Chronic stress and gonadectomy decrease the levels of neurotrophin-3 and brain-derived neurotrophic factor in adult mouse brain

LIAN Xiao-Yuan[1], ZHANG Yan[2], ZHOU Chang-Man[3], ZHANG Jun-Tian[1*]

(1. *Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing* 100050, *China*; 2. *Department of Pharmacy, Guang An Men Hospital, Chinese Academy of Traditional Chinese Medicine, Beijing* 100055, *China*; 3. *Department of Anatomy, Beijing Medical College of PLA, Beijing* 100071, *China*)

Abstract: The present study was to determine the impact of exposure to chronic stress on the levels of neurotrophic factors in the mouse brain, and investigate whether the stress-induced decline in reproductive hormone may implicate this process. Intact adult male mice and gonadectomized ones were exposed to the scheduled stress episode for 60 consecutive days. The protein levels of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) were significantly decreased in the dentate gyrus, CA1, CA2 and CA3 subfields of the hippocampus and in the cerebral cortex, consistently, these brain regions showed significantly neurodegeneration in intact stressed mice. Interestingly, the stress-induced diminution of BDNF and NT-3 protein levels in these brain regions were significantly intensified by gonadectomy. Particularly, NT-3 level in the dentate gyrus of stressed mice was preferentially decreased by gonadectomy. Likewise, the stress-induced neurodegeneration in these brain regions, especially the dentate gyrus was also aggravated by gonadectomy. The results demonstrate that chronic stress decreases NT-3 and BDNF protein levels and produces neurodegeneration in the hippocampus and the cerebral cortex, and gonadectomy exacerbates these deleterious effects of chronic stress on the brain, indicating that gonadectomy increases the vulnerability of neurons to neuropathology of stress, and suggesting that stress-induced testosterone deficit play a contributive role in neurodegeneration. This study also suggests that the diminishments of BDNF and NT-3 protein levels contribute to the neurodegeneration in the brain during chronic stress.

Key words: stress, chronic; gonadectomy; hippocampus; cerebral cortex; immunohistochemistry; brain-derived neurotrophic factor; neurotrophin 3

CLC number: R963
Document code: A
Article ID: 1000-3002(2001)04-0245-06

At least three members of neurotrophic factors are expressed in the hippocampus: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3)[1], and they are necessary for the normal development, survival and plasticity of neurons[2,3]. Stress and corticosterone were reported to be capable of affecting mRNA statement of neurotrophins as evidenced by the facts that BDNF mRNA levels are reduced, whereas NT-3 mRNA levels increased in the hippocampus by repeated stress[4], and that BDNF and NT-3 mRNA levels are downregulated in the hippocampus after 1 week of daily injections with high doses of corticosterone, which is equivalent to the plasma level of corticosterone released from adrenal during high intensive stress[5].

It is well known that stress accelerates reproductive endocrine degeneration, which means sex hormone deficits, and the aging of the brain. And reproductive endocrine dysfunction is often accompanied by degeneration of cognitive processes in middle and late life. So we hypothesized that chronic stress may influence expression of neurontrophins at protein level, and this event may be involved in the neuropathological effects of chronic stress on the brain, and that some interaction or relationship exists between brain aging and reproductive endocrine degeneration. This study was to observe the impact of chronic stress, which pro-

Received date: 2000-03-09　**Accepted date**: 2001-03-15

Biography: LIAN Xiao-Yuan (1962 –), female, native of Jinxi, Jiangxi Province, associate professor, PhD, main research field is neuropharmacology.

* Corresponding author. Tel: (010) 63165179, Fax: (010) 63017757, E-mail: zjtian@public.bta.net.cn

duced impairment of learning and memory and testosterone deficit, on BDNF and NT-3 protein levels in the hippocampus and the cerebral cortex. And the influence of gonadectomy on the stress-induced changes in the neurotrophic levels was also to be observed.

1 MATERIALS AND METHODS

1.1 Stress paradigm

Adult male Kun-Ming mice (3 – 4 month of age) were purchased from Animal Center of Chinese Academy of Medical Sciences and maintained in our laboratory for at least 1 week prior to experimental use. These animals were housed in a temperature regulated (25.0 ± 0.5)℃, light-controlled (14:10 h light:dark, lights at 5:00 am), and allowed free access to mouse chow and water. Mice were divided into three groups 〔control, stress, and gonadectomy(GDX) plus stress〕 of 12 animals each. Mice of the gonadectomy group were gonadectomized under ether anesthesia and injected intramuscularly with 0.1 mL Combiotic (procaine penicillin G plus dihydrostreptomycin sulfate, 0.1 GU·L^{-1}) for 5 weeks prior to the stress episode.

The stress used in these studies was always carried out between 9:00 am and 3:00 pm. The chronic stress was accomplished by alternatively using four stressors including hanging tail, swimming in cold water, periodic food deprivation and restraint. In order to avoid habituation for stress, the stress intensity was increased step by step with progression of the stress episode. In d 1 – 3 of the episode, animals in groups of stress and GDX + stress were exposed to hanging tail (2 h·d^{-1}) daily. In d 4 – 6 the animals were forced to swim daily (5 min·d^{-1}) in cold water with 20 cm deep and a regulated temperature (10.0 ± 0.5)℃. In d 7 – 9 the animals were deprived of their food for 72 h. In d 10 – 12 the animals were exposed to restraint daily (2 h·d^{-1}), which was accomplished by placing the animals in the glass bottles of 50 mL volume. In d 13 – 15 animals were again subjected to tail-hanging daily for the time 1 h longer (3 h·d^{-1}) than the last hanging, in d 16 – 18 to the swimming with a temperature 1℃ lower (9 ± 0.5)℃ than the last stress, in d 19 – 21 deprived of their food for 72 h, in d 22 – 24 to the restraint for the time 1 h longer (3 h·d^{-1}) than the last ones. In this pattern, the stress intensity was progressively increased until the scheduled 60 d stress episode was finished. During the whole episode, animals in control group were not disturbed in cages.

1.2 Immunohistochemistry

After 24 h of termination of the final scheduled stress episode, mice under anesthesia were perfused with saline, followed by 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) through heart. Brains were post-fixed. Thirty-five micrometer -thick coronal sections were continuously cut through the hippocampus with a vibratome. The every third and fourth sections were used for examination of NT-3 and BDNF immunoreactivity, respectively. Adjacent sections were stained with Nissl for examination of neuronal structure.

Immunohistochemistry was performed on free-floating sections with a standard avidin-biotin-peroxidase (ABC) protocol (Vectastain Elite ABC kit, Vector Labs, Burlingame, CA). The peroxidase reaction was visualized with diaminobenzidine with nickel intensification for BDNF and NT-3. The primary antibodies (Santa Cruz Biotechnology, Delaware, CA) were used at dilutions of 1:200, 1:500, respectively. The secondary antibody was employed at a dilution of 1:200. The specificity of the immnuoreaction was tested by incubating a few sections without the primary antibody or with nonspecific rabbit IgG. Tissue from control and treated animals was processed in parallel to eliminate day-to-day variation in the staining procedure.

Average optical density ($A \cdot mm^{-2}$) of BDNF- and NT-3-immunoreactivity in the curtain area was quantified with computer-assisted image analyst. Average optical density correlates positively with the number and staining density of

BDNF- or NT-3-immunoreactive granules and with the average level of BDNF or NT-3 protein in the brain area measured. After shading correction, the cerebral cortical and hippocampal images were corrected for film background, and the average of the cell layers of the CA3 and CA4 regions and the dentate gyrus and the cerebral cortex was measured. Three to four sections from each piece of tissue were used for measurement of NT-3 and BDNF protein levels. Animals in each group were 5 – 6.

1.3 Statistical analysis

Data were expressed as the mean average optical density of BDNF- or NT-3-immunoreactivity and the mean percentage of the control protein levels $\bar{x} \pm s$ as indicated in the table captions. Differences between groups were tested statistically by analysis of variance (ANOVA) according to Student-Newman-Keuls test.

2 RESULTS

2.1 Chronic stress-induced changes in NT-3 and BDNF protein levels in the cerebral cortex and hippocampus

Chronic stress for 60 consecutive days produced impairment of learning and memory in male mice (results were reported in other paper). Tab 1 shows the effects of chronic stress and GDX on NT-3 protein level shown as the absorbance of NT-3-immunoreactivity, which correlates positively with NT-3 protein level, in the cerebral cortex and the hippocampus. The dark-granules, which were in solid circles, depict NT-3 positive granules in cells. In control (unstressed mice), NT-3 positive granules were abundantly present in the granular layer of the dentate gyrus, and the CA3 and CA4 layers of the hippocampus, and the cerebral cortex. But in mice subjected to chronic stress, the positive granules were decreased in number and density, and the positive granules were further decreased in number and density in GDX mice throughout the brain (Fig 1). Consistent results were found in the quantitative analysis of NT-3-immunoreactivity. NT-3 levels in the cerebral cortex and the hippocampal dentate gyrus, CA3 and CA4 layers of stressed mice were markedly decreased compared with control as demonstrated by that the average optical density of NT-3-immunoreactivity in these brain regions of stressed mice was significantly reduced compared with control. Furthermore, the stress-induced diminution of NT-3 levels in all these brain regions were exacerbated by gonadectomy as evidenced by that the average optical density of NT-3-immunoreactivity in GDX mice was significantly diminished compared with intact-stressed mice (Tab 1). And it is noticeable that NT-3 level in the dentate gyrus was preferentially decreased in GDX mice because the relative average optical density of NT-3-immunoreactivity to control in the dentate gyrus (25.6 ± 3.0)% was obviously decreased compared with the cerebral cortex (40.0 ± 9.0)%, the hippocampal CA3 (40.7 ± 11.0)% and CA4 (34.4 ± 7.0)% subfields in the same group, respectively.

The stress-induced alterations in BDNF protein levels were similar to changes in NT-3 levels.

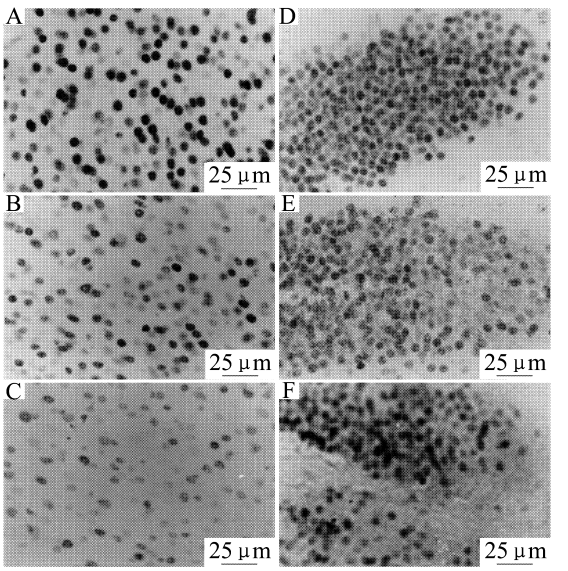

Fig 1. The expression of NT-3 protein in the brain with immunohistochemical ABC-stained method. The dark-granules depict NT-3 positive granules in cell nucleus of the cerebral cortex (A – C) and the dentate gryus (D – F). Note abundantly positive granules in control (A and D), decreases in NT-3-immuoreactivity in the stressed mice (B and E) and further decreases in the mice gonadectomized prior to exposure to the stress (C and F).

Tab 1. Effects of chronic stress and gonadectomy on NT-3 protein levels in brain

Group	NT-3 protein level			
	Cortex	Dentate	CA3	CA4
Control	0.099 ± 0.018	0.325 ± 0.028	0.182 ± 0.033	0.352 ± 0.022
Stress	0.056 ± 0.013**	0.162 ± 0.027**	0.107 ± 0.018**	0.184 ± 0.024**
GDX + stress	0.040 ± 0.009**#	0.083 ± 0.010**#	0.074 ± 0.021**#	0.121 ± 0.025**#

Data in Tab 1 are the average optical density ($A \cdot mm^{-2}$) of NT-3-immunoreactivity in cerebral cortex, dentate, CA3 and CA4 subregions of hippocampal dentate gyrus. Animals in stress were exposed to the controlled stress episode. Animals in "GDX + stress" were gonadectomized for 5 weeks prior to the same stress episode. Three to four brain sections from each animal were analyzed. $\bar{x} \pm s$, $n = 5-6$. ** $P < 0.01$, compared with control; # $P < 0.05$, compared with stress.

The average optical density of BDNF-immunoreactivity in the cerebral cortex and the hippocampus of stressed mice was significantly decreased compared with control. And the stress-induced diminution of the average optical density of BDNF-immunoreactivity was also aggravated by gonadectomy (Tab 2). But the relative average optical density of BDNF-immunoreactivity to control in the dentate gyrus(41.5 ± 5.0)% was not preferentially decreased compared with cortex(45.0 ± 4.0)% and CA3(51.9 ± 6.0)% in GDX mice. The results demonstrated that chronic stress significantly reduced BDNF protein level in the cerebral cortex and the hippocampus, and this change was intensified by gonadectomy.

2.2 Chronic stress-induced change in morphology of cortical and hippocampal neurons

In order to understand the relationship between the diminution of NT-3 and BDNF and neuronal structure degeneration, the sections from the same piece of brain tissue that were used to observe immunoreaction of neurotrophic factors were stained with Nissl. Fig 2 shows the deleterious effects of chronic stress on neuronal structure in the cerebral cortex and the dentate gyrus. It is intriguing that neurodegeneration in the dentate gyrus is the most obvious among all the hippocampal subregions in GDX mice, that is consistent with the preferential diminution of NT-3 level in this region of GDX mice, suggesting that neurotrophin deficit be involved in stress-induced neurodegeneration. Furthermore, other morphological degeneration such as neuronal apoptosis in the dentate gyrus was shown in the 6 μm-thick sections cut by rotary microtome in these animals (results not shown).

3 DISCUSSION

The present results demonstrate that the 60 consecutive days of chronic stress not only decreases BDNF and NT-3 protein expressions throughout the brain especially in the hippocampus, but also causes structural changes and neuronal damages as well as cognitive impairment (results shown in other papers). Chronic stress-induced diminution of BDNF protein levels in this study is supported by the study that immobilization and administration of corticosterone diminished BDNF mRNA levels in the hippocampus[5]. And the reductions in NT-3 protein level especially in

Tab 2. Effects of chronic stress and gonadectomy on brain-derived neurotrophic factor protein levels in brain

Group	Brain-derived neuntrophic factor protein level		
	Cortex	Dentate	CA3
Control	0.244 ± 0.023	0.609 ± 0.053	0.602 ± 0.059
Stress	0.158 ± 0.019**	0.390 ± 0.025**	0.425 ± 0.051**
GDX + stress	0.110 ± 0.010**#	0.253 ± 0.030**#	0.312 ± 0.036**#

Data in Tab 3 are the average optical density ($A \cdot mm^{-2}$) of BDNF-immunoreactivity. See Tab 1 for the treatment. $\bar{x} \pm s$, $n = 5-6$. ** $P < 0.01$, compared with control; # $P < 0.05$, compared with stress.

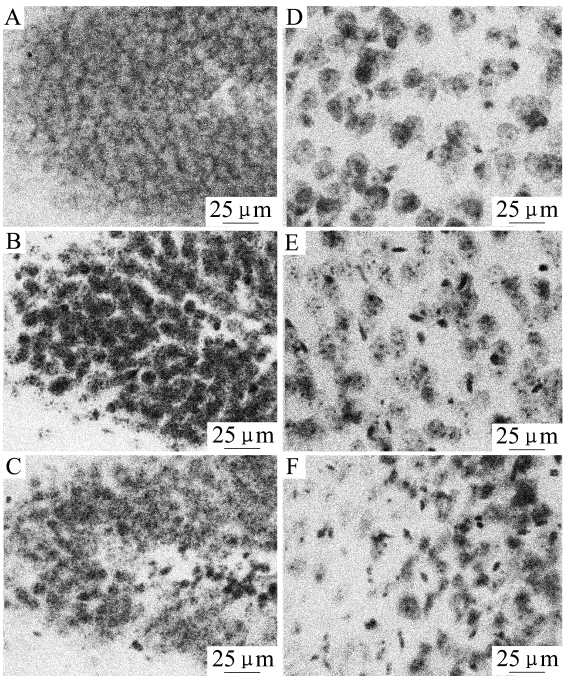

Fig 2. Nissl-stained cells in dentate gyrus of hippocampus and cerebral cortex. Hippocampus (A, B, C), cerebral cortex (D, E, F), control (A, D), stress (B, E), GDX + stress (C, F). Note marked changes in necrosis of neurons in the sections of the groups of stress and "GDX + stress".

the dentate gyrus are consistent with the results that seizures or ischemia reduced NT-3 mRNA levels in the dentate gyrus[6-8], but opposite to the enhancement of NT-3 mRNA levels in the hippocampus during 7 consecutive days of repeated immobilization[4]. These facts imply that the neurons exposed to different duration of stress or different insults may show different expression of neurotrophic factors.

Corticosterone is found to downregulate BDNF and NT-3 statement[4, 5]. In the present study, the diminution of BDNF and NT-3 protein levels in the brain of GDX mice is also related to corticosterone because the stress-induced enhancement of adrenal weight and adrenal/body weight ratio were further significantly increased by gonadectomy prior to the stress. Androgen was found to inhibit the increase in hypothalamic corticotrophin-releasing hormone (CRH) and CRH-immunoreactivity was increased following gonadectomy[9], so it is reasonable that gonadectomy exacerbated the stress-induced enhancement of adrenal/body weight ratio in this study. The preferential diminution of NT-3 protein level in the dentate gyrus of GDX mice indicated that gonadectomy increased the vulnerability of neurons in the dentate gyrus to stress damage, but the mechanisms underlying this process are not clear. This study provides a more direct evidence for the fact that stress plays an important role in negative regulation of neurotrophic factors statement, and indicates that testosterone is involved in a regulation of neurotrophins levels at least via indirect pathway, suggesting that it may be useful to delay the aging of the brain to maintain normal testosterone level during late life in male.

Neurotrophic factors have dramatic effects on the function and the morphology of mature neurons. For example, BDNF simulates synaptic transmission in hippocampal neurons[10-12], and BDNF knockout mice display attenuated LTP in the CA1 region of the hippocampus[13,14]. In addition, BDNF and NT-3 both decrease the vulnerability of cultured hippocampal neurons to glucose deprivation, and NGF and BDNF reduce glutamate neurotoxicity in these neurons[4]. So the low levels of BDNF and NT-3 protein statement (in dentate gyrus, CA3, and CA4) observed in this study would increase the vulnerability of neurons in these subfields to various insults from chronic stress. And it is conceivable that the decreases in BDNF and NT-3 protein level play a contributive role in the stress-induced impairment of cognition and neurodegeneration.

In summary, this study demonstrates that chronic stress reduces NT-3 and BDNF protein levels in the hippocampus and the cerebral cortex, and these suppressive actions of chronic stress on BDNF and NT-3 are aggravated by gonadectomy. But how gonadectomy exacerbated stress-induced diminishment of neurotrophins in the brain and why NT-3 level in the dentate gyrus of stressed brain was preferentially reduced by gonadectomy are not clear.

4 REFERENCES:

[1] Lindsay RM, Wiegand SJ, Altar CA, DiStefano PS. Neurotrophinc factors: from molecule to man[J]. *Trends Neurosci*, 1994, **17**(5):182 – 190.

[2] Lindvall O, Kokaia Z, Bengzon J, Elmer E, Kokaia M. Neurotrophins and brain insults[J]. *Trends Neurosci*, 1994, **17**(11):490 – 496.

[3] Lo DC. Neurotrophic factors and synaptic plasticity[J]. *Neuron*, 1995, **15**(5):979 – 981.

[4] Smith MA, Makino S, Kvetnansky R, Post RM. Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus[J]. *J Neurosci*, 1995, **15**(3):1768 – 1777.

[5] Schaaf MJ, Hoetelmans RW, de Kloet ER, Vreugdenhil E. Corticosterone regulates expression of BDNF and trkB but not NT-3 and trkC mRNA in the rat hippocampus[J]. *J Neurosci Res*, 1997, **48**(4):334 – 341.

[6] Rocamora N, Palacios JM, Mengod G. Limbic seizures induce a differential regulation of the expression of nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3, in the rat hippocampus[J]. *Mol Brain Res*, 1992, **13**(1 – 2):27 – 33.

[7] Takeda A, Onodera H, Yamasaki Y, Furukawa K, Kogure K, Obinata M, *et al*. Decreased expression of neurotropin-3 mRNA in the rat hippocampus following transient forebrain ischemia[J]. *Brain Res*, 1992, **569**(1):177 – 180.

[8] Takeda A, Onodera H, Sugimoto A, Kogure K, Obinata M, Shibahara S. Coordinated expression of messenger RNAs for nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 in the rat hippocampus following transient forebrain ischemia[J]. *Neuroscience*, 1993, **55**(1):23 – 31.

[9] Bingaman EW, Magnuson DJ, Gray TS, Handa RJ. Androgen inhibits the increases in hypothalamic corticotrophin-releasing hormone (CRH) and CRH-immunoreactivity following gonadectomy[J]. *Neuroendocrinology*, 1994, **59**(3):228 – 234.

[10] Kang H, Schuman EM. Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus[J]. *Science*, 1995, **267**(5204):1658 – 1662.

[11] Levine ES, Dreyfus CF, Black IB, Plummer MR. Brain-derived neurotrophic factor rapidly enhances synaptic transmission in hippocampal neurons via postsynaptic tyrosine kinase receptors[J]. *Proc Natl Acad Sci USA*, 1995, **92**(17):8074 – 8077.

[12] Lessmann V, Gottmann K, Heumann R. BDNF and NT-4/5 enhance glutamatergic synaptic transmission in cultured hippocampal neurons[J]. *Neuroreport*, 1994, **6**(1):21 – 25.

[13] Korte M, Carroll P, Wolf E, Brem G, Thoenen H, Bonhoeffer T. Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor[J]. *Proc Natl Acad Sci USA*, 1995, **92**(19):8856 – 8860.

[14] Patterson SL, Abel T, Deuel TAS, Martin KC, Rose JC, Kandel ER. Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice[J]. *Neuron*, 1996, **16**(6):1137 – 1145.

慢性应激与去睾丸减少成年小鼠脑中 NT-3 和 BDNF 的蛋白表达

连晓媛[1], 张 岩[2], 周长满[3], 张均田[1]

(1. 中国医学科学院和中国协和医科大学药物研究所, 北京 100050; 2. 中国中医研究院广安门医院制剂科, 北京 100050; 3. 中国人民解放军北京军医学院解剖学教研室, 北京 100071)

摘要: 研究了慢性应激对小鼠脑中神经营养因子蛋白表达的影响, 并探讨了性激素降低是否参与此过程. 性成熟完整雄性小鼠和去睾丸小鼠接受慢性应激 60 d, 完整动物海马和大脑皮层中神经营养因子 3(NT-3)和脑源性神经营养因子(BDNF)蛋白水平显著降低, 而且 NT-3 在海马齿状回的降低呈现某种程度的特异性, 此外, 齿状回颗粒细胞的形态退化在整个脑中也最为明显; 去睾丸应激动物海马和大脑皮层中 BDNF 和 NT-3 蛋白水平较完整动物进一步降低, 尤其是海马齿状回 NT-3 蛋白水平的降低与其他脑区相比表现出明显的特异性, 同样, 慢性应激引起的大脑皮层和海马特别是齿状回的神经元退化进一步被去睾丸恶化. 结果表明, 慢性应激可抑制脑中 NT-3 和 BDNF 蛋白表达, 去睾丸可使这种变化进一步加剧, 海马齿状回对应激神经毒性较为敏感, 提示: 脑中 BDNF 和 NT-3 蛋白水平降低与慢性应激过程中大脑形态退化密切相关; 性激素降低在应激诱导的大脑退化过程中可能扮演着重要角色.

关键词: 应激, 慢性; 去睾丸; 海马; 免疫组织化学; 脑源性神经营养因子; 神经营养因子 3

(本文编辑 乔 虹)