00584653$, *PDYN*: $P = 0.0000000247$, *GRIN2A*: $P = 0.001655659$, *GABRA2*: $P = 0.005809766$, *CAMK4*: $P = 0.00000117$). The expression levels of *UBC*, *GRM2*, *NRXN1*, *PDYN*, *GRIN3A*, *ITGAM*, *GRIN2A*, *GABRA2*, and *CAMK4* are significantly different in SCZ patients compare to controls (*UBC*: $P = 0.019168799$, *GRM2*: $P = 0.00225209$, *NRXN1*: $P = 0.017605625$, *PDYN*: $P = 0.001230074$, *GRIN3A*: $P = 0.032131135$, *ITGAM*: $P = 0.000325919$, *GRIN2A*: $P = 0.022841965$, *GABRA2*: $P = 0.000902351$, *CAMK4*: $P = 0.009384225$). These results are in agreement with our analyses, supporting that the expression levels of these genes are potentially important for ASD.

Discussion

The genetic mechanisms underlying ASD have been regarded as highly heterogeneous, and an extensive number of genes have been reported to be associated with the disease. Since a large number of identified ASD risk genes were reported to be related to gene expression regulation, it is possible that a number of overlapped downstream genes may participate in the pathology of the disease. Therefore, we conducted an integrated transcriptome analysis of ASD mouse models to identify the overlapped downstream genes and pathways, which may be important for understanding the molecular mechanisms of ASD, which would potentially benefit the future development of novel diagnosis and treatment methods.

To this end, we characterized the alterations of expression profiles in multiple ASD mouse models and identified overlapped DEGs of various data sets in several subgroups. Indeed, a number of overlapped DEGs were identified in these subgroups, supporting our hypothesis. Thus, we demonstrated the role of the overlapped DEGs play in the etiology by ASD-related genes enrichment analyses, GO and KEGG enrichment analyses in each subgroup. ASD related genes enrichment analyses showed the significant overrepresentation of known ASD susceptibility genes, indicating the reliability of our data and potential notogenesis in common. As for GO and KEGG enrichment analyses, we found that structural and functional descriptions related

to synapse and cell adhesion were clearly associated with the role of the overlapped DEGs in the three subgroups, consistent with the previous studies [Gokoolparsadh et al., 2016]. This further suggested that the defects of synapse might be a universal and important cause of ASD, especially the glutamatergic synapses. In addition, our analyses revealed that pathways related to drug addiction may also be involved in ASD (e.g., Amphetamine addiction and Nicotine addiction), and the underlying rewarding mechanisms may be important for both processes. Interestingly, the convergence of pathways between ASD and drug addiction was actually proposed before [Rothwell, 2016]. Moreover, it has been reported that chronic drug exposure could cause changes in the structure and function of synapses, and the abnormal synaptic plasticity then contributes to a variety of behavioral deficits in rodents [Grueter, Rothwell, & Malenka, 2012]. Besides, several functional annotations in the corresponding subgroups are worthy of further in-depth characterization, such as cell morphogenesis involved in differentiation, NADH dehydrogenase activity and oxidative phosphorylation in the Cortex-Adult subgroup; G-protein alpha-subunit binding, acetylcholine receptor signaling pathway and calcium signaling pathway in the Cortex-Juvenile subgroup; intrinsic apoptotic signal, MAPK cascade and cAMP signaling pathway in the Hip-Adult subgroup. These biological processes have been reported to be essential for various aspects of the nervous system, such as neurodevelopment, behavior, synaptic plasticity, axon pathfinding, and so forth, many of which have been associated with ASD. Interestingly, dysfunction of mitochondria, calcium and MAPK and cAMP signaling pathway have been reported to associate with abnormal synaptic plasticity [Fernandez et al., 2019; Booker et al., 2018; Vithayathil, Pucilowska, & Landreth, 2018; Cui et al., 2008; Zamarbide et al., 2019]. It is possible that the different transcriptional factors may result in ASD by the final dysfunction of synapses. It is interesting that different GO terms and pathways are enriched in the three subgroups. The functional divergence of transcription factors may contribute to the phenomena. It would be ideal to analyze the transcriptome of the same group of transcriptional factors in different brain regions. However, due to data availability, the composition of transcriptional factors varies among these groups. In addition, the age and tissue specificity may also contribute to the differences in the transcriptional networks. For instance, the genes expression profile in the brain has been proved to undergo dramatic changes with cortical development and aging [Colantuoni et al., 2011].

Furthermore, we constructed the PPI networks of the overlapped DEGs and then identified in total eight modules in the subgroups based on the topological interaction structure. We then performed GO and KEGG enrichment analyses for these modules and the common functions of the genes in each module are mainly linked to nervous system development and function, indicating that these modules may potentially form biological complexes that are involved in ASD. Next, we isolated the hub genes in these modules based on 11 algorithms. *Sdc4*, *Vegfa*, and *Cp* are the hub genes in the Cortex-Adult subgroup, which have been shown to execute various neurological functions. *Sdc4*, which encodes a cell-surface heparan sulfate proteoglycan, regulates gastrulation, neural tube closure and directed neural crest migration by wnt/PCP signaling pathway [Escobedo et al., 2013; Lin, Lu, Chen, Cheng, & Lin, 2015], *Vegfa* is related to Alzheimer's disease and neuronal apoptosis [Lanke, Moolamalla, Roy, & Vinod, 2018; Zhang, Wang, & He, 2018]. *CP*, which encodes a ceruloplasmin, is related to oxidative stress. It has been implicated that the decreased expression level of *CP* diminishes the protective capacity against brain damage in ASD patients [Yui, Imataka, Kawasak, & Yamada, 2016]. *Gria1* is the hub gene in the Cortex-Juvenile group, and it has been associated with ASD susceptibility. It encodes an AMPA receptor subunit and is essential for glutamatergic synaptic transmission [Salpietro et al., 2019]. For the Hip-adult group, there are 12 hub genes identified, including *Kdr*, *S1pr1*, *Ubc*, *Grm2*, *Grin2b*, *Nrxn1*, *Pdyn*, *Grin3a*, *Itgam*, *Grin2a*, *Gabra2*, and *Camk4*. Among them, *Grin2b*, *Nrxn1*, *Grin2a*, *Gabra2*, and *Camk4* have been associated with ASD based on human genetics evidence, indicating that this integrated analysis indeed enriched genes that are important for ASD [Gonzalez-Nunez, 2015; Leblond et al., 2019; Tarabeux et al., 2011; Zech et al., 2018]. Moreover, the other genes including *Kdr*, *S1pr1*, *Ubc*, *Grm2*, *Pdyn*, and *Itgam* are possible candidates for ASD due to the related molecular functions. For examples, *S1pr1* regulates multiple aspects of sensory neuron and immune system functions [Healy & Antel, 2016]; *Ubc* encodes a ubiquitin C and participates in neuronal development and maintenance; *Grm2* is associated with depression and memory network related to glutamatergic synapse [Jin et al., 2018; Lyon et al., 2011]; *Pdyn* encodes a prodynorphin, which is closely related to drug abuse and synaptic plasticity [Henriksson et al., 2014]; and *Itgam* has been reported to be involved in the debris clearance of microglia [Norris et al., 2018]. Therefore, it is possible that the misregulation of these genes contributes to certain phenotypes of ASD.

Finally, we used human brain transcriptional data sets to examine whether the DEGs we isolated are aberrantly regulated cross-species. Most of these genes expressed differentially in human ASD or SCZ samples. Notably, the expression levels of *SDC4*, *CP*, *S1PR1*, *UBC*, *PDYN*, *GRIN2A*, *GABRA2*, and *CAMK4* are significantly altered in ASD patients. Among these genes, *UBC*, *PDYN*, *GRIN2A*, *GABRA2*, and *CAMK4* are also differentially expressed in SCZ patients. These genes may be involved in both ASD and SCZ since the two disorders are both related to neurodevelopment and may be caused by the disruption of some common processes. In addition, *GRM2*, *NRXN1*, *GRIN3A*, and *ITGAM* are misregulated in SCZ. Moreover, some of the hub genes have been linked to other neurobehavioral diseases, including

drug abuse, bipolar disorder, and ID. Taken together, our integrated transcriptome analysis with mouse models has highlighted a group of genes, which are evidently associated with human ASD and other related neurological diseases.

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Conflict of Interest

The authors declare no competing interests or other interests that might be perceived to influence the results and/or discussion reported in this article.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Table S1. GO and KEGG enrichment analysis of the overlapped genes in Cortex-Adult subgroup.

Supplementary Table S2. GO and KEGG enrichment analysis of the overlapped genes in Cortex-Juvenile subgroup.

Supplementary Table S3. GO and KEGG enrichment analysis of the overlapped genes in Hip-Adult subgroup.