HSP70 Expression in the Hippocampal CA3 Subfield in Different Chronic Stress Models

Qi Li[1], Fang Pan[2], Xiao-yang Chen[2], Hong Jiang[2], Hong-jing Zhang[2], Hong-luan Yu[3], and Cui-yan Lu[4]

[1]*Brain Research Center, LG111, Anatomy Department*
The Chinese University of Hong Kong
Sha Tin, Hong Kong
[2]*Institute of Medical Psychology*
Shandong University Medical School
Jinan 250012, Shandong, P.R.C.
[3]*Psychology Department of Qi Lu Hospital*
Shandong University
Jinan 250012, Shandong, P.R.C.
and
[4]*Psychology Consultation Center for Students*
Shandong University
Jinan 250012, Shandong, P.R.C.

Abstract

This study examined the heat shock protein 70 (HSP70) expression in hippocampal CA3 subfield of rats in different chronic stress models. The chronic restraint stress (CRS) and the chronic mild stress (CMS) models were used in this study. Observation of exploratory behavior in an open field test indicated stress level. The expression of HSP70 in hippocampal CA3 subfield was measured by immunohistochemical methods. The results showed that the number of quadrant crossing in both CRS and CMS groups decreased more than that of the control ($P < 0.01$). CRS group crossing decreased more than CMS group. Damage in the hippocampus of the CMS group occurred later and to a less extent than that of the CRS group. Compared with CMS group, the expression of HSP70 was greater in the CRS group. Moreover, increased stress duration enhanced these effects. These results show that the CRS model affects both exploratory behavior and HSP70 expression in the hippocampus more dramatically than the CMS model.

Key Words: chronic restraint stress, chronic mild stress, hippocampal CA3 subfield, heat shock protein 70, open field test

Introduction

Chronic stress can induce excitable toxic damage in hippocampus (18, 23). There is a large body of evidence supporting the close association between heat shock proteins (HSPs) expression and physical and emotional stresses (10). The HSPs expression in brain, heart and blood vessel, liver, spleen and other tissues increases rapidly during stress (13). Among heat shock proteins, HSP70 is the most sensitive molecular marker to temperature changes and stress (11). HSP70 acts as a molecular chaperone inside cells, which can promote the correct folding of nascent polypeptide chain, molecular rearrangement, protein disaggregating and polypeptide transmembrane transport (17, 27). It was reported that the continuous HSP70 expression protected cells against cytolysis induced by nitric oxide (NO). The cytolysis induced by NO was similar to the cell injury after heat stress, thus HSP70 is thought to be a protective anti-NO factor and improve the defense ability to stresses like ischemia (2, 21). Stress mode and period are the

Corresponding authors: Dr. Fang Pan and Dr. Xiao-yang Chen, Institute of Medical Psychology, Shandong University Medical School, Jinan 250012, Shandong, P.R.C (Both authors have equal contribution). Tel: +86-531-88383396 / +86-531-88382086, Fax: +86-531-88382502, E-mail: panfang2003@hotmail.com / chenxy@sdu.edu.cn
Received: March 8, 2005; Revised (Finally): September 8, 2005; Accepted: October 4, 2005

main factors influencing HSP70 expression, with strengthened expression as the stress enhanced. The enhanced expression of HSP70 may indicate a dangerous situation of tissue cells (4). Although HSP70 and heat shock cognate protein 70 (HSC70) mRNA expression can be induced by psycho-physical stress (6), little research has been done to study the expression of HSP70 under different stress types. Using chronic restraint stress and mild stress to build two chronic stress animal models, we observed the exploratory behavior of rats and HSP70 expression in hippocampus under these two stress types. The exploratory behaviors and locomotor activities during open field test can reveal the rat's adaptation to a new environment (6, 14). Therefore, we attempt to explain and differentiate the underlying mechanisms for the possible effects of HSP70 under different stress types through studying the brain and behavioral changes in different stress models.

Materials and Methods

Animals

Experiments were performed on 84 male Wistar rats (2.5 months, 180-230 g). Rats were randomly divided into three groups: control group (n = 20), chronic restraint stress (CRS) group (n = 32) and chronic mild stress (CMS) group (n = 32). The colony room and laboratory temperature was set to 20±3°C with a 12 h light / 12 h dark cycle (8 a.m. ~ 8 p.m.). Rats were allowed to free access to food and water in the first week, afterwards they were fed at fixed time (8 a.m. ~10 a.m.; 5 p.m. ~7 p.m.) with a fixed amount of food.

Chronic Stress Models

[1] Chronic restraint stress model: This model was adopted from Sunanda *et al*. (25). Rats were restrained in a 25×7 cm^2 small wire mesh cage with no food and water available for 6 hrs each day (10 a.m. to 4 p.m.), lasting for 3 consecutive weeks.

[2] Chronic mild stress model: This model was adopted from Willner *et al*. (28) with a minor modification. Six kinds of stress in combination were used, including physical restraint for 1hr as mentioned above, reversed light/dark cycle for 24 hrs, noise exposure (1500 Hz, 92 dB, 1 hr/d), fasting and water deprivation for 24 hrs, tail clamping for 30 min. One kind of stress was randomly applied on a daily basis lasting for 3 weeks.

Open Field (OF) Test

OF was used to observe the exploratory behavior and emotional reaction (23). The OF test box was a 90×90×45 cm^3 painted wooden box, its inner side painted in deep gray, and the bottom area divided into thirty six 15×15 cm^2 squares. The squares along the 4 sides were peripheral squares, and others were central squares. During the 3-week stress period, OF test was performed on the day before stress, and right after the stress was finished on the 2^{nd} (D2), 7^{th} (D7), 14^{th} (D14) and 21^{st} (D21) day of stress. Eight rats were randomly selected in each experiment group and five in the control group. Each rat was placed in the center of the OF box, and observed for 5 min. The following indices were recorded and observed by two persons who did not know the objective of this test. The indices included square crossing numbers (horizontal movement scores, defined as numbers of 3 paws or half of the body cross into nearby squares), number in grooming, vertical movement scores (indexed as both forelimbs raise at least 1cm above the ground), time spending of stay in central squares, number of stools. Urine and feces were washed out after each test session to avoid the odor interference to the next rat put in this box.

Brain Slice Preparation

After the OF tests were finished on the 2^{nd}, 7^{th}, 14^{th} and 21^{st} day of stress, the selected rats were anesthetized with sodium pentobarbital (60 mg/kg) by intraperitoneal injection. We then cut the thorax wall open to expose the heart, perform aortic cannulation, and cut the right atrium open. A total of 100 ml normal saline (4°C) was perfused to wash out blood, followed by 4% paraformaldehyde phosphate buffered solution (PBS) (4°C, pH adjusted to 7.4) 500 ml perfusion to fix the tissues for 30 min. We then opened the skull and took the brain out. Two close pieces of coronal sections containing hippocampus at the thickness of 2 mm were removed. The brains were further sliced into 5 μm sections to perform hematoxlin and eosin (HE) stains and HSP70 immunohistochemistry assay.

HSP70 Assay

HSP70 was measured by immunohistochemical methods. The sections were incubated with fresh 3% H_2O_2 for 10 min under room temperature, antigen hot restoration in 0.01 M citrate buffer (pH 6.0), blocked with normal goat serum for 20 min, and incubated with rabbit anti-HSP70 antibody (1:80, NeoMarker, Fremont, CA, USA) at 4°C overnight in refrigerator. Biotinylated goat anti rabbit IgG was added to the sections, incubated at 37°C for 30 min, followed by Streptavidin- Biotin Complex (SABC) for 30 min at 37°C, then staining use diaminobenzidine under microscope. Brain slices from negative controls followed the same procedure except that the 1^{st} antibody

was replaced by PBS. Sections from corresponding location were selected in each group, and five different visual fields (50×50 μm^2) of HSP70 express areas in hippocampus were selected in each section under high power lens. The pictures were converted into electronic signals through camera, then turned into digital data in computer where the number and optical density (OD) value of HSP70 stained cells will be analyzed. The total HSP70 positive cells number and OD level in slices were measured by BI-2000 photo analyzing software.

Statistical Analysis

SPSS 10.0 was used to perform statistical analysis, and SigmaPlot 2000 was used to plot the results. Student's *t*-test was used to analyze the number of HSP70 positive cells and OD level results of CRS and CMS groups. One-way ANOVA followed by LSD-*t* test were employed to examine the differences of behaviors and the pyramidal cell number in CA3 subfield among different groups.

Results

Exploratory Behavior Changes in Different Stress Models

All three displayed groups exhibited no significant difference in behavioral indices before stress. After the stress regimens, the stress groups displayed less square crossing, more staying in central squares and less grooming than those of control group. As stress was applied, the differences became more obvious. The exploratory behavior of CRS rats was much less than that of CMS rats. The square crossing number of CRS and CMS groups decreased significantly than the control group on 2nd (D2), 7th (D7), 14th (D14) and 21st (D21) day. And the square crossing numbers of CRS group were less than those of CMS group on 2nd and 7th day (Fig. 1A). There was no obvious difference in the vertical movement scores (Fig. 1B). The grooming numbers of CRS and CMS groups both had decreasing trends than control group (Fig. 1C). The CRS and CMS rats had more central square stay time than control rats, especially on 7th (D7), 14th (D14) and 21st (D21) day of stress. The increasing trend of central square stay time in CRS group was obvious, while the CMS group only had a slight increasing trend (Fig. 1D). The number of feces had no obvious difference except an increase on 14th day in CRS rats (Fig. 1E).

Shape and Number of Hippocampus CA3 Neurons in Different Stress Models

The shape and number of pyramidal cells in hippocampus CA3 subfield in HE staining sections were observed under a high power microscope. The pyramidal cells in CA3 subfield of control group rats had normal shape, tidy arrangement, and no shrink or melting in nucleus. The stressed rats in CRS and CMS groups had similar appearance on the 2nd day. The pyramidal cells displayed swelling, light staining of cell body, sparse cell arrangement on the 7th day. The pyramidal cells shrank, cell nucleus pycnosis and intercellular space increased on 14th day. These became worse on the 21st day. Pyramidal cells in 0.5×0.5 mm^2 hippocampus CA3 subfield were counted (Fig. 2). The pyramidal cells in CA3 area in CRS rats were decreased during the stress period, and had significant differences with control ones (D2: $F = 4.423$, $P < 0.05$, D7: $F = 53.090$, $P < 0.01$, D14: $F = 31.499$, $P < 0.01$, D21: $F = 40.729$, $P < 0.01$). There was a significant difference between the CRS and CMS rats on 21st day of stress. However, cells in CMS rats were decreased during the first 3 weeks and after that there was an increasing trend of pyramidal cells in CA3 area. The results showed that both CRS and CMS produced decreases on CA3 neurons. Meanwhile there was a significant difference of cell numbers on the 7th day and 21st day between the CMS rats and control ones. Taken together, the damage of hippocampus in CMS rats was less than the CRS ones. Because the start of cells reducing at one week in CMS rats while the CRS rats began at the 2nd day of stress, the hippocampus CA3 neurons damage in CMS group occurred later than that of CRS group, and the duration of cells damage were longer in CRS group than in CMS group.

HSP70 Expression in Hippocampus CA3 Area in Different Stress Models

The HSP70 expression was observed under microscope after the paraffin sections performing immunohistochemical staining. The CA3 subfields of stress groups showed typical brown stains, indicating HSP70 positive expression. The stained neurons were mainly pyramidal cells in this area. The stained area mainly located in the cytoplasm of the positive neurons (Fig. 5). No typical positive stain was found in control group.

HSP70 positive cells in CA3 subfield in hippocampus were increased in CRS rats. In CMS rats the cells increased during the first 2 weeks of stress, however, in 3rd week the HSP70 positive cells decreased. There was a significant difference of HSP70 positive cells between CRS and CMS on the 2nd, 14th, and 21st day of stress. (D2: $t = 3.549$, $P < 0.01$, D14: $t = 5.495$, $P < 0.01$; D21: $t = 13.138$, $P < 0.01$), but no difference on the 7th day of stress. (Fig. 3)

From the start of the stress the OD value of HSP70 in hippocampus was increased in CMS group

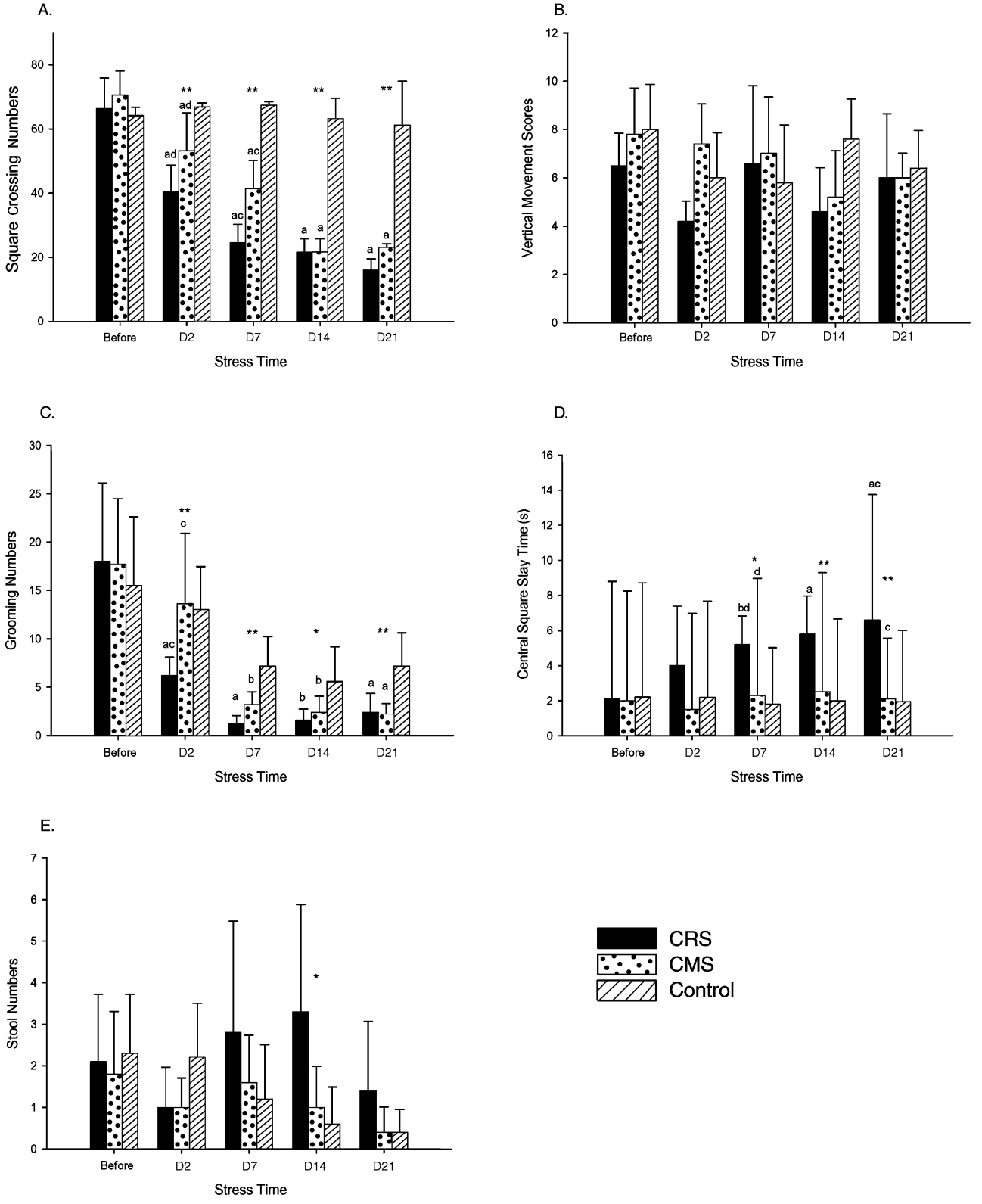

Fig. 1. Behavior changes of rats in different stress models (mean ± SD). D2 = Day 2, D7 = Day7, D14 = Day 14, D21 = Day 21. There were 32 rats in each stress group and 20 rats in the control group, and 8 rats of each stress group and 5 rats of control group were used in every stress period. $^{**}P < 0.01$,$^{*}P < 0.05$ (ANOVA); $^{a}P < 0.01$, $^{b}P < 0.05$, difference compared with control group (ANOVA, LSD-*t* test); $^{c}P < 0.01$, $^{d}P < 0.05$, difference between CRS and CMS (ANOVA, LSD-*t* test).

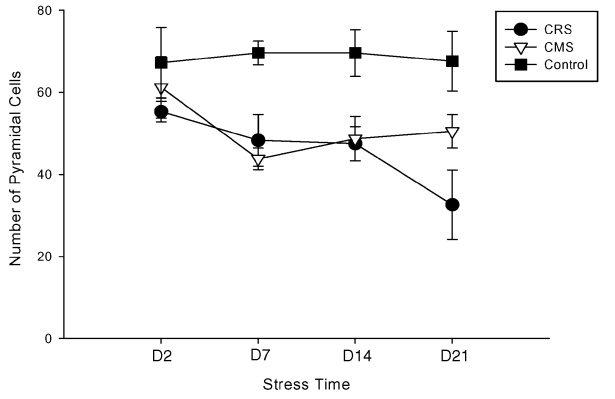

Fig. 2. Pyramidal cells in hippocampus CA3 area of rats in different stress models (mean ± SD).

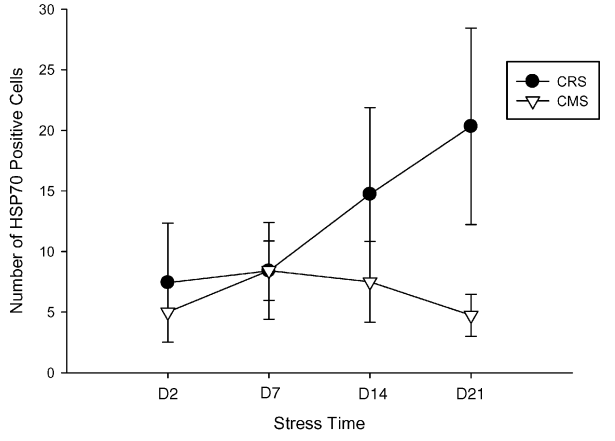

Fig. 3. HSP70 positive cells number in CA3 area of rats in different stress models (mean ± SD)

and CRS group, but decreased on 21st day of stress (Fig. 4). Meanwhile there was a significant difference of OD value between CRS and CMS groups on the 7th,14th, and 21st day of stress, but no difference on the 2nd day of stress (D7: $t = 2.233$, $P < 0.05$, D14: $t = 3.786$, $P < 0.01$, D21: $t = 4.977$, $P < 0.01$). The results indicated that HSP70 expression increases at the beginning of stress in both CMS and CRS groups, with a slight decrease at 3rd week.

Discussion

Open field test was used to evaluate the rat behaviors under stress. The quadrant crossing is a locomotor activity that shows the rat's movement in open field, and the staying time in central squares is the time before the rats took exploratory in a new environment. These indices help us evaluate the behavioral changes of rats in response to stress (8, 20). Besides the behavior disturbance can be induced by

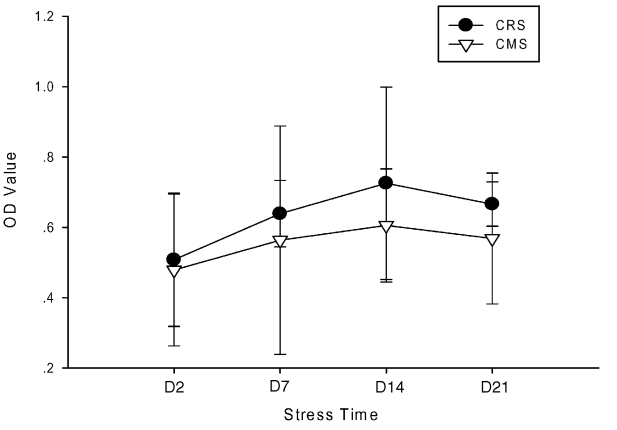

Fig. 4. HSP70 OD value of CA3 subfield of rats in different stress models (mean ± SD).

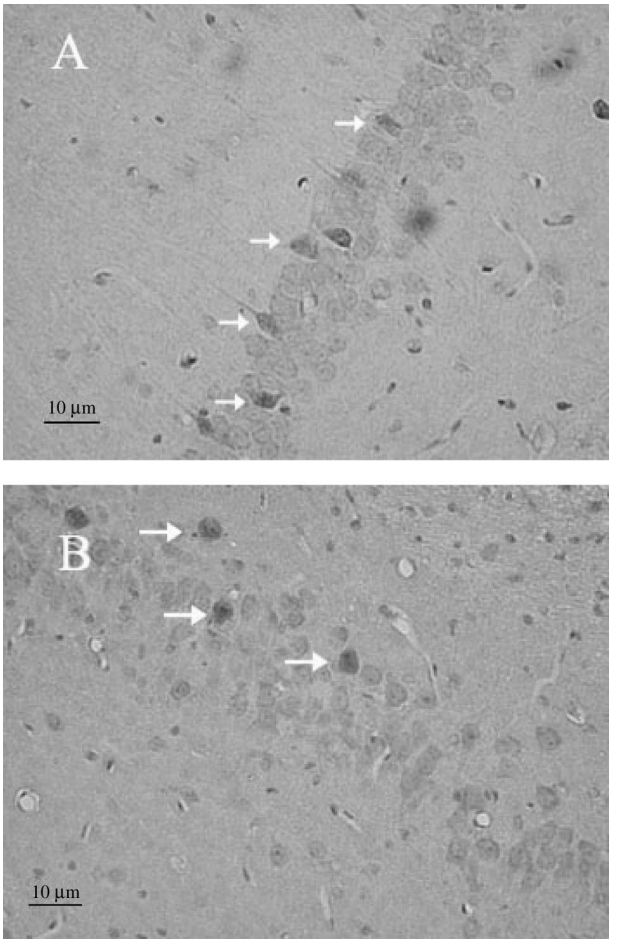

Fig. 5. HSP70 immunostaining in the CA3 subfield in hippocampus of a CRS rat (A) and a CMS rat (B) on the 14th day of stress. The arrows indicated HSP70 staining positive neurons.

many kinds of stress. Furthermore behavior manifests itself differently depending on the duration of stress. Acute stress results in an increase of movements while

chronic stress results in behavioral and emotional disorders (5, 7, 23). Under prolonged stress, the behavior of rats turns from exciting state to inhibitory state. Although this change had been recognized by many researchers, the mechanism of the change still remains unclear.

In agreement with previous results, we found that rats under stress showed less active than the control group, thus displayed a decrease of square crossing, an increase of stay time in central square and the decrease of grooming. The exploratory activities of CRS group decreased significantly compared to those of CMS group. The CRS rats had a severer damage during stress and the decrease of exploratory activities. Vyas *et al.* (26) found that chronic immobilized stress (CIS) rats expressed greater anxiety than the chronic unexpected stress (CUS) and the control ones. Taken together, we came to a conclusion that the changes of rats behaviors are dependant on the period and mode of stress (9, 24).

Sapolsky (22) reported that chronic immobilized stress or chronic multiple stress could cause apical dendrite shrinkage of the pyramidal neurons in CA3 area in hippocampus, characterized by the significant loss of neuron branches and total length of the apical dendrite. The CA3 area is the most sensitive subfield of the hippocampus (19), and chronic stress may induce the neurons to have a shape change, cell space enlargement, loose cell arrangement, the Nissle body disappearance and cell counts decrease (23, 29). Researchers have found significant shrinkage of pyramidal cells in hippocampus CA3 subfield in CIS compared to CUS (3). We observed a similar results of damage to the pyramidal neurons in hippocampus CA3 subfield caused by prolonged stress. The CA3 subfield pyramidal neurons damage in CRS group was much more severe than that of the CMS group.

The mechanism of HSP70 expression in brain especially in the hippocampus still eludes researchers. It had been found that psycho-physical stress will induce the HSP70 and HSC70 mRNA expression in rat brain(11, 12). Abe *et al.* (1) and Kawagoe *et al.* (15) have reported that transient cerebral ischemia can induce the HSC70 mRNA expression in hippocampus. The CA3 subfield of hippocampus has the highest HSP70 mRNA expression in different stress models (16, 19). A possible mechanism of HSP70 under stress is that stress caused hyperfunction of hypothalamic-pituitary-adrenocortical axis to release large amount of glucocorticoids (GCs). GCs combines with glucocorticoid receptor (GR), and then the HSP70 that combined with GR is released out to protect the cells. Filipovic *et al.* (10) reported that a significant decrease in cytosol GR and HSP70 was observed after acute stress, while chronic stresses led to negligible changes in both these proteins and caused a reduced responsiveness to a novel acute stress. We found that the hippocampal CA3 subfield in CRS and CMS rats had an increased HSP70 expression while under prolonged stress. The shrinkage and necrosis of pyramidal cells in hippocampus CA3 area of CRS group continues though stress induced HSP70 expression. A possible explanation is that HSP70 can combine with abnormal proteins when the damage to the neuron is mild, keeping the expression of HSP70 from being obstructed. However, when the stress damage is so severer that the transcription and/or the translation progress of HSP70 are obstructed, further HSP70 cannot be generated. Without the protecting effects of HSP70, the neurons will suffer severe damage as extreme as death.

Above all we found that HSP70 expression increased as the stress prolonged, and reached the peak at the later period of stress. Meanwhile the up-regulation of HSP70 is responsible for neuronal damage. However, the up-regulation of HSP70 occurred later than neuron damage, which probably indicates that cell loss and the behavior disturbance could be prevented if we use HSP70 directly or induce HSP70 expression before the stress. This preventive mechanism of HSP70 needs further study before it can be applied to clinical use.

References

1. Abe, K., Kawagoe, J., Aoki, M. and Kogure, K. Dissociation of HSP70 and HSC70 heat shock mRNA inductions as an early biochemical marker of ischemic neuronal death. *Neurosci. Lett.* 149: 165-168, 1993.
2. Bellmann, K., Jaattela, M., Wissing, D., Burkart, V. and Kolb, H. Heat shock protein hsp70 overexpression confers resistance against nitric oxide. *FEBS Lett.* 391: 185-188, 1996.
3. Blake, M.J., Udelsman, R., Feulner, G.J., Norton, D.D. and Holbrook, N.J. Stress-induced heat shock protein 70 expression in adrenal cortex: an adrenocorticotropic hormone-sensitive, age-dependent response. *Proc. Natl. Acad. Sci. U.S.A.* 88: 9873-9877, 1991.
4. Chrousos, G.P., Loriaux, D.L., Gold, P.W. and National Institutes of Health (U.S.) Mechanisms of physical and emotional stress. New York: Plenum Press, 1988.
5. Conrad, C.D., LeDoux, J.E., Magarinos, A.M. and McEwen, B.S. Repeated restraint stress facilitates fear conditioning independently of causing hippocampal CA3 dendritic atrophy. *Behav. Neurosci.* 113: 902-913, 1999.
6. de Cabo de la Vega, C., Pujol, A. and Paz Viveros, M. Neonatally administered naltrexone affects several behavioral responses in adult rats of both genders. *Pharmacol. Biochem. Behav.* 50: 277-286, 1995.
7. Dunn, A.J. and Swiergiel, A.H. Behavioral responses to stress are intact in CRF-deficient mice. *Brain Res.* 845: 14-20, 1999.
8. Elliott, J.M., Heal, D.J. and Marsden, C.A. Experimental approaches to anxiety and depression. Chichester; New York: John Wiley, 1992.
9. Ely, D.R., Dapper, V., Marasca, J., Correa, J.B., Gamaro, G.D., Xavier, M.H., Michalowski, M.B., Catelli, D., Rosat, R., Ferreira, M.B. and Dalmaz, C. Effect of restraint stress on feeding behavior of rats. *Physiol. Behav.* 61: 395-398, 1997.

10. Filipovic, D., Gavrilovic, L., Dronjak, S. and Radojcic, M.B. Brain glucocorticoid receptor and heat shock protein 70 levels in rats exposed to acute, chronic or combined stress. *Neuropsychobiology* 51: 107-114, 2005.
11. Fukudo, S., Abe, K., Hongo, M., Utsumi, A. and Itoyama, Y. Brain-gut induction of heat shock protein (HSP) 70 mRNA by psychophysiological stress in rats. *Brain Res*. 757: 146-148, 1997.
12. Fukudo, S., Abe, K., Hongo, M., Utsumi, A. and Itoyama, Y. Psychophysiological stress induces heat shock cognate protein (HSC) 70 mRNA in the cerebral cortex and stomach of rats. *Brain Res.* 675: 98-102, 1995.
13. Fukudo, S., Abe, K., Itoyama, Y., Mochizuki, S., Sawai, T. and Hongo, M. Psychophysiological stress induces heat shock cognate protein 70 messenger RNA in the hippocampus of rats. *Neuroscience* 91: 1205-1208, 1999.
14. Gray, J.A. The psychology of fear and stress. Cambridge; New York: Cambridge University Press, 1987.
15. Kawagoe, J., Abe, K., Sato, S., Nagano, I., Nakamura, S. and Kogure, K. Distributions of heat shock protein (HSP) 70 and heat shock cognate protein (HSC) 70 mRNAs after transient focal ischemia in rat brain. *Brain Res*. 587: 195-202, 1992.
16. Kitraki, E., Karandrea, D. and Kittas, C. Long-lasting effects of stress on glucocorticoid receptor gene expression in the rat brain. *Neuroendocrinology* 69: 331-338, 1999.
17. Lindquist, S. and Craig, E.A. The heat-shock proteins. *Annu. Rev. Genet*. 22: 631-677, 1988.
18. Makino, S., Smith, M.A. and Gold, P.W. Increased expression of corticotropin-releasing hormone and vasopressin messenger ribonucleic acid (mRNA) in the hypothalamic paraventricular nucleus during repeated stress: association with reduction in glucocorticoid receptor mRNA levels. *Endocrinology* 136: 3299-3309, 1995.
19. Mizoguchi, K., Kunishita, T., Chui, D.H. and Tabira, T. Stress induces neuronal death in the hippocampus of castrated rats. *Neurosci. Lett.* 138: 157-160, 1992.
20. Pellow, S., Chopin, P., File, S.E. and Briley, M. Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J. Neurosci. Methods* 14: 149-167, 1985.
21. Powers, S.K., Locke, A.M. and Demirel, H.A. Exercise, heat shock proteins, and myocardial protection from I-R injury. *Med. Sci. Sports Exerc*. 33: 386-392, 2001.
22. Sapolsky, R.M. Why stress is bad for your brain. *Science* 273: 749-750, 1996.
23. Sarkisova, K. and Kulikov, M.A. Prophylactic actions of the antioxidant agent AEKOL on behavioral (psychoemotional) disturbances induced by chronic stress in rats. *Neurosci. Behav. Physiol.* 31: 503-508, 2001.
24. Silveira, P.P., Xavier, M.H., Souza, F.H., Manoli, L.P., Rosat, R.M., Ferreira, M. B. and Dalmaz, C. Interaction between repeated restraint stress and concomitant midazolam administration on sweet food ingestion in rats. *Braz. J. Med. Biol. Res*. 33: 1343-1350, 2000.
25. Sunanda, Rao, B.S. and Raju, T.R. Restraint stress-induced alterations in the levels of biogenic amines, amino acids, and AChE activity in the hippocampus. *Neurochem. Res*. 25: 1547-1552, 2000.
26. Vyas, A., Mitra, R., Shankaranarayana Rao, B.S. and Chattarji, S. Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *J. Neurosci*. 22: 6810-6818, 2002.
27. Welch, W.J. and Suhan, J.P. Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. *J. Cell Biol*. 103: 2035-2052, 1986.
28. Willner, P., Wilkes, M. and Orwin, A. Attributional style and perceived stress in endogenous and reactive depression. *J. Affect. Disord*. 18: 281-287,1990.
29. Woolley, C.S., Gould, E. and McEwen, B.S. Exposure to excess glucocorticoids alters dendritic morphology of adult hippocampal pyramidal neurons. *Brain Res.* 531: 225-231, 1990.