Original Research

Expressional of neurotrophins on neural tube defects the hub BDNF and NGF signaling pathways

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Abstract

Objective: This study uses bioinformatics to analyze the differentially expressed genes (DEGs) in the NTDs. Methods: R software screens differentially expressed genes, and the WebGestalt functional enrichment analysis tool conducts Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomics (KEGG) pathway analysis. The Search Tool for the Retrieval of Interacting Genes/Proteins constructs protein interaction networks, and the cytoHubba plug‐ in in the Cytoscape software predicts core genes. Subsequently, the NTD model of SD mouse was established and the tissues were isolated. BDNF, NGF, TrkA and TrkB were verified by immunofluorescence staining. Real-time polymerase chain reaction assay and Western blotting assay were used to detect the mRNA and protein expression of BDNF, NGF, TrkA and TrkB. Results: A total 2057 DEGs were identified in the brain remnant tissue samples, including 1415 upregulated and 642 downregulated. A total of 2161 DEGs were identified in the spinal cord tissue samples, including 994 upregulated and 1067 regulated genes, a total of 239 commonly DEGs. GO and KEGG revealed among the 2057 DEGs in the brain remnant tissue samples, KEGG analysis found that they were enriched in the has0472 neurotrophic factor pathway, etc. GO analysis showed that the 2057 DEGs in the brain remnant tissue samples were involved in biological such as cognition, learning and learning or memory. Has04064 NF-κB signaling pathway, GO analysis showed that the 216 DEGs in the spinal cord tissue samples were involved in biological processes such as type I interferon signaling pathway. The BDNF/TrkB-MAPKPI3K-NF-κB signaling pathway is a classical signaling pathway that may play an important role in NTDs and deserves further exploration. 29 DEGs in the two groups. Upregulated and downregulated genes were mapped using the Search Tool for The Retrieval of Interacting genes /Proteins (STRING) (https://string-db.org/) based on the PPI network. Cytoscape (version 3.6, Beijing, China) software was used to the hub genes. The differential gene protein network of the neurotrophic factor signaling pathway and the NF-κB signaling pathway 35 nodes, 199 edges, an average node degree of 11.4, top10 genes obtained by the MCC algorithm are HRAS, TP53, PIK3R1, TRKA, NGF, BDNF and TRKB. Morphological changes in spinal cord neural tube malformations, PCR and WB results showed that BDNF, NGF, TrkA and TrkB were higher in the malformed group than in the control group. The BDNF and NGF signal pathways play an important role in the process of NTDs.

Keywords: neural tube defects; rat embryo; apoptosis; BDNF; NGF

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Introduction

During embryonic development, a series of congenital birth defects caused by incomplete closure of the neural tube are called NTDs. NTDs can occur anywhere along the neural axis, mainly manifesting as anencephaly, craniorachischisis and spina bifida, and are one of the most common structural birth defects in humans [1-2]. Neurotrophic factors are a family of proteins, including NGF, brain-derived neurotrophic factor (BDNF), neurotrophic factor 3 (NT-3) and neurotrophic factor 4 (NT-4), which regulate the development and function of the nervous system [3]. They transduce signals through two different transmembrane receptors: the Trk receptor tyrosine kinase [4] and $p75^{NTR}$ [5]. The latter is a member of the tumour necrosis factor (TNF) receptor superfamily, which has been shown to cooperate with neurotrophic factors (NTFs) to induce survival and differentiation. $p75^{NTR}$ also induces apoptotic signaling, which is initiated in vivo by immature neurotrophic factor precursors [6]. NGF, as an important member of neurotrophic factors (NTFs), is well known to promote neuronal survival, differentiation, neurite growth and nerve regeneration, including in the development of neural tube. NGF regulates cell survival and cell death by binding to two different receptors, TrkA and p75NTR [7]. In contrast, proNGF selectively induces apoptosis via $p75^{NTR}$, but not TrkA [8]. However, not all $p75^{NT}$R-expressing cells respond to proNGF, Sortilin creates a signaling complex by simultaneously binding to $p75^{NTR}$ and proNGF, acts as a co-receptor and molecular switch governing the $p75^{NTR}$-mediated pro-apoptotic signal induced by proNGF [9]. Some evidence suggests that it is associated with NRIF nuclear translocation and JNK activation following NRIF phosphorylation [10]. Indeed, proNGF is the main form of NGF in brain [11] and has been shown to induce apoptosis in different diseases [12-13]. High-affinity binding of proNGF to $p75^{NTR}$ appears to bemediated by the interaction of the “pro” domain of the former (pro-peptide) with Sortilin [14], a transmembrane receptor containing a Vps10p domain [15]. In different cell systems, $p75^{NTR}$ can act as an essential co-receptor to promote apoptosis [16-17]. Furthermore, the proNGF/Sortilin/$p75^{NTR}$ complex has been shown to be involved in neurodegenerative processes, including Alzheimer's disease[7] and age-related neurodegeneration [18]. We therefore hypothesized that Sortilin plays a role in NTDs models in which proNGF/$p75^{NTR}$ is known to trigger apoptosis. Therefore, the aim of this study was to assess the possible role of proNGF in the development of NTDs in rats after ATRA treatment.

Materials and Methods

Data Extraction

Retrieve "neural tube defects" (keyword) and "human" (biological sample) from the GEO database of the National Center forotechnology Information (https://www.ncbi.nlm.nih.gov/geo/). The raw gene expression profiles of GSE33111 were obtained, integrated, analyzed. The gene sequences were sequenced using the GPL6884 Illumina Human WG-6 v3.0 expression beadchip, with samples being brain remnants and spinal cord tissues of human midgestation fetuses with neural tube defects.

DEGs Analysis

Preprocessing of the original GSE33111 dataset included normalization and log2 conversion. The thermal heat map was then drawn based on the amount of DEGs, and a volcano plot was created based on the log2 fold change and p value ($|log2FC| > 1$, $p < 0.05$). R software was used to draw the figures.

Functional Enrichment Analysis of DEGs

Analysis of DEGs using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) was performed using the WebGestalt (http://www.webgestalt.org/, accessed on 16 June 2022) database. Genes related to molecular function, biological process, and cell composition were found using GO analysis. A signaling pathway-basedvisualization of the enrichment analysis is displayed.

Protein-Protein Interactions (PPIs) Analysis of DEGs

The PPIs of DEGs were analyzed, the gene data were integrated, and upregulated and downregulated genes were mapped via a PPI network diagram using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (https://string - db.org/, accessed on 16 June 2022). Core genes were screened using Cytoscape (Ver 3.6, Beijing, China).

Animal grouping and model preparation

Adult Sprague-Dawley rats (weighing 0.2-0.22 kg, age 3.5-4 months) were provided by the Laboratory Animal Center of Kunming University. Every effort was taken to reduce the number of animals and suffering during the experiments. The animal grouping and the number of animals were described in detail in each portion of method. At 18: 00 P.M, male and female (proportion 1:2) rats were caged and take out at 8:00 A.M the next day. After that put the female rats in another empty cage (avoid the other rats affected), while clearing the hair wood dunnage, holding the female's tail with the thumb and index finger, the rest three fingers press thereafter back (this method is more suitable for active rats), turning the tail to reveal the vaginal mouth. The feces near the vulva should be shaken off. First go to check whether there is a vaginal suppository, generally not visible. Then stay slightly in the vaginal mouth with normal saline cotton branch, first let the rat to adapt, then gently insert the rat vagina and slowly rotate, slowly withdraw the cotton branch, the mucus on the cotton branch is evenly coated on the glass slide, naked eye observation and microscopic examination. Check sperm or vaginal suppository as the first day of pregnancy embryo, the day before 18:00 is the 0 day, and so on. The pregnant SD rats were randomly divided into 2 groups, control and experimental group, which were divided into 14, 15, 16, 17, 18, 19, 20 and 21 days groups, each group of 10 fetuses, 5 for immunohistochemical experiment, 5 are used only for RT-PCR experiment. Each rat was under standard conditions of humidity and temperature with 12 h light/dark cycles and given free access to food and water. During pregnancy, the ATRA dissolved in olive oil, according to the 50 mg/kg at E10d when the disposable stomach feeding experimental group of pregnant rats, resulting in pregnant rat NTDs. The control group is without any treatment.

Tissue preparation

At the time point in each group, with cervical dislocation method executed in 2 groups of pregnant rats, isolated rat embryos under a dissecting microscope, record the total number of embryos, the number of live births, stillbirths count, absorbed fetus number, check the appearance to determine whether there is any appearance of visible deformity and record. For immunohistochemistry, five embryonic neural tube or spinal cord tissues from each group were fixed into 4% paraformaldehyde solution fixation for more than 24 h, paraffin section machine will make cross sectioned embryos, film thickness of 5 μm. For RT-PCR, the remaining five in each group isolated the neural tube or spinal cord tissue of the embryos under a dissecting microscope, immediately put into 1 ml EP tubing containing TRIZOL, ice bath homogenates induced uniform, not sticky, no particles, -80℃ conservation.

Immunofluorescence Staining

The tissues were washed with

phosphate-buffered saline (HyClone, Logan, Utah, USA), mixed with 0.3% Triton X-100 (Sigma Aldrich, St. Louis, MO, USA), and then incubated at room temperature for 1 h. The primary antibodies used were BDNF (1:1000, Abcam, Cambridge, UK), NGF (1:1000, Abcam, Cambridge, UK), TrkA (1:1000, Abcam, Cambridge, UK), TrkB (1:1000, Abcam, Cambridge, UK). A combination containing the primary antibody and 5% goat serum albumin (1:1000, Santa Cruz, Dallas, Texas, USA) was combined and incubated at 4 ◦C overnight. The following day, the tissue were incubated at room temperature for 30 min, washed with phosphate - buffered saline with Tween 20 (PBST), and then incubated with a secondary antibody immunoglobulin G (1:1000, Abcam, Cambridge, UK). The second antibody was diluted with 5% goat serum albumin (Gibco, Grand Island, NY, USA) at room temperature for 2 h. First, tisses were washed with PBST and stained with nuclear dye 4', 6-diamino-2-phenylindole (DAPI) (1:1000, Sigma Aldrich, St. Louis, MO, USA), which was diluted with 2% goat serum albumin. Finally, the cells were washed with PBST. Images were captured using fluorescence microscopy (DS-Vi1 and Az100, Nikon, Tokyo, Japan). Image J (Ver 1.8.0, Bethesda, MD, USA) was used to count the number of positive immunofluorescent cells.

Real-Time (RT) Polymerase Chain Reaction Assay

Tissues were treated with the TRIzol solution (Invitrogen, Carlsbad, CA, USA) to extract their total RNA. Complementary (cDNA) was reverse-transcribed using SuperScript III (Takara, Osaka, Japan). GADPH was used as a negative control, and the SYBR quantitative qPCR kit (Takara, Osaka, Japan) was used to measure the relative expression of mRNA. The ABI Prism 7500 Rapid Sequence detection system (Applied Biosystems, Carlsbad, CA, USA) was used to perform qRT-PCR. The relative expression levels of mRNA were calculated and quantified using the $2^{-\Delta\Delta Ct}$ method.

Western Blotting Assay

Total protein was extracted from tissues, and each tube contained 200 μL of a detergentlysate (containing 2 μL of phenylmethylsulfonyl fluoride and 2 μL of phosphatase inhibitor). A microplate analyzer (DG-3022A, Tecan, Männedorf, Switzerland) was used to determine the protein concentration after diluting the samples. The extracted protein supernatant and 5× protein loading buffer (4:1, Solarbio, Beijing, China) were placed in boiling water for a 10-min denaturation process. After electrophoretic gel preparation, the primary antibodies were β-actin (1:500, Bioss, Beijing, China). We scanned the film for recovery value analysis (Bio-Rad, Hercules, CA, USA). Image J (Ver 1.8.0, Bethesda, MD, USA) was used to count gray value.

Statistical Analysis

Prism software (Ver 7.0, GraphPad Software, San Diego, CA, USA) was used for data analysis. All data are expressed as mean ± standard deviation (S.D.). Analysis of variance (ANOVA) was used, followed by Bonferroni post- hoc test between groups. $p < 0.05$ was considered statistically significant.

Results

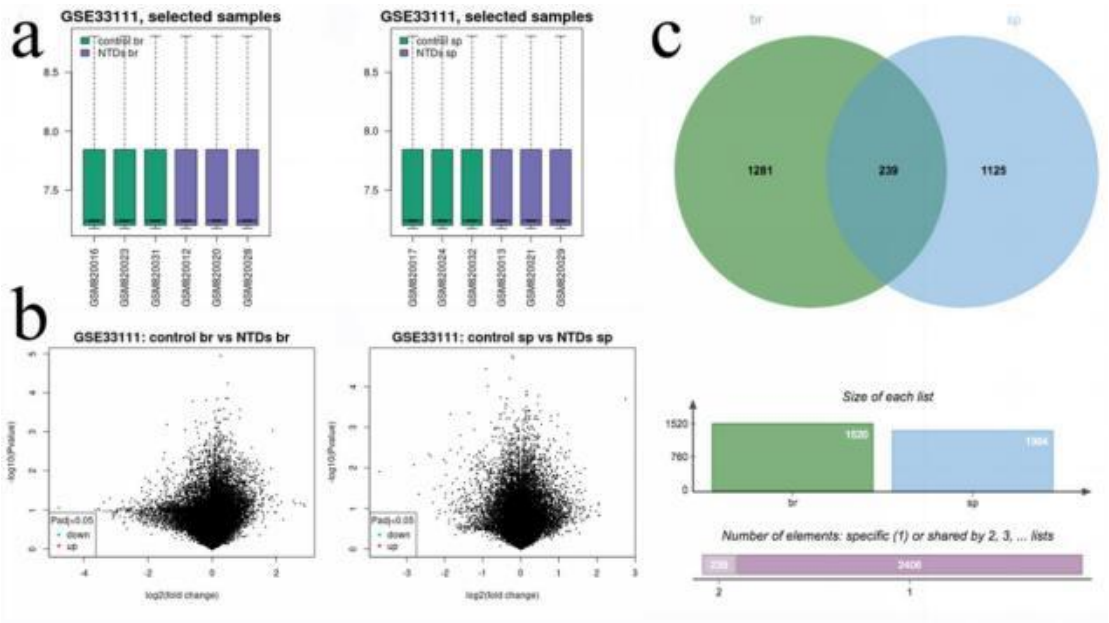

Figure 1 Analysis of differentially expressed genes in human NTDs. (a) GSE33111 includes two types of samples (fetal brain remnants of human midimester neural tube defects; spinal cord tissues of human mid-trimester neural tube defects). (b) Visualization of differentially expressed genes in theSE33111 dataset (volcano plot of differentially expressed genes in fetal brain remnants of human mid-trimester neural tube defects; b volcano plot of differentially expressed genes in spinal cord tissues of human mid-trimester neural tube defects). (c) Differentially expressed genes co-expressed two samples of the GSE33111 dataset (Green br: fetal brain remnants of human mid-trimester neural tube defects; blue sp spinal cord tissues of human mid-trimester neural tube defects).

Analysis of differentially expressed genes in human NTDs

Preprocessing of the original GSE33111 dataset (Figure 1-a) included normalization and log2 transformation. Volcano plots were drawn based on log fold change and P value (|log2FC|>1, $P < 0.05$) using R software. A total 2057 DEGs were identified in the brain remnant tissue samples, including 1415 upregulated and 642 downregulated. A total of 2161 DEGs were identified in the spinal cord tissue samples, including 994 upregulated and 1067regulated genes (Figure 1-b). Venn diagrams showed that there were a total of 239 commonly DEGs (Figure 1-c).

Functional enrichment analysis of differentially expressed genes

Gene sequence analysis using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) revealed among the 2057 DEGs in the brain remnant tissue samples, KEGG analysis found that they were enriched in the has0472 neurotrophic factor pathway, etc. GO analysis showed that the 2057 DEGs in the brain remnant tissue samples were involved in biological such as cognition, learning, learning or memory, regulation of synaptic transmission signaling, and modulation of chemical synaptic transmission; in cellular component pathways, they were involved inynaptic density, asymmetric synapses, synapses between neurons, postsynaptic specialization, and synaptic membranes; in molecular functions, they were

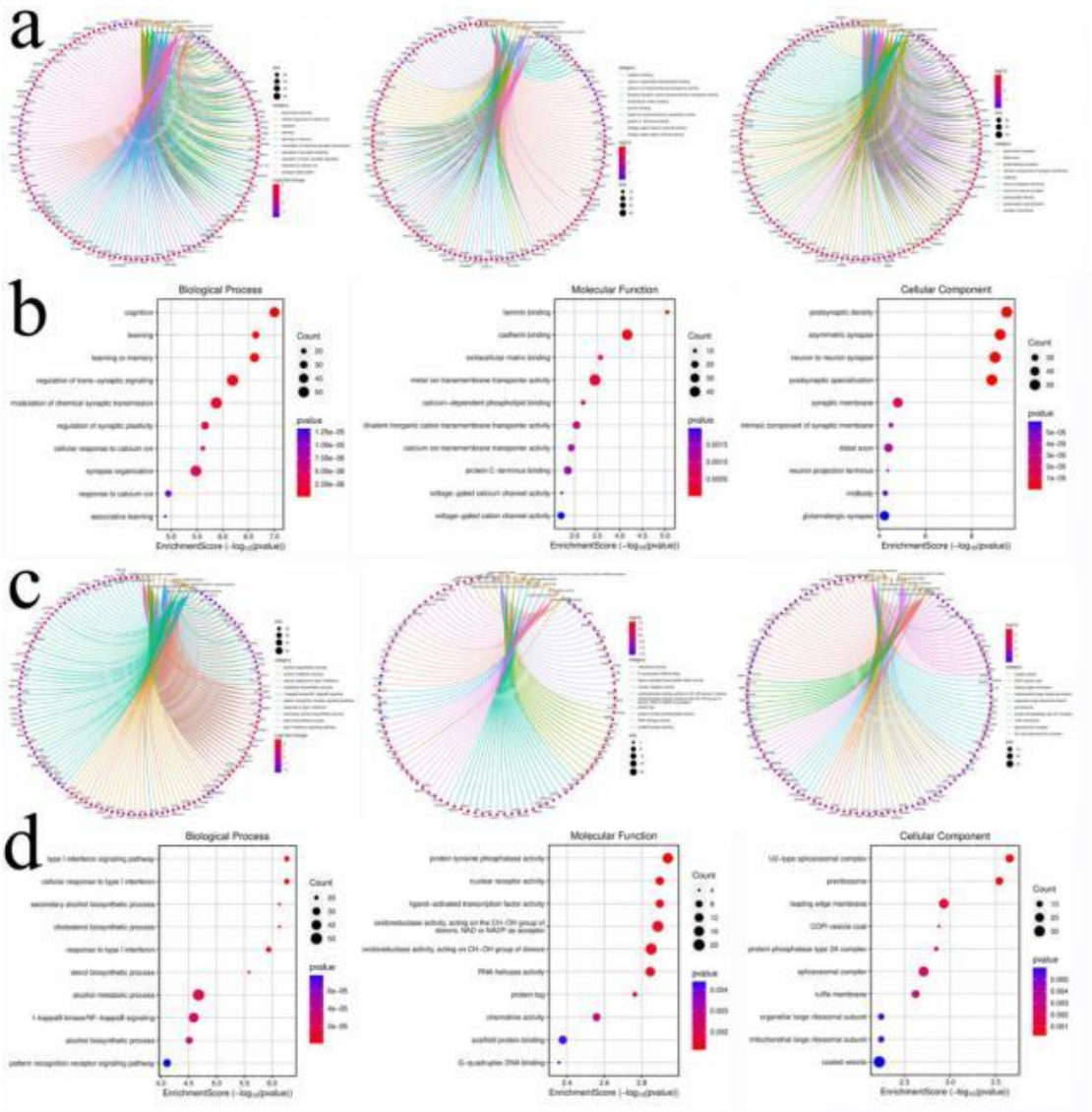

Figure 2 Functional enrichment analysis of differentially expressed genes.

involved in laminin, cadherin binding, extracellular matrix binding, metal ion transmembrane transporter activity, and calcium-dependent phospholipid binding (Figure 2), gene sequence analysis using GO and KEGG was performed, and KEGG analysis found that among the 2161 DEGs in the spinal tissue samples, they were enriched in the has04064 NF-κB signaling pathway, etc. GO analysis showed that the 216 DEGs in the spinal cord tissue samples were involved in biological processes such as type I interferon signaling pathway, cholesterol biosynthetic process, and response to type interferon; in cellular component pathways, they were involved in U2-type spliceosome complex, vesicle coat, and protein phosphatase 2A complex in molecular functions, they were involved in protein tyrosine phosphatase activity, nuclear receptor activity, ligand-activated transcription factor activity, oxidoreduct activity, and RNA helicase activity (Figure 2). As can be seen from Figure 4a, the BDNF/TrkB-MAPKPI3K-NF-κB signaling pathway is a classical signaling pathway that may play an important role in NTDs and deserves further exploration.

Functional enrichment analysis of key genes

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were also performed for the 29 DEGs in the two groups. KEGG analysis showed that the 239 DEGs were enriched in adrenergic signaling in cardiomy, T cell receptor signaling pathway, chemokine signaling pathway, lysosome, dopaminergic synapse, NF-kappa B signaling pathway, and toll-like signaling pathway. GO analysis showed that the 239 DEGs were involved in cellular response to monoamine stimulus, cellular response to catecholamine stimulus response to monoamine, etc. in biological processes; axon growth cone, smooth endoplasmic reticulum, E3 ubiquitin ligase complex mitochondrial membrane gap, etc. in cellular components; and rRNA methyltransferase activity, catalytic activity acting on rRNA, chloride channel inhibitor activity, RNAtransferase activity, etc. in molecular functions (Figure 3).

PPI analysis on the differentially expressed genes in the neurotrophic factor signaling pathway and the NF-κB signaling pathway

Upregulated and downregulated genes were mapped using the Search Tool for The Retrieval of Interacting genes/Proteins (STRING) (https://string-db.org/) based on the PPI network. Cytoscape (version 3.6, Beijing, China) software was used to the hub genes. As shown in Figure 7, the differential gene protein network of the neurotrophic factor signaling pathway and the NF-κB signaling pathway 35 nodes, 199 edges, an average node degree of 11.4, an average local clustering coefficient of 0.64, and PPI enrichment statistics of $p < 1.0$ e-16. The Cytoh-

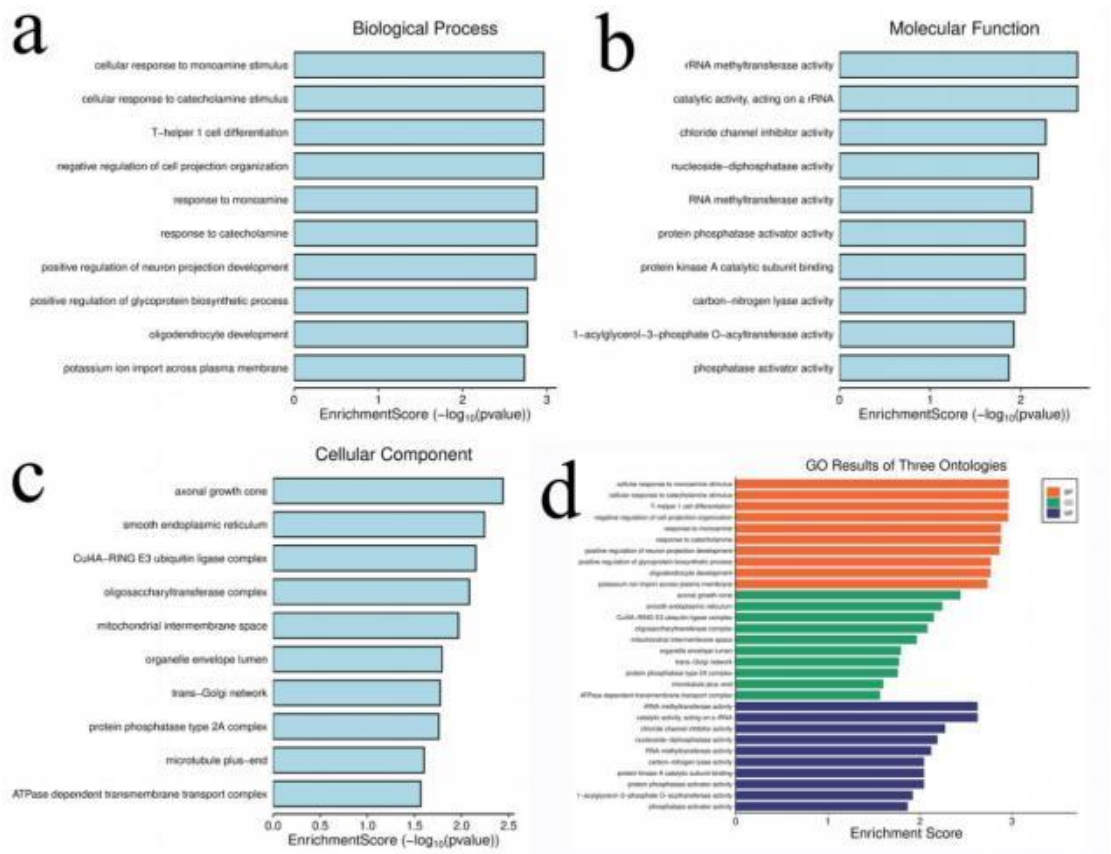

Figure 3. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses.

ubba plug-in shows that the top10 genes obtained by the MCC algorithm are HRAS, TP53, PIK3R1, NTRK1 (TRKA), NGF,DNF, NTRK2 (TRKB), etc. (Figure 4).

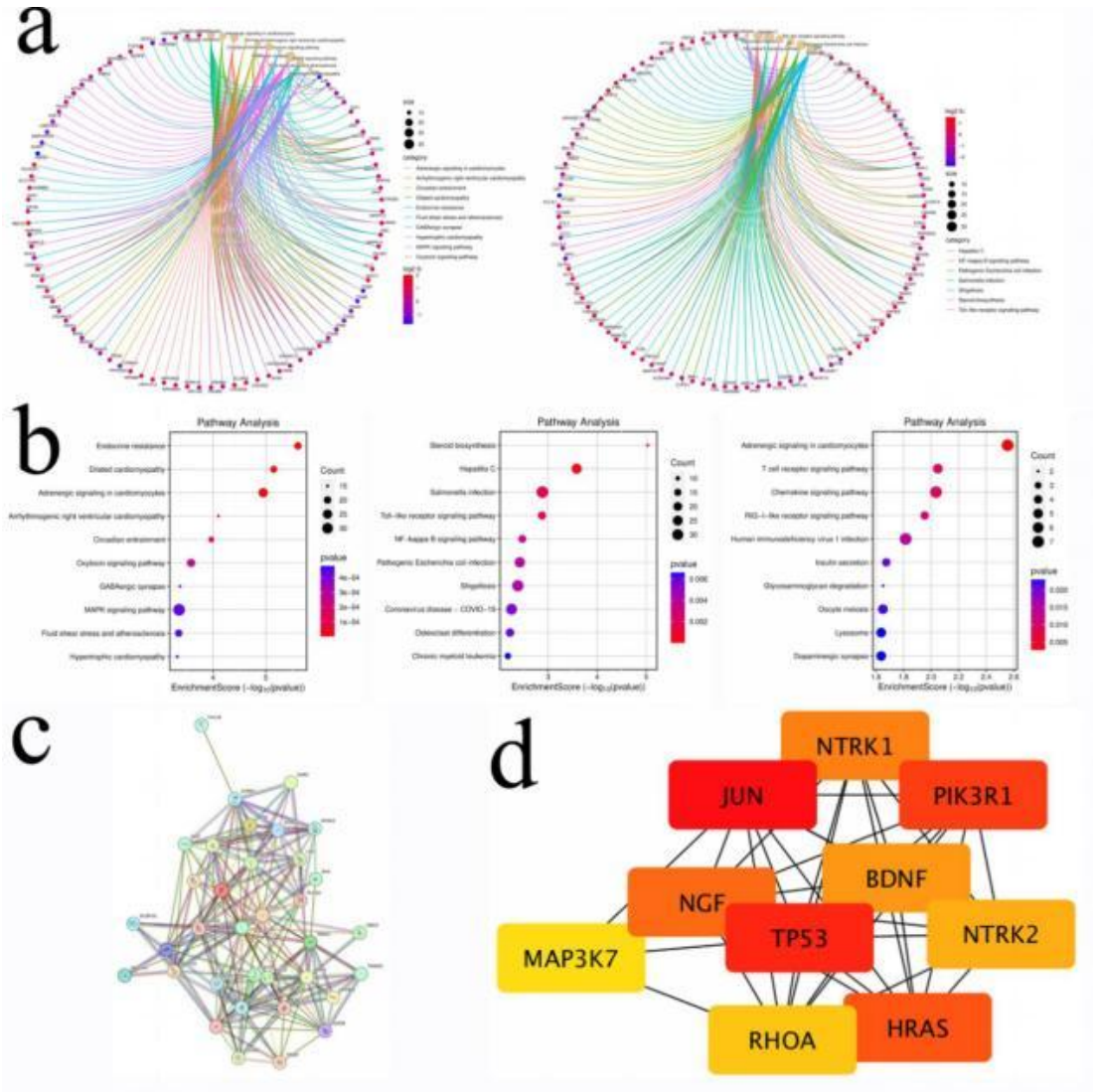

Figure 4 Interaction of differential gene proteins in the neurotrophic factor signaling pathway and the NF-κB signaling pathway. 8 Top 10 key genes in the interaction of differential gene proteins in the neurotrophic factor signaling pathway and the NF-κB signaling pathway.

SD rat neural tube defect model

compared with the NC group, overexpression of Morphological changes in spinal cord neural tube malformations In the normal group and control group, the morphology of the tissues the spinal cord was normal and the structure was intact. In contrast, in the malformation group, the morphology of the dorsal spinal cord tissue was abnormal, with inward indentation (Figure 5-a,b). Changes in neuronal morphology in spinal cord neural tube malformations In the normal group and control group, the morphology of the nerve was normal and the outlines of the cells and nuclei were clearly visible. In contrast, in the malformation group, the neurons in the

spinal cord tissue of the were disordered, the structure of the neurons was destroyed, and the nuclei disappeared (Figure 5-c).

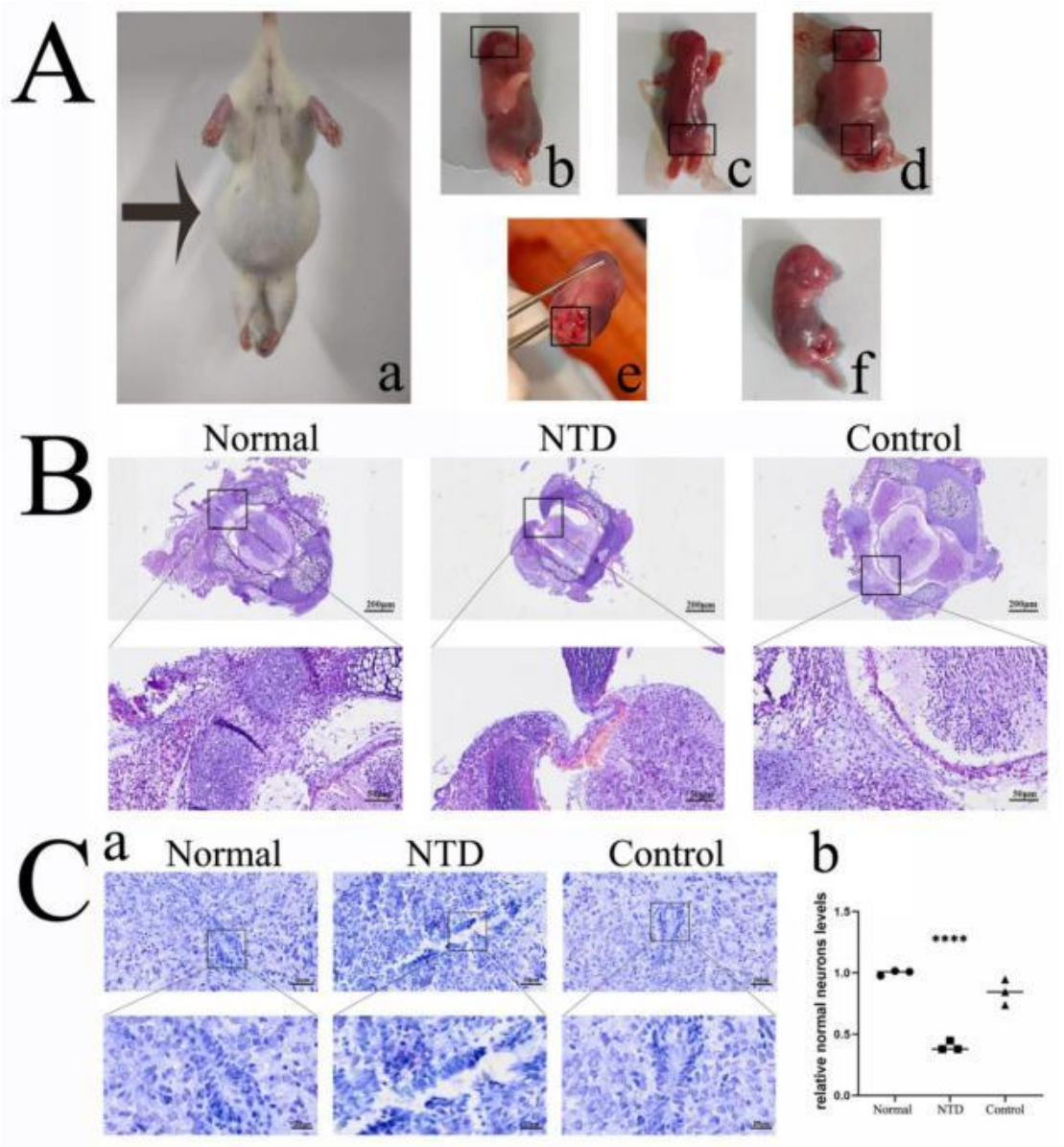

Figure 5. SD rat model of neural tube defects. a: Pregnant rat; b: Anencephaly; c: Aplasia of tail; d: Anencephaly Aplasia of tail; e: Spina bifida; f: Normal rat. B: HE staining. Nl staining. B: Morphological changes in spinal cord neural tube defects. C: Morphological changes in neurons of spinal cord neural tube.

Immunohistochemical staining results

The expression of BDNF, NGF, TrkA and TrkB were higher in the dysplasia group than in the normal (Figure 6).

Changes in factor genes and proteins

PCR results There was no significant difference between the normal group and the control group. The expression of BDNF, NGF, TrkA and TrkB were higher in the malformed group than in the control group (Figure 7-a). Western Blot results There was no significant difference between the normal group and the control group. The

expression of BDNF, NGF, TrkA and TrkB were higher in the malformed group than in the control group (Figure 7-b).

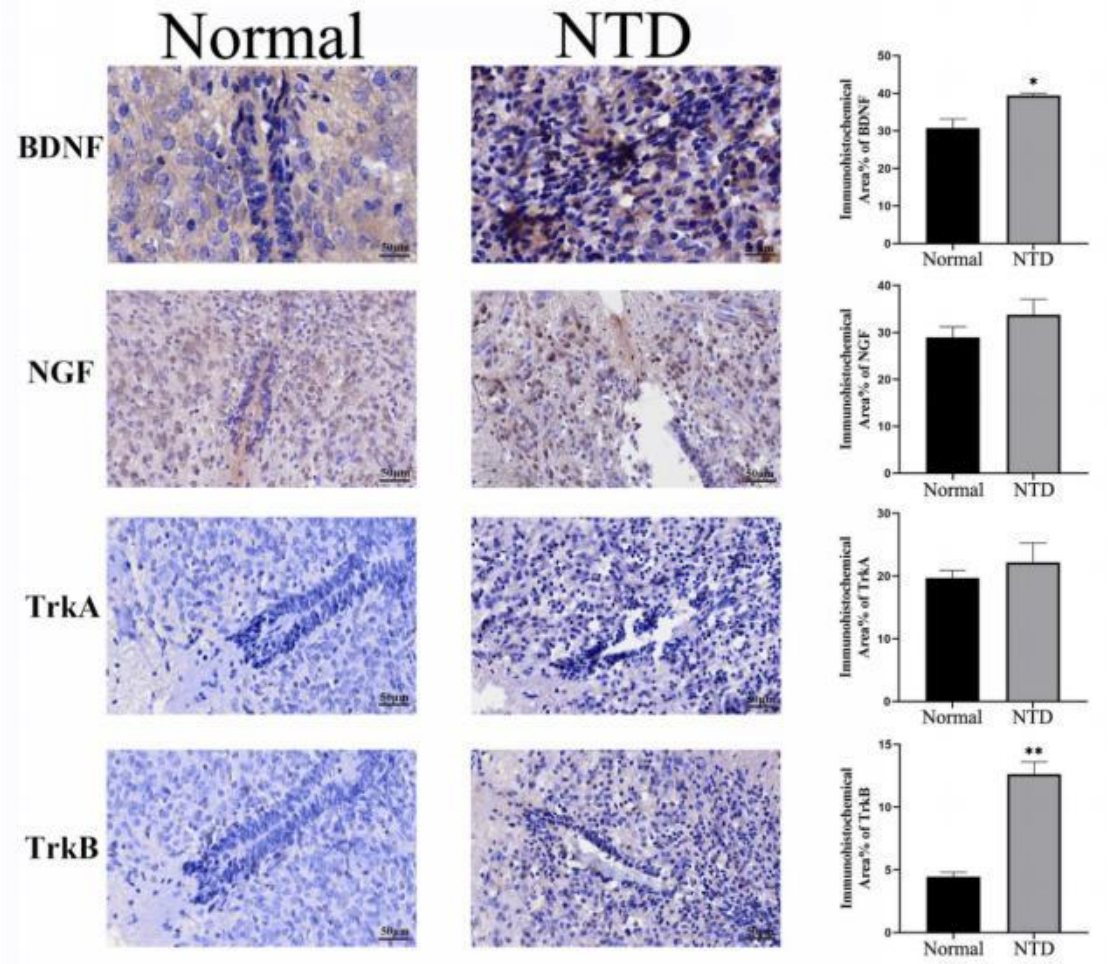

Figure 6. Immunohistochemical staining results.

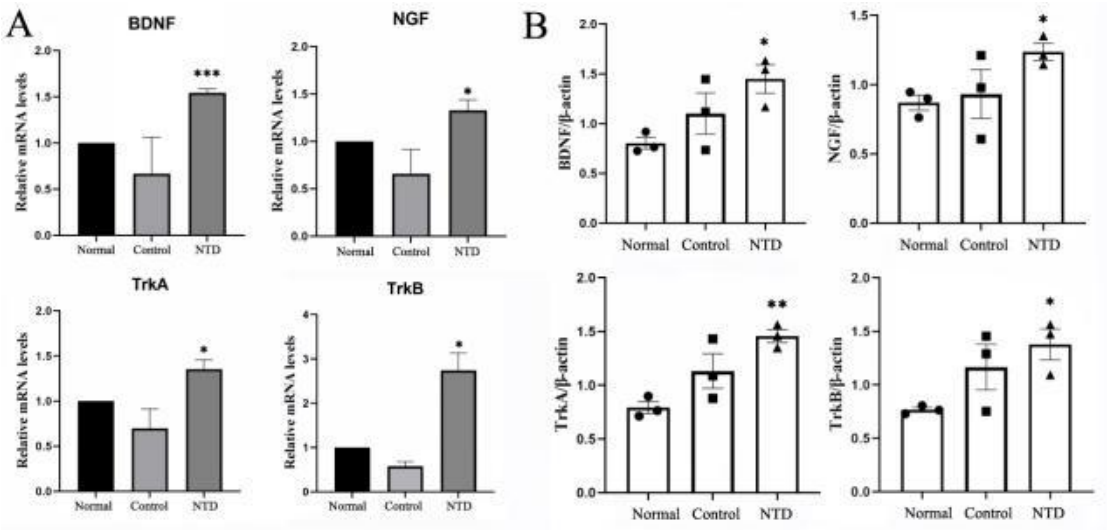

Figure 7. Changes in factor genes and proteins.

Discussion

One of the most common and serious congenital defects is neural tube defect in the world, and are deformities of the central nervous system (CNS) that occur during fetal embryonic development due to abnormal neurulation process and incomplete closure of the neural tube [19]. It presents with a wide spectrum of clinical features including anencephaly, spina bifida, encephalocele, myelomeningocele, and others. NTD is the second most common type of congenital defect after congenital heart defects. Its etiology is multifactorial, a composite of genetic and environmental causes, of which the most common is folic acid deficiency during the first trimester of pregnancy. The chromosomal anomalies associated with NTD are trisomy, trisomy, and triploidy among which triploidy with spina bifida being the most common NTD associated with genetic chromosomal abnormality [20-21]. As per a 10-year prospective observational study done in India, the burden of NTDs is 3.9 per 1000 live births [22]. However, there are geographical variations within the country, with incidence varying from 7.48 per 1000 live births in northern India compared to 3.6 per 1000 live births in South India which may be attributed to dietary, genetic, and health infrastructure differences [23]. Contrary, the incidence of NTD in the United States is 1 per 1000 live births which is 3 times lower than that observed in India and 3 to 5 times lower than in Northern China [24]. The consequences of NTD include live births, stillbirths, and second-trimester abortions. Studies have reported live birth and stillbirth prevalence were 1.3 and 1.7 per 1000 births, respectively, among NTD cases [25].

Neural tube defects (NTDs) are the second most common congenital defects in humans and are characterized by impaired development of the central nervous system [26]. NTDs are the result of the interaction of multiple mechanisms, and their etiology is related to genetic inheritance, maternal environment in utero, and teratogenic agents, but the mechanisms are still inconclusive and need to be further investigated [27]. Studies have confirmed that vitamin A and retinoid A derivatives are morphogens that control body bone formation and are essential for normal embryonic development, leading to congenital malformations or death in mammals (including humans) [28]. The teratogenic effects of RA overdose may be related to inappropriate activation or inhibition of certain morphogenesis-related regulatory genes by RA in time and space. In this experiment, the teratogenic agent trans retinoic acid was used for animal modelling. The results of this experiment showed that a single high-dose trans retinoic acid of 50mg/kg in rats on day 10 of pregnancy could cause the embryonic neural tube defects. It is assumed that the reason is that when the rat embryo reaches day 10, it is at the critical stage of neural sulcus atresia, and the administration of high doses of all-trans retinoic acid at this time may paradoxically activate or inhibit directly or indirectly certain regulatory genes related to neural tube morphogenesis, which may affect the closure of the neural sulcus and eventually result in incomplete or partial closure, and if the embryo can continue to develop after partial closure, then the neural tube defects occurs.

BDNF has been studied in detail with respect to its function on neuronal survival, formation, metabolism, and axonal growth [29-31]. BDNF injection can inhibit neural apoptosis and promote survival of sensory neurons in the rat fetuses with SBA [32]. BDNF has also been shown to protect neurons and improve neurological function in models of spinal cord injury [33] retinal injury [34] and hearing diseases [35]. NGF can directly repair injured nerves and regulate neuronal activity, neural connections, and synaptic plasticity [36].

NTDs are highly debilitating and many a times life-threatening conditions which can be prevented at all primary, secondary, and tertiary levels of prevention. Preconceptional administration of folic acid and screening for genetic diseases can prevent NTDs from occurring in a population. The secondary level of prevention constitutes antenatal radiological screening and management of such cases in utero, if possible, to reduce the severity of the cases. If incompatible with life, pregnancy can be terminated as early as possible to reduce the mental, physical, and social bearing on the pregnant woman and family. Once diagnosed after birth, cases of NTD can be treated surgically and physical rehabilitation can be done thereafter. Also, grass root level workers should be educated about this so that they can encourage early antenatal visits and screening of pregnant women. Hence, we see that with adequate and proactive participation from the government side and policy makers, this problem can be sorted out, and the incidence of NTDs can be brought down.

Ethics statement

All samples were approved and authorized by the First People’s Hospital of Yunnan Province Ethics Committee, and all participants provided informed consent. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants ’ legal guardians/next of kin.

Author contributions

Jinwei Yang and Wei Ma: Conceptualization, Project administration, Writing-original draft. Kuangping Liu and Li Shen: Conceptualization, Formal Analysis, Writing-original draft.

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