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*Original Article*

# The gene expression patterns of neuronal cells reveal the pathogenesis of autism

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**Abstract:** Objective: To explore the effects of early comprehensive and specific behavioral interventions on improving social communication and attenuating anxiety in autism patients. Methods: This study was based on the modular comprehensive analysis method and explored the neurotransmitter conduction mechanism in the pathogenesis of autism. We downloaded autism-related data from the Gene Expression Omnibus (GEO) database and performed a differential analysis, a co-expression Network Analysis (WGCNA) analysis, a GO function, and a KEGG pathway enrichment analysis. Finally, we predicted the non-coding RNAs (ncRNAs) and transcription factors (TFs) that regulate the module on the basis of hypergeometric testing. Results: We obtained five co-expression modules, in which the active regulatory effects of the MTA3, PHB2, TNXB, DCTN2, and RBM23 genes in the dysfunction modules were obtained. The module genes were predominantly involved in biological processes and significantly regulated the mRNA monitoring and calcium signaling pathways. In addition, we identified a prominent regulation effect of the ncRNA and TF pivots on the dysfunction modules. Conclusions: This study deciphered a comprehensive network of key genes involved in autism, and it reveals the main dysfunction modules, the latent regulatory factors, and the driver genes for autism, contributing to our further understanding of the mechanisms of autism neuronal conduction disorders.

**Keywords:** Autism, key genes, enrichment, co-expression analysis, regulatory factors

## Introduction

Originally, autism was considered to be an innate incapability of normal, biologically determined emotional contact with other people [1]. Subsequently, two domains of impairment were identified, namely social communication and interaction, and restricted repetitive behavior. Its global prevalence is estimated to be about 1%, and 70% of patients have greater odds of complications [2]. Genetic and epidemiological studies demonstrate that autism is a complex disease related to genetic and environmental factors [3]. The environmental factors include genetic mutations or deletions after vaccination, viral infections, and encephalitis, while inflammation is the main cause of placental defects, immature blood-brain barriers, the mother's immune response to infection during pregnancy, preterm birth, encephalitis in postnatal children, or a toxic environment [4]. In autism, a highly heritable disease of universal neurodevelopmental disorders and genetic factors are considered the major etiology. Dual specific phosphatase 15 (DUSP15) has been recognized as a key regulatory gene for oligodendrocyte differentiation and is probably a susceptibility gene for autism in the Chinese Han population [5]. Risk alleles and haplotypes near MEGF10 TSS may regulate the transcriptional activity and leave it susceptible to autism [6]. Moreover, it has been proved that PCDHA acts as a potential candidate gene for autism [7]. To date, scientists have found some potential therapeutic targets for autism, such as miR-486-3p [8], miR-132 [9], MSNP1AS [10], and THRIL [11]. Universally, early intervention using various techniques is beneficial to the treatment of autism, yet it still fails to remove the radical cause [12]. Likewise, drugs at present cannot secure a complete cure of the disease and instead simply relieve its symptoms, including self-harm, aggression, repetitive and stereotyped behavior, inattention, hyperactivity,

and sleep disorders [13]. Despite the growing body of research on autism, the exact pathogenesis remains unclear. To deeply investigate the pathogenesis of autism, we determined the dysfunction modules and core molecules using a systematic modular analysis, further exploiting the key genes related to autism neuronal conduction disorders.

## Materials and methods

### *Data resource*

The NCBI GEO Dataset [14] has abundant and high-throughput experimental data that contains single- and dual-channel microarray-based assays for mRNA abundance, genomic DNA, and protein molecules. Additionally, this study included data from non-array-based high-throughput functional genomics and proteomics technologies. First, the related gene expression profiles for autism neuronal cells were collected from GEO, numbered GSE67528 [15]. Then, the ncRNA-mRNA interaction pairs with a score ≥ 0.5 were screened out from the RAID v. 2.0 database [16], in which 431937 interaction pairs with 5431 ncRNAs are involved. Over 5.27 million RNA-related interactions were enrolled in the RAID v. 2 database, with over 4 million RNA-RNA interactions and more than 1.2 million RNA-protein interactions comprising 130,000 RNA/protein symbols, which is conducive to observing various RNA-related interactions. Among them, overall human TF target data were downloaded from the TRRUST v. 2 database and employed in this study [17], involving 2492 TFs and 9396 interaction pairs.

### *Differentially expressed genes*

In the present study, the R language DESeq2 package [18, 19] was employed for a differential expression analysis of the data. The R language DEseq2 analysis process consisted of 3 main steps, normalization, dispersion estimation, and differential expression testing. Using a weighted conditional likelihood and by simulating the dependence of dispersion on the average expression, we alleviated the dispersion estimate of each gene, making it tend to be a general estimate of global genes, or a local estimate of genes with similar expression strengths, measured and corrected for low dispersion to estimate the intensities of all the samples. BBSeq was used to simulate dispersion on the mean, and the average absolute deviation of the dispersion estimate was used to drive down the influence of the outliers. The Bayesian method was utilized to estimate the dispersion of a single gene, which can explain the heterogeneity of the dispersion value of different genes. BaySeq and ShrinkBayes estimated the priors of the Bayesian model of all the genes, leaving the posterior probability or the false discovery rate (FDR) for the differential expression.

### *Co-expression analysis*

To evaluate the drivers of neuronal conduction disorders in autistic patients, we separately targeted the ASD-derived iPSC-derived neurons and the normal iPSC-derived neurons, the ASD-derived iPSC-derived neural progenitors, and the normal iPSC-derived neural progenitors. Our ASD differential analysis of the IPSCs and the normal IPSCs yielded differential gene expression profiles for autism. Furthermore, we used WGCNA for an analysis of the differential expression matrix of autism and for a search for the co-expressed gene modules. Initially, the correlation coefficient between any two genes was obtained after applying the gene correlation coefficient to the power of N. The subjection of the connections in the network to scale-free networks resulted in a significant algorithm in terms of biology. A hierarchical clustering tree was established. The diversity of the gene modules was represented by the diverse branches of the clustering tree, and the varying colors were indicative of the various modules. Therefore, based on the gene regulatory power in each dysfunctional module, the genes that give rise to dysfunctional modules were evaluated and considered to be the key genes in the pathogenesis of neuronal conduction disorders.

### *Patient and blood samples*

All the blood samples were confirmed by experienced pathologists. All the patients signed the informed consent forms after being fully informed of the study. The human tissue samples were collected following the *International Ethical Guidelines for Biomedical Research Involving Human Subjects*. This study was approved by the Department of Psychiatry, Qingdao Municipal Hospital, and carried out according to the regulations of the Department of Psychiatry, Qingdao Municipal Hospital. Ethics approval number: 2018-12-21.

*Verification of key genes by qPCR*

A reverse transcription kit was used for the extraction of blood total RNA and cDNA transcriptions, and a SYBR qPCR Detection Kit was for the qPCR reactions. The hot-start iTaqTM DNA polymerase was activated through a 3-minute denaturation at 95°C, followed by 45 denaturation cycles at the same temperature for 10 seconds. Subsequently, annealing and extension at 60°C for 45 seconds were performed. β-actin was taken as the internal reference source.

*Enrichment analysis*

The determination of the functions and signaling pathways in gene expressions invariably yields favorable effects on studying the disease mechanisms at a molecular level, and an enrichment analysis of the dysfunctional modules, including gene functions and pathways, is considered effective at evaluating the pathogenesis of neuronal conduction disorders in autism. Therefore, the Go language function and the R language ClusterProfiler package for the KEGG pathway enrichment analysis were selected for the five modular genes of autism [21]. As a bioconductor software package, ClusterProfiler can statistically analyze and visualize the functional clustering of gene sets or clusters.

*TFs and ncRNAs that regulate dysfunctional modules*

ncRNA and TF, in most cases, serve as the main impetus for gene transcription and post-transcriptional regulation. Therefore, their effects on the autism dysfunction module were scientifically predicted and tested. The pivot regulators were specified as modulators with a crucial regulatory impact on modules during the onset of neuronal conduction in autism, including ncRNA and TF. Having over two control connections between the regulators and the modules was indispensable, with a *P*-value of the enrichment target less than 0.01 through the hypergeometric test.

*Statistical analysis*

The autism-related data in the GEO database were downloaded, and a differential analysis, a WGCNA analysis, and a GO function, as well as a KEGG pathway enrichment analysis, were performed.

## Results

*Identification of the autism-related expression disorder molecules*

In terms of the genetic characterizations of the ASD patients, there are at least two categories of ASD patients: qualitative trait inheritance patients (single gene, symptomatic ASD) and quantitative trait inheritance patients (multigene, classic ASD). Classic ASD: The cause is unknown (polygenic); the early development seems to be normal, the appearance is normal, the early intelligence is mostly normal, the intervention is social-centric, and the early intervention is effective. Symptomatic ASD: The cause is clear, early developmental delays are common, there are special external characteristics, most of the patients have mental retardation, etc., comprehensive training and treatment are commonly seen, and the effects of the training are unsatisfactory.

Various studies on the pathogenesis of autism have been conducted by biologists who then determined the potential pathogenic genes for the condition. However, the connections among these genes at the molecular level and their overall effects are not clear. To inspect the molecular changes in the progression of neuronal conduction disorders in autistic patients, we performed a microarray analysis to further identify the differential expressions of the genes between the APS-derived iPSC-derived neuronal cells and the normal iPSC-derived neuronal cells, between the ASD-derived iPSC-derived neural progenitor cells and the normal iPSC-derived neural progenitor cells, between the ASD-derived IPSCs and the normal IPSCs (DEG), and 2058, 1460, and 1101 differentially expressed genes (**Figure 1A-C**) were obtained. We believe that some of these genes cause neuronal conduction disorders in autistic patients. For the Venn diagram showing the overlapping differential genes, we found that 17 of the three sets of differential results overlapped (**Figure 1D**).

*Identification of the autism-related functional disorder modules*

The combination of the three sets of the differential genes resulted in 4192 differential genes

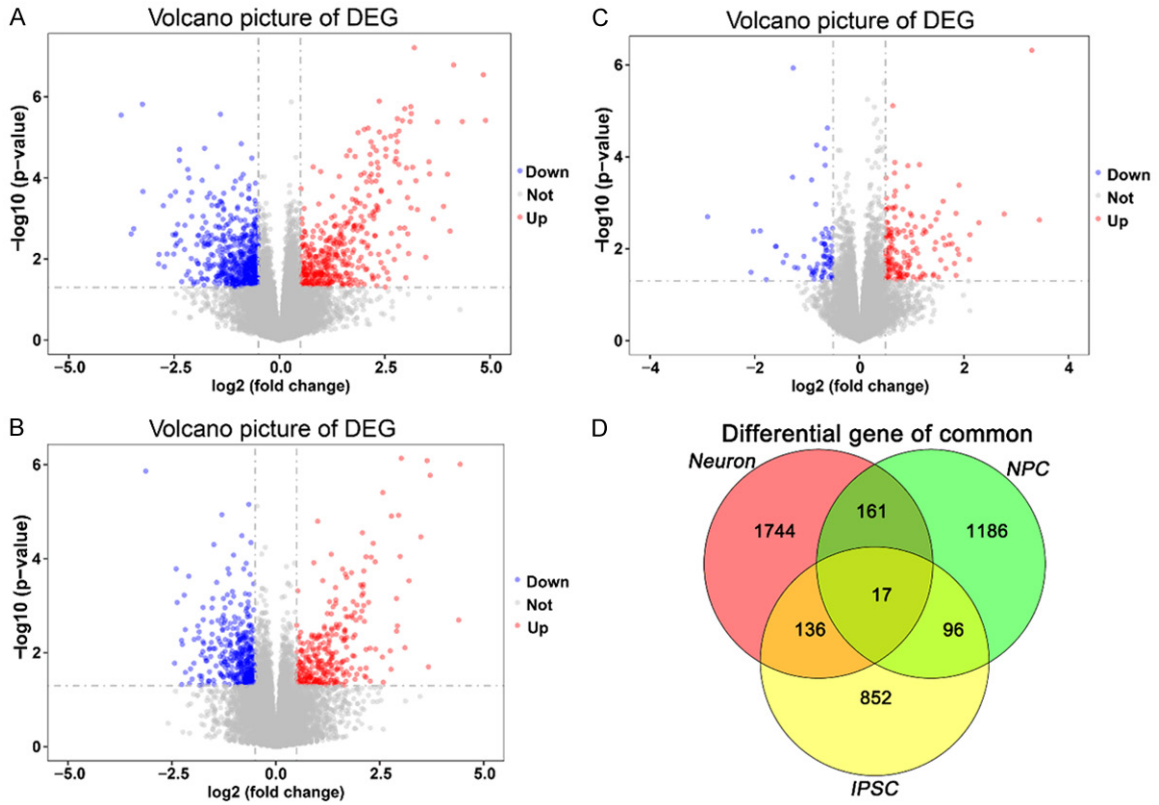


**Figure 1.** A-C. Volcanic maps of the three sets of differential genes for autism. D. A Wayne diagram of the three different sets of genes for autism.

that led to neuronal conduction disorders in autistic patients. Based on the 4192 differential genes and their interacting genes in the autism and patient samples, an expression profile matrix was built. With WGCNA, an apparent inclination towards gene co-expression in groups was observed. The global complex systems can be handled by Modularity and divided into more elaborated and coordinated subsystems with their functions. As for the elemental genes, genes with a synergistic expression relationship and genes with a consistent expression were collected by the module. Also, each module also interacts with its effect standing for the gross characteristics, constructing a bridge for each gene to operate. In terms of the expression pattern, this is an effective method that can aggregate the autism expression behavior in patient samples into modules to determine the complicated cooperative correlations among the genes. Accordingly, using the co-expression panel as a module to make the determination, five functional disorder modules for autism were collected, including 3395 differential genes (**Figure 2**). According to the dysfunction module, the key genes of each module were clarified, and the core genes, such as MTA3, PHB2, TNXB, DCTN2, and RBM23, were obtained (**Table 1**). In addition, the key genes' expression trend, confirmed using qPCR (**Figure 3**), was consistent with the results of prior studies (**Table 1**).

*Functions and pathways involved in the genes of interest*

The exploration of the functions and pathways in the dysfunctional module gene was successful, at the molecular level, in determining the upstream and downstream relationships of the gene in the same pathway of the module, contributing to bridging the module and the disease in systematic biology, and enhancing the knowledge regarding the disease mechanism. Five modules were analyzed for GO function and KEGG pathway enrichment, with 2997 biological processes, 553 cells, 768 molecular functions, and 80 KEGG pathways found (**Figure 4**). We found that the module gene was

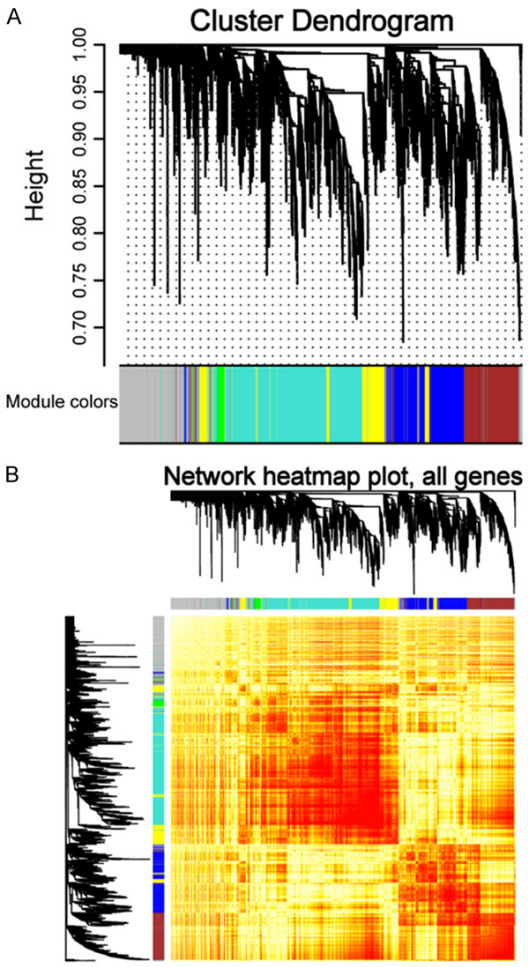


**Figure 2.** A synergistic expression of the differential genes of autism in the patient samples. A. The 5 co-expression groups via clustering were identified as modules, and the 5 colors represent 5 co-expression modules. B. Here are heat maps of all the gene expressions in the sample, and their expression behaviors are clustered into 5 co-expression modules.

notably drawn in biological processes such as ion channel activity, substrate-specific channel activity, active regulation of the cellular amide metabolism, and small molecule catabolism. These processes were observed to be active in the mRNA monitoring pathways, RNA degradation, the calcium signaling pathways, and the signaling pathways such as neuroactive ligand-receptor interactions and ECM-receptor interactions.

*TFs and ncRNAs that drive the neuronal conduction disorders in autistic patients*

In terms of systematic biology and genetics, TFs and ncRNAs are frequent regulators of expression and function. Notwithstanding the research hotspot among biologists on the single or multiple TF and ncRNA regulating of the pathogenic mechanisms of autistic patients, the total effect on the dysfunctional mechanisms and disease progression has been rarely studied. Thus, in this study, a co-module analysis was performed to assess the main transcriptional regulators in the neuronal transmission disorders in autism. The predicted results showed 433 ncRNAs involved in 583 ncRNA-module regulatory pairs and 31 TFs involved in 32 TF-module target pairs. Moreover, we statistically analyzed the quantity of the pivot control modules and obtained ncRNA (CRNDE, miR-103a-3p, miR-106b-5p, etc.) and dysfunctional modules TF (MYC) most regulated. These TFs might regulate the progression of neuronal conduction disorders in autistic patients using the signaling pathways involved in gene involvement (**Figure 5**). Therefore, the latent regulatory factors were identified as dysfunctional molecules of the neuronal conduction disorder.

## Discussion

Autism, a complex neurodevelopment disorder, is characterized by weakened social interactions and communication as well as restricted, repetitive, or stereotyped behavior [22]. There are many controversies about the etiology of autism, but the underlying pathogenesis remains unclear. In this study, the genes of iPSC-derived neuronal cells, the iPSC-derived neural progenitor cells, and the IPSCs in autism patients in the NCBI GEO Dataset were collected in order to analyze the autism dysfunction modules driven by the TFs and ncRNAs regulators based on the differential gene expression profile data for autism, to understand, from a molecular point of view, the mechanisms of neuronal conduction disorders in autistic patients. At the module level, we first noticed that the two modules of the gene were significantly involved in the mRNA monitoring pathways, RNA degradation, the calcium signaling pathways, and the signaling pathways such as neuroactive ligand-receptor interactions and ECM-receptor interactions. Additionally, mRNA monitoring promotes the detection and destruction of mRNAs containing premature stop codons through a process called nonsense-mediated decay, thereby preventing the synthesis of truncated and other abnormal proteins, which might exert dominantly negative

**Table 1.** The core genes within the autism-related dysfunction module

| colour | HubGenes | Module |
|---|---|---|
| blue | PHB2 | m2 |
| F | 5'-GGGGTACCCCCCACCATGGACTACAAAGACGATGACGACAAGATGGCCCAGAACTrGAA-3' | |
| R | 5'-GGAATTCCTCATTTCTTACCCTT-3' | |
| brown | MTA3 | m3 |
| F | 5'-CAGUGUAGAUUAUGUGCAATT-3' | |
| R | 5'-AGAUAAGCAUGcuAAAGAATT-3' | |
| green | TNXB | m5 |
| F | 5'-TCACCGTGCAGTACAAGGAC-3' | |
| R | 5'-CCCGAGACTCCAAGCACTAC-3' | |
| turquoise | DCTN2 | m1 |
| F | 5'-CTAGCTAGCATGACCGCAGAGGATTCCACC-3' | |
| R | 5'-GGAATTTAAATTCAGTATGTCTGGTAGACGTCG-3' | |
| yellow | RBM23 | m4 |
| F | 5'-CTAGCTAGCATGGCATCTGATGACTTTGAC-3' | |
| R | 5'-CGCGGATCCTTACATGGTCTGGGGGGTAAA | |

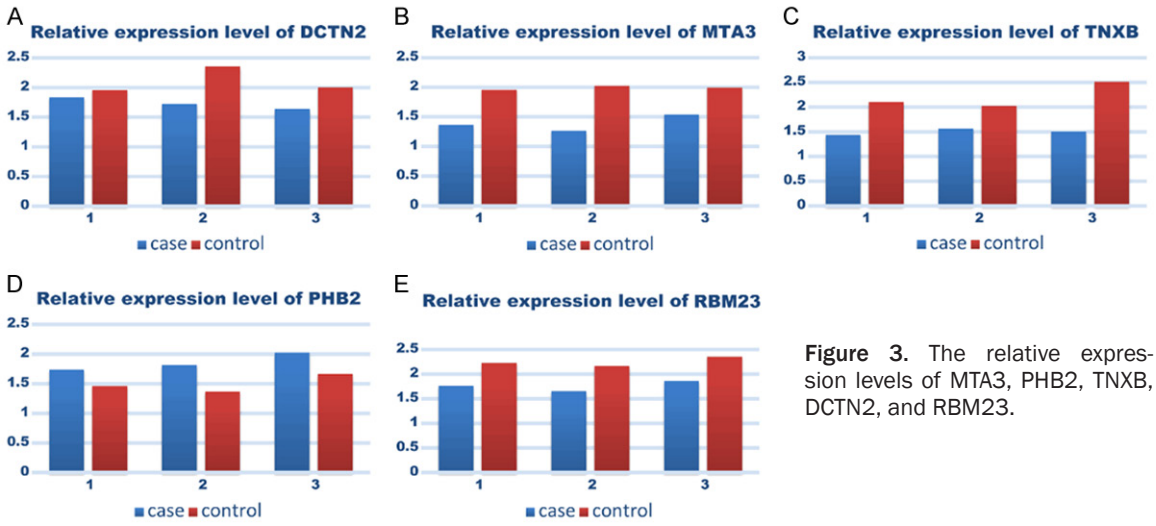


**Figure 3.** The relative expression levels of MTA3, PHB2, TNXB, DCTN2, and RBM23.

and harmful effects [23]. Any interruption of the RNA degradation process can seriously affect the neurons [24]. Calcium is one of the most versatile biological signaling molecules, regulating physiological systems from membrane potential and ion transporters to various levels of kinases and TFs [25]. Moreover, $Ca^{2+}$ signaling is involved in synapse formation and dendritic growth in proteins associated with ASD, while several voltage-gated and ligand-gated ion channel mutations that regulate neuronal excitability and $Ca^{2+}$ signaling are also associated with Related [26]. The signaling of neuroactive ligand-receptors is correlated with the process of synaptic transmission [27]. In addition, the activation of ECM receptors regulates the downstream signaling cascade, which controls the cytoskeletal dynamics and the synaptic activity to regulate neuronal structure and function, thus affecting animal behavior [28].

We predicted 433 ncRNAs that mediate neuronal conduction disorders in autism via a mediator module. It was demonstrated that CRNDE, miR-103a-3p, and miR-106b-5p, etc. exert significant effects on three dysfunctional modules. LncRNA CRNDE gives rise to inflammation through the TLR3-NFκB-cytokine signaling

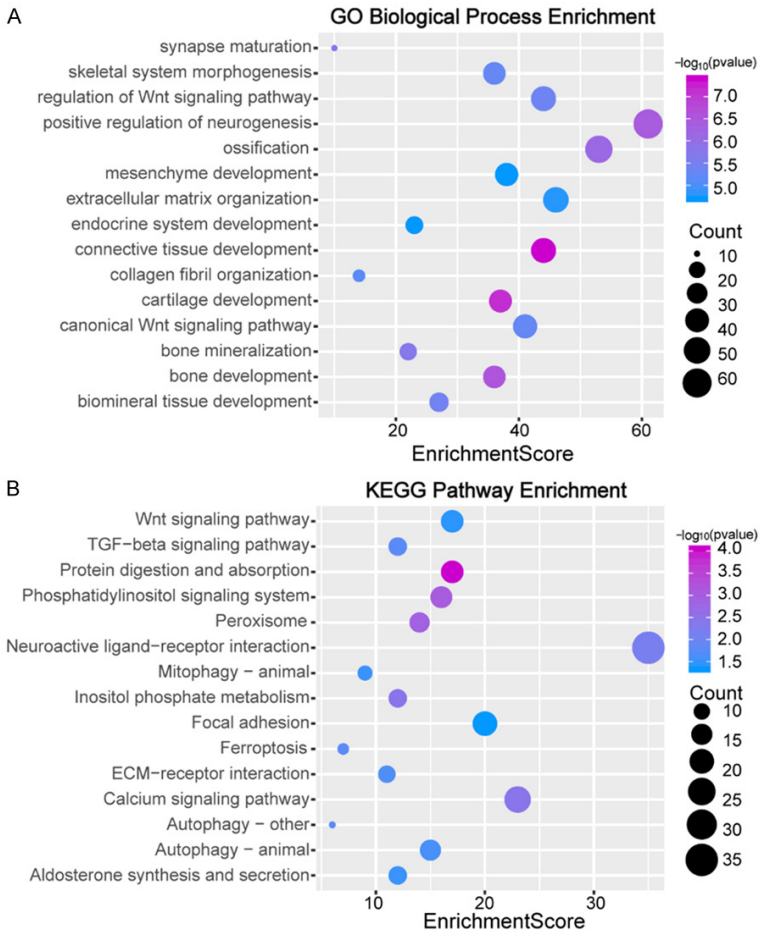


**Figure 4.** Functional and pathway enrichment analysis excerpts of the module gene. A. A functional enrichment analysis excerpt of the module gene GO. From blue to purple, the enrichment increases significantly. The larger the circle, the greater the proportion of the module gene in the GO function entry gene. B. A functional enrichment analysis excerpt of the module gene KEGG. From blue to purple, the enrichment increases significantly. The larger the circle, the greater the proportion of the module gene in the KEGG function entry gene.

pathway [29]. CRNDE boosts the proliferation, invasion, and migration of osteosarcoma cells by regulating Notch1 signaling and the epithelial-mesenchymal transition [30]. Moreover, miR-103a-3p acts as a tumor suppressor by binding to Linc00152 to regulate the malignant behaviors of glioma stem cells [31]. MiR-106b-5p promotes invasiveness and the stem cell-like phenotype of renal cell carcinoma by activating Wnt/β-catenin signaling [32]. The potential function of the above regulators in ASD has rarely been studied. However, this study clearly indicates that CRNDE, miR-103a-3p, and miR-106b-5p, as ncRNAs that regulate the most dysregulated ASD dysfunction, may be potentially crucial in the regulation of neuronal conduction disorders in ASD patients. They are latent candidates for further molecular experimental validation studies. Other ncRNAs that substantially participate in the regulation of the autism dysfunction module may be possibly drawn in the main process of autism neuronal conduction disorders.

Last but not least, we evaluated 31 TFs which were expressed in different degrees and evidently regulate the dysfunctional module of autism. The regulatory analysis showed that MYC predominantly regulates two modules, which differs from autistic patients. Pam, an MYC-associated protein, regulates cell growth and proliferation and neuronal function through the TSC/mTOR pathway in mammalian cells [33]. MYC, primarily a transcription factor, accelerates the expression of many target genes to organize death, proliferation, and metabolism [34]. More importantly, the downregulation or inactivation of MYC leads to impaired cell cycle progression [35]. Parikshak has emphasized that [36] the substantial genetic contributions which ASD involves are predominantly heterogeneous, and they may converge on common pathways that are not yet well understood. Therefore, through our post hoc genome-wide transcriptome analysis of the largest sample size, the non-coding transcriptome, the alternative splicing, and the upstream molecular regulators were interrogated to deepen our knowledge of molecular convergence in ASD. The contribution of SOX5 (a TF in the regulation of neuronal fate) to this reduction lies in regional differences. In addition, the results indicated that through genetic mechanisms, the defined subtype of ASD, chromosome 15q11.2-13.1 duplication syndrome,

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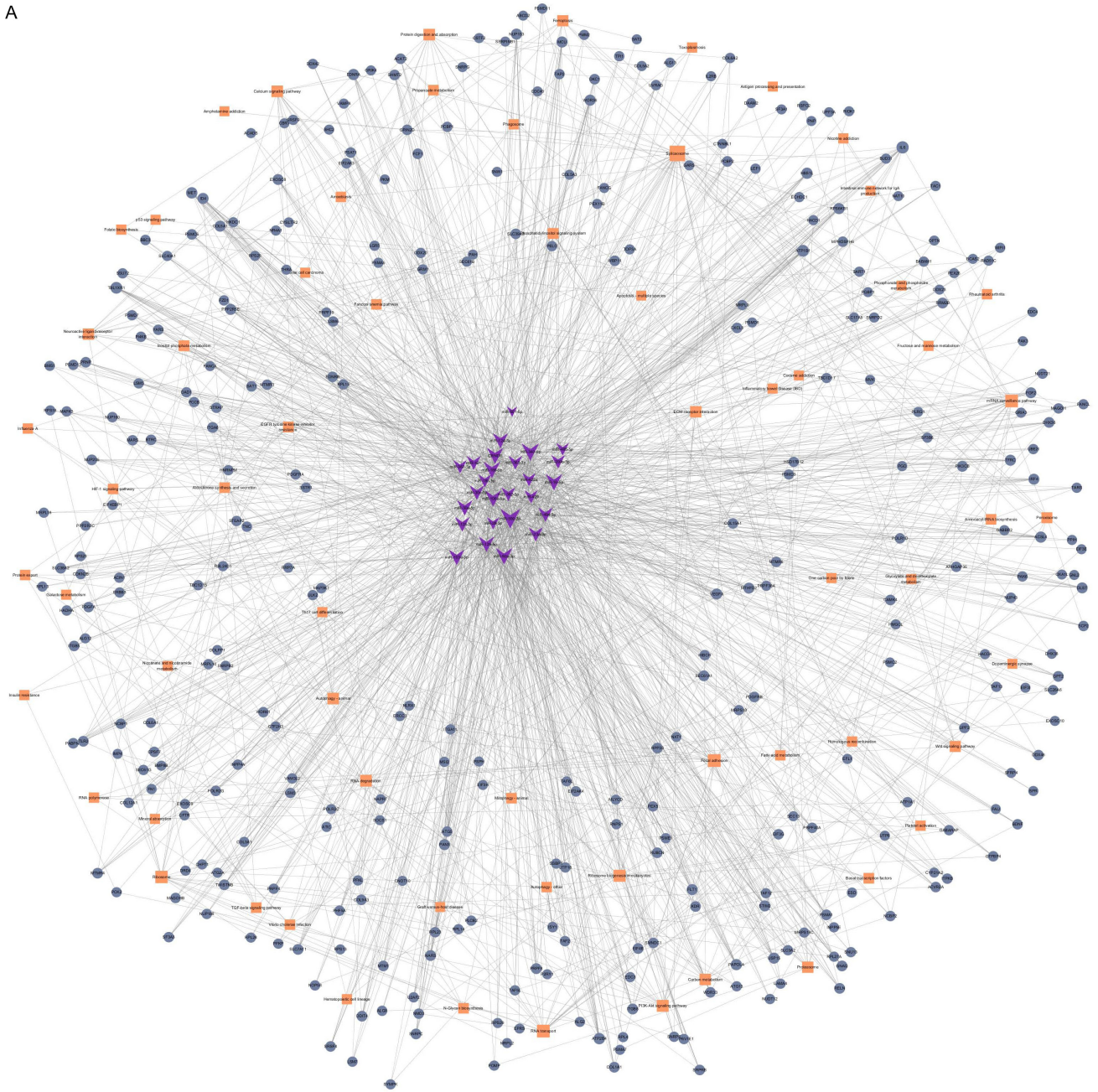

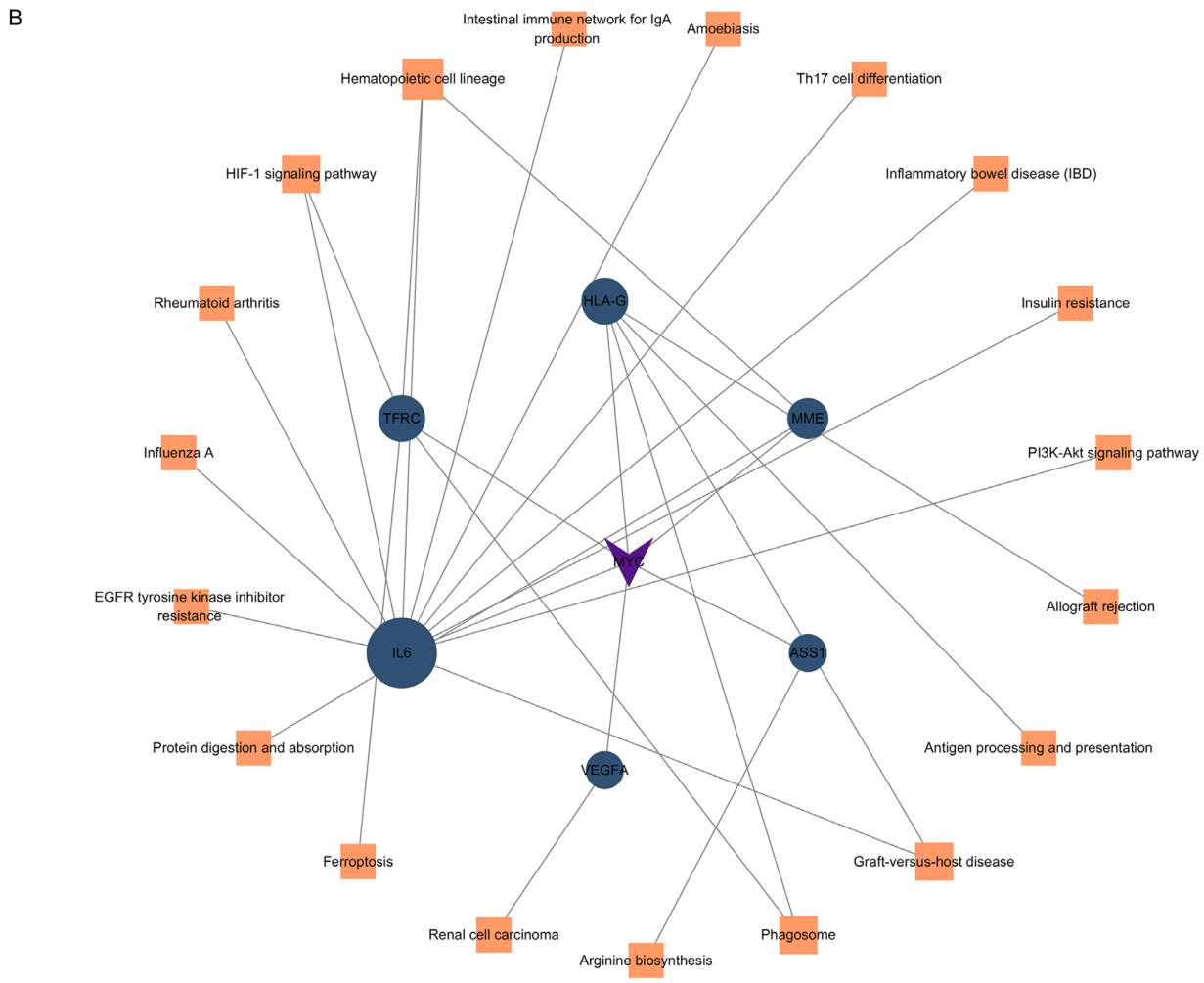


**Figure 5.** Signaling pathways by regulator-mediated genes involved in disease. The purple arrow represents the regulator, the blue circle stands for the gene, and the orange square symbolizes the participation pathway.

shares the core transcriptome characteristics obtained in idiopathic ASD. It also revealed age-related changes in the track of microglial and synaptic function in individuals with ASD in the first twenty years, which suggested the possible impact of genetic risk for ASD on the regional cortical gene expression changes. The results illustrated that the approach of diverse genetic perturbations results in phenotypic convergence at multiple biological levels in a complex neuropsychiatric disorder. This research is limited by the absence of the analysis of the condition and the clinical phenotype of ASD, and an in-depth study on the distribution of allele frequencies in mild, moderate, and severe cases of ASD. Moreover, the differences in the intelligence scores of different genotypes have not been taken into consideration. These will be further investigated in future studies to obtain more clinical evidence of ASD.

## Conclusion

No studies have found the effect of MYC on neuronal conduction in autistic patients, but our analysis shows that MYC regulates the dysfunctional modules at the most, suggesting its potential role in neuronal conduction disorders in ASD patients and indicating that it can be a candidate for further molecular experiments. Furthermore, other TFs that evidently regulate the autism dysfunction module may also be involved in the basic process of neuronal conduction disorders in ASD patients, which needs to be confirmed experimentally.

## Disclosure of conflict of interest

None.

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