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Changes of brain-derived neurotrophic factors in rats with generalized anxiety disorder before and after treatment

X.-L. YIN, Y.-Y. MA, Y.-L. LIU, L.-X. WANG, N. DU, L. YANG

Department of Neurology, Clinical Medical College of Dali University, Dali, China

Xiaoling Yin and Yiyan Ma contributed equally to this work

Abstract. – OBJECTIVE: The aim of the current study was to investigate the association between brain-derived neurotrophic factor (BDNF) and generalized anxiety disorder (GAD).

MATERIALS AND METHODS: A total of sixty male adult Wistar rats with similar body weight and age were randomly divided into 3 groups the blank control group (CON, n=20), the saline control group (SAL, n=20), and the combined medication group (Deanxit +fluoxetine, DF, n=20), then rats in group SAL and group DF were prepared for model of anxiety disorder for 14 days. The body weight, center-retention time (CRT) and square-crossover number per unit time (SCN) were compared during modeling to define the anxiety of rats on day 1, day 7 and day 14; the BDNF mRNA in brain were detected by RT-PCR and the protein of BDNF in brain were detected by immunohistochemistry before and after intervention. The body weight, CRT and SCN in group SAL and DF after modeling were decreased with time compared with CON ($p<0.05$). The rats were taken euthanasia after 14 days, the BDNF mRNA showed significant decrease in SAL group (0.58±0.07) compared with group CON (2.87±0.23), while in DF group (1.76±0.21), the BDNF mRNA were higher than SAL group but lower than CON ($p<0.05$); the BDNF positive cells in group CON was highest (90%), then was group DF (75%) and group SAL was the lowest (35%).

RESULTS: The changes in the indexes of the rats among different groups before and after modeling showed that after modeling, the body weights of the rats in group SAL and group DF were lower than group CON, the CRT decreased, and the SCN increased. The differences were statistically significant ($p < 0.05$), indicating that the combined medication (Qilixin + fluoxetine) can improve anxiety symptoms (body weight, CRT, and SCN).

CONCLUSIONS: Anti-anxiety drugs (Deanxit+fluoxetine) can improve anxiety symptoms of rats and increase the expressions of BDNF mRNA and protein in rat brain cells. Anxiolytic drugs (Deanxit+fluoxetine) may achieve the treatment of anxiety disorders through improving the 5-HT nervous system and the expressions of BDNF mRNA and protein. BDNF can be used as a biochemical indicator for the diagnosis and efficacy evaluation of GAD.

Key Words:
Generalized anxiety disorder, Brain-derived neurotrophic factor (BDNF), Anxiolytic drugs (Deanxit, fluoxetine).

Introduction

Generalized anxiety disorder (GAD), also known as chronic anxiety disorder, is a chronic disease characterized by excessive nervousness[1], or anxiety, as well as being accompanied by autonomic dysfunction and exercise stress[2]. The current conceptualization of the etiology of anxiety disorders includes an interaction of psychosocial factors and the pathogenesis of GAD has not yet been fully clarified[3], and there are numerous hypotheses in biochemical mechanisms, but there is no clear gold standard[4]. The main hypotheses include the neurotransmitter hypothesis and the neuroendocrine dysfunction hypothesis[5], covering gamma-aminobutyric acid (GABA), serotonin (5-HT), norepinephrine (NE), etc[6]. Moreover, the pharmacological effects of most anti-anxiety drugs are at the level of neurotransmitters, and most are related to 5-HT and its receptors (the core hypothesis of the pathological mechanism of anxiety disorders). Selective 5-HT reuptake inhibitors (SSRIs) have anti-anxiety effects and have been used as first-line drugs for the treatment of anxiety disorders. However, if anxiolytics only work by increasing the concentration

Corresponding Author: Lin Yang, MM; e-mail: linyangyn@yeah.net

of 5-HT and/or receptors, they should work in a short period of time (1 week); however, it takes a long time for anti-anxiety drugs to fully function (4 weeks), and the depletion of 5-HT and/or receptors in normal population does not cause anxiety disorders. Therefore, the hypothesis of "depletion of 5-HT and/or receptors" does not fully explain the biological manifestations of anxiety disorders.

As research progresses, the hypothesis of "neurotrophic factor expression" in GAD has attracted the attention of scholars[7]. Scholars have found that the expression of BDNF plays an important role in improving anxiety symptoms and protecting the growth, maintenance, and regeneration of specific neurons in adult brain tissue[8]. This study detected the expression of BDNF in brain tissue of rats with anxiety disorder and the expression change after anti-anxiety medication, aiming to investigate the association between BDNF and GAD, exploring the pathogenesis of GAD, and find objective biochemical indicators for clinical diagnosis and treatment.

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Materials and Methods

Animals

Adult male Wistar rats, weighing 200 ± 20 g, were selected and bred using ordinary granule-type rat feed, together with high-temperature disinfection tap water, at 17-24°C, 60%-70% relative humidity, and normal circadian rhythm (lighting cycle as 12 hours) for 1-week environment adaptation. The rats were assessed behaviors using the open field test and body fluid consumption test. After removed the rats with abnormal results, a total of 60 rats were selected for the study and randomly divided into three groups, namely group CON, group SAL, and group DF, with 20 rats in each group. The rats in group SAL and DF were given chronic mild unpredictable stress stimulation (CUMS) and sole breeding. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. This study was approved by the Animal Ethics Committee of Dali University Animal Center.

Detection of BDNF mRNA by RT-PCR

All the rats were killed at the same time point, placed on ice, quickly dissected, and sampled the brain. Certain fresh brain tissue was sampled, placed in sterile tubes treated with diethylpyrocarbonate (DEPC) solution (Beyotime, Shanghai, China), and cryopreserved at -80°C for detecting the quantitative expression of corresponding neurotrophic factor mRNA by RT-PCR experiments; the remaining specimens were placed in 4% paraformaldehyde phosphate buffer for 24-h fixation, followed by routine dehydration, paraffin embedding, and median sagittal slicing. Each specimen shall be sliced continuously as much as possible, with the slice thickness as 4 μm, for qualitatively detecting the expression of corresponding neurotrophic factors by immunohistochemistry. The quantitative expression of BDNF in the hippocampus of dominant hemispheres of rats in each group was detected by Real-Time fluorescent PCR. The results of RT-PCR were analyzed by ABI's Real-time detector software. When the reaction was terminated, the instrument automatically displayed the threshold cycles (CT) when the fluorescence threshold was achieved. The results were automatically calculated by computer software and generated PCR amplification product curves using RT fluorescent PCR reaction software ABI Prism 7300SDS (Applied Biosystems, Foster City, CA, USA). The CT value of BDNF mRNA was normalized by the CT value of the internal reference β-actin mRNA, and the relative expression of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method. BDNF: forward primer: CCTCTTGGGGTTAGGAGAAGTCA; reverse primer: GCCACTTTGTTTCACCCTTTCC.

Detection of BDNF Protein by Immunohistochemical Analysis

Immunohistochemical SP method (streptavidin-peroxidase ligation method) is used to detect the expression of BDNF protein in rat hippocampus. Preparation and observation of immunohistochemical specimens (assisted by the staff of the Department of Pathology, First Affiliated Hospital of Dali University), hypersensitive SP (rat) kit (GR-81452); PBS buffer was used to replace the primary antibody as a negative control. The immunohistochemical staining was observed under 10×40× light microscope. The cytoplasmic brown staining was judged as BDNF-positive cells, and those without brown staining were defined as negative.

Statistical Analysis

Data statistics and analysis were performed using Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA). The statistical description of measurement

data was expressed as mean ± standard deviation ($\bar{x} \pm s$), and the count data were expressed as %. Differences between two groups were analyzed by using the Student›s *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ indicated the significant difference.

Results

Modeling of GAD

The Open-field Test: Chamber (80 cm 80 cm 40 cm) had black circumferential wall and 25 squares with the same area at the bottom. The rats were placed in the central square and observed for 3 minutes. The rats were exposed to strong light in the Open Field Chamber and put into the central grid to observe the activity for 3 minutes. The main outcome measures were central stopping time (Unit: SEC), number of crossing cells (unit:), number of Standing Times (unit:), and number of fecal grains (unit: Grains). Analysis of open field after open field experiment. The data were recorded by three observers at a time, and the three-person mean values were taken for each rat.

Open Field Test Analysis specific indicators:

- Central Parking Time (Unit: Sec): reflects the cognitive ability and the incubation period of activation, normal rats will avoid the open environment, quickly left the central grid.
- The number of traversing Lattice (unit: Units): more than three paws into the adjacent lattice can be recorded as traversing a lattice, reflecting the excitability of rats.
- Times of Standing (Unit: Times): 1 cm off the ground for two forelimbs can be recorded as standing once, reflecting the adaptation of rats to the environment.
- FECAL Granule Number (unit: Granule): reflect the tension degree of rats.

The experiment of liquid consumption: during the period when the rats were familiar with the environment, the animals were trained to adapt to drinking water with sugar. Two water bottles were placed in each cage at the same time. On the first day, the two bottles were both 1% sucrose water. The experiment of basic sugar water and pure water consumption was carried out 1 day before the stress experiment, 15 days after the start of stress and 2 days after the end of gastric lavage. The total liquid consumption, sugar water consumption and pure water consumption were calculated Finally, the rat's sugar water preference was calculated as sugar water consumption / total liquid consumption of 100%. In order to protect the drinking water from the stress events, no fasting for 48 hours in the last 2 days and 24 hours in the last 1 days were adopted. Normal rats did not suffer from anhedonia and liked to drink sugar water, but anxious rats suffered from anhedonia and the ratio of drinking sugar water was significantly lower than normal rats.

The weight of each group was measured at 1-week intervals after the beginning of the chronic stress experiment.

After 2 weeks of various CUMS stimulation, the rat models of anxiety were successfully prepared, appearing body weight loss, appetite loss, interest decrease, related exploratory activity and spontaneous activity decrease (Table I, $p<0.05$), indicating the success of preparing the rat anxiety model after chronic unpredictable stress stimulation and sole breeding.

The changes in the indexes of the rats among different groups before and after modeling showed that after modeling, the body weights of the rats in group SAL and group DF were lower than group CON, the CRT decreased, and the SCN increased. The differences were statistically significant ($p<0.05$), indicating that the combined medication (Qilixin + fluoxetine) can improve anxiety symptoms (body weight, CRT, and SCN).

Table I. Changes in various indexes of rats in different groups before and after modeling.

	Body weight (unit: g)		CRT (unit: s)		SCN (unit: n)	
Group	Before modeling	After modeling	Before modeling	After modeling	Before modeling	After modeling
CON	208.40±6.17	212.60±5.17	5.11±0.47	4.89±0.42	10.80±1.01	10.50±0.93
SAL	211.70±4.98	190.40±4.91*	4.93±0.39	4.20±0.34*	10.93±1.11	11.98±1.31*
DF	209.60±6.57	192.40±6.23*	5.03±0.41	4.17±0.37*	10.54±0.89	11.38±0.76*

Note: *$p<0.05$, compare with before modeling.

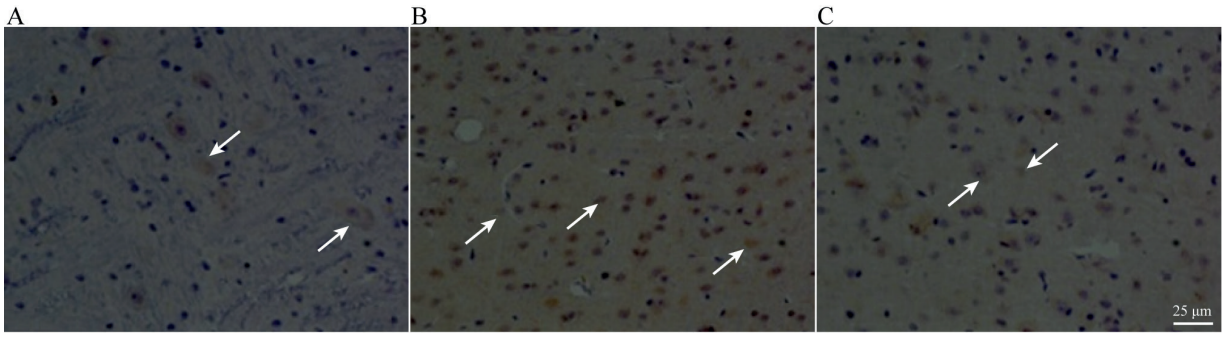

Figure 1. Cytoplasmic BDNF staining of neurons in hippocampus of dominant hemispheres in rats. **A,** Group CON, the brown stained particles represent the BDNF protein (as indicated by arrows). **B,** Group SAL, the brown stained particles represent the BDNF protein (as indicated by arrows). **C,** Group DF, the brown stained particles represent the BDNF protein (as indicated by arrows). Cytoplasmic BDNF staining of neurons in hippocampus of rat dominant hemispheres in different groups (magnification: 400×): formalin fixation, paraffin embedding, DAB color development, and brown stained particles represent the BDNF protein (as indicated by arrows). The immunohistochemical expression contents of BDNF in different groups were as follows: group CON> group DF> group SAL.

Changes in Body Weight, CRT, and SCN

In summary, on Day 7 and 14, the body weight, CRT, and SCN in group DF showed statistical significance than those in group CON and group SAL (p<0.05, Table II).

Comparison of Relative Content of BDNF mRNA in Hippocampus of Dominant Hemispheres

Compared with group CON, the BDNF mRNA levels in the brain cells of the rats in group DF and group SAL decreased, and the differences were statistically significant (p<0.05); compared with group SAL, the expression of BDNF mRNA in the rat brain cells in group DF increased, and the difference was statistically significant (p<0.05, Table III). The above results indicated that Deanxit+fluoxetine can increase the expression of BDNF mRNA in the hippocampus of rat hemisphere.

Comparison of Immunohistochemical BDNF Expression in Hippocampal

The cytoplasmic BDNF staining was observed under light microscope (Table IV): the numbers of specimens with positive expression in different groups were: group CON (18/20), group SAL (7/20), and group DF (15/20); the degrees of staining were group CON > group DF> group SAL (Figure 1); the positive rate in group DF showed statistical significance from group SAL (p<0.05), indicating that the combined medication (Deanxit + fluoxetine) can upregulate the BDNF mRNA in the brain.

Discussion

Most of current pharmacological studies of anti-anxiety drugs are related to transmitters and receptor regulation of the monoamine system.

Table II. Changes in body weight, CRT and SCN in each group at different time points.

Index	Group	1 d	7 d	14 d
Body weight (g)	CON	212.60±5.17	217.60±6.65	220.20±5.35
	SAL	190.40±4.91[a]	187.10±4.42[a]	182.80±5.86[a]
	DF	192.40±6.23[ab]	202.80±9.70[ab]	213.60±8.18[ab]
CRT (s)	CON	4.89±0.42	5.01±0.47	4.84±0.45
	SAL	4.20±0.34[a]	4.23±0.37[a]	4.16±0.32[a]
	DF	4.17±0.37[ab]	4.55±0.35[ab]	4.76±0.37[ab]
SCN (n)	CON	10.50±0.93	10.75±1.28	10.20±1.35
	SAL	11.98±1.31[a]	11.75±1.46[a]	11.88±1.24[a]
	DF	11.38±0.76[ab]	10.15±1.04[ab]	9.88±0.83[ab]

Note: [a]p<0.05, compare with group CON; [b]p<0.05, compare with group SAL

Table III. Comparison of relative content of BDNF mRNA in hippocampus of dominant hemispheres among different groups (n=20, $\bar{x}$±s).

Group	BDNF mRNA(2-ΔCT)
CON	2.87±0.23
SAL	0.58±0.07[a]
DF	1.76±0.21[ab]

Note: [a]p<0.05, compare with group CON; [b]p<0.05, compare with group SAL.

Selective 5-HT reuptake inhibitors (SSRIs) are currently the first-line drugs for the treatment of anxiety disorders. However, SSRIs shall work within 1 week, but it normally takes 3-4 weeks to fully function in clinics, and the depletion of 5-HT and/or receptors in normal population does not cause anxiety disorders. Therefore, the hypothesis of "depletion of 5-HT and/or receptors" does not fully explain the biological manifestations of anxiety disorders. Therefore, looking for objective clinical biochemical diagnostic indicators against GAD, authoritative evidence-based medical evidence, and changes in the pathological mechanisms of GAD at the molecular level have become problems that clinicians and experimental researchers are trying to explore and solve.

With the deepening of studies, the relevance between BDNF and GAD has been increasingly valued by scholars. The human BDNF gene is located at 11p13, is about 744 bp in length, and consists of 11 exons. BDNF is mainly secreted by the neurons and astrocytes, has long-term nutritional regulation, regulates the synaptic transmission of neurons, and exerts neurotrophic effects by binding to its specific high-affinity receptor TrkB. It has physiological functions, such as promoting the growth and development of nerve cells, synaptic conduction, and neural plasticity. The prefrontal cortex of the brain is an important emotional integration center in the brain and is related to emotions, abstract thinking, and high-level neural activity; the marginal lobes (including the corpus callosum, hippocampus, amygdala, hypothalamus, etc.) are the basic centers of human alertness and fear, and BDNF is the most abundant neurotrophic factor in the brain and widely distributed in the above brain tissue, especially in the hippocampus and prefrontal cortex[10]. Studies have found that 5-HT and its receptors are closely related to BDNF. 5-HT can promote the expression of BDNF, and BDNF can promote the growth and survival of 5-HT neurons[11]. Long-term use of SSRIs can upregulate the BDNF protein[12]. The BDNF-TrkB (tyrosine kinase receptor B) signaling pathway plays an important role in neuronal development, integration, and apoptosis in the hippocampus of animals. Hippocampus neurons play an important role in the production and regulation of anxiety in animals. When neurons lack the Trk B receptor, the expression of BDNF is reduced, which can increase the occurrence of anxiety-like behaviors[13,14]. Daftary et al[15] found that BDNF decreased in the almond of basal nucleus of animal models, which may result in a decrease in the 5-HT receptors and inhibitory neurotransmitter gamma-aminobutyric acid (GABA) while an increase in the excitatory neurotransmitters (glutamic acid), thus inducing anxiety. McEwen et al[16] reported that acute and chronic stress can lead to a decrease in the expression of BDNF in the hippocampus, which may in turn cause anxiety-like behaviors. Siuciak et al[17] injected BDNF into the dorsal raphe nucleus of rats and found that it can improve inviolable electric shock-resulted acquired helplessness and forced swimming-caused emotional disabilities. Ueyama et al[18] found that the downregulating BDNF can increase the sensitivity of hippocampal neurons to noxious stress, thus indirectly causing neurotoxicity and leading to atrophy and even death of hippocampal neurons. Nibuya et al[19] used SSRIs

Table IV. Comparison of immunohistochemical BDNF expression in hippocampal cytoplasm of dominant hemispheres of rats among different groups.

Group	Cases with positive expression	Cases with negative expression	Sum	Positive rate (%)
CON	18	2	20	90
SAL	7	13	20	35[a]
DF	15	5	20	75[ab]
Sum	40	20	60	67

Note: [a]p<0.05, compare with group CON; [b]p<0.05, compare with group SAL.

antidepressants to treat mice with stress and found that anxiety symptoms in mice were improved, and the expressions of BDNF mRNA and protein in the cerebral cortex were increased, suggesting that BDNF content in the brain is associated with anxiety disorders. Pandey et al[20] directly injected the no-effect chain of BDNF into mouse central amygdala (CeA) and medial amygdala (MeA), causing decreased expressions of BDNF mRNA and protein, and the anxiety-like symptoms appeared in mice, which were relieved after BDNF injection into the same site. So, it directly suggests that downregulating BDNF in the amygdala may be the molecular pathological mechanism leading to anxiety disorders. In addition, BDNF is closely related to 5-HT: 5-HT can promote the expression of BDNF, and BDNF can promote the growth and survival of 5-HT neurons[21], but the exact mechanism between them still needs further studies. Lu et al[22] study reported that BDNF can improve anxiety symptoms by affecting the gamma-ammonia station butyrate system, indicating that BDNF can affect the atrophy or apoptosis of corresponding nerve cells and produce anxiety symptoms. Stress events in GAD patients also change the levels of BDNF in the hippocampus and other parts through different pathways, which can further promote the changes of nerve cells, and these two factors interact with each other[23]. In the complex pathological mechanisms of GAD, BDNF plays important roles which may possibly protect the neurons, stabilize the intracellular free Ca^{2+} level, regulate the enzyme protein and gene expressions, and reduce the apoptosis through multiple pathways, thereby reducing anxiety symptoms.

Current studies have indicated that genes associated with "anxious behaviors" exist in both humans and mice, and the gene sequences are identical, which means that clinical studies of human anxiety disorders are capable of being tested through animal experiments. In this test, a total of 60 male Wistar rats with similar weight and age were selected and randomly divided into 3 groups (group CON, group SAL, and group DF). After successfully prepared the rat model of anxiety disorder, different groups were applied different treatment so as to compare the expression of BDNF in the brain tissue of rats among different groups by RT-PCR and immunohistochemistry, as well as its change after intervention with anti-anxiety drugs.

Deanxit is a tricyclic anti-anxiety and antidepressant (including 0.5 mg of flurazepam and 10 mg of melitracen per tablet); flupirtine is a nerve blocker, and small doses mainly act on the dopamine D2 receptor in the presynaptic membrane of nerves, which can promote the synthesis and release of dopamine and increase the content of dopamine in the synaptic cleft, thus exerting the anxiolytic effect; melitracen belongs to 5-HT reuptake inhibitor, which can inhibit the reuptake of norepinephrine and 5-HT by the presynaptic membrane, thus increasing the content of monoamine transmitters in the synaptic cleft; Fluoxetine is a selective serotonin (5-HT) reuptake inhibitor (SSRIs), which can increase the extracellular 5-HT level by inhibiting the reabsorption of 5-HT by the synaptic cells[24]. Both Deanxit and fluoxetine used in this study can inhibit the reuptake of 5-HT, thereby increasing the content of 5-HT in the synaptic cleft and alleviating the symptoms of anxiety. At the same time, the expressions of BDNF mRNA and protein were detected, which indicated certain correlation between BDNF reduction and GAD.

The results of this study showed that after chronic unpredictable stress stimulation, the body weight and CRT in each experimental group decreased while SCN increased, indicating that the rats were in an anxious state, and the establishment of an anxious rat model was successful[25]. After drug treatment, the weight gain, CRT increase, and SCN decrease in each treatment group indicated that the activity and excitement degree of the rats were lower than that before treatment (the anxiety state was alleviated). The expression of BDNF mRNA in the brain tissue of rat anxiety model was lower than that in group CON. The expression of BDNF mRNA in the brain tissue of rats in group DF was higher than that in group SAL, and the positive rate of BDNF protein expression in the brain tissue of rats in group DF was higher than that in group SAL, indicating that the combination of anti-anxiety drugs (Deanxit and fluoxetine) can increase the expressions of BDNF mRNA and protein in the brain. One study has shown that SSRIs can increase the serum BDNF levels in human[26]. It has been found that the plasma BDNF concentration in patients with GAD was lower than that in normal controls, and its concentration increased after treatment but still lower than normal controls, indicating that BDNF plays an important role in the pathogenesis of GAD[27]. After the administration of anti-anxiety drugs, the concentration of BDNF can be increased. Although there are differences in animal and human studies, it can at least explain the correlation between BDNF and GAD,

but the deep interaction between them remains further studies.

BDNF may act through NMDA receptors. BDNF enhances AMPA receptor-dependent intersynaptic signals in the hippocampus through downstream pathways mediated by NMDA receptors. Repetitive transcranial magnetic stimulation (TMS) method enhances the BDNF/TrkB signaling pathway. At the same time, the activity of NMDA receptors in the cerebral cortex has a great correlation with the activation of TrkB. In cultured hippocampal neuron cells and rat neocortical cells, activation of TrkB or chronic administration of BDNF can increase the expression of NMDA receptors NR1 and NR2A/2B through transcriptional activation, and BDNF can also signal transduction through presynaptic receptors. The pathway promotes the release of glutamate, and at the same time enhances the activity of AMPA receptor and NMDA receptor through the postsynaptic receptor pathway, thereby participating in and promoting the formation of LTP. Leptin is a lipid-derived hormone encoded by the Obese gene. It can cooperate with other growth factors such as BD-NF and insulin-like growth factors to participate in the new generation of the body.

The novelty of this study is that both Deanxit and fluoxetine used in this study can inhibit 5-HT reuptake, thereby increasing 5-HT content in synaptic fissures and reducing anxiety symptoms. At the same time, it was detected that the expression of BDNF mRNA and BDNF protein increased, indicating that the decrease of BDNF has a certain correlation with GAD.

Limitations and Solution of this Experiment

During preparing the rat anxiety model, the rats with significant differences had been removed, and only male rats were used, but clinical epidemiological data have shown that there is a certain gender difference in the incidence of anxiety disorders. Therefore, certain number of female rats should be selected for further studies under the same conditions to lay the foundation for further research.

Conclusions

CUMS and sole breeding can successfully prepare rat anxiety model. Anti-anxiety drugs (Deanxit+fluoxetine) can improve anxiety symptoms of rats and increase the expressions of BDNF mRNA and protein in rat brain cells. Anxiolytic drugs (Deanxit+fluoxetine) may achieve the treatment of anxiety disorders through improving the 5-HT nervous system and the expressions of BDNF mRNA and protein. BDNF can be used as a biochemical indicator for the diagnosis and efficacy evaluation of GAD.

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Conflicts of Interest

The authors declare no conflicts of interest.

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