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Hippocampal gene expression in a rat model of depression after electroacupuncture at the *Baihui* and *Yintang* acupoints

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Abstract

Preliminary basic research and clinical findings have demonstrated that electroacupuncture therapy exhibits positive effects in ameliorating depression. However, most studies of the underlying mechanism are at the single gene level; there are few reports regarding the mechanism at the whole-genome level. Using a rat genomic gene-chip, we profiled hippocampal gene expression changes in rats after electroacupuncture therapy. Electroacupuncture therapy alleviated depression-related manifestations in the model rats. Using gene-chip analysis, we demonstrated that electroacupuncture at *Baihui* (DU20) and *Yintang* (EX-HN3) regulates the expression of 21 genes. Real-time PCR showed that the genes *Vgf*, *Igf2*, *Tmp32*, *Loc500373*, *Hif1a*, *Folr1*, *Nmb*, and *Rtn* were upregulated or downregulated in depression and that their expression tended to normalize after electroacupuncture therapy. These results indicate that electroacupuncture at *Baihui* and *Yintang* modulates depression by regulating the expression of particular genes.

Key Words: nerve regeneration; acupuncture; traditional Chinese medicine; depression; gene expression profiling; gene-chip; electroacupuncture; Baihui (DU20); Yintang (EX-HN3); chronic stress; behavior; NSFC grant; neural regeneration

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Introduction

Chronic stress presents macroscopic changes in psychoactivity, causing stress-related mood disorders. Microscopically, it shows structural and functional changes in neurites and neural circuits. In essence, chronic stress leads to abnormal expression of stress-induced genes[1-3]. There is strong evidence that abnormal gene expression in turn worsens the disease, and this vicious circle makes it difficult to cure conditions such as depression[4-7]. Recently, a large number of genes, such as the 5-hydroxytryptamine receptor, 5-hydroxytryptamine transporter, and tryptophan hydroxylase, have been found to be related to depression[8-10]. Depression is a complex, clinically common mental disorder, which involves various brain nuclei, multi-region neuronal associations, and diverse transmitters[11-14]. It cannot be fully explained in terms of single-gene disorders. A previous study found evidence for multiple gene imbalances in the brain of patients with depression, and proposed this as a pathological factor. Various nerve cells and multiple brain functions lead to a complex interaction between gene expression and the clinical manifestations of depression[16].

The hippocampus is a brain region closely associated with learning and memory abilities and emotion. It is composed of the hippocampal gyrus and the dentate gyrus. The former consists of CA_{1-4} regions and is made of pyramidal cells; the latter is composed of granular cells and can produce new neurons throughout life[17-19].

Chronic stress can persistently activate the hypothalamic-pituitary-adrenal (HPA) axis, leading to an increase in glucocorticoid levels. The hippocampus regulates this axis and is a major target for glucocorticoids. It is selectively affected by the high levels of glucocorticoids in stress, resulting in decreased hippocampal synaptic plasticity, and neuronal atrophy and loss, which consequently influences learning and memory abilities. Therefore, damage to hippocampal neuronal structure and function plays a key role in the onset of depression[20-27].

There is strong ancient and modern evidence for the utility of electroacupuncture in the treatment of depression. However, most studies report the underlying mechanism at the single gene level[28-31]. To the best of our knowledge, there are no studies reporting the underlying mechanism at the

whole-genome level. This study was the first to use a rat genome gene-chip to investigate changes in hippocampal gene expression after electroacupuncture therapy.

Results

Quantitative analysis of experimental animals

After habituation for 1 week, 45 Wistar rats were randomly and evenly divided into normal control, model, and electroacupuncture groups. A model of chronic unpredictable mild stress-induced depression was established in the latter two groups. Rats in the electroacupuncture group only received electroacupuncture at acupoints *Baihui* (DU20) and *Yintang* (EX-HN3). All animals were included in the final analysis.

Electroacupuncture did not influence body weight

Compared with control rats, the rats that received chronic unpredictable mild stress and the rats that received electroacupuncture both exhibited significantly decreased body weight ($P < 0.01$ or $P < 0.05$; Table 1).

Electroacupuncture ameliorated the manifestations of depression in the model rats

Electroacupuncture increased open-field test scores

Compared with control rats, the rats that received chronic unpredictable mild stress exhibited significantly decreased scores for both horizontal and vertical activities in the open-field ($P < 0.01$ or $P < 0.05$). However, after electroacupuncture, the scores for both were increased ($P < 0.05$; Table 2).

Electroacupuncture increased sucrose consumption in the depression model rats

Before and 21 days after chronic unpredictable mild stress, sucrose consumption was significantly lower in the model group than that in the control group ($P < 0.05$). However, after electroacupuncture, sucrose consumption was significantly increased in the depression model rats ($P < 0.05$; Table 3).

Electroacupuncture shortened the time spent in an immobile state in the depression model rats

Compared with control rats, the rats that received chronic unpredictable mild stress spent a significantly longer time immobile in the forced swimming test ($P < 0.01$ or $P < 0.05$). However, after electroacupuncture, the time spent immobile was shortened ($P < 0.05$ or $P < 0.01$; Table 4).

Electroacupuncture influenced hippocampal depression-related gene expression in the depression model rats

A total of 21 genes (Table 5), *Trim32*, *Tm2d1*, *Sumo2*, *Spcs2*, *Serinc3*, *Rtn4*, *Rnf103*, *Loc500373*, *Rnf103*, *RGD1311493*, *Psmd4*, *Ppap2a*, *Vgf*, *Tmp32*, *Nmb*, *Lsm8*, *Igf2*, *Hif1a*, *H3f3b*, *Gpm6b*, *Gng10*, *Folr1*, and *XM_231625* were identified to be differentially expressed in the hippocampus of the depression model rats compared with control rats. After electroacupuncture, the expression level of these genes was normalized. Among the 21 differentially expressed genes, *RGD1311493*, *Nmb*, *Folr1*, and *Chmp4B* were upregulated, *i.e.*, gene expression in the model group was more than twice that in the control group. The remaining 17 genes were downregulated, *i.e.*, gene expression in the model group was less than 0.5-times that in the control group. These 21 genes encode stress-response proteins, DNA injury and repair proteins, apoptotic proteins, ion channel receptors, growth factors, and cell cycle-related proteins.

After 21 days of electroacupuncture, the expression of 21 genes (all except *Folrl*) was closer to a normal level. Moreover, 14 genes (*Trim32*, *Tm2d1*, *Sumo2*, *Spcs2*, *Rnf103*, *Psmd4*, *Vgf*, *Ppap2a*, *Nmb*, Lsm8, *Igf2*, *H3f3b*, *Gng10*, and *Chmp4B*) showed close-to-normal expression after electroacupuncture (*i.e.*, a ratio of gene expression in the electroacupuncture group to that in the normal control group of 1.0 ± 0.2). The genes *Vgf*, *Igf2*, *Tmp32*, *Loc500373*, *Hif1a*, *Folr1*, *Nmb*, and *Rtn4*, were selected for PCR analysis. Amplification bands were obtained for all of them (Figure 1).

Table 1 Effect of electroacupuncture on body weight (g) in a rat depression model

		Time after depression induction (day)		
Group	Before experiment	7	14	21
Normal control	156.88±6.20	221.28±9.22	243.50±10.95	286.20±14.40
Model	157.76±10.47	164.50±11.78[a]	202.90±11.11[b]	215.70±13.65[a]
Electroacupuncture	153.10±11.08	169.00±17.12[a]	198.60±12.08[b]	244.80±16.23[b]

Data are expressed as mean ± SD of 15 rats in each group. The paired *t*-test was used to compare two groups. [a]$P < 0.01$, [b]$P < 0.05$, *vs.* normal control group.

Table 2 Effect of electroacupuncture on open-field test scores in a rat depression model

	Horizontal activities		Vertical activities	
Group	Before test	After test	Before test	After test
Normal control	71.5±4.4	55.4±3.5	19.8±2.6	16.5±1.8
Model	69.7±5.6	26.2±3.8[a]	17.1±1.6	7.0±3.7[a]
Electroacupuncture	70.3±6.2	45.7±4.7[bc]	19.4±2.3	12.5±2.1[bc]

The number of line crosses with all four paws and the number of entries into the central square within 3 minutes were taken as the number of horizontal activities. The number of times the rats stood on their hind legs within 3 minutes was taken as the number of vertical activities. Data are expressed as mean ± SD of 15 rats in each group. [a]$P < 0.01$, [b]$P < 0.05$, *vs.* normal control group; [c]$P < 0.05$, *vs.* model group.

Table 3 Effect of electroacupuncture on sucrose consumption (g/kg) in a rat depression model

		Time after depression induction (day)		
Group	Before experiment	7	14	21
Normal control	304.2±22.6	286.4±26.5	278.9±16.7	271.3±15.3
Model	290.8±20.1	288.2±14.6	263.1±12.3	221.3±10.2[a]
Electroacupuncture	289.5±10.6	300.4±13.5	272.3±15.4	281.7±12.1[b]

Sucrose consumption is expressed as the quantity of sucrose consumed per kilogram of body weight. Data are expressed as mean ± SD of 15 rats in each group. The paired *t*-test was used to compare two groups. [a]$P < 0.05$, *vs.* normal control group; [b]$P < 0.05$, *vs.* model group.

Table 4 Effect of electroacupuncture on time spent immobile (seconds) in a rat depression model

		Time after depression induction (day)		
Group	Before experiment	7	14	21
Normal control	96.7±6.4	97.1±5.8	96.3±8.2	98.6±7.9
Model	97.6±8.6	124.8±7.9[b]	139.9±8.5[b]	152.3±13.0[a]
Electroacupuncture	99.0±8.9	111.4±9.4[b]	107.5±6.5[d]	92.3±7.1[c]

Time spent immobile (seconds) refers to the time that the rats spent in struggling or maintaining an immobile state with their head above water within 5 minutes. Data are expressed as mean ± SD of 15 rats in each group. The paired *t*-test was used to compare two groups. [a]$P < 0.01$, [b]$P < 0.05$, *vs.* normal control group; [c]$P < 0.01$, [d]$P < 0.05$, *vs.* model group.

Table 5 Effect of electroacupuncture on hippocampal depression-related gene expression in a rat depression model

Genbank_ _ID	Name	M/NC	E/M	E/NC	Gene description
BC081980	*Trim32*	0.4669	2.0518	0.9579	A protein maintaining stemness of neural stem cells/neural stem cells inhibit related protein
XM_342866	*Tm2d1*	0.3152	2.9805	0.9395	Beta-amyloid peptide-binding protein
XM_346730	*Sumo2*	0.4786	2.3407	1.1203	Ubiquitin protein 2
BC098782	*Spcs2*	0.4725	2.0139	0.9517	Signal peptidase complex subunit 2, coding a glutamine-aminocyclitol transferase
BC085853	*Serinc3*	0.4490	2.8999	1.3019	Serine incorporator 3 (tumor differentially expressed protein)
AJ242961	*Rtn4*	0.4175	7.6642	3.2000	Encoding endoplasmic reticulum protein
AF306394	*Rnf103*	0.4954	2.135	1.0578	Ring finger protein 103, binding to cell membrane protein in the central nervous system
XM_213493	RGD1311493	2.4319	0.2266	0.5511	A recognition site for interaction between attachment protein and cell surface specific receptor protein
BC060559	*Psmd4*	0.4483	2.3303	1.0447	26S proteasome non-ATPase regulatory subunit 4, selectively binding to ubiquitin protein and mediating protein degradation
BC063835	*VGF*	0.4093	2.7789	1.1375	Nerve growth factor inducing protein
NM_001423	*Tmp32(Emp1)*	0.4548	2.8697	1.3050	Epithelial membrane protein 1 gene
CF110609	*Ppap2a*	0.3765	2.9636	1.1158	Phosphatidic acid phosphatase 2a
XM_574481	*Nmb*	2.2217	0.4122	0.9158	Neuromedin B
CB314239	*Lsm8*	0.2990	2.858	0.8546	U6 snRNA-associated Sm-like protein
BF555443	*Igf2*	0.4512	2.3277	1.0502	Insulin-like growth factor 2, cell proliferation-regulating factor
AF057308	*Hif1a*	0.4674	3.7739	1.7638	Hypoxia-inducible factor 1a, organism cells adapt for hypoxia-induced transcription factor
NM_053985	*H3f3b*	0.4341	2.2658	0.9837	H3 histone
AB036421	*Gpm6b*	0.4721	6.4456	3.0427	Encoding a membrane glycoprotein that belongs to the proteolipid protein family
NM_053660	*Gng10*	0.4401	2.5078	1.1036	Guanine-nucleotide-binding protein (G protein)
AF219904	*Folr1*	2.0259	5.2077	10.5504	Folic acid-binding protein
XM_231625	*Chmp4B*	5.5960	0.1738	0.9726	Nuclear chromatin modified protein 4B

M: Model group; NC: normal control group; E: electroacupuncture group.

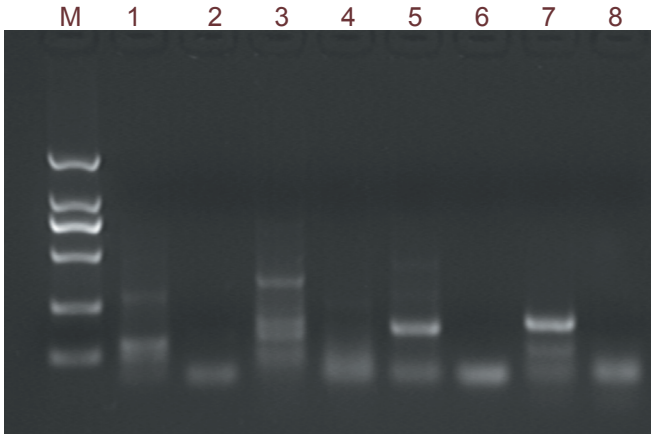

Figure 1 Electrophoresis results of PCR amplification products of genes from rat hippocampal tissue.
The band size in the marker lane, from top to bottom, is 2,000 bp, 1,000 bp, 750 bp, 500 bp, 250 bp, and 100 bp. M: Marker DL2000; 1: *Vgf*; 2: *Igf2*; 3: *Tmp32*; 4: *Loc500373*; 5: *Hif1a*; 6: *Folr1*; 7: *Nmb*; 8: *Rtn4*.

Each gene showed good amplification specificity upon examination of the dissociation curve in real-time quantitative PCR. The quantitative PCR findings were consistent with those of the gene chip analysis (data not shown).

Discussion

Our present results suggest that (1) depression is attributable to comprehensive regulation of multiple hippocampal genes that are concerned with metabolism, signal transduction, and cell growth. In the diseased state, genes with altered expression are closely related to the pathological mechanism and are the main factors leading to depression; and (2) electroacupuncture can treat depression by modifying or regulating the expression of various genes. The mechanism underpinning the effect of electroacupuncture on depression is related to gene regulation. The known function of the eight genes selected here is as follows.

(1) *Tmp32* and *Vgf*: *Tmp32* and *Vgf* regulate steroid hormone levels. These genes were significantly downregulated in depression and upregulated after electroacupuncture. Neuroactive steroids are the active steroids in the nervous system. They are endogenous neuromodulators, which can be synthesized in the brain, adrenal gland, ovary, and testis, and mainly comprise progesterone, deoxycortone, dehydroepiandrosterone, testosterone, and the latter's metabolites[23]. By binding to intracellular receptors, neuroactive steroids regulate synaptic inhibitory transmission, inflammation, myelination, central nervous system development and post-injury repair, and the HPA axis and its stress effect[32-33]. Recently, increasing attention has been paid to the role of the stress hypothesis in the onset of depression. According to the stress hypothesis, depression is caused by excessive stress mechanisms in the brain, in which the HPA axis plays a key role. The HPA axis is considered the common pathway of many symptoms and signs of depression, and the stress hypothesis is opening new and exciting avenues for the treatment of this condition[34].

(2) *Trim32*: *Trim32* is a widely expressed ubiquitin ligase of 655 amino acids that is closely involved with cellular apoptosis[35]. In this study, we found that *Trim32* expression was downregulated in depression and returned to normal after electroacupuncture. This warrants further study.

(3) *Nmb*: *Nmb* encodes a 117-amino-acid protein that functions in: (i) estrogen release and regulation during individual maturation; (ii) energy metabolism in adipocytes; (iii) thyroxine release; and (vi) neural *C-fos* gene expression and nerve cell growth[36]. Energy metabolism and nerve cell growth are both closely associated with depression, suggesting that *Nmb* is a depression-related gene that deserves further study.

(4) *Igf2*: *Igf2* expression is likely to be related to the Haxis and the activity of signal transduction pathways. Here, *Igf2* gene expression decreased in the brain of depression model rats and its expression returned to normal after electroacupuncture. This might relate to normalization of the endocrine system by electroacupuncture, and of the HPA axis in particular. In addition, *Igf2* can stimulate the phosphatidylinositol 3-kinase/protein kinase B pathway, and the products downstream of this signal transduction pathway overlap considerably with those of 5-hydroxytryptamine metabolism. *Igf2* promotes nerve cell proliferation, increased transmitter levels between synapses, and synaptic plasticity [37].

(5) Cytochrome c oxidase: Cytochrome c oxidase is located at the terminus of the cytochrome system of cellular respiration: it transmits electrons onto oxygen molecules to bind to protons to form water molecules. Cytochrome c oxidase carries four protons simultaneously to form a difference in transmembrane chemical potential energy, which contributes to ATP formation. The active cytochrome c oxidase molecule is an assembly of many subunits and cofactors[38]. We found that expression of the related gene *Loc500373* was decreased in the brain tissue of depression model rats. This suggests that energy metabolism is inhibited and ATP formation is reduced in this rat model. However, after electroacupuncture therapy, the increased *Loc500373* gene expression would promote ATP formation, thus benefiting cell function.

(6) Ribosomal protein: Ribosomal proteins are an important component of the ribosome that plays an essential role in protein synthesis. We demonstrated that *Rtn4* expression was decreased in the brain tissue of depression model rats, indicating a deficit in protein biosynthesis. After electroacupuncture, *Rtn4* expression was increased, which would facilitate protein biosynthesis[39].

(7) *Hif1a*: *Hif1a* is a receptor that mainly functions in cellular apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). After specifically binding to TRAIL, it stimulates and transmits a death signal via its death domain, and activates the caspase cascade that leads to cellular apoptosis. *Hif1a* causes tumor cell death and is also related to thymocyte and nerve cell apoptosis[40]. We found that *Hif1a* expression was downregulated in depression model rats, which would suggest a reduction in hippocampal nerve cell apoptosis. *Hif1a* expression recovered after electroacupuncture, which suggests that it is a depression-related gene that warrants further study.

In the model group, we demonstrated decreased expression of genes related to transcription/translation, neurotransmission/signal transduction, inflammation/the immune system, metabolism, enzymatic reactions, metabolism, and protein biosynthesis. Together, this would be predicted to lead to impaired hippocampal structure and function, which might even result in cell death. We believe that these gene changes underlie the manifestations of depression in this model. The upregulated genes in the model group/normal control group and the downregulated genes in the electroacupuncture group/model group are related to inflammation/immunity and neurotransmission/signal transduction. The downregulated genes in the model group/normal control group and the upregulated genes in the electroacupuncture group/model group are related to cell cycle/cell structure, neurotransmission/signal transduction, and transcription.

In the model group, the upregulated genes are related to inflammation/immunity and oxidative stress, and therefore the model rats would be expected to exhibit increased inflammation/immunity, oxidative stress, injury responses, and clotting. The downregulated genes are related to the cell cycle, signal transduction/neurotransmission, and metabolism (in particular protein metabolism). After electroacupuncture, the expression of these genes tended to return to normal. This is highly important for maintaining tissue structure, restoring cell function, and alleviating the symptoms of depression. The mechanism underlying the effect of electroacupuncture on depression relates to the regulation of multiple genes. This adds experimental evidence to the observed clinical effect of electroacupuncture on depression. We validated eight genes by real-time PCR, and the results were consistent with the gene-chip findings. This indicates that the data determined by gene-chip technology are reliable.

It should be emphasized that knowledge of gene function is still limited. The post-genomic era has only just begun. Many genes have multiple effects and their functions interact. The function of these and other depression-related genes should be studied further.

Materials and Methods

Design

A cytological gene level-based contrast observation animal study with bioinformatics analysis of high-throughput gene-chip data.

Time and setting

This study was performed in the Laboratory Animals Facility, Chinese PLA General Hospital, China, between September 2011 and September 2012.

Materials

Forty-five adult healthy male Wistar rats of specific pathogen-free grade, aged 5–6 weeks, weighing 160–180 g, were provided by SPF (Beijing) Laboratory Animals Science & Technology Co., Ltd., China (license No. SCXK (Jing) 2011-0001). Rats were raised in an artificially illuminated environment, with a 12-hour day-night cycle and a controlled temperature of 22°C. The animal experiment protocols were in strict accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals* issued by the Ministry of Science and Technology of China[41].

Methods

Establishment of a depression model in rats using chronic unpredictable mild stress

We established a rat model of chronic unpredictable mild stress-induced depression using a slight modification of the method of Willner[42-43]. In summary, after a 7-day habituation, rats received 21 days of unpredictable stimuli, including movement restriction, illumination, day-night reversal over a 24-hour period, electrical stimulation on the sole of the foot (1.0 mA, 10-second stimulation once every other minute, a total of 30 times), swimming in iced water (4°C, 5 minutes), thermal stress (45°C, 5 minutes), vibration (once per second for 15 minutes), tail clamping (1 minute), water deprivation (24 hours), and food deprivation (48 hours). One of the above-mentioned stimuli was performed every day. Each stimulus was performed four times on average. No stimulus was performed on any two or more successive days, so that the rats could not predict which stimulus would be given. The depression model rats were raised separately, one rat per cage. The control group rats were housed five to a cage and did not receive any stimulus.

Electrical stimulation

In accordance with *The Rat Brain in Stereotaxic Coordinates*[44], electroacupuncture at rat acupoints *Baihui* (the center of parietal bone) and *Yintang* (the middle point of the line between the brow bones) was performed from the day of establishing the depression model to day 21 of treatment. The acupuncture needle (HWATO Brand, No. 30, 0.5 cun; Suzhou Medical Instrument Factory, Suzhou, Jiangsu Province, China) was pricked into the skin at *Baihui* and *Yintang* at a depth of 2 mm. A forward oblique needling was required for *Baihui* and an upward oblique needling for *Yintang*. Electroacupuncture was carried out with a cluster-shaped wave, a frequency of 2 Hz and an intensity of 1 mA, once a day for 20 minutes. The level was such that the animals did not struggle or vocalize.

Body weight measurement

Body weight was measured 1 day before and on days 7, 14, and 21 of the treatment.

Open-field test

One day before the induction of depression and on day 21 of the treatment, rats were placed into the central square of an open-field apparatus. The number of line crosses with all four paws, number of entries into the central square, and the frequency with which the rats stood on their hind legs were counted during a 3-minute trial[45].

Sucrose consumption test

The sucrose consumption test was performed 1 day before

the induction of depression and on days 7, 14, and 21 of the treatment. Following water deprivation for 24 hours, rats were given 1% (w/v) sucrose solution and were simultaneously food deprived. Twenty-four hours later, the sucrose solution-containing bottle was weighed and the sucrose consumption was calculated. The data were expressed as sucrose solution mass per kilogram of body weight (g/kg)[46].

Forced swimming test

Rats were placed into a plastic cylinder (100 cm high, 40 cm diameter) filled with 25 ± 1°C water at a depth of 75 cm. After two minutes, the rats were observed for a further 5 minutes, and the time that the rats spent struggling or maintaining an immobile state with their head above water was recorded[47]. The forced swimming test was performed 1 day before the induction of depression and on days 7, 14, and 21 of the treatment. In the electroacupuncture group, the forced swimming test was performed 12 hours after electroacupuncture. This was to avoid false positives, *i.e.*, reduced immobility because of transient increases in mobility after stimulation of the cortical motor area.

Measurement of rat hippocampal gene expression by gene-chip analysis

The rats were decapitated on day 21 after the induction of depression. Following washes with pre-cooled 4°C physiological saline, a cruciform incision was made at the base of the skull. The cranial bone was separated using blood vessel forceps and the entire brain was harvested. The brain tissue and fascia on the surface of the hippocampus[44] was stripped using ophthalmic surgical forceps. Then the entire hippocampus was gently harvested and its wet weight recorded. The hippocampus was placed immediately into a pre-tagged 1.5-mL freezing tube. The entire procedure of sample harvesting was performed in an ice bath. The freezing tube was stored in liquid nitrogen. For RNA extraction, the hippocampus was cut into small blocks and ground in a pre-cooled mortar. After the addition of 2–3 mL Trizol reagent (Invitrogen, Gaithersburg, MD, USA), total RNA was extracted by the one-step method. Total RNA was precipitated from the aqueous phase by isopropanol and then further purified with a Nucleospin® RNA Clean-up kit (Macherey-Nagel, Düren, Germany). The RNA was quantified using a spectrophotometer (Beijing Huashengpuxin Instrument Co., Ltd., Beijing, China).

The RNA sample was then fluorescently labeled using a Crystal Core® cRNA amplification labeling kit (CapitalBio Corporation, Beijing, China). The labeled sample was dissolved in 80 μL hybridization solution (3 × SSC, 0.2% SDS, 5 × Denhardt's solution, 25% fluoroamine) and hybridized to a 27K Rat Genome Array gene-chip (CapitalBio Corporation; Rat Genome Version 3.0.5) at 42°C overnight. This chip contains 26,962 70-mer oligo DNAs representing approximately 22,012 genes and 27,044 transcripts. After hybridization, the sample was washed for 5 minutes in a 42°C solution containing 0.2% SDS, 2 × SSC, for 5 further minutes in 0.2 × SSC, and then dried. The chip images were scanned using a double-channel laser scanner (CapitalBio Corporation). The typing was determined according to the difference in mass spectrum molecular weight.

Validation of differentially expressed genes

The candidate genes that appeared repeatedly in the experiment were selected for further analysis. The candidate genes were selected depending on their high frequency, their extent of up-regulation or down-regulation, and their expression ratio relative to the normal control group or the model group[47]. Differentially expressed genes were validated by real-time quantitative PCR. The genes chosen for validation and their corresponding primer sequences are in Table 6.

Table 6 Prime sequences of the chosen genes

Gene	Sequence
Vgf	Upstream: 5'-GGA GGA GGA GGA CGA CGA GG-3' Downstream: 5'-TTC TTC CGC TTC CGC TTC TC-3'
Igf2	Upstream: 5'-GGG GAA GTC GAT GTT GGT GC-3' Downstream: 5'-CAC TCT TCC ACG ATG CCA CG-3'
Tmp32	Upstream: 5'-CCG AAG CTG TGG TTT GGT GT-3' Downstream: 5'-GCC TTC CTC CTT TGC AGC TC-3'
LOC500373	Upstream: 5'-TAC AGC GAC TTC GGG ACA CG-3' Downstream: 5'-GGG TGC CAT CAA TCT GTG CC-3'
Nmb	Upstream: 5'-ACT TGG CTG CTC CGT GGT CT-3' Downstream: 5'-TGA AGT GAC CAG TCG CCC AG-3'
Hif1a	Upstream: 5'-TCA TCC AAG GAG CCT TAA CC-3' Downstream: 5'-ATA GTA GGG GCA CGG TCA CC-3'
Rtn4	Upstream: 5'-GCT TTG CCC ATC ATT TGA GG-3' Downstream: 5'-GGG GGT TTT CAG GCT CAA GC-3'
Folr1	Upstream: 5'-CTG ATG TGG ATG GCC GAA TG-3' Downstream: 5'-TTG TGT TGG TGG AGC AGC AG-3'
β-Actin	Upstream: 5'-GCT CGT CGT CGA CAA CGG CTC-3' Downstream: 5'-CAA CAT GAT CTG GGT CAT CTT CTC-3'

For real-time PCR, 2 μg RNA sample was mixed with 5 μg diethyl pyrocarbonate-treated RNase-free water, then incubated at 70°C for 10 minutes and at 4°C for 5 minutes in a PCR instrument (Hangzhou Jingle Scientific Instrument Co., Ltd., Hangzhou, Zhejiang Provcince, China). The sample was then reverse transcribed in a 20-μL volume containing 4 μL moloney murine leukemia virus 5 × buffer, 2 μL 10 mmol/L dNTPs, 0.5 μL recombinant RNase inhibitor, 1 μL moloney murine leukemia virus reverse transcriptase (CapitalBio Corporation; 200 units), and 0.5 μg primer at 25°C for 10 minutes, 37°C for 60–120 minutes, 99°C for 5 minutes, and 4°C for 5 minutes, before being stored at –20°C for later use.

A fragment of the rat β-actin gene was PCR amplified from each reverse-transcribed template. The PCR products were separated on a 1% agarose gel. Eight genes with high frequency were selected for PCR analysis. Amplification was performed at a temperature 3–5°C lower than primer annealing temperature, for 40 cycles. The PCR reagent mix included SYBR Green dye (CapitalBio Corporation). An IQ5P-

CR instrument (Bio-Rad, Hercules, CA, USA) was used.

Statistical analysis

SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All data were expressed as mean ± SD. The paired *t*-test was used for comparison between groups and the *F* test for comparison among groups. A level of $P < 0.05$ was considered statistically significant.

Author contributions: *Duan DM was responsible for experimental data analysis and validation and also wrote the paper. Yang XY performed the pre-experiments and informatics analysis. Tu Y and Chen LP designed the study and provided advice on the techniques. All authors approved the final version of this paper.*
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