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# Treatment with Phytoestrogen α-Zearalanol Might Protect Neurons of Hippocampus in Ovariectomized Rats

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**Although neuroprotective effects of estrogen on postmenopausal women have been recognized, an associated increased incidence of uterine and breast tumors has jeopardized the clinical use of estrogen. This study was designed to evaluate the neuroprotective effects of a novel phytoestrogen α-zearalanol (α-ZAL), on ovariectomized (OVX) rats. Adult Wistar rats were ovariectomized or sham-operated and treatment with equivalent doses of 17β-estradiol or α-ZAL for 5 wk. Uteruses have been weighted and stained by hematoxylin and eosin for morphology analysis. The expression of synaptophysin and parvalbumin in hippocampus were evaluated by immunohistochemistry assays. Our experiments indicated that the synaptophysin and parvalbumin-positive areas were significantly decreased in the OVX group compared to the sham group, α-ZAL or 17β-estradiol administration can reverse the effects. Although α-ZAL and 17β-estradiol treatments reconciled uterus weight loss which was induced by ovariectomy, the effect of α-ZAL was less than 17β-estradiol. This result suggests that α-ZAL may effectively abate neurons loss in the hippocampus while slightly promoting weight gain of the uterus.**

**Key Words:** Ovariectomized rats; α-zearalanol; hippocampus; synaptophysin; parvalbumin.

## Introduction

Estrogen has several effects that extend beyond regulation of the reproductive functions; in addition to its classic role in reproduction, the neuroprotective properties of estrogen have become one of the major focuses over the last decade. Accumulating evidence in vivo *(1–4)* suggests that estrogen plays a neuroprotective role in neurodegenerative processes. Moreover, in vitro studies demonstrate that 17β-estradiol enhances neuronal survival in response to a variety of neuronal injuries including oxidative stress *(5)*, excitotoxic insults *(6)*, and β-amyloid toxicity *(7)*, related to $Ca^{2+}$ imbalance. Epidemiological studies also suggest that estrogen replacement therapy in postmenopausal women lowers the risk of Alzheimer's disease *(8)*. However, the clinical use of estrogen was limited by the risk of increasing the cases of mammary cancer and carcinoma of endometrium *(9)*. Therefore, it is the clinical focus to search low side-effect estrogen-like medicine.

Recently, a plant-derived phytoestrogen, α-zearalanol (α-ZAL), has been proposed as a potential replacement for estrogen. α-ZAL is a reductive product of the *Gibberella zeae* metabolite, which is abundant in plants and vegetables including soybean, wheat, rape, radish, celery, spinach, and apple *(10)*. Mounting evidence has shown that α-ZAL is highly efficient and safe for use in animal husbandry in the United States and Canada *(11)*. More important, it has been reported that α-ZAL was able to alleviate DMBA-induced mammary gland tumor formation, and decrease the incidence of estrogen-dependent cancer *(12)*. Thus, attention has been draw to the value of clinical potential of this phytoestrogen as a "safe estrogen" with less risk of tumorigenesis. Preliminary evidence from different groups indicated that α-ZAL may effectively prevent atherogenesis and endothelial dysfunction *(13–15)*. The benefit of α-ZAL on cardiovascular system has been proved *(16,17)*, but there are only limited reports about the effect of α-ZAL on the nervous system. The present study was designed to evaluate the potential neuroprotective effect of phytoestrogen α-ZAL. Because the ovariectomized (OVX) rat has been shown to be suitable for the studies of estrogen *(18)*, we investigated the effect of α-ZAL on this in vivo animal model.

## Results

### *Uterus Weight and Morphology*

The weights of uteruses in the OVX-group were significantly decreased compared with the sham-operated group ($p < 0.05$). However, the weights of uteruses increased 2.6

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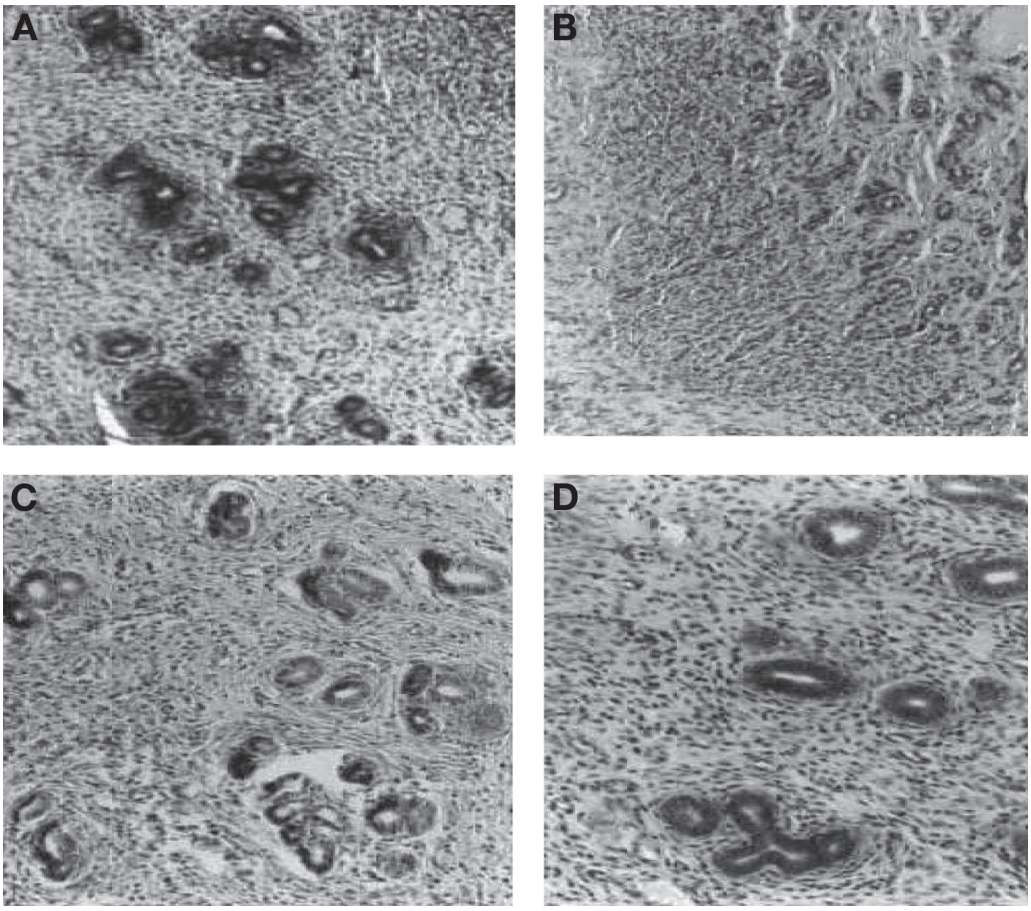


**Fig. 1.** Normal gland and interstitial cells were seen in sham-operated rat (**A**); adenocytes were lost and atrophic in OVX rat (**B**); 5 wk after 17β-estradiol (**C**) or α-ZAL (**D**) treatment, there are no notable changes observed in the uterus (×200).

**Table 1**
Effects of 17β-Estradiol and α-ZAL
on the Weight of Uterus and Serum Estrogen

| Group ($n$ = 7) | Uterus (g) | Estrogen (pg/mL) |
|---|---|---|
| Sham | 0.776 ± 0.205 | 215.66 ± 11.85 |
| OVX | 0.256 ± 0.077 | 135.50 ± 12.72 |
| OVX+17β-estradiol | 1.628 ± 0.483# | 344.86 ± 60.28# |
| OVX+ α-ZAL | 0.815 ± 0.134* | 196.72 ± 13.18* |

*$p < 0.05$; #$p < 0.01$, compared to OVX group.

times ($p < 0.01$) and 1.5 times ($p < 0.05$) after 17β-estradiol or α-ZAL treatment compare to OVX-group, respectively. This indicates that α-ZAL is less potent in stimulating uterine growth than estrogen (Table 1).

There are normal proliferative or secretory glands without hyperplasia and atrophy and normal-shape interstitial cells in the endometria of the sham-operated rats. In the OVX rats, the epithelial cells in gland shrunk; the interstitial cell partially degenerated and disappeared, the hyperplastic fibrous tissue took its place. After treatment with 17β-estradiol or α-ZAL for 5 wk, the shape of gland and the epithelial cells in the OVX rats were similar to that of the sham-operated rats (Fig. 1).

### *Serum Estrogen*

Serum estrogen levels decreased sharply in OVX-group compare to sham-group. Five weeks after 17β-estradiol or α-ZAL treatment, estrogen levels were significantly increased, however, the increase induced by α-ZAL was not as prominent (Table 1).

### *Expression of Synaptophysin*

Immunocytochemical studies revealed that a synaptophysin-positive area is dispersed in hippocampus CA1 region and dentate gyrus (DG) .The mean optical density and area density of synaptophysin immunoreactivity in hippocampus CA1 region and dentate gyrus of OVX group decreased significantly compared with sham-group, after treatment with 17β-estradiol or α-ZAL for 5 wk; these changes were reversed and there was significantly difference compared with OVX group (Figs. 2 and 3).

### *Parvalbumin Analysis*

In the sham-operated rats group, there are large, deep-filemot bodies and long axons with many nodular nerve buckles on the PV-positive neurons of hippocampus CA1

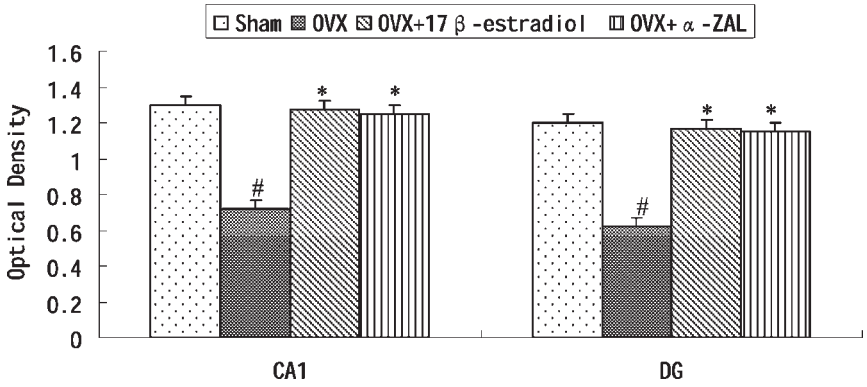


**Fig. 2.** The effects of estrogen and α-ZAL on the optical density of synaptophysin in hippocampus of OVX rats. $^{\#}p < 0.01$ compared with sham-group; $^{*}p < 0.01$ compared with OVX group.

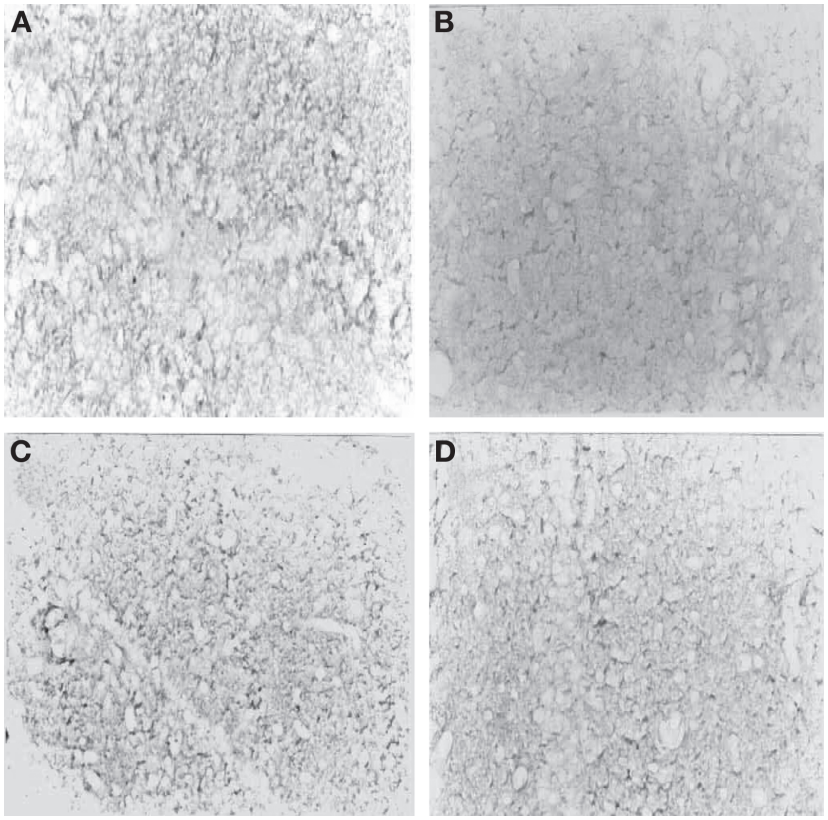


**Fig. 3.** Neuroprotective effect of α-ZAL identified with immunoreactivity for synaptophysin in hippocampus CA1 region. Synaptophysin-positive area in sham-operated rat (**A**) is larger than OVX rat (**B**); the area of synaptophysin-positive increased with treatment by 17β-estradiol (**C**) or α-ZAL (**D**) (×400).

region. The same phenomena were observed in dentate gyrus. Five weeks after ovariectomy, the number of PV-positive neurons and density of nerve fibers in hippocampus CA1 region and dentate gyrus of the OVX group decreased significantly; these changes were lessened by treating with 17β-estradiol or α-ZAL for 5 wk (Figs. 4 and 5).

## Discussion

Our results demonstrated that phytoestrogen α-ZAL may alleviate neuron loss in the hippocampus, which was induced by low-estrogen levels; the effect was similar to that of 17β-estradiol. Moreover, α-ZAL restored the ovariectomy-induced uterus weight loss but did not induce uterine overgrowth elicited by 17β-estradiol, indicating that α-ZAL does not have the uterus tissue growth–promoting property of 17β-estradiol.

Synaptophysin is a 38-kDa glycoprotein found in the membranes of neurotransmitter-containing presynaptic vesicles *(20)* and increases in synaptophysin immunoreactivity have most often been interpreted as reflecting an increase the density of synapse *(21,22)*. The present experiments clearly demonstrate that the synaptophysin immunoreactivity in hippocampus CA1 region and dentate gyrus of

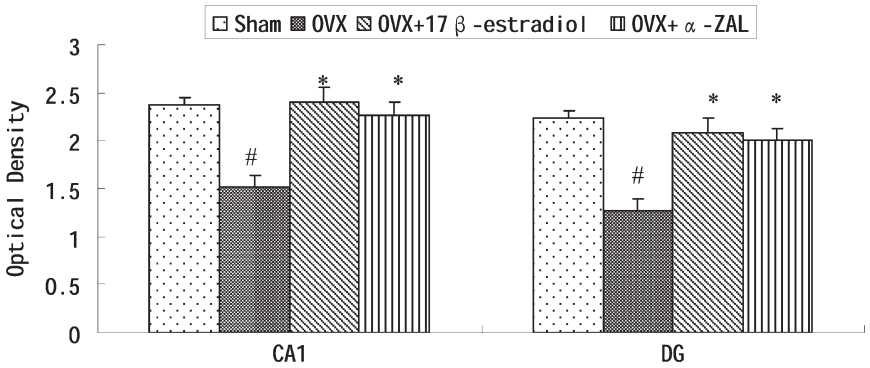


**Fig. 4.** The effects of estrogen and α-ZAL on the optical density of PV in hippocampus of OVX rats. $^{\#}p < 0.01$ compared with sham-group; $^{*}p < 0.01$ compared with OVX group.

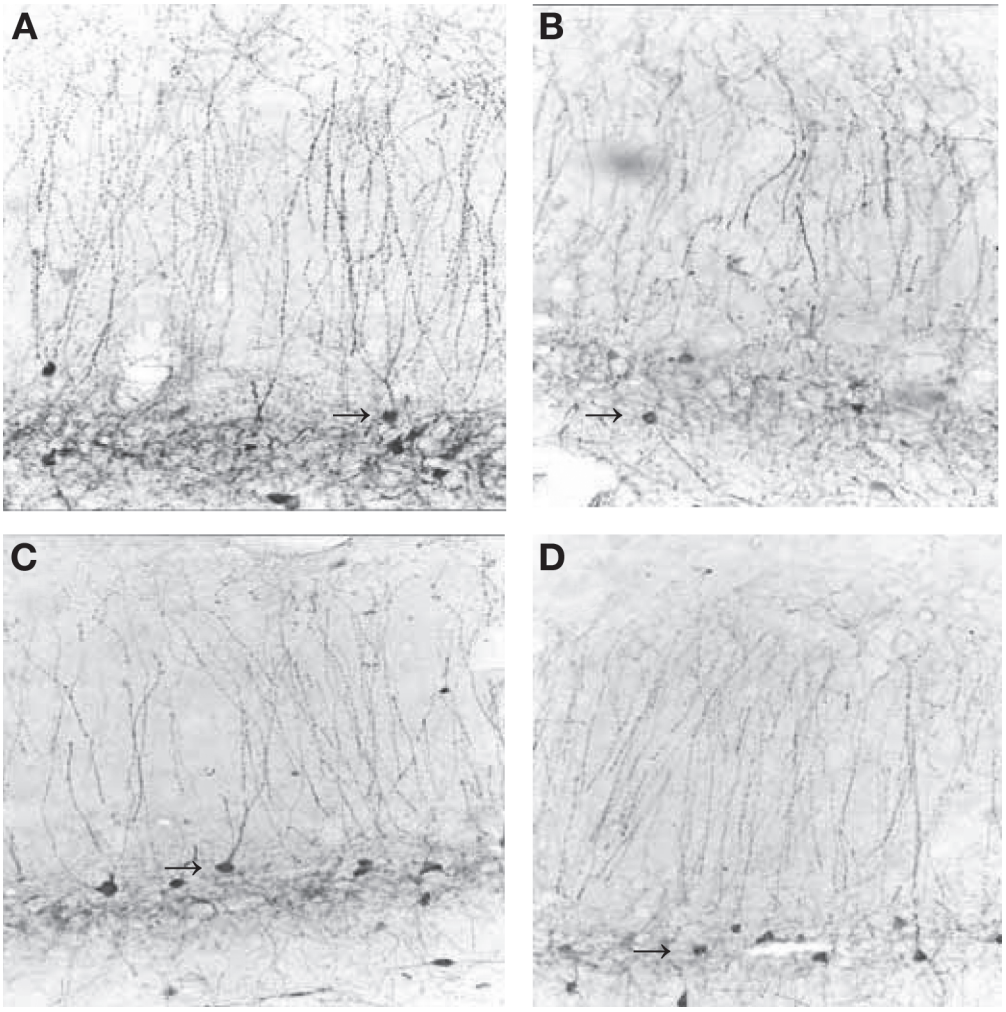


**Fig. 5.** Positive immunoreactivity for PV is seen easily in the hippocampus CA1 region in sham-operated rat (**A**); PV-positive neurons decreased in the OVX group (**B**); the rats were treated with 17β-estradiol (**C**) or α-ZAL (**D**), and there was apparently an increase of PV-positive neurons (×400).

OVX group decreased significantly; after treating with 17β-estradiol for 5 wk, these changes were reversed. These data are consistent with prior results found in rats and mice *(23, 24)*, while expanding the role of estrogen in its synaptogenic effects to higher-order mammals. Our findings also support a recent Golgi study which showed that estrogen treatment increased spine density in the hippocampus of ovariectomized adult female African green monkeys *(25)*. Simultaneously, synaptophysin-positive area increased after administration α-ZAL compare with OVX rats; this means that

α-ZAL reduced neuron loss in hippocampus just like 17β-estradiol. Thus, we were able to speculate that α-ZAL may counteract the neurons loss induced by lack of estrogen.

Parvalbumin (PV) belongs to a family of intracellular proteins that have high affinities for calcium and possesses the ability to regulate the cellular $Ca^{2+}$ homeostasis *(26)*. The intracellular calcium concentration ($[Ca^{2+}]_i$) at rest, or peak $[Ca^{2+}]_i$ during a $Ca^{2+}$ transient, is not affected by PV *(27)*, but PV accelerates the initial decay of $[Ca^{2+}]_i$ *(28)*. Therefore, PV has been implicated in protecting against high levels of intracellular $Ca^{2+}$. Results of our investigation indicated that PV-positive neurons decreased in hippocampus CA1 region and dentate gyrus in OVX rats, while increased significantly in OVX rats administered 17β-estradiol or α-ZAL. There is no statistical difference between in 17β-estradiol group and α-ZAL group, suggesting that α-ZAL alleviated neuron damage partly depend on promoting the expression of PV. Although the cellular mechanisms of α-ZAL remain to be elucidated, there is evidence to support α-ZAL's ability to interact with the estrogen receptor, while the affinity of binding to the estrogen receptor for α-ZAL is estimated to be only one-tenth of that for 17β-estradiol *(13)*; perhaps this is one of the reasons that α-ZAL regulates the expression of PV. Thus, the cross-action between α-ZAL and estrogen receptor is worthy of more research.

In addition, the data in our research shows that α-ZAL increased the OVX rat uterus weight but to a significantly lesser extent than that of 17β-estradiol. The uterus weight increased by α-ZAL was only approx 50% of the uterus weight gain induced by 17β-estradiol. However, morphology analysis demonstrated that administration of α-ZAL may abate the gland shrinking in uterus, just like 17β-estradiol, these data are consistent with that found in rabbit *(13)*. Obviously, this kind of action will benefit to long-term estrogen replacement treatment therapy.

Our study showed that phytoestrogen α-ZAL effectively abates neuron loss in the hippocampus with fewer growth-promoting effects in the uterus compared with estrogen. The present results may provide useful information for future clinical use α-ZAL in postmenopausal women suffering neurodegenerative diseases.

## Materials and Methods

### *Experimental Animals and Treatment*

Female Wistar rats (8- to 10-wk-old, 240 ± 10 g) were purchased from the Laboratory Animal Center of the Chinese Academy of Medical Sciences. All of the rats were housed in groups of five on a 12-h light/dark schedule. Rats were allowed free access to mouse lab chow and water. All animal experiments were approved by the Laboratory Animal Center of the Chinese Academy of Medical Sciences. After a 2-wk adaptation period, bilateral ovariectomy or sham-operation performed under sodium pentobarbital anesthesia (50 mg/kg ip). Animals completely recovered 2 wk after surgery. Then, the rats were assigned to one of the following four groups ($n = 7$ in each group): Group A: Sham-operated + olive oil; Group B:OVX + olive oil; Group C: OVX + 17β-estradiol (0.5 mg/kg); Group D: OVX + α-ZAL (0.5 mg/kg).

17β-Estradiol (Shanghai 9th Pharmaceutical Co., Shanghai, China) and α-ZAL (gift from Prof. Shunling Dai at Perking Union Medical College) were dissolved in olive oil (Beijing Shenzhou Olive Development Corporation, China). Rats from groups A and B received an equal volume of the olive oil. The intraperitoneal injections were given every 3 d. Treatment continued for 5 wk.

### *Tissue Processing*

Rats were deeply anesthetized by sodium pentobarbital (50 mg/kg ip) and killed 12 h after the last dose. After behavioral checks were made to ensure the disappearance of deep reflexes, animals were transcardially perfused with 100–200 mL of phosphate-buffered saline flush, followed by 300–500 mL of 4% paraformaldehyde in 0.1 *M* phosphate buffer (PB). The brains were removed immediately after perfusion and were post-fixed for 8 h at 4°C in the same fixative. Then, the brains were placed in 0.1 *M* PB containing 10%, 20%, and finally 30% sucrose for cryoprotection. After the brains had sunk in the most concentrated sucrose solution, they were rapidly frozen in powdered dry ice, and stored at −70°C until use.

### *Morphological Observation*

The uterus were removed quickly and weighted. Then uterus were fixed in formalin at 4°C for 36 h, embedded in paraffin wax, cut into 5 μm in coronal sections, and stained in hematoxylin and eosin for light microscopy examination.

### *Determination of 17β-Estradiol*

Blood samples were obtained from heart. Serum 17β-estradiol (estrogen) levels were determined by radioimmunoassay with a commercially available kit (China Institute of Atomic Energy, Beijing, China). Assay sensitivity was 1.0 pg/mL, intra-assay and interassay coefficients of variation for estradiol were 5.3% and 6.4%, respectively.

### *Immunohistochemistry and Image Analysis*

Brain coronal sections (20 μm) were cut with a Vibratome (Technical Products, St. Louis, MO). Series of sections from each brain were immunohistochemically examined using the avidin–biotin–peroxidase (ABC) method, as described by Reddy *(19)*. Briefly, endogenous peroxidase activity was blocked with 3% $H_2O_2$ in PBS for 20 min. Incubation of the sections in 3% normal goat serum blocked nonspecific reaction for 1 h at room temperature and sequentially incubated with anti-synaptophysin (1:200; mouse monoclonal antibody; Sigma) or anti-parvalbumin (1:200, mouse monoclonal antibody; Oncogene Research Products, Boston, MA)

overnight at 4°C. After washing with PBS, the sections were incubated with a secondary biotinylated antibody (goat anti-mouse IgG, Vector Laboratories, Burlingame, CA) for 30 min at 37°C. After incubation with secondary antibody, sections were treated with ABC kit (Vector Laboratories) to form avidin/biotin peroxidase complex. Visualization was done by 3,3-diaminobenzidine (DAB; Vector Laboratories). The sections were washed with PBS, dehydrated in graded ethanol solutions, clear in xylene, and coverslipped.

To quantify the protein expression, the integral optical density of synaptophysin and PV immunoreactivity in hippocampus CA1 region and dentate gyrus were estimated by using multifunctional pathologic image analyses system (Beihang University, Beijing, China). Six sections from the same brain were evaluated and the mean optical density was used to express the date of every animal.

### *Statistical Analysis*

Data were expressed as mean ± SEM. Statistical analyses were performed with one-way ANOVA followed by SPSS software (SPSS Science, Chicago, IL,USA). *P* value less than 0.05 denoted the presence of a statistically significant difference.

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