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SPECIAL SECTION ON NEW TRENDS IN BRAIN SIGNAL PROCESSING AND ANALYSIS

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Transcriptome Study of Brain Nerve Impairment Induced by Pb Exposure

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ABSTRACT With the introduction of the global brain science program, the brain science and cognitive science are listed as one of the important research areas in China, the goal of which is to understand the function of the brain. In the human brain plan, the neurodegeneration and neurodevelopment in diseases and aging are one of the key areas of concern. However, lead is an environmental heavy metal neurotoxin, which can cause neurodegenerative diseases and affect brain health. At present, the mechanism of the lead-induced nervous system damage mainly focuses on the damage of lead to neurons, while the researches related to the repair of damaged nerve cells induced by the lead exposure have not been reported. The Transcriptome sequencing technology is direct detection of transcripts by the deep sequencing technology, which can more accurately measure the expression of transcripts, and is an inevitable link between genomic genetic information and the proteome of biological functions. In addition, the regulation of the transcription level is the most important regulation mode of organisms. Based on the characteristics of neurogenesis for nerve repair, the current study applied transcriptional sequencing technology to screen the differentially expressed genes in the choroid plexus of rats and to explore the changes in the expression of neurotrophic factors regulating neurogenesis in the choroid plexus of the lead-exposed rats. The results showed that the differentially expressed genes on choroid plexus of the lead exposed rats were related to neurogenesis. In the process of neurogenesis, the expression of nutritive and growth-promoting factors IGF1, EGFr, IGF1r, EGFr1, VEGFα, and TGFβ2 decreased, which suggested that these cytokines may be involved in the process of lead exposure neurogenesis, which needs further experimental verification. The findings of this study provide a new target for the mechanism research and prevention and control measures of brain nerve injury caused by the lead exposure.

INDEX TERMS Brain nerve impairment, transcriptome, Pb.

I. INTRODUCTION

Lead (Pb), an environmental heavy metal neurotoxin, is widely used in production and living fields. Lead can enter the body through air, water and food by the respiratory tract, digestive tract, skin contact and other ways, and has an impact on various organs and systems of the body [1]. Among them, the central nervous system is one of the main target organs of its toxic effects [2]. After the lead enters the body, it can be stored in different parts of brain tissue, such as cortex, hippocampus, choroid plexus and cerebellum, as blood circulation passes through the brain barrier [3], [4]. As a neurotoxic substance, the early symptoms of lead neurotoxicity are mainly headache, irritability, inattention, forgetfulness, slow reaction, etc., while serious lead poisoning can cause persistent coma, irreversible consciousness, ataxia disorder. Long-term and low-dose lead exposure can also lead to neurological impairment such as decreased learning, decreased memory and cognitive abilities, attention deficit, and increased aggressive behavior [5]. With the rapid development of China's industrialization, lead pollution and the health threats caused by lead exposed have always been a hot spot of public health.

Lead exposure mainly causes degenerative diseases of the nervous system, which are mainly learning, memory and cognitive dysfunction. As is known to all, hippocampus and cortex are important brain regions for learning and memory function, and are also the main target sites of nerve injury

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caused by lead exposure. The mechanism of lead neurotoxicity is mainly focused on the damage of lead to neurons: lead exposure can promote the apoptosis of hippocampal and cortical neurons, which leads to cognitive impairment. However, repair studies about nerve cells damaged by lead exposure have not been reported [6].

It is known that neural stem cells with neurogenic function exist throughout life in adult mammalian brains in subventricular zone(SVZ) and the hippocampal sub-granular zone (SGZ). Stem cells in the adult brain can serve as a reserve for neuronal cells and glial cells to replace cells which die for life due to various injuries or diseases. At present, the maintenance, repair and reconstruction of brain structures based on neural stem cells (NSCs) has become one of the key issues in the field of brain science research [7], [8]. Under the stimulation of some physiological or pathological factors, NSCs can proliferate and differentiate into neurons or glial cells. Under the stimulation of some physiological or pathological factors, NSCs can proliferate and differentiate into neurons or glial cells, and migrate to the injured brain area to replace damaged cells and establish a new neural circuit [9]. To maintain the normal function of the brain, this process is also defined as neurogenesis. Neurogenesis has also been shown to be closely related to brain health, such as Parkinson's disease, Alzheimer's disease (AD), and manganese poisoning the development of neurodegenerative diseases. However, whether adult brain neurogenesis has been inhibited has not been reported after subchronic lead exposure.

Neurogenesis is regulated by endogenous or exogenous cytokines such as growth factors, neurotransmitters, hormones, and inflammatory factors [10]–[12]. Among them, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor 1 (IGF-1), transforming growth factor (TGF) and other factors have important regulatory effects on the proliferation and differentiation of neural stem cells [13]–[15]. SVZ and hippocampal regions are closely related to cerebrospinal fluid (CSF), and studies have found that CSF containing a variety of bioactive peptides plays an important regulatory role in neurogenesis and brain health [16]. Cerebrospinal fluid is mainly secreted by the choroid plexus, and the choroid plexus is also the first barrier for exogenous substances to enter the brain. Besides secreting cerebrospinal fluid, choroid plexus is also responsible for the transport of substances between peripheral blood circulation and CSF, and plays a key role in maintaining the dynamic balance of microenvironment in the brain [17], [18]. The first barrier of lead into the central nervous system with blood circulation is the choroid plexus. Studies have shown that lead can accumulate in the choroid plexus, which may cause damage to the choroid plexus, and its permeability, transport and binding functions may be affected or altered, which leads to imbalance of ion homeostasis and composition of CSF, which in turn causes damage to nerve tissue in the brain. Whether lead has damaged the structure, secretion and transport function of the choroid plexus during the process of entering the central nervous system (CNS), and affected the regulation of the choroid plexus on the growth factor affecting neurogenesis into the CNS, were not known.

Through establishing a model of lead exposure in adult rats, the current study explored the changes in cognitive function and neurogenesis of adult rats after subchronic lead-exposed and provided new ideas for the study of lead-induced central nervous system injury mechanisms. Using transcriptome sequencing technology and Gene Ontology (GO) functional enrichment analysis, the choroid plexus transcript information was excavated deeply and accurately, and the significant differentially expressed genes of rat nerve injury after lead exposure were screened for early identification of lead-induced nerve injury and the development of control measures provides new targets.

II. TRANSCRIPTOME SEQUENCING TECHNOLOGY

In the 1990s, the Human Genome Project (HGP) was proposed, DNA sequencing, gene chips, mass spectrometry and high-throughput sequencing technologies were popularized. The life science, which was based on experimental and empirical science, was promoted to the high-throughput research era of multi-dimension and large samples. Large amounts of data were gathered together to produce the concept of omics. Genomics, transcriptome, proteome, metabolome and other omics data have greatly broadened people's horizons and deepened people's understanding and understanding of life sciences [19], [20]. These omics technology were applied in the field of life research, which has greatly promoted the development of medical research and made people have a deeper understanding of the pathogenesis of diseases [19], [21], [22]. At the same time, the comprehensive application of these omics has also helps people to find new targets for the diagnosis, treatment and prognosis of new diseases [23]. With the development of sequencing technology, transcriptome sequencing has been further developed. Transcriptome is the sum of all RNA transcribed under the state that is the specific tissue or cell at a certain stage of development or function [24]. By using computational system biology method, the difference of RNA expression between disease and normal individuals can be studied, and the RNA pre-screening can be realized from the perspective of high throughput, and even new RNA related to physiology and pathology can be found. On this basis, in-depth discussion of physiological and pathological mechanisms will greatly promote the study of disease mechanisms.

RNA-sequencing (RNA-Seq) is a high-throughput sequencing method for studying transcriptomic, which is based on second generation sequencing technology. Compared with traditional chip methods, RNA-seq can accurately and quantitatively express transcripts, discover new transcripts, identify alternative splicing events, and detect differentially expressed genes and their functions. Thus, the dynamic changes of transcriptome in different time and space are revealed. In addition, RNA-seq has prominent advantages, such as digital signal, low background

noise, small sample size, large number of detection genes, wide detection threshold, high sensitivity and good repeatability [25].

Transcriptome research can study gene function and gene structure at an overall level, reveal specific biological processes and the molecular mechanisms in which diseases occur, and provide comprehensive and rapid access to almost all transcripts in a certain state of specific particular organ or tissue. Transcriptome is the first and most widely used technology in the post-gene era. Therefore, transcriptome technology is also widely used in the field of nervous system injury and disease research. Some studies have shown that the overexpression of genes is related to the structure and prominent function of neurons in AD patients' brain tissue. The transcription isomers of apolipoprotein E gene (Apoe-001, Apoe-002 and Apoe-005) in normal brain tissue and brain tissue of AD patients are controlled by different promoters [26]. It can be seen that high-throughput total transcriptome sequencing technology will help identify the level of gene differential expression and identify genes related to neurodegenerative diseases. Fillman detected differentially regulated transcription factors at 798 in patients with schizophrenia (SZ) and pathway analysis showed that SZ was closely related to the inflammatory response of the body. Subsequently, he verified that these candidate cytokines and immunoregulatory factors include IL-6, IL-8, IL-1β and serpina3 [27].

RNA-seq technology mainly acquires differentially expressed genes (DEGs). The analysis process was as follows: the count of reads corresponding to the gene or statistical transcript; reads count was standardized to accurately compare the level of differential expression between samples and within samples. Reads distribution after standardization was fitted with a statistical model. Statistical test was used to evaluate the differential expression of genes, and the corresponding P value and difference multiple (fold change, FC, False Discovery Rate, FDR) were obtained. Multiple tests and corrections were completed. Differentially expressed genes were screened according to specific thresholds (for example, FC$>$2 and FDR$>$0.05). Functional enrichment analysis included hierarchical clustering analysis of differentially expressed genes. Functional classification and enrichment analysis were carried out with GO database of differentially expressed genes. Based on the existing gene annotation results, this study identified and analyzed the differential expression of genes in different treatment tissues, screened the significantly differentially expressed genes, which was a gene for neurogenesis injury induced by lead exposure in rats, and provided theoretical basis for early identification and timely prevention of lead exposed nerve injury.

III. RESEARCH METHODS

A. SAMPLE ACQUISITION

60 healthy adult SPF Fisher 344 rats, male, average weight (277.72 + 18.28g), were purchased from Beijing Weitonglihua experimental animal technology co., LTD. (animal certificate no.: SCXK (Beijing) 2011-0009). One week after adaptive feeding, rats were randomly divided into control group, low lead exposure group and high lead exposure group according to body weight, with 20 rats in each group. Rats in the low-lead exposure group and the high-lead exposure group were fed lead acetate solution containing 300mg/L and 600mg/L respectively, and the rats in the control group were fed with a sodium acetate solution containing 600mg/L; the time of exposure was 9 weeks. The study was conducted out in compliance with standard animal use practices and approved by the Animal Care Welfare Committee of North China University of Science and Technology (Permit Number: 201577).

The experimental rats were anesthetized by intraperitoneal injection with ketamine/toluenthiazide. After the rats were deeply anesthetized, the brain was decapitated. The brain tissue was quickly stripped away and the choroid plexus tissue was obtained on the ice-sheet. Five rat choroid plexus tissues were one sample, and each group had 3 samples, a total of 9 samples, which were frozen at -80ř.

B. PRETREATMENT FOR TRANSCRIPTOME SEQUENCING SAMPLES

Total RNA of choroid plexus tissue samples was extracted. After digesting the DNA with DNase I, the mRNA was enriched with magnetic beads with Oligo (dT); interrupt reagents were added to the constant temperature homogenizer to break mRNA into short fragments. The stranded cDNA was synthesized using the interrupted mRNA as a template, and then a two-stranded synthesis reaction system was used to synthesize the double-stranded cDNA, purification and recovery with the kit, sticky end repair, and addition of base ''A'' to the 3 'end of cDNA and connection of the joint were performed. Then, the fragment size was selected. After the final PCR amplification, it was quickly frozen and stored in liquid nitrogen, and then submitted to Shenzhen Huada Gene Technology Service Co., Ltd. for subsequent transcriptomics sequencing analysis. Illumina HiSeqTM 2000 sequencing platform was used for sequencing after the constructed library passed the quality inspection. As shown in Figure 1.

C. TRANSCRIPTOME SEQUENCING DATA ANALYSIS PROCESS

The transcriptome sequencing data analysis process was showed in Figure 2. Raw reads were first analyzed for quality control (QC), unqualified reads were removed, and the filtered clean reads were compared to the reference sequence. The quality of the comparison results (QC of alignment) was judged by statistical comparison ratio, distribution of reads on the reference sequence. Then, a series of follow-up analyses were conducted on the quality control data, which are quantitative analysis of genes and transcripts and analysis based on gene expression level (correlation, conditional specific expression, differential gene screening, etc.). The differentially expressed genes between the selected samples were analyzed for significant enrichment of GO function.

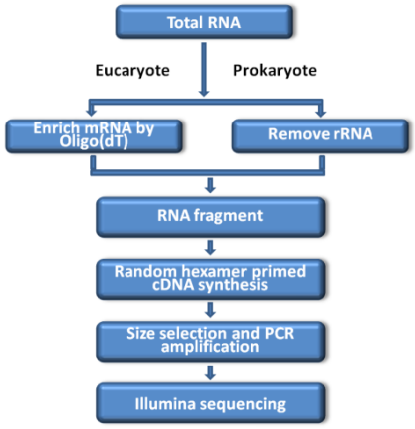


FIGURE 1. The samples processing used for transcriptome sequencing.

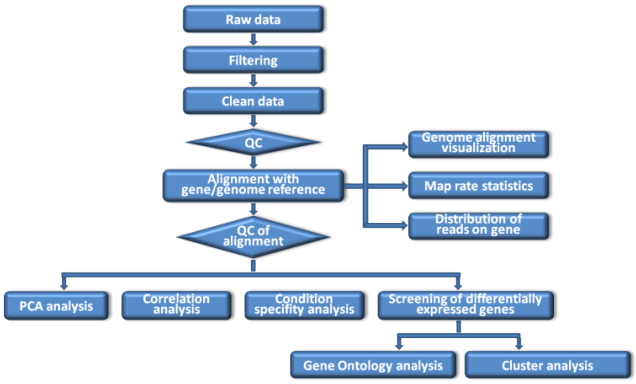


FIGURE 2. The analysis process of transcriptome sequencing data.

Filtering steps were as follows: 1) remove reads with adapters; 2) remove reads in which unknown bases are more than 10%; 3) remove low quality reads (the percentage of low quality bases is over 50% in a read, we defined the low quality base to be the base whose sequencing quality was no more than 10).

Comparison with reference genes: clean reads was aligned to the reference genome using the alignment software BWA and Bowtie. Reads were compared with each reference sequence and the numbers of reads at different positions of the gene were counted. And the distribution of reads on genes was analyzed.

D. SAMPLE QUALITY CONTROL ANALYSIS

1) CORRELATION ANALYSIS BETWEEN SAMPLES

According to the quantitative results of FPKM, the correlation between two samples was calculated by correlation analysis and scatter plot. According to the standard recommended by the Encode program, two samples with $R^2 \geq 0.92$ were biological replicates with good correlation.

2) CLUSTERING ANALYSIS BETWEEN SAMPLES

The distances of expressed gene in samples were calculated by euclidean method. Meanwhile, the algorithm of Sum of Squares of Deviations was used to calculate the distance between samples so that cluster tree can be build. It is the true distance between two points in two-dimensional space, and the formula for calculating the Euclidean distance between two gene expression profiles is as equation (1):

$$D(X, Y) = \frac{1}{m}\sqrt{\sum_{i=1}^{m}(x_i - y_i)^2} \tag{1}$$

3) PCA ANALYSIS OF SAMPLES

Principal component analysis (PCA) was used to reduce the complexity of data, deeply explore the relationship between samples and the size of variation, and find outliers and identify clusters with high similarity.

E. ANALYSIS OF SAMPLE GENE EXPRESSION LEVELS

1) GENE AND ISOFORM EXPRESSION

RSEM (RNA-Seq by Expectation Maximization) was adopted to achieve quantitative expression of genes and transcripts. RSEM used the paired-end relationship, the length of the reads, the length distribution of the fragments, the quality values, etc. to distinguish the different subtypes of the transcript. And RSEM used abundance estimation models, which was based on the maximum expected algorithm to establish maximum likelihood to distinguish different subtypes of transcripts. Quantification of gene expression was in FPKM, which refers to the number of transcripts per thousand bases per million fragments in double-ended sequencing.

The specific calculation formula is as equation (2):

$$FPKM = \frac{10^6 C}{NL/10^3} \tag{2}$$

FPKM (A) was the expression level of gene A, C was the only number of fragments that were aligned to gene A, N was the number of fragments that were unique to the reference gene, L was the number of bases in the coding region of gene A. FPKM method could eliminate the influence, which was influence of gene length and sequencing difference on the calculation of gene expression. The calculated gene expression could be directly used to compare the gene expression differences between different samples.

2) DIFFERENTIAL EXPRESSION GENE SCREENING (DEGS)

Based on the Poisson distribution analysis method, it was assumed that the number of reads corresponding to gene A was x, and it was known that in a large library, the expression of each gene was only a small fraction of the expression of all genes. In this case, the distribution of x obeys Poisson distribution, just as equation (3):

$$p(x) = \frac{e^{-\lambda}\lambda^x}{x!} (\lambda \textit{ is the true transcript of gene } A) \tag{3}$$

The total clean tag number of the sample 1 was N_1, and total clean tag number of sample 2 was N_2; gene A hold x tags in sample 1 and y tags in sample 2. The probability of gene

A expressed equally between two samples can be calculated with equation (4):

$$2\sum_{i=0}^{i=y} p(i\,|x)$$
$$\text{or} \quad 2\times(1-\sum_{i=0}^{i=y} p(i\,|x))(\text{if}\sum_{i=0}^{i=y} p(i\,|x)\ >0.5)$$
$$p(y\,|x)\ =(\frac{N_2}{N_1})^y\frac{(x+y)!}{x!y!(1+\frac{N_2}{N_1})^{(x+y+1)}} \tag{4}$$

Differentially expressed genes (DEGs) were screened by comparing FPKM values of different genes in each group. Multiple hypothesis tests were performed to correct the P value of the difference test, and the domain value of P was determined by controlling the FDR. The FDR cannot be preset to exceed 0.05. When the difference test FDR value was obtained, the differential expression multiple of the gene between different samples was calculated according to the gene expression amount (FPKM value). The smaller FDR value and the larger difference multiplier indicated that the difference in expression was more pronounced. In the present study, DEGs followed the criteria: differentially expressed genes were defined as genes with FDR$\leq$0.001 and expression change folds | Fold-Change| $\geq$ 2.

3) CO-EXPRESSED GENES AND DEGS ANALYSIS

Venn diagrams were used to demonstrate the expression of shared genes and unique genes between samples. Using |log2(Fold Change)| $\geq$0.5, log2(P value)$\geq$12.5 as the screening condition, the distribution of differential genes between different groups of samples was demonstrated by volcano map.

4) CLUSTER ANALYSIS OF DEGS

Using cluster3.0 software, the Euclidean distance was used as the distance matrix calculation formula. The differentially expressed genes and experimental conditions were simultaneously clustered and analyzed. The clustering results were displayed by java treeview5.1.6.

5) GENE ONTOLOGY ANALYSIS OF DEGs

Gene Ontology (GO), which was an international standard gene functional classification system, GO had three ontologies: molecular function, cellular component and biological process. Comparing to a genome background, GO enrichment analysis provided all GO terms that significantly enriched in a list of DEGs, and filtered the DEGs that correspond to specific biological functions. This method firstly mapped all DEGs to GO terms in the database (http://www.geneontology.org/), calculating gene numbers for every term, then used hypergeometric test to find significantly enriched GO terms in the input list of DEGs, based on 'GO:: Term Finder' (http://smd.stanford.edu/help/GOTermFinder/GO_TermFinder_help.shtml/), we had developed a strict algorithm to do the analysis, and the method used was described as equation (5):

$$P=1-\sum_{i=0}^{m-1}\frac{\binom{M}{t}\binom{\text{N-M}}{\text{N-t}}}{\binom{N}{n}} \tag{5}$$

M was the number of all genes that were annotated to certain GO terms; taking corrected $P\leq 0.05$ as a threshold. This analysis was able to recognize the main biological functions that DEGs exercise.

Where N was the number of genes with GO annotation in all genes; n was the number of differentially expressed genes in N; M was the number of genes annotated as a particular GO term in all genes; m was the number of DEGs in M. The calculated P-value went through Bonferroni correction, the corrected $P\leq 0.05$ was used as a threshold. GO terms fulfilling this condition were defined as significantly enriched GO terms in differentially expressed gene. This analysis was able to recognize the main biological functions that DEGs exercise.

IV. RESULTS

A. THE SEQUENCING QUALITY ANALYSIS ON CHOROID PLEXUS OF LEAD-EXPOSED RATS

Transcriptome sequencing of 9 samples of choroid plexus exposed to lead yielded 487420978 reads, and 466941938 clean reads were obtained after low-quality reads and reads containing joints were removed. The number of clean reads accounted for 94.37% to 96.48% of the original data, as shown in Table 1. After filtering the data of each group, the average value of (G + C)% was 50.04%; the average value of Q20 was 97.49%, and the average value of Q30 was 91.62%. After filtering, the sequencing data could be compared to the reference genes. The number of reads that could be compared to the unique position of the reference gene accounted for 81.34%, as shown in Table 2. It indicated that the sequencing data of each group was generally high and could be used for subsequent analysis.

TABLE 1. The sequencing quality analysis on choroid plexus of lead-exposed rats based on Illumina HiSeqTM 2000.

Samples	Raw reads	Clean reads	Clean data GC(%)	Clean data Q20(%)	Clean data Q30（%）
XC1	57037142	54591798	49.98	97.10	90.78
XC2	57037106	54556616	50.01	97.18	90.99
XC3	57036962	54641118	49.56	97.18	90.00
LPb1	51854576	49962288	50.31	97.76	92.39
LPb2	51854624	50028820	49.92	97.81	92.55
LPb3	51854676	49817230	50.21	97.74	92.34
HPb1	51854442	48934996	50.46	97.69	92.20
HPb2	51854328	49980586	50.18	97.77	92.43
HPb3	57037122	54428486	49.73	97.17	90.94

Note: XC1-3 was a control sample, LPb1-3 was a low lead exposure group, and HPb1-3 was a high lead exposure group.

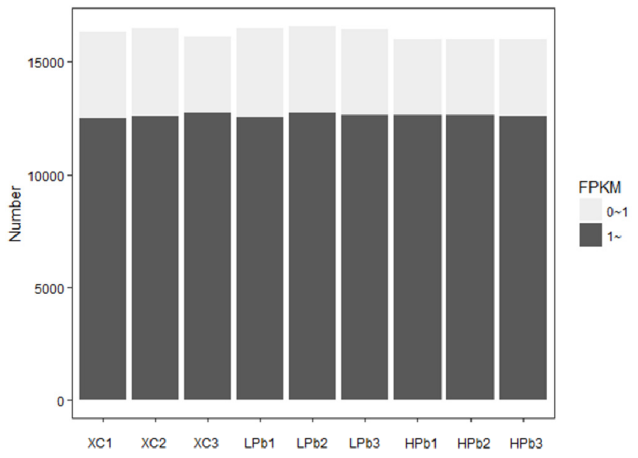
B. SEQUENCING SAMPLES STABILITY AND CORRELATION ANALYSIS ON CHOROID PLEXUS OF LEAD-EXPOSED RATS

Figure 3 showed that the number of tissue genes identified by each sample was 16009-16574, among which the number

TABLE 2. The comparisons analysis on reference genome of mRNA in choroid plexus of lead-exposed rats.

Samples	Cleac reads	The mapped reads		The unique match reads	
		Total	%	Total	%
XC1	54591798	42644522	88.12	38994028	81.43
XC2	54556616	42371295	87.66	38863847	81.24
XC3	54641118	43013045	88.72	39065644	81.49
LPb1	49962288	39289961	88.64	35656302	81.37
LPb2	50028820	39332604	88.62	36130543	82.22
LPb3	49817230	38608964	87.50	35360169	80.98
HPb1	48934996	38436770	88.55	34665338	80.84
HPb2	49980586	38932895	87.90	35644178	81.32
HPb3	54428486	42556613	88.19	38716077	81.13

Note: XC1-3 was a control sample, LPb1-3 was a low lead exposure group, and HPb1-3 was a high lead exposure group.



Note: XC1-3 was a control sample, LPb1-3 was a low lead exposure group, and HPb1-3 was a high lead exposure group.

FIGURE 3. The amount of identified genes in choroid plexus of lead exposed rats. Note: XC1-3 was a control sample, LPb1-3 was a low lead exposure group, and HPb1-3 was a high lead exposure group. Note: XC1-3 was a control sample, LPb1-3 was a low lead exposure group, and HPb1-3 was a high lead exposure group.

of genes with FPKM<1 was 3388-3929, and the number of genes with FPKM greater than 1 was 12511-12744. It was suggested that the number of genes identified in each sample was relatively stable.

Three kinds of sample quality control methods had been adopted in this article. The correlation of gene expression level among samples is a key criterion to test whether the experiments are reliable and whether the samples chosen are reasonable.The euclidean method is used to calculate the distance between samples so that cluster tree can be build, which can directly reflect the distance relationship and difference relationship between samples. Principal component analysis can reduce the complexity of the data, and dig deep the relation between sample size and variation. For two or three principal components axis graphed, which can see the distance of the relationship between each sample, including visual effect of clusters groups. In this study, the three methods being used, the purpose is to determine that the samples in the same group have good repeatability and small difference, which can reflect the effect of Pb exposed and the obtained data can be used for subsequent data analysis.

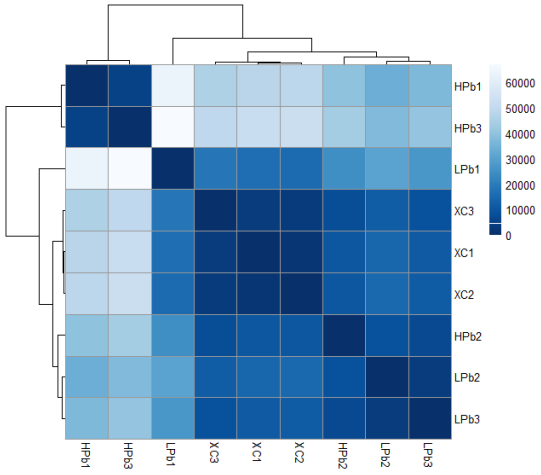


FIGURE 4. The heat map of tissue samples correlation analysis in choroid plexus of lead-exposed rats. Note: XC1-3 was a control sample, LPb1-3 was a low lead exposure group, and HPb1-3 was a high lead exposure group.

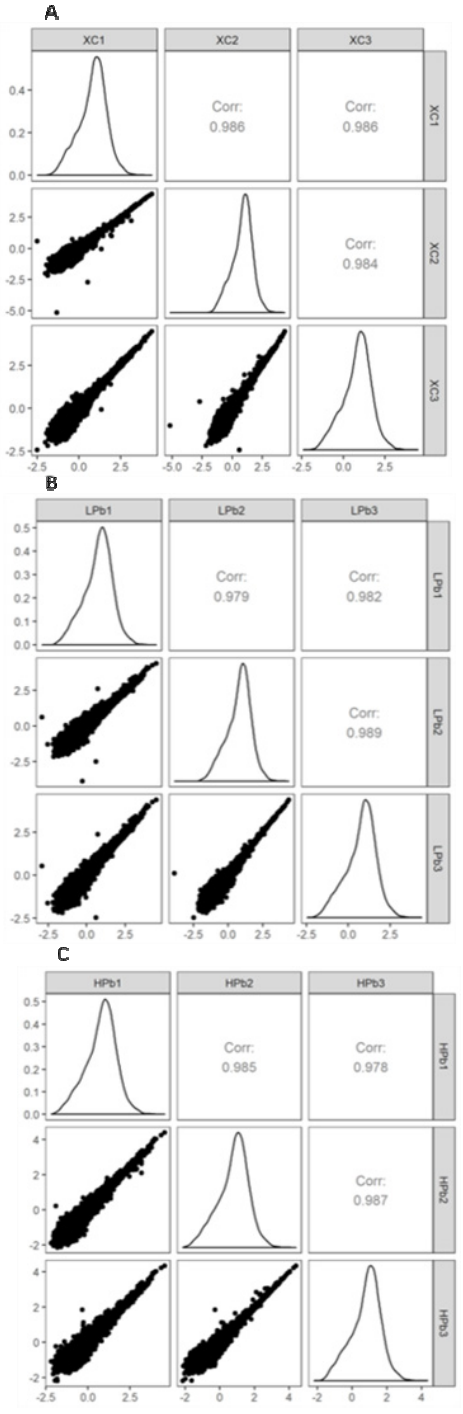
The correlation analysis of the gene expression level on choroid plexus of lead exposed rats was showed in Figure 4; and the correlation analysis results of the same group of samples were showed in Figure 5(A-C). The same group of samples $R^2 \geq 0.92$ indicated that the samples in the group had good biological repeatability. Further analysis of sample clustering and principal components, as shown in Figure 6 and Figure 7, shown that the differences between the same groups of samples were small, the homogeneity was better, and the differences between the different groups were larger, so in the subsequent analysis took the average value of gene expression of each group.

C. CLUSTER ANALYSIS ON CHOROID PLEXUS TISSUE EXPRESSION OF LEAD EXPOSED RATS

Taking Euclidean distance as the calculation formula of distance matrix, the identification genes and lead exposure were simultaneously stratified and clustered. It was found that all the identified genes were divided into 10 clusters, and the gene of the 7th gene cluster was enriched in the GO_BP entry related to neuronal cell proliferation and differentiation. Figure 8 shows that the functional gene cluster showed a low expression trend in the lead exposure group compared with the control group, and the high lead exposure group appeared in the low lead exposure group.

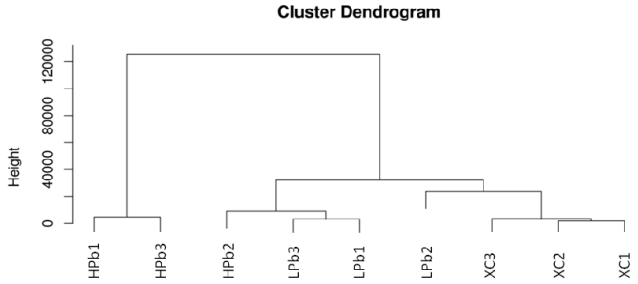
D. DEGS ANALYSIS ON CHOROID PLEXUS OF LEAD-EXPOSED RATS

Through analysis, it was found that there were 135 differentially expressed genes in different groups, of which 65 were up-regulated and 70 were down-regulated. The differentially expressed genes in different groups were compared as shown



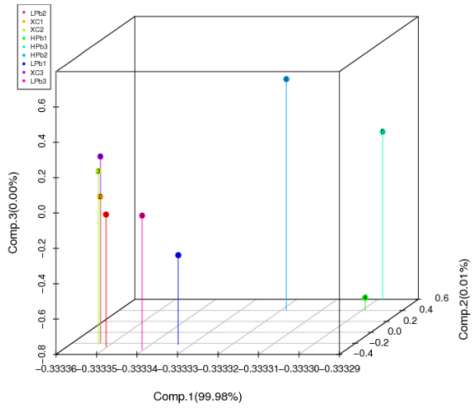
Note: XC1-3 was a control sample, LPb1-3 was a low lead exposure group, and HPb1-3 was a high lead exposure group.

FIGURE 5. **The correlation analysis of tissue samples in choroid plexus of lead-exposed rats. Note: XC1-3 was a control sample, LPb1-3 was a low lead exposure group, and HPb1-3 was a high lead exposure group.**



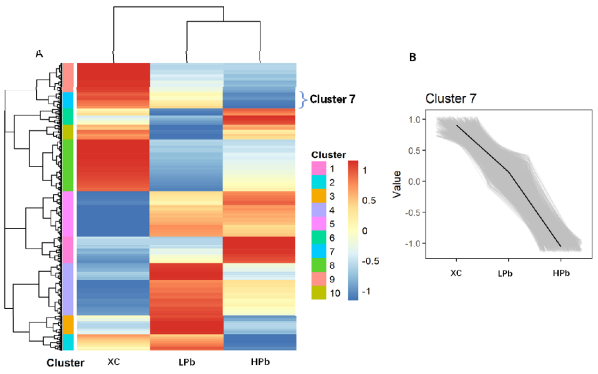
Note: XC1-3 was a control sample, LPb1-3 was a low lead exposure group, and HPb1-3 was a high lead exposure group.

FIGURE 6. **The tree diagram of tissue samples correlation analysis in choroid plexus of lead-exposed rats. Note: XC1-3 was a control sample, LPb1-3 was a low lead exposure group, and HPb1-3 was a high lead exposure group.**



Note: XC1-3 was a control sample, LPb1-3 was a low lead exposure group, and HPb1-3 was a high lead exposure group.

FIGURE 7. **The PCA diagram of tissue samples correlation analysis in choroid plexus of lead-exposed rats. Note: XC1-3 was a control sample, LPb1-3 was a low lead exposure group, and HPb1-3 was a high lead exposure group.**



Note: XC was a control sample, LPb was a low lead exposure group, and HPb was a high lead exposure group.

FIGURE 8. **The heat-map of gene expression in choroid plexus of lead exposed rats. Note: XC was a control sample, LPb was a low lead exposure group, and HPb was a high lead exposure group.**

in Table 3. There were 35 differentially expressed genes in the low lead exposure group and the control group, including 25 up-regulated genes and 10 down-regulated genes. There were 86 differentially expressed genes in the high lead exposure group and the control group. There were 28 up-regulated genes and 58 down-regulated genes. There were 14 differentially expressed genes in the high lead

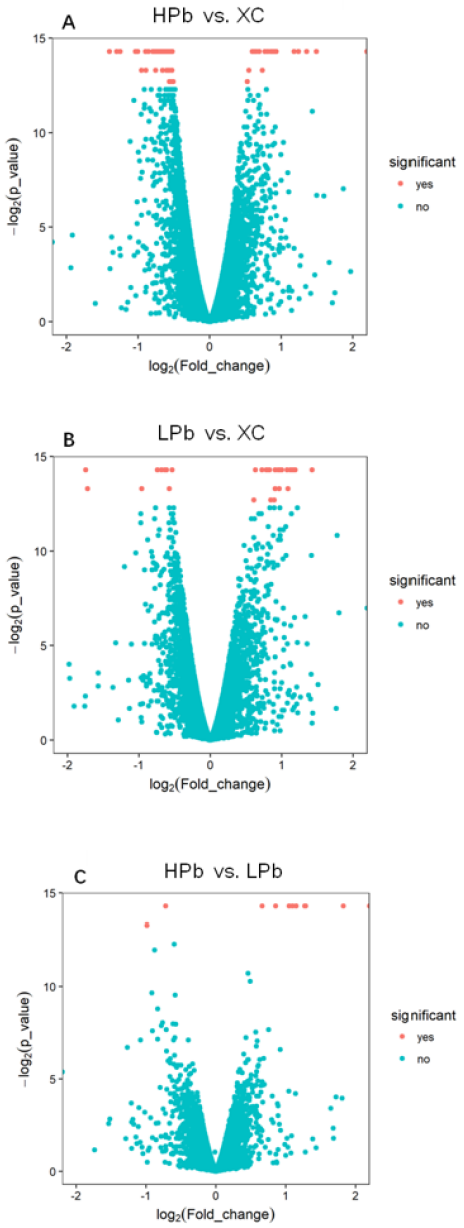
TABLE 3. Analysis of differentially expressed genes in choroidal plexus of lead-exposed rats.

Group	n(DEGs)	DiffGene(Up)	DiffGene(Down)
LPb vs. XC	35	25	10
HPb vs. XC	86	28	58
HPb vs. LPb	14	12	2

exposure group and low lead exposure group, including 12 up-regulated genes and 2 down-regulated genes. Using $|\log2(FC)| \geq 0.5$, $\log2(P \text{ value}) \geq 12.5$ as the screening condition, the volcano map was used to visually display the distribution of differential genes between samples, as shown in Figure 9(A-C).

E. ENRICHMENT ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES ON CHOROID PLEXUS OF LEAD EXPOSED RATS

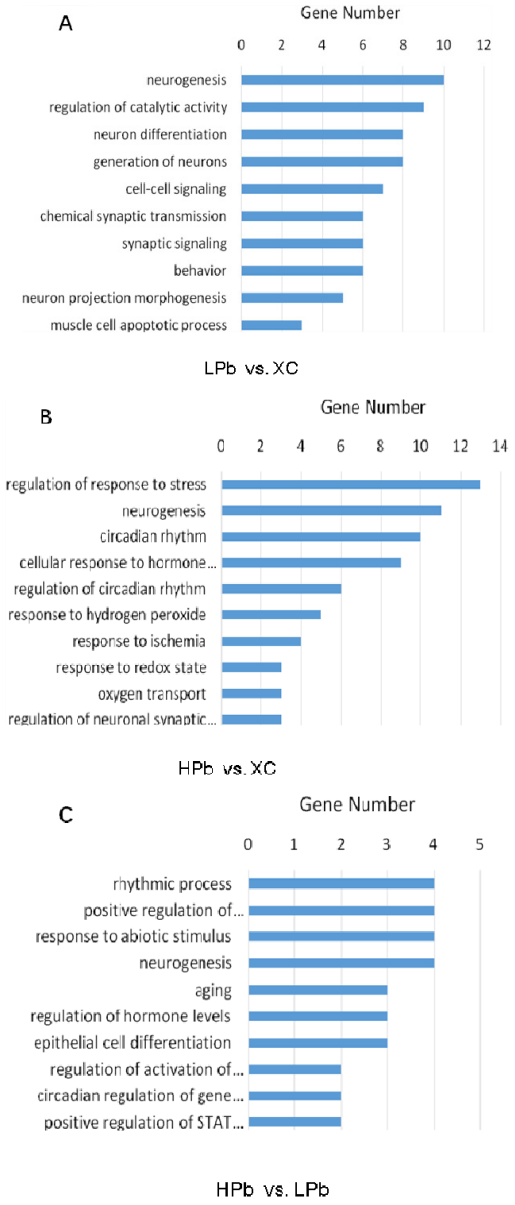
Using FC≥2 as the screening condition, the GO function enrichment analysis was performed on the differentially expressed genes of choroid plexus tissue. By comparing the results of the top ten differentially expressed genes significantly enriched in each two groups, it was found that: compared with the control group, the functions of differentially expressed genes in choroid plexus tissues in the low-lead exposure group were mainly enriched in neurogenesis ($P = 4.72E\text{-}04$, Count = 10, 32.25%), regulation of catalytic activity ($P = 8.96E\text{-}03$, Count = 9, 29.03%), differentiation of neurons ($P = 3.62E\text{-}03$, Count = 8, 25.81%), neuron generation ($P = 6.38E\text{-}03$, Count = 8, 25.81%), cell and cell signal transduction ($P = 1.03E\text{-}02$, Count = 7, 22.587%), chemical synaptic transmission ($P = 1.78E\text{-}03$, Count = 6, 19.35%), synaptic signal ($P = 1.78E\text{-}03$, Count = 6, 19.35%), behavior ($P = 3.04E\text{-}03$, Count = 6, 19.35%), neuron projection morphology ($P = 1.08E\text{-}02$, Count = 5, 16.13%), and apoptosis process of muscle cells ($P = 7.12E\text{-}03$, Count = 3, 9.68%). The functions of differentially expressed genes in choroid plexus tissues in the high-lead exposure group were mainly concentrated in the regulation of stress response ($P = 1.59E\text{-}03$, Count = 13, 17.33%), neurogenesis ($P = 9.86E\text{-}02$, Count = 11, 14.67%), circadian rhythm ($P = 1.25E\text{-}07$, Count = 10, 13.33%), cellular response to hormone stimulation ($P = 3.78E\text{-}03$, Count = 9, 12.00%), and circadian rhythm regulation ($P = 6.65E\text{-}05$, Count = 6, 8.00%), reaction to hydrogen peroxide ($P = 4.41E\text{-}03$, Count = 5, 6.67%), reaction to ischemia ($P = 7.53E\text{-}04$, Count = 4, 5.33%), reaction to REDOX state ($P = 9.15E\text{-}04$, Count = 3, 4.00%), oxygen transport ($P = 2.09E\text{-}03$, Count = 3, 4.00%), and regulation of synaptic plasticity ($P = 3.14E\text{-}02$, Count = 3, 4.00%). Compared with the low-lead exposure group, the function of differentially expressed genes in choroid plexus tissues in the high-lead exposure group was mainly enriched in rhythm process ($P = 3.70E\text{-}04$, Count = 4, 50.00%), positive regulation of catalytic activity ($P = 1.11E\text{-}02$, Count = 4, 50.00%), response to non-biological stimuli



Note: XC was a control sample, LPb was a low lead exposure group, and HPb was a high lead exposure group.

FIGURE 9. The volcano diagrams of differentially expressed genes in choroid plexus of lead exposure rats. Note: XC was a control sample, LPb was a low lead exposure group, and HPb was a high lead exposure group.

($P = 1.13E\text{-}02$, Count = 4, 50.00%), neurogenesis ($P = 2.30E\text{-}02$, Count = 4, 50.00%), and aging ($P = 1.22E\text{-}02$, Count = 3, 37.5%), hormone level regulation ($P = 1.61E\text{-}02$, Count = 3, 37.50%), epithelial cell differentiation ($P = 2.31E\text{-}02$, Count = 3, 37.50%), JAK2 kinase activity activation regulation ($P = 2.82E\text{-}03$, Count = 2, 25.00%), circadian rhythm regulation of gene expression ($P = 2.43E\text{-}02$, Count = 2, 25.00%), and positive regulation of STAT cascade ($P = 3.06E\text{-}02$, Count = 2, 25.00%). The result is showed in Figure 10(A-C).



Note: XC was a control sample, LPb was a low lead exposure group, and HPb was a high lead exposure group.

FIGURE 10. GO functional enrichment analysis in choroid plexus of lead-exposed rats. Note: XC was a control sample, LPb was a low lead exposure group, and HPb was a high lead exposure group.

F. EXPRESSION OF GENES RELATED TO NEUROGENESIS IN CHOROID PLEXUS OF LEAD-EXPOSED RATS

From the above GO functional enrichment analysis results, it could be seen that the experimental rat choroid plexus tissue and biological process related genes were enriched. And this process was a biological process of neurogenesis or neuronal cell growth and differentiation. In this study, the top 10 up-regulated genes and the top 10 down-regulated genes related to neurogenesis were analyzed. The results were showed in Table 4 and Table 5. The expression levels of *Igf1*, *Igf1r* and *Egfr* genes in choroid plexus of lead exposed rats were significantly lower than those in the control group, so this study was further verified as a target gene.

TABLE 4. The differentially expressed genes related with neurogenesis in choroid plexus of lead-exposed rats (up-regulated).

No	Gene ID	Symbol	Up/Down	Description	Function
1	25742	S100b	Up	S100 calcium binding protein B	cell proliferation, neurogenesis
2	29423	Gap43	Up	growth associated protein 43	Neurogenesis, neuron differentiation
3	29545	Uchl1	Up	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	cell proliferation, neurogenesis
4	29246	Stmn3	Up	stathmin-like 3	Neurogenesis
5	246118	Tubb3	Up	tubulin, beta 3 class III	Neurogenesis
6	24179	Agt	Up	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	cell proliferation, neurogenesis
7	25400	Camk2a	Up	calcium/calmodulin-dependent protein kinase II alpha	Neurogenesis, neuron differentiation
8	63840	Per2	Up	period circadian clock 2	
9	65218	Agap2	Up	ArfGAP with GTPase domain, ankyrin repeat and PH domain 2	cell proliferation, neurogenesis, neuron differentiation
10	-	LOC100912228	Up	-	neurogenesis

As is known to all, it was well known that CSF contains a variety of growth factors that regulate neural precursor cells and regulate neurogenesis [28], [29], changes in the composition and properties of CSF could directly or indirectly affect neurogenesis [30]. *Igf1* and *Egf* were key genes which regulate cell proliferation and differentiation networks. The results showed that the mRNA expression levels of *Igf1* and its receptor *Igf1r*, *Egf* and its receptor *Egfr* decreased on chorionic plexus tissues of lead-exposed rats. The down-regulation of these genes might be related to the decrease of cytokine levels in cerebrospinal fluid, which might inhibit the proliferation and differentiation of neural stem cells.

G. IGF-1

Insulin-like growth factor-1 (IGF-1) was a peptide neurotrophic factor with insulin homology. It had the biological effects of nutrition and neuroprotection. IGF-1 was widely used in CNS and was almost present in brain tissue cells in all developmental processes. The expression level of IGF-1 in the adult brain tissue was significantly decreased, but at this time, IGF-1 secreted by extra-brain tissues can be taken up by the IGF-1 receptor on the choroid plexus epithelial cells. Mediated by megalin protein, it transported cerebrospinal

TABLE 5. The differentially expressed genes related with neurogenesis in choroid plexus of lead-exposed rats (down-regulated).

No	Gene ID	Symbol	Up/Down	Description	Function
1	24482	Igf1	Down	insulin-like growth factor 1	Regulation of cell proliferation, neuron development, neurogenesis
2	24329	Egfr	Down	epidermal growth factor receptor	neuron development
3	25718	Igf1r	Down	insulin-like growth factor 1 receptor	Regulation of cell proliferation, neuron development, neurogenesis
4	59323	Erbb4	Down	v-erb-b2 avian erythroblastic leukemia viral oncogene 4	cell proliferation, neurogenesis, neuron differentiation
5	24330	Egr1	Down	early growth response 1	
6	79114	Fgfr1	Down	Fibroblast growth factor receptor 1	Regulation of cell proliferation
7	83785	Vegfa	Down	vascular endothelial growth factor A	Regulation of cell proliferation
8	116660	Ptprn	Down	protein tyrosine phosphatase, receptor type, N	cell proliferation
9	685059	Tgf 2	Down	transforming growth factor, beta 2	Regulation of cell proliferation
10	-	LOC100911402	Down	-	Neurogenesis

fluid or nerve tissue through endocytosis and became the main source of igf-1 in the brain [31].

IGF-1 entering the cerebrospinal fluid has a corresponding biological effect as the cerebrospinal fluid circulates to different brain regions. IGF-1 is involved in the cognitive function of the brain. When IGF-1 binds to the IGF-1 receptor on nerve cells, it can activate downstream related proteins through MAPK, PI3K/Akt or MAPK/Erk, JAK-STAT signaling pathways. It has an impact on biological functions such as protein synthesis, autophagy, apoptosis and anti-oxidation. IGFs can also play a strong role in promoting mitogens under the regulation of receptors and IGFBPs, promote cells from the G1 phase of the cell cycle to the S phase [32], stimulate DNA synthesis and inhibit apoptosis to promote cell proliferation and differentiation [33], and play an important role in nerve growth.

The transcriptome sequencing analysis of this study found that the mRNA expression of Igf1 or Igf1r on choroid plexus of lead exposed rats was lower than the control group, It is suggested that after lead exposure, IGF-1 enters the central nervous system channel - the expression of receptors on the choroid plexus was decreased. The decrease of IGF-1, which caused blood to enter the cerebrospinal fluid, led to a decrease in the level of IGF-1 in the brain. It affected the proliferation and differentiation of neural stem cells in the SVZ region and affects neurobehavioral changes.

IGF-1 played a very important role in regulating the growth, differentiation, repair, regeneration, and other aspects of nerve tissue, and then affects the function of the brain [34]. IGF-1 was expressed in the early stage of the central nervous system. For example, the deficiency of igf-1 expression can widely affect the occurrence, survival and differentiation of central nervous cells, the formation of synapses and the directional migration of axons, thus lead to the dysfunction of the neural network [35]. During the development of the central nervous system, insulin-like peptides (ILPs) can regulate neuronal differentiation's time, axonal occurrence, synaptic formation and other related events through the PI3k-Akt-Mtor signal transduction pathway [36].

In the late development of the central nervous system, IGF-1 established hippocampal synaptic connections by regulating the occurrence and migration of γ-aminobutyric acid and glutamatergic neurons, and participates in the construction of neural fiber connections in hippocampus, olfactory bulb and cerebral cortex [37], [38]. IGF-1 also promoted postnatal hippocampal neurogenesis and synapse formation [38], [39]. Aberg's study founded that regulation of neural stem cell proliferation and directed differentiation is closely related to IGF-1.

In ischemic and hypoxic brain damage, IGF-1 was involved in the pathophysiological process of brain injury, had a nutritional protective effect on central nervous cells, and contributes to functional recovery after nerve cell damage [39]. In the process of central neurogenesis, the deficiency of IGF1 receptor led to obvious impairment of brain function, the deficiency of IGF1 receptor expression on neurons significantly increases the mortality of mice, and the surviving mice also showed severe small-head deformity [40]. IGF1 also regulated the differentiation of astrocytes and oligodendrocytes [41].

H. EGF

Epidermal growth factor(EGF) is a known mitogen with a wide range of functions. It is an active polypeptide composed of 53 amino acids. When combined with its receptor, it can mediate mitosis signal transduction pathway, promote cell division and proliferation, and regulate cell growth. There is increasing evidence from in vivo and in vitro studies that EGF plays an important role in the proliferation, maturation and differentiation of neural stem cells [42]–[45]. EGF mRNA can be detected in many regions of the CNS, and Teramoto has found that EGF can promote the further differentiation, maturation, migration and replacement of neurons necrotic due to tissue ischemia.

EGF can also induce proliferation of glial precursor cells and increase the number of progenitor cells that migrate from SVZ to the striatum [46]. Injecting EGF into the brain can cause rapid proliferation of SVZ endogenous precursor cells [47], so EGF increases the number of new nerve cells in the striatum by promoting the proliferation of local neural progenitor cells or stimulating the migration of neural stem cells in the SVZ region [48].

In summary, studies had shown that EGF provided important extracellular signals during CNS development. As IGF-1, the level of endogenous EGF in the brain of adult mammals was also significantly reduced. The EGF in the

brain was mainly derived from the secretion of extra-brain tissues. After the choroid plexus binds to the EGF receptor, a clathrin complex was formed, which passed through the choroid plexus by endocytosis, entered the cerebrospinal fluid from the blood, reaches the target site with the cerebrospinal fluid circulation, and phosphorylated tyrosine by binding to its receptor. Ras/Raf/MEK/MAPK, PI3K-AKT, and PLC-γ1 signaling pathways were involved in biological processes such as cell proliferation, differentiation, cell movement, nutrient metabolism, anti-apoptosis, and receptor endocytosis. EGF mainly promotes cells from G0 to S phase. In G2 phase, growth factor treatment of cells can delay the transfer of G2 to M phase, leading to delayed cell cycle regulatory protein Cyclin B1 activity and nuclear transport, and then regulate cell proliferation and differentiation [49].

In this study, it was founded that after lead exposure, EGFr expression in rat choroid plexus tissue decreased significantly, suggesting that EGF transport disorder resulted in decreased EGF level in the brain and affected the proliferation and differentiation of neural stem cells. EGF could stimulate the proliferation and migration of neural stem cells, and high expression of EGF in the brain could stimulate the division and proliferation of neural stem cells in the SVZ region [42], [50], [51]. At the same time, in vitro studies had shown that the neural stem cells grow rapidly and the cell synapses are prolonged, when EGF was added to the medium. Studies had shown that EGF could promote the survival and growth of nerve cells, improved cognitive and behavioral ability [46]. Down-regulation of EGF expression could reduce the binding of EGF to the extracellular domain of EGFr, which blocked receptor dimerization, activation of intrinsic PTK (protein tyrosine kinase), tyrosine self-phosphorylation and recruitment of various signaling proteins to these self-phosphorylation sites, and then inhibits the proliferation of neural stem cells [52].

V. CONCLUSION

In this study, transcriptome sequencing technology was used to detect the expression of choroid plexus transcripts in lead-exposed rats, and differential expression factors related to nerve damage induced by lead exposure were determined from the gene level. The results showed that the rats in the low-dose group were compared with the control group, and DEGs of choroid plexus were up-regulated by 23 and down-regulated by 8. In the high-dose group, 28 differentially expressed genes were up-regulated and 58 were down-regulated. Twelve genes were up-regulated in the high-dose group compared with the low-dose group, and the two genes were down-regulated. The GO functional enrichment analysis showed that the choroid plexus of the low lead exposure group was associated with 32.25% neurogenicity and 25.81% of neuronal differentiation and neuronal development.14.67% of the differentially expressed genes in the high-lead exposure group and the control group were closely related to neurogenesis, which was consistent with the result that lead exposure could damage the neurogenesis in SVZ and DG region of the hippocampus in this study, that is, lead exposure could lead to neurogenesis disorder. Further analysis of differentially expressed genes related to neurogenesis. Further analysis of the DEGs related to neurogenesis showed that the expression of nutritive and growth-promoting factors *Igf1*, *Egfr*, *Igf1r*, *Egfr1*, *Vegfα* and *Tgfβ2* in the neurogenesis process decreased during lead exposure, suggesting that this may be involved in the process of lead exposure neurogenesis injury.

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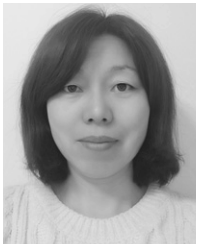

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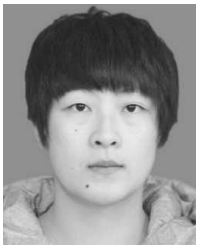

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