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Catalpol regulates function of hypothalamic-pituitary-adrenocortical-axis in an Alzheimer's disease rat model

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Aims: To investigate the regulating effects of catalpol on the hypothalamic-pituitary- adrenocortical-axis (HPA) in an Alzheimer's disease (AD) rat model. *Methods:* Healthy male Wistar Rats were selected. The AD model was generated by orthotopic injection of β-amyloid 25–35 (Aβ25–35) into the right lateral ventricle. The animals were divided into five study groups: Catalpol at low dose (5 mg/kg), Catalpol at high dose (10 mg/kg), model control group and sham surgery control group, n = 9 respectively. The serum concentration of hydrocortisone (HYD), adrenocorticotropin (ACTH) and corticotropin releasing hormone (CRH) determined by Enzyme-Linked Immunosorbent Assay (ELISA). Structural alterations of the hypothalamus were examined by H&E stain and electron microscope. The CRH receptor 1 (CRHR1) positive neurons were detected with immunohistochemistry. *Results:* Serum HYD level was significantly increased ($p < 0.01$), and both ACTH and CRH were dramatically decreased ($p < 0.01$) in the AD model group rats compared with normal control rats at day 7. Catalpol treatment was able to improve the hormone secretion disorder in AD model group rats compared with the model group ($p < 0.01$ or $p < 0.05$) in particular at 21 days. Structure damage of hypothalamus in the AD rat as evidenced less CRHR1 positive neurons, rough endoplasmic reticulum dilation and degranulation, and mitochondrial swelling under electron microscope. Catalpol treatment at both high and low doses was able to alleviate the structure damage of the hypothalamus in the AD rats. *Conclusions:* Catalpol could improve the endocrine function of the HPA and alleviate the structural damage of hypothalamus in AD rats.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease in the elderly. The disease is pathologically featured with the senile plaques caused by beta-amyloid protein deposit and tangled neurofibril inside neurons, neuronal degeneration and loss of hippocampus and cortex areas (Blennow 2006; Hardy and Selkoe 2002). Clinical studies showed there were neuroendocrine function disorders in AD patients, especially dysfunction of the hypothalamic-pituitary-adrenal axis (HPA). At present, it is considered that neuroendocrine disorders could lead to memory drop and cognitive dysfunction (Csernansky 2006; Van Osch 2004), and high concentration of glucocorticoids in serum can lead to brain aging, extensive degradation and loss of neurons in the brain. When malfunction developed in AD sufferers, the protection function of corticotropin releasing hormone (CRH) to the neurons would be attenuated, which caused neuron loss in hippocampus, gradual diminution of memory ability and cognitive function problems (Umegaki 2000; Pardon 2011), promoting AD progress.

Current available treatment for AD is to improve the cholinergic nerve function with cholinesterase inhibitors such as donepezil. However, AD is a chronic progressive disease, and a long-term application of cholinesterase inhibitors results in severe side effects and reduced therapeutic effects (Winblad 2004). Therefore it is essential to develop new drugs for more effective AD treatments. Traditional Chinese medicines possess advantages in the treatment of the chronic degenerative neural disease such as AD and PD. These traditional Chinese medicines are able to demonstrate improvements of body physiological function and fewer side effects. In this study we focus on identifying the effective components in a natural product of traditional Chinese medicine for AD therapy.

Rehmannia root has been used as an anti-ageing traditional Chinese medicine, whose main chemical components are iridoid glycosides with catalpol as the most important one. During the past years, many studies indicated that catalpol may have a protective function in neural degenerative diseases (Li 2005; Liu 2006; Jiang 2004). Consistent with these reports, our previous studies (Wang 2007) demonstrated the catalpol showed protective activity in an AD cell model. In several preliminary experiments however, we found that catalpol showed better effects in AD animal models than in cell models. Together with other studies that proved the regulation effect of catalpol with AD and a diabetes rat model (Xia 2012; Huang 2010), we believe that catalpol may regulate the body network especially the nerve–endocrine network, in which HPA is an important part. Effects of catalpol on HPA function in AD have not been reported. Thus, our current research interest is to evaluate the effects of catalpol on HYD, ACTH, CRH and hypothalamus morphology.

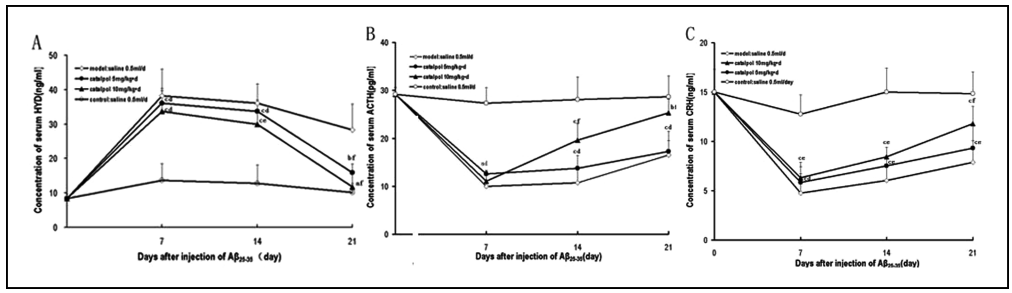

Fig. 1: Serum concentration of HPA related hormone of AD rats by ELISA. The numerals in the abscissa indicate the days after injection of Aβ25–35. Agents are continuously injected intraperitoneally from the 2nd day for 7 days. Each point represents the average concentration ($x \pm SD$) of 10 ras in each group. Blood was taken at 8–9 am, serum concentration of HYD(A), ACTH(B) and CRH(C) was measured by ELISA procedure. $aP > 0.05$, $bP < 0.05$, $cP < 0.01$ as compared with control. $dP > 0.05$, $eP < 0.05$, $fP < 0.01$ as compared with the model group.

2. Investigations and results

The results of the Y-maze behavior test were the same than those reported by other researchers (Wang 2007), learning ability of the rats in the model group was decreased markedly, catalpol improved the behavior ability of rats. Effects of catalpol on HPA were as follows:

2.1. Catalpol regulates the balances of serum HYD-ACTH-CRH in AD rats

The serum hormone concentration in the model group was changed dramatically compared to sham operation group; serum HYD was significantly increased ($p < 0.01$), however, ACTH and CRH decreased greatly ($p < 0.01$) 7 days after injection of $A\beta_{25–35}$ compared to the normal control group (Fig. 1).

Catalpol improved hormone secretion disorder, serum CRH concentration was increased by low (5 mg/kg) and high doses (10 mg/kg) of catalpol ($p < 0.01$ and $p < 0.05$ respectively) in comparison to the model group; at the 21st day, CRH concentrations in the catalpol 10 mg/kg group reached normal levels ($p > 0.05$ vs control group). However, its concentration in the 5 mg/kg group remained at the same level than the model group ($p > 0.05$). Those diversities may be related to weak effects of catalpol at 5 mg/kg and rapid CRH self-healing recovery. Compared to the model group, catalpol 5 mg/kg did not change both HYD and ACTH concentrations at day 7, but those varied hugely in the catalpol 10 mg/kg group ($p < 0.01$) from 7 to 21 days. Also catalpol continued to rise ACTH concentration and decrease HYD ($p < 0.01$). Catalpol 10 mg/kg even declined HYD concentration to a normal state after 21 days ($p > 0.05$ vs control group).

These results prove that catalpol is able to regulate the endocrine disorders of AD rats. Catalpol at 10 mg/kg ameliorates HYD, ACTH and CRH disorder markedly, for there is no difference in serum HYD concentration between catalpol high dose and control until the 21st day, also ACTH and CRH recovered prominently.

2.2. Catalpol protects morphology changes of rats hypothalamus

Compared with normal hypothalamus morphology, injuries were shown by a less number of neurons, widened neuron intercellular spaces, and karyopyknosis in partial neurons. Treatment with catalpol led to more normal neurons with clear and intact nuclear membranes and the morphology in the catalpol 10 mg/kg group was much closer to the normal structure with more neurons compared to the model (Fig. 2).

2.3. Catalpol protects expression of CRHR1 at hypothalamus and hippocampus

CRH mediates the majority of its functions upon binding to the CRH receptor type 1 (CRH-R1)(De Souza 1995), so the effect of catalpol on CRH-R1 was observed. The immunoreactivity distributes on membrane and dendrites of neurons. Expression decreased in hypothalamus and hippocampus of model rats, moreover partial neurons have the normal morphology without stain. Further investigations need to be done to figure out the relations between those morphological and functional changes of the neurons. High doses of catalpol (10 mg/kg) increased the expression significantly, positive neurons showed darker stain in membrane and longer dendrites (Fig. 3).

2.4. Catalpol protects ultrastructure injuries of the hypothalamus

Ultrastructure of normal rats showed most neurons with clear cell boundary and synapses, lots of normal mitochondria, rough endoplasmic reticulum and Golgi complex. Nucleus of neurons was big with scattered chromatin and nucleolus. Injuries of model rats showed neuron pyknosis with increased electron density, swelling and degranulation of rough endoplasmic reticulum, lots of lipofuscin particles in the cytoplasm. Also there were lots of swelling mitochondria and crista degranulation, some vacuolar degeneration. Nucleus showed irregular pyknosis, a locally widened perinuclear space and concentrated heterochromatin in the nucleus. The other obvious change is that there are abundant Golgi complex around the lipofuscin particles areas, indicating some relationship between the production of the lipofuscin particles and function of the Golgi complex.

Low doses of catalpol (5 mg/kg) can partly improve these injuries with less nucleus pyknosis, widened perinuclear space, concentrated heterochromatin, and mitochondrial crista disorder. Catalpol (10 mg/kg) can ameliorate the injuries significantly with abundant mitochondrion and rough endoplasmic reticulum, and rarely mitochondrial crista widen, very few degranulation of rough endoplasmic reticulum and less heterochromatin. These obvious changes proved the protective effect of catalpol on hypothalamus ultrastructure in rats (Fig. 4).

3. Discussion

Alzeimer's Dieseas (AD) is a common degenerative disease of the central nervous system that affects the life of the patients seriously. Because its pathogenesis is not well understood, there is no cure for this disease. Current available therapeutic measures such as Aβ vaccine, NGF intracerebral injection only showed limited effects, therefore it is important to develop more efficacious medicines.

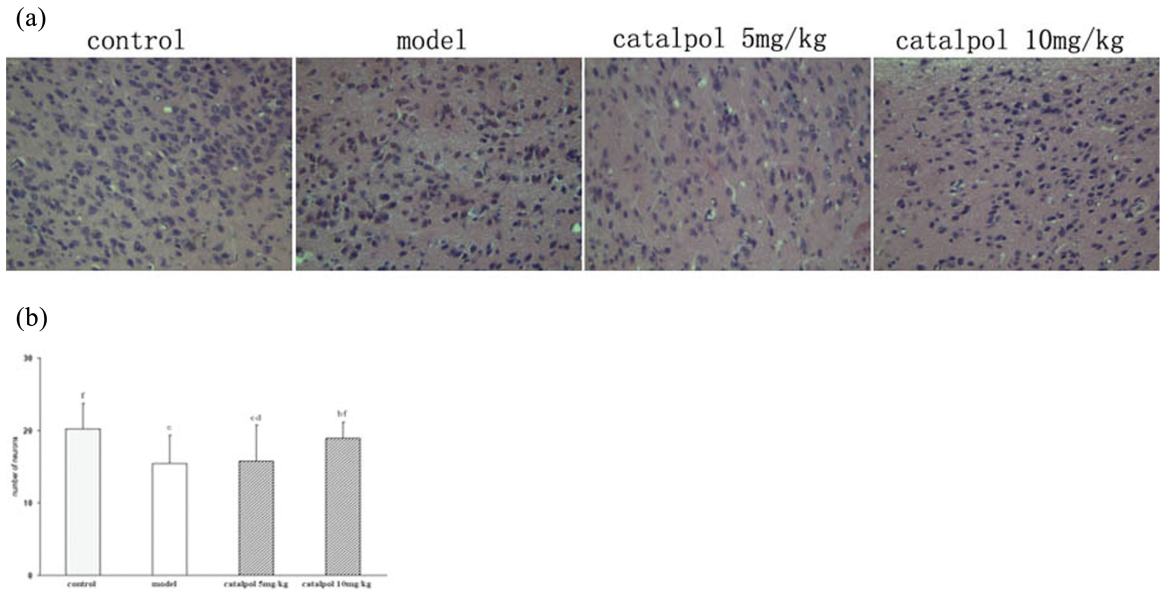

Fig. 2: Evaluation of the hypothalamus morphology by hematoxylin & eosin (H&E) staining. Representative examples of rats in each group(A). Neurons in control group were round or oval with high nucleocytoplasmic ratio, and staining of neuron nuclei was homogeneous. Sample in model group showed neurons with condensed cytoplasm and some abnormal hyperchromatic neurons. Catalpol at high dose(10 mg/kg) improved these injuries significantly. Number of neurons were calculated from 3 independent field of vision of each section, 5 sections for each group under the microscope(B). $aP > 0.05$, $bP < 0.05$, $cP < 0.01$ as compared with control. $dP > 0.05$, $eP < 0.05$, $fP < 0.01$ as compared with the model group.

The hypothalamic-pituitary-adrenocortical-axis (HPA) is a major regulatory pathway of the endocrine system and plays a vital role in maintaining the internal environment stabilization of the human body. Neurons of the hypothalamus were stimulated by the signals secreted CRH, which binds to CRH receptor type 1 (CRHR1) and activates adenohypophysis to synthesize and secrete ACTH, further the adrenal cortex is stimulated to synthesize and release glucocorticoids. The latter exerts extensive physiological or pathophysiological functions as the end hormone. Last century, Landfield (1978, 2007) presented the glucocorticoid/brain aging theory and believed that serum-increased glucocorticoid accelerates brain aging, which starts from the hippocampus (rich with glucocorticoid receptors) and displays degeneration, loss and hypofunction of the neurons. In the normal status, hippocampi possess inhibitive regulation effects on HPA axis. However, when brain aging happens, the hippocampus will be denatured and the binding forces with glucocorticoid will be attenuated, which result in HPA axis hyperactivity and increased serum glucocorticoid levels. Meanwhile, the higher serum glucocorticoid levels will boost the degeneration and loss of the brain neurons. O'Brien (1996) reported that abnormal HPA axis regulation usually was accompanied by hippocampus atrophy in elderly people while the latter is worsening the former. The majority of AD patients performed hyperactive HPA axis regulation and had significantly increased glucocorticoid levels in the cerebrospinal fluid. The relations of cause and result between functional brain degeneration and nerve endocrine disorder especially abnormal HPA regulation are still controversial. It may be a good idea to break the vicious circles of hormone disorder and neuron degeneration through improving nerve endocrine disorder.

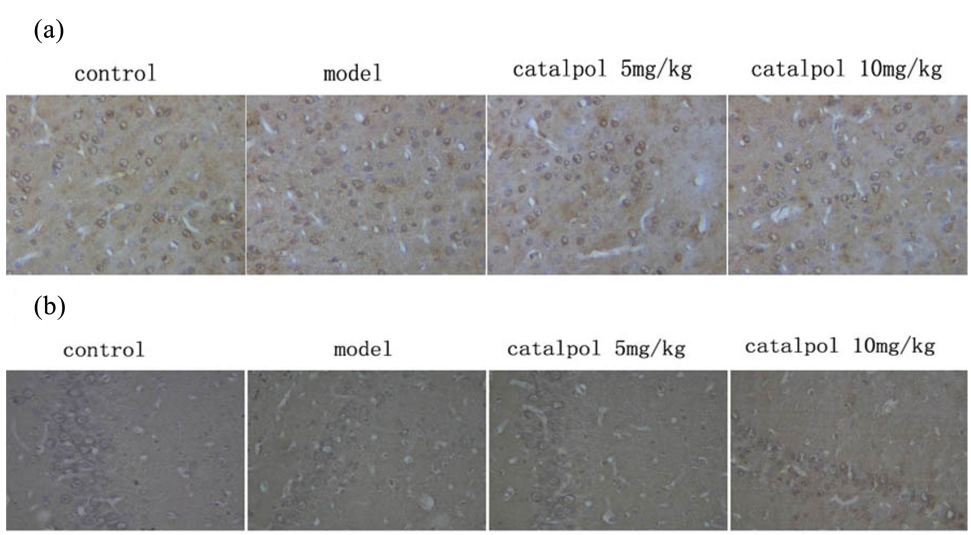

Fig. 3: Representative examples of CRHR1 expression by immunohistochemistry. Positive reactivity stain distributes mainly on membrane of the neurons both in hypothalamus(A) and hippocampus(B). Expression decreased in model group, and treatment of catalpol improved the expression.

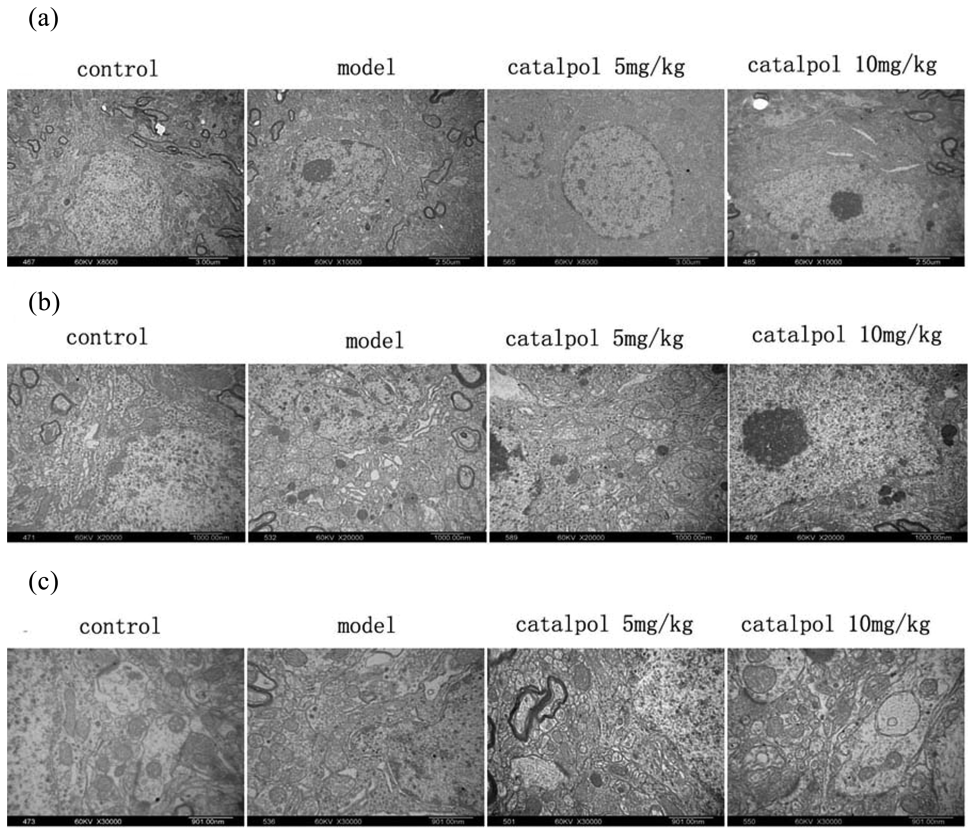

Fig. 4: Morphology observation of rats hypothalamus by transmission electron microscope. (A) Ultrastructure of hypothalamusat under low magnification (× 8000 or × 10000), (B) magnification (× 20000), (C) magnification(× 30000). Structure of model rats showed lots of lipofuscin particles in the cytoplasm with swelling and degranulation of rough endoplasmic reticulum, swelling mitochondrion and crista degranulation. Locally widen perinucler space and concentrated heterochromatin in the nucleus. Catalpol ameliorated these changes especially at high dose.

The effects of catalpol were investigated in our study on the basis of the following aspects: (1) Catalpol is one of the most important effective ingredients of Rehmannia root used for the treatment of senile dementia in traditional Chinese medicine. Recently more and more studies showed it has functions of regulating the endocrine and nerve growth factor, anti-inflammatory, anti-oxidative damage and so on (Huang 2010; Shieh 2011; Wang 2010), protecting nerves from injury as well (Li 2005; Liu 2006; Jiang 2004; Hu 2010); (2) previous studies proved that Catalpol was not able to inhibit the activity of cholinesterase and to combine M receptor. Therefore, its mechanism of action must be different from currently available AD medicines, so catalpol is worth to be further investigated (Xia 2012; Wang 2006). Doses of catalpol (5 mg/kg and 10 mg/kg) resulted from our trial experiments and literature reports (Liu 2006; Huang 2010).

Rats model injected with $A\beta_{25-35}$ into the lateral ventricle is a commonly used AD animal model. This results in many symptoms of AD, such as the weakened reactive potency, disorder of serum hormones, morphological injuries and decreased expression of CRHR1 at the hypothalamus.

However, the deregulation of endocrine hormones in the AD rat model gradually recovers over time, the level of CRH is even close to normal levels after 21 days. This indicates that the endocrine symptoms in the rat model are not maintained for a long period. Therefore, for long term studies on AD suitable stable phenotype animal models should be developed. In addition, research data demonstrated that the expression of CRH in both AD and depression patients is much higher than normal (Raadsheer 1995). We consider that this is due to the application of different animal models and the differences of brain area sample collections. Regardless the AD patients or animal mod els those studies were usually accompanied by depression or insulin resistance. The AD rat model adopted in our study has been widely used for AD studies. We found the serum HYD elevated rapidly leading to the levels of ACTH and CRH dropped down due to the negative feedback and the potential damages to the hypothalamus and hippocampus (O'Brien 1994). So we assume that it will be feasible to develop a chronic AD model by utilizing the neural toxic function of cortical hormone.

The results of this study affirmed the protective effect of catalpol on HPA of AD rats. The learning ability of the rats was improved greatly, especially at 21 days after treatment with catalpol by successive intraperitoneal injection, and the hormone secretion disorder was also improved obviously.

The structural injuries observed at hypothalamus with H&E stain (40 ×) ameliorated after the treatment with catalpol. The ultrastructure of hypothalamus showed karyopyknosis, a great quantity of lipofuscin granules, widened perinuclear space, and the intranuclear concentrated heterochromatin, catalpol at both low and high doses was able to improve these degenerative changes, so to promote HPA function recovery. Meanwhile, immunohistochemistry stain showed decreased expression of CRHR1 and shorter neuron dendrites in hypothalamus of AD rats. In contrast, after the treatment with catalpol, the expression increased in lots of neurons and longer dendrites displayed.

The results above proved that catalpol can ameliorate the disorder of HPA endocrine function and abnormal morphology of hypothalamus, but its functional targets and exact mechanisms need further investigations.

Other results of our study indicated that catalpol could regulate not only HPA function, but also exerted effects on the hypothalamic-pituitary-gonadal axis. Together with the neural protection effect from reports about catalpol, it is clear that

catalpol may have particular advantages in the treatment of AD. It not only protected neurons from injury but also improves the recovering from nerve endocrine disorder. Moreover, the traditional Chinese medicine has fewer side effects. Therefore, catalpol will be a kind of prospective anti-AD medicine and worth further studying and developing. So we will next use PS1/APP mice, a kind of transgenic mouse model of AD with accelerated Aβ production to study the effect of catalpol.

4. Experimental

4.1. Animals

To avoid the potential effects of the physiological hormone variations in female rats, male rats were used in this study. SPF (Specified Pathogen Free) healthy male Wistar rats, body weight 250 g ∼ 300 g, were purchased from Shandong Lukang Medicine Inc. (License No. scxkLu20080102). Rats were fed under conventional laboratory conditions at a temperature 20–25 °C, humidity between 50%–60%, natural rhythmical illumination during the day and night.

4.2. Establishment of AD rat model

The AD rat model was generated by injection of A β_{25-35} (Sigma, Cat# 050M4765) intracerebroventricularly. Aβaccumulation caused by imbalance of production and degradation of the protein has been believed to be the general pathologic basis for AD development regardless of initiation causes, and damage neurons especially the cholinergic neurons. The main toxic effective fragment of accumulated Aβ is $A\beta_{25-35,}$ which has been used as the tool agent to establish AD animal and cell model (Tran 2002), so $A\beta_{25-35}$ lateral ventricle injection was applied to develop the AD rat model for our study. The formulation of injection solution was prepared as below: the original $A\beta_{25-35}$ was diluted with sterile saline at 2 μg/μl, then incubated at 37 °C for 7 days to condensed configuration and stored at 4 °C for future use.

Y-maze escape reaction (Xue 2012) was performed for rat screening. Rats that took too long to escape in response to normal stimulation, and that were unable to escape at a high voltage stimulus were excluded for the following tests. The working voltage of Y-maze was set up at the range of 35–45 V based on the primary screen. Twenty tests were performed to each rat every day at the interval of 5 s. The accurate reaction times and the total reaction time among the twenty tests were recorded and the ratio of both indexes was defined to measure the learning ability.

Lateral ventricle orthotopic injection: rats after an overnight fast were anaesthetized with chloral hydrate, and then fixed to the stereotactic apparatus. With the hair removed and the skin sterilized around the operation site, the sculp was cut open with a 2 cm incision following the middle line of the calvaria, then the periosteum was separated to expose the skull and the anterior fontanelle. The procedure of the operation was referred to the Rat Brain Stereotaxic Atlas (Bao and Shu 2005).

After the right ventricle was pitched at 1.0 mm behind and 1.8 mm of the right anterior fontanelle and 3.8 mm deep under the *dura mater*, the skull was drilled open to expose the *dura mater*. The microinjector was inserted vertically and 5 μl $A\beta_{25-35}$ (10 μg) were injected slowly during 5 min, and the needle was kept on site for another 3 min, then pulled out slowly. Correspondingly, the equal volume of physiology saline was injected intracerebroventricularly in the control group rats. Y-maze escape reaction was performed again to evaluate the rat model and for the next consecutive 15 days to evaluate the animal behaviors.

4.3. Drug administration

Rats having died during the operation and model development were excluded, and the eligible rats were randomly grouped with 9 rats each group. Catalpol (purchased from National Institutes for Food and Drug Control of China, Cat#110808–201009) was injected at 5 mg $kg^{-1}\cdot d^{-1}$ and 10 mg $kg^{-1}\cdot d^{-1}$, respectively. Equal volumes of physiological saline was injected in rats of the model group and control groups, all agents injected intraperitoneally from the 2nd day after injection of $A\beta_{25-35}$, once a day for the following seven days.

4.4. Enzyme-linked immunosorbent assay (ELISA) to detect hormone concentration

In order to reduce the effects of the hormone rhythm around the clock, blood was taken through the rat eye posterior vein at 8–9 am. The serum was separated immediately and the following ELISA procedure was operated according to the instructions of the kits (Lichen Biological Technology Co., Ltd, Shanghai). The concentration gradients of the standard sample were 160, 80, 40, 20, 10, 5 ng/ml, and the samples wells were designed such as the standard, experimental and blanks wells. Different concentrations of the standard sample 50 μL and experimental sample 10 μL were added into the according wells respectively. The HRP (horseradish peroxidase) labeled antibody was added at 37 °C for 60 min. After complete rinse, substrate was added at 37 °C for 15 min. Finally, stop buffer (50 μL) was added and OD (optical density) was obtained at 450 nm. The standard linear regression curve was made with the standard sample concentration as Y axis and it's according OD value as X axis and the experiment samples concentration was calculated.

4.5. Hematoxylin & eosin (H&E) staining and transmission electron microscope to observe the structure of hypothalamus

To preserve tissue in a life-like state, directly perfusing fixative through the circulatory system was carried out according to a literature method (Gage 2012). Briefly, heart was displaced through an operation after rats were anesthetized. Then make a small incision to the posterior end of the left ventricle and pass a perfusion needle into the ascending aorta, clamp the heart with a hemostat. Make an incision to the animal's right atrium as an outlet, buffer infusion firstly with a certain steady pressure. After the fluid running clear and the clearing of the liver, 4% paraformaldehyde infusion started and finished until the rat was stiff. The brain tissue was removed and fixed in 4% paraformaldehyde for post-fixation 4 °C.

For HE staining, a paraffin section of hypothalamus supraoptic nucleus tissue (5 μm thick) was prepared. The paraffin sections were stained with haematoxylin for 15 min, then screened with a microscope under 1% hydrochloric acid alcohol, immersed in 1% ammonia for 2–3 min, fully rinsed with distilled water and stained with 1% eosin for 3 min, and finally mounted with neutral gum.

The hypothalamus supraoptic nucleus tissue was trimmed at a size of 1 mm × 1 mm × 1 mm square, immersed in 3% glutaraldehyde at 4 °C, then fixed in 1% osmium tetroxide (OsO_4) and embedded with epoxy resin. Sections at 70 nm thick stain with uranyl acetate and citric acid lead, then observed through a transmission electron microscope

4.6. Immunohistochemistry to observe the expression of CRHR1 positive neurons

Paraffin sections of hypothalamus tissue (5 μm thick) were prepared. Blocking was done in a mixture of 5% bovine serum albumin (BSA), 0.2% Triton X-100, and 1% H_2O_2 in PBS. After washing with PBS, primary antibody anti-CRHR1 (1:200, Zhongshan Co limited, China) was used and incubated at −4 °C overnight (negative control by PBS instead). Immunoperoxidase labeling was performed using a DAB kit (Boster, Wuhan, China), and slides were evaluated using an Olympus BX51 microscope (Olympus, Japan).

4.7. Statistical analysis

Data were presented as mean ± S.E.M of five independent experiments. The significance of differences between groups was tested using single factor analysis of variance (ANOVA). SPSS ver. 12.0 was used, $P < 0.05$ was considered statistically significant.

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