Action of ZDY101 on passive avoidance test and brain M-receptor density after intracranial co-injection of Aβ plus ibotenic acid

HU Ya-Er, XIA Zong-Qin, SUN Qi-Xiang, Li Ai-Ming, Chen Qin

(Research Laboratory of Cell Regulation, Shanghai Second Medical University, Shanghai 200025)

Abstract The aim of this study was to establish a model by single injection of $A\beta_{1-40}$+IA into rat brain basal ganglion and examine the effect of ZDY101, an active component of a Yin tonic from Chinese traditional medicinal herb. The results showed that the amnestic effect of co-injection of Aβ and IA lasted for at least 2 months. At same time, the total M-receptor density in model brain was significantly lower than blank control, indicating the change is profound enough for long term pathological studies or drug screening. It can be clearly seen that the decreased brain M-receptor density caused by Aβ was significantly increased by ZDY101. Such an elevation effect was significantly correlated with dose of ZDY101 when the dose was examined in a certain reasonable range. It can also be clearly seen that such an elevation of M-cholinergic receptor density was significantly correlated with the improvement of memory which indicated that the increase of M-cholinergic receptor density was an important factor in improving the memory of such animal model.

Keywords Alzheimer's disease, Animal model, β-amyloid, Co-injection, M-receptor

CLC numbers R749.1+6 A

1 INTRODUCTION

Animal models are very important for the study of both pathological mechanism of Alzheimer's disease (AD) and screening of drugs for this disease. Many different models have been tried but none has been proved satisfactory. At present, in addition to naturally aged rats or mice, two animal models have attracted our attention. One is mouse transgenic with familial AD-associated mutant genes of amyloid precursor protein, the pathological changes of which have been reported to be most close to familial AD. The other is based on the intracranial injection of the toxic substance found in senile plagues of AD patients, the beta-amyloid (Aβ). However, pathological changes following simple injection of Aβ is usually transient, hence not suitable for long-term drug screening. In 1998, two papers in literature reported that some modification of the injected material

Supported by the National Natural Science Foundation of China (39570835)

Manuscript received date: 2001-07-06

could successfully prolong the pathological changes. One was the use of aggregated $A\beta$,[1] the other was the use of a mixture containing $A\beta$ and ibotenic acid (IA, an extrinsic neuro-excitatory amino acid).[2] In this work, we established a model by single injection of $A\beta_{1-40}$+IA into rat brain basal ganglion and examined the effect of ZDY101, an active component of a Yin tonic from Chinese traditional medicinal herb.

2 MATERIALS AND METHODS

2.1 Production of animal models

The method of Morimoto *et al*[2] was followed with some modification (unilateral instead of bilateral). 60 SD Rats were all obtained from SIPPR-BK Co. in Shanghai. They were all 3 months old, and were randomly divided into 5 groups (12 for each group), each having half female (250- 300g body weight) and half male (280-350g body weight). One group served as normal control (i.e., operated but not injected with $A\beta_{1-40}$+IA), one group served as model control (i.e., operated, injected with $A\beta_{1-40}$+IA, but not treated with ZDY101), and three groups were used to test different doses of ZDY101. Injection of $A\beta_{1-40}$+IA (both from Sigma) (or saline for normal control group) was accomplished by the aid of a stereotaxic instrument (Stoelting Co.) when the animal was anaesthetized with choral hydrate. The coordinates of injection were AP=0.5 mm (right to medial line), L=−2.8 mm (backward from bregma), H=−7.0 mm (ventral to dura). This is the position of basal ganglion according to Paxinos and Watson[3]. The dose for each rat was $A\beta_{1-40}$ 4 μg and IA 1 μg in 1 μL of saline (1 μL of saline for normal control group). The injection was completed in 20 min, and the needle was withdrawn 5 min later. Then the skin was sutured.

2.2 Drug administration

ZDY101 was an active component of a single Yin tonic from Chinese traditional medicinal herbs prepared by our own laboratory. When examined with gas chromatography, infrared spectrometry, nuclear magnetic resonance spectrometry, and mass spectrometry, it was a single compound, with chemical purity better than 95%. It was administered to the 3 tested groups as stable suspensions in 0.5% CMC-Na once daily through a gastric tube at doses 3.6, 18, 90 mg/kg per day respectively, starting from the day next to the operation and lasted for 60 d. The operated controls and the model controls were given same volume of 0.5% CMC-Na once daily. 18 mg/kg of ZDY101 per day was the dose suggested for clinical trial after surface area transformation from men to rats. The step-through test was carried out on the 59 th and 60 th day and animals were sacrificed on the 61st day for measurement of M receptor density. From each group, 10 animals were taken by randomization and used for step-through test and M receptor

density measurement

2.3 Step-through test

A $60\times15\times15\,cm^3$ box was divided into 2 equal size rooms, one dark room with cooper rod base which was electrically charged (40V AC) when in use, while the other was a lighted room but not electrically charged. Between the two rooms there was a hole for the rat to go through. The experiment was carried out for each rat in two consecutive days. The first day was for training. The rat was adapted in the box for 3 min first, then put in the lighted room, with its back toward the hole, and the dark room was charged for 5 min. The second day was for test. The latent time for the first cross from the lighted to dark room as well as the number of crosses in 5 min was recorded. The longer the latent period and the smaller the number of crosses, the better was the memory.

2.4 Measurement of total M-receptor density

The single-site method with ^{3}H-QNB as the ligand was used. The brain samples were homogenized in PBS with 0.25 mol/L sucrose, centrifuged, the pellet of centrifugation at $800\times g$ was discarded and the pellet of the second centrifugation at $27000\times g$ was collected, re-homogenized in PBS without sucrose and used for measurement of M-receptor density. The concentration of ^{3}H-QNB was chosen at the saturation range (i.e., 1.0 nmol/L), and each sample was measured in triplicates. After incubation at 37°C for 30 min and separation by glass fibre filter, the bound portion was measured by liquid scintillation counter. The non-specific binding was obtained from parallel samples with the addition of 1000 times of unlabeled QNB. Protein contents of the samples was measured by Lowry's micro-assay.

2.5 Statistics

Test of normality and test of variance homogeneity were carried out first. When normal distribution and homogeneity of variance was verified, ANOVA was used followed by paired t-test, This is the case of M receptor density. In case of non-normality and non-homogeneity of variance, non-parametric univariate procedure was used followed by Wilcoxon's test. This is the case of step through test. All the above statistic procedures were carried out with SAS software package.

3 RESULTS

3.1 Results of step through test

As can be seen in Table 1, the number of wrong response in 5 min was significantly

higher in model group than normal control group, indicating a real impairment of memory. ZDY101 was effective in decreasing the number of wrong response, although the small dose group was not significantly different from the model group. Also shown in table 1 are the data of latent time. The latent period was significantly shorter in model group than normal control group. ZDY101 was effective in prolonging the latent time. If the number of wrong response was divided by latent time for each rat to obtain a combined index, the differences among various groups and model group were all very significant.

Table 1 Results of step through test (numbers of animals were 10 for each group)

Group	Wrong response in 5 min	Latent time /min	Combined index WR/LT
Sham-operated (Blank)	$0.50\pm0.53^{(2)}$	$4.59\pm0.46^{(2)}$	$012\pm0.13^{(2)}$
Model	3.30 ± 1.25	1.84 ± 0.64	2.09 ± 1.15
Model+ZDY101 (3.6 mg/(kg·d))	2.50 ± 0.97	$2.88\pm0.97^{(2)}$	$0.95\pm0.46^{(2)}$
Model+ZDY101 (18 mg/(kg·d))	$2.20\pm0.97^{(1)}$	$2.95\pm0.90^{(2)}$	$0.88\pm0.55^{(2)}$
Model+ZDY101 (90 mg/(kg·d))	$1.90\pm1.10^{(1)}$	$3.34\pm1.04^{(2)}$	$0.65\pm0.41^{(2)}$

Superscripts $^{(1)}$ and $^{(2)}$ denote $p<0.05$ and $p<0.01$, respectively, when compared with the model group. Non-homogeneity of variances, data were analyzed with non-parametric univariate analysis followed by Wilcoxon signed rank test.

3.2 Results of M-receptor measurement

As shown in Table 2, the total M-receptor density in model brains was significantly lower than blank control. ZDY101 has significant elevation effect on brain M-receptor density.

Table 2 Results of M-receptor measurement (10 animals per group)

Group	Total brain M-receptor density (fmol/mg prot)
Sham-operated (Blank)	1423 ± 536
Model	$827\pm262^{(2)}$
Model+ZDY101 (3.6mg/(kg·d))	$1149\pm253^{(2)}$
Model+ZDY101 (18mg/(kg·d))	$1413\pm180^{(2)}$
Model+ZDY101 (90mg/(kg·d))	$1425\pm294^{(2)}$

Superscripts $^{(1)}$ and $^{(2)}$ denote $p<0.05$ and $p<0.01$. Normal distribution, homogeneous variances, ANOVA followed by paired comparison.

When tested with linear regression taking Log dose as X-axis coordinate, the change of M-receptor density by the administration of ZDY101 was significantly correlated with

its dose (Fig.1).

3.3 Correlation of M-receptor density and results of step through test

When the M-receptor density and the combined index (number of errors/latent time) of step through test of 5 groups were tested for their correlation by linear regression, it was found that there was a significant correlation between these two parameters (Fig 2).

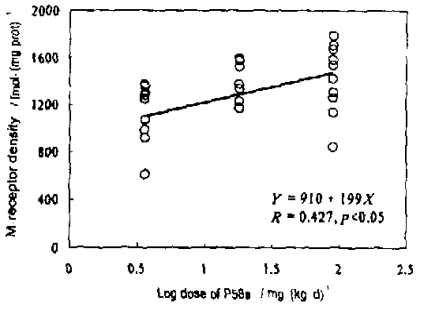

Fig.1 Correlation of M-receptor density and dose of ZDY101

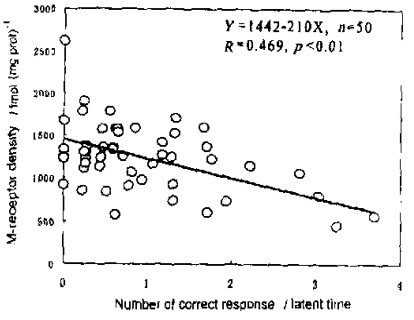

Fig 2 Correlation of M-receptor density and combined index of step through test

4 DISCUSSION

(1) Aβ has been repeatedly reported to be toxic to cultured neuronal cells in in vitro experiments. It has also been reported that intracranial injection of small amount of Aβ can produce amnestic effects on mice.[4,5] However, in mice or rats, the neurotoxic effect of intracranial injection of extrinsic Aβ is not always reproducible,[6,7] possibly because extrinsic Aβ only caused minor and transient degenerative effects. Morimoto et al reported that combined injection of Aβ and IA caused much profound degenerative effect in rats than single injection of Aβ or IA alone.[2] In the present work, we found that the amnestic effect of combined injection of Aβ and IA lasted for at least 2 months, indicating the change is profound enough for long term pathological studies or drug screening. Although it has been reported that Aβ can cause a significant decrease of ChAT activity as examined by immunochemical technique,[1] so far as we know, no report about the change of M-receptor density has been found regarding the in vivo effect of Aβ. In this work, we found that the decrease of M-receptor density was significant and lasted for at least 2 months. In previous experiments, we reported that M-receptor density was also significantly decreased in aged mice and rats, however, whether such a decrease in aged animals is related to Aβ is unknown. In this respect, if we want to study the relation of pathological changes or drug actions to Aβ, the animal models produced by combined intracranial injection of Aβ and IA seems to be more suitable.

(2) ZDY101 is an active component extracted from one of commonly used Yin tonics of Chinese traditional medicinal herbs. In previous experiments, we have proved that it can suppress the density of β-adrenergic receptors in case the density is higher than normal in several animal models, and it can also raise the density of M- cholinergic receptors in case the density is lower than normal[8]. In aged animals, the brain M-cholinergic receptor density is usually significantly lower than young animals, and ZDY101 can significantly increase the density, making it approaching normal. However, we have never proved whether such an effect could also be seen in animals, of which the M-cholinergic receptor density was decreased by the toxic effect of Aβ. In this work, it can be clearly seen that the decreased brain M-receptor density caused by Aβ was significantly increased by ZDY101. Such an elevation effect is significantly correlated with the dose of ZDY101 when the dose was examined in a certain reasonable range. It can also be clearly seen that such an elevation of M-cholinergic receptor density was significantly correlated with the improvement of memory which indicated that the increase of M-receptor density was an important factor in improving the memory of such animal model.

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