Original Research

The improvement of spatial memory deficits in APP/V717I transgenic mice by chronic anti-stroke herb treatment

Kaiyu Yang[1,2,*], Yan Tan[1,*], Feng Wang[1,*], Qi Zhang[1], Ping Sun[1], Ying Zhang[1,3], Na Yao[1], Yanan Zhao[1], Xu Wang[1], Angran Fan[1] and Qian Hua[1]

[1]Beijing University of Chinese Medicine, School of Preclinical Medicine, No.11 N.3rd Ring Eastern Road, Beijing 100029, China; [2]Dalian No.4 people hospital, Pharmacy, Dalian 210200, China; [3]Nankai hospital, Tianjin 300100, China

*The authors contributed equally to this paper.

Corresponding author: Qian Hua. Emails: hqianz@aliyun.com and huaq@bucm.edu.cn

Abstract

In China, herbal medicine has an extensive history for the treatment of cerebrovascular diseases. Clinical studies have shown that stroke patients are more likely to experience significant memory decline in comparison to their healthy counterparts. Cognition is improved in stroke patients treated with herbal medicine active components, Geniposide (GP) and Geniposide Rg1 (GRg1) (together, called TLJN). However, the effect of TLJN in Alzheimer disease remains unknown. Therefore, we investigated the behavioral effect of TLJN in male and female APP/V717I transgenic (Tg) mice. We conducted two different treatment strategies: (1) pretreatment strategy: medically treated at the age of 3 months which lasted for 3 months; (2) early treatment strategy: medically treated at the age of 6 months which lasted for 4 months. In open field test, locomotor activity and anxiety-like behavior were not affected after TLJN administration in Tg mice. In Morris Water Maze test, spatial learning processes in both genders were improved by TLJN treatments. Furthermore, retrieval processes were significantly improved in the pretreatment strategy for only male mice, which also showed a trend for improved retrieval processes with early treatment. In the inhibitory avoidance test, TLJN enhanced learning processes. In addition, gender differences were found in Tg mice exposed to TLJN treatments. In Tg male mice, significant efficacy was seen at high and middle doses, and in Tg female mice, a low dose was more effective.

Keywords: Alzheimer disease, APP/V717I Tg mice, behavioral test, gender differences, TongLuoJiuNao

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Introduction

Alzheimer disease (AD) is a neurodegenerative disease consisting of cognitive and memory impairment. AD is often accompanied by high cortical dysfunction, such as aphasia, agnosia, or apraxia and other non-cognitive psychiatric symptoms. Symptoms usually develop slowly with memory loss or other neurasthenia symptoms occurring at the early stage, followed by delusions or hallucinations, decline of mind activities, and spatial disorientation, and sever long-term memory loss and incontinence at the later stages, eventually leading to the failure of bodily functions and death. AD patients become increasingly dependent on the assistance of others as AD progresses.

In the current study, we used APP/V717I Tg mice, which overexpress the ''London'' mutant of APP.[1,2] These mice develop amyloid plaques between 12 and 14 months of age, accompanied by vascular deposits.[1] Furthermore, neuroinflammation, cognitive impairment, reduced long-term potentiation, and neophobia occur as early as three months.[3] Therefore, these responses occur earlier than the accumulation of amyloid peptides. In the current study, we aimed to compare gender differences and explore the efficacy of TongLuoJiuNao (TLJN) based on behavioral responses in APP/V717I Tg mice, medical treatments of which begun with the age of 3 months and 6 months.

TLJN consists of two main active components (GRg1 and GP), which are extracted from two herbs.[4] GRg1 is a main bioactive ingredient in *Panax noto-ginseng*, which has been popularized as a hemostatic herb that invigorates and produces blood. Pharmacological research shows that GRg1 reduces brain amyloid protein level in an animal model of aging[5,6] and restores brain function.[7] GP is extracted from *Gardenia jasminoides*, whose fruit has been used in Traditional Chinese Medicine for more than a millennium to treat certain febrile conditions. Recent studies have shown that GP inhibits microglia-mediated inflammatory responses, reduces the production of pro-inflammatory cytokines, decreases the cytotoxicity of Aβ,[8] and induces neurotrophic effects.[3,9]

Stroke and AD are both common in elderly individuals; however, a possible relationship between the two remains

controversial. Clinical studies[10,11] have shown that stroke patients are more likely to experience significant memory decline compared with their healthy counterparts. Recently, our phase II and III clinical trials have showed that cognition is improved when ischemia stroke patients are treated with TLJN. Therefore, our group was interested in exploring whether TLJN may be beneficial in AD therapy. Therefore, in this study, we used APP/V717I Tg mice to observe the neuroprotective effect of TLJN, with both genders.

Materials and methods

Chemicals

GRg1 and GP were purchased from Tianjin HeYi Biotechnology Company (Tianjin, China). High-performance liquid chromatography for GRg1 and GP is described previously.[4] Aricept was purchased from Eisai Pharmaceutical Company (Lot number: 070624A, Beijing, China). Beta-amyloid (Aβ) 17-42 monoclonal antibody was obtained from Signet Laboratories (clone 4G8, Lot number: SIG-39220, Dedham, MA, USA). The secondary antibody against mouse IgG was purchased from COVANCE (Lot number: CW0102, Beijing, China). SuperPolymer Rabbit & Mouse horseradish peroxidase (HRP) Kit (Lot number: CW0117) and 3,3′-diaminobenzidine (DAB) kit (Lot number: CW0125) were bought from COVANCE.

Animals

The APP/V717I Tg mice we used over-express a ''London'' mutated human APP (V717I) under control of *platelet-derived growth factor* promoter. The heterozygous Tg mice in C57BL/6 background and non-Tg littermates were provided by the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences, Beijing, China.[12,13] After arrival, the animals were individually caged and maintained at a constant temperature on a 12-h light–dark cycle with accessing to food and water freely. After 2 weeks of habituation to the facilities, experimental procedures begun. All experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Efforts were made to minimize animal suffering and to reduce the number of animals used.

Treatment

Wild type littermates of C57BL/6 J mice were used as negative control group and described as normal. The APP/V717I Tg mice were randomly divided into five groups: a model group was given saline solution, a positive drug group was administered a therapeutic dose of Aricept (also called Donepezil) and the drug TLJN of high dose, middle dose, or low dose. For male mice, there are male negative control group (MNC), male model group (MM), male Aricept group (MA), male low dose of TLJN group (MLD), male middle dose of TLJN group (MMD), and male high dose of TLJN group (MHD). For female groups, there are female negative control group (FNC), female model group (FM), female Aricept group (FA), female low dose of TLJN group (FLD), female middle dose of TLJN group (FMD), and female high dose of TLJN group (FHD). The drugs were dissolved in saline solution, fed by self-administered eating way.

Based on clinical practice knowledge of Traditional Chinese Medicine, the dosage of *Panax notoginseng* and *Gardenia jasminoides* in stroke treatment was 5 g and 8.5 g, respectively. Measuring by HPLC, the concentrations of GRg1 and GP were 141 mg/g and 122 mg/g. With reference to the book of Pharmacology Experimental Method (page 203, edited by Shu-Yun Xu, etc.), we transferred the amount used in human to mice: dosage (mg/kg) $= d_A{*}K_B/K_A$. d_A is the dosage treated in AD patient (mg/kg) and K_B/K_A is the dosage conversion factor of mice to human. Hence, according to this theory, we calculated that in mice GRg1 was 15.844 mg/kg·d and GP was 18.311 mg/kg·d. Then, we defined this dosage as a middle dose, and high-dose set of GRg1 and GP was as twice as that of middle dose and low-dose set was a half of middle dose. Positive drug administration for the group is 0.64935 mg/kg·d of Aricept.

Treatment strategies are showed in Figure 1. In brief, in pretreatment strategy, we medically administrated mice at the age of 3 months which lasted for 3 months; in early treatment strategy, we started the treatment at the age of 6 months which lasted for 4 months.

Open field

The task is performed in a plexiglas open field (reference in a book: Methods of Behavior Analysis in Neuroscience, page 35, edited by Sidney A. Simon and Miguel A.L. Nicolelis), diametric 30 cm at the bottom, 40 cm at the top and 40 cm in height, with black wall and white bottom. The apparatus was dimly illuminated by a 60-W lamp placed above the box. Experiments were conducted in a sound-attenuated room. After each trial, the box was wiped by 96% ethanol to eliminate olfactory cues. A camera connected to the computer was used to record animal activities.

On the first day of training, mouse was submitted to a habituation session by placing it in the empty open field for 5 min. There were three habituation training for each mouse with a 4-h interval delay. After 24 h of the last training, mice were submitted to a 5-min trial. The circle was with diameter of 15 cm, half of which was considered as middle zone. The locomotor activity was recorded as total distance within 5 min, average speed, and time spent moving; the anxiety was concerned as time spent in the center.

Morris Water Maze

To evaluate hippocampal-dependent spatial learning and memory, mice were tested in a standard Morris Water Maze task (reference in a book: Methods of Behavior Analysis in Neuroscience, p. 147, edited by Sidney A. Simon and Miguel A.L. Nicolelis). A 6 cm diameter platform was submerged in a 100 cm circular pool of water, 1 cm below. The water temperature was maintained at $27 \pm 1^{\circ}C$ throughout the course of testing. All trials were monitored by a video camera placed about 2 m above the central of the pool. The water was made opaque by the

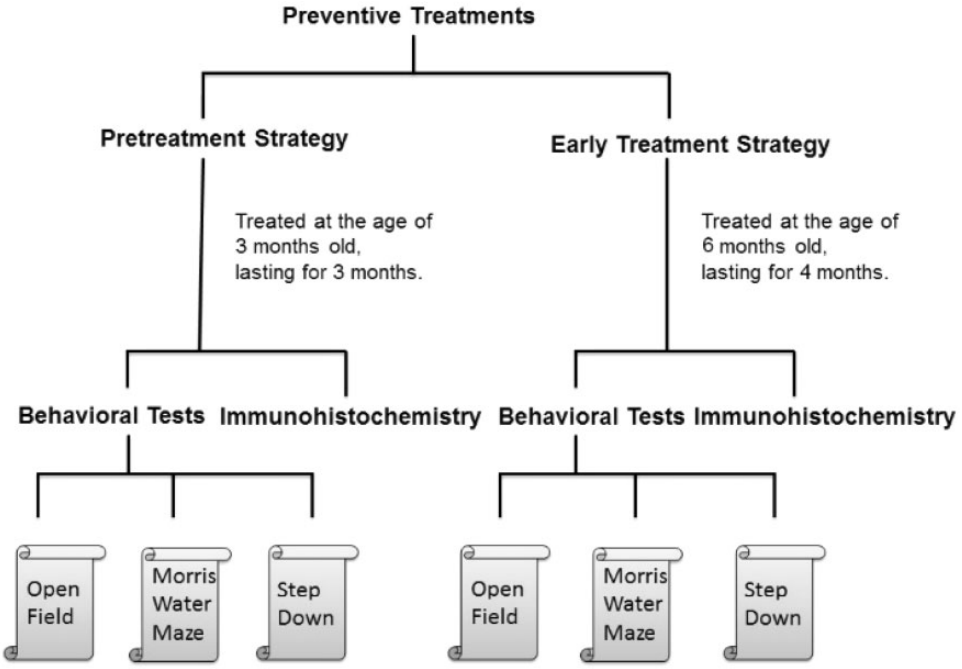

Figure 1 Two treatment strategies on APP/V717I Tg mice – pretreatment and early treatment. For pretreatment, the mice were administrated at the age of 3 months which lasted for 3 months of medication. For early treatment, the mice were treated at the age of 6 months old which lasted for 4 months. At the end of each treatment, mice were tested in three different behavioural experiments – open field test, Morris Water Maze, and step-down test, and following with immunohistochemistry assessments

addition of nontoxic white paint. The pool was placed in a room surrounded by fixed spatial cues.

1. Habituation trial: each mouse received one habituation trial (one trial/day) in the Morris Water Maze prior to the spatial training.
2. Spatial training: Training on the hidden platform began within 24 h after the habituation trial. Hidden platform training was carried out over five consecutive days with three trials per day. The platform was set to the center of the target quadrant of the pool and remained in a fixed position throughout spatial training. During a given trial, the mouse was placed on the platform for 15 s, which allowed it to be familiar to surroundings and reference location. Then the mouse was gently introduced into the pool, at one of four quadrants randomly as start quadrant, and was allowed 1 min to find the platform. If the animal did not find the platform after 1 min, it was placed on the platform for 10 s. After each trial, the mouse was patted gently with a towel to dry. Dependent measures acquired each trial are escape latency (in sec), swimming distance (in cm).
3. Spatial memory testing: 24 h after the last trial of spatial training, a probe test was conducted in the same spatial training pool with the platform removed. All mice started the probe test from the opposite quadrant of the target quadrant. The dependent measures were percentage of escape latency and percentage of dwelling time in target quadrant.

Step-down test

The inhibitory avoidance apparatus was an acrylic box with length 17 cm, width 13.5 cm and height 32 cm, whose floor consisted of a grid of parallel copper network with diameter 0.3 cm and spacing 1 cm. A 5 cm diameter, 5 cm high insulated platform was placed in the center of the floor. The behavioral performance of animals was recorded by infrared sensors and was transformed into digital signal. After each mouse was removed out, the box was cleaned with alcohol to avoid the smell hint to the later mouse (reference in a book: Methods of Behavior Analysis in Neuroscience, p. 142, edited by Sidney A. Simon and Miguel A.L. Nicolelis).

1. Training test: the animal was put into the box for 3 min to habituate, then placed to the floor. An electric current of 36 V was delivered to the copper grid. The mice subjected to electric shocks would jump back to the platform to avoid noxious stimuli. The cut-off time was 300 s. Their escape latency to step up to the platform with all four paws was measured.
2. Retention test: it was carried out 24 h after the training test, in a manner similar to training test except that the animal was first put on the central platform. Their escape latency to step down to the floor with four paws was measured.

Histological assessment of β-amyloid burden

After the behavioral tests, mice were sacrificed, brains dissected and fixed with 4% formaldehyde in phosphate-buffered saline. Serial 8 μm thick paraffin sections were cut

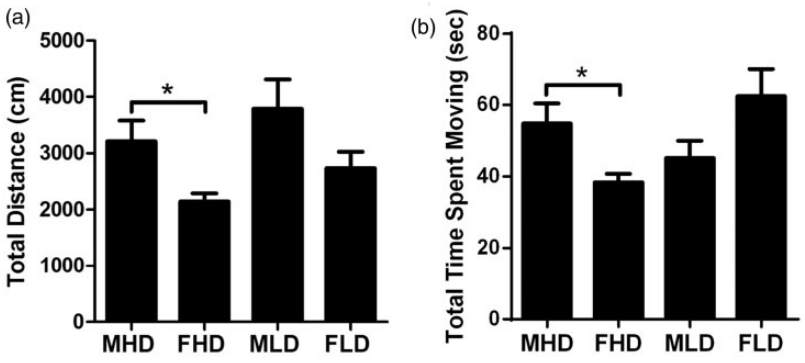

Figure 2 Gender differences of locomotor activity in open field tests. Gender differences of total distance (a) and total time spent moving (b) under high-dose and low-dose treatments of TLJN in pretreatment strategy. Total distance in cm, total time spent moving in seconds. Results are expressed as mean ± SEM of the mice in each group (n = 8), by Student's t-test, *P < 0.05

throughout the right hemisphere and mounted on polylysine-coated slides. After sections were dewaxed, they were pretreated with citrate buffer for antigen retrieval, followed by incubation with 0.3% H_20_2 in 80% methanol for 15 min to eliminate endogenous peroxidase activity. Sections were exposed to monoclonal anti-Aβ 17-42 (1:500) at 4°C, overnight. Secondary antibody against mouse IgG was then added (1: 10,000) for 30 min at room temperature. The SuperPolymer Rabbit & Mouse HRP Kit and DAB kit were used to enhance and visualize the signal, respectively. Finally, the hematoxylin and eosin stain was added. Light microscopic images (using the Nikon Eclipse 50i) were taken from hippocampus from five series of sections. Two brains from each group were examined.

Statistics

Results are expressed as mean ± standard error of the mean (S.E.M.). Student's *t*-test was used to compare two groups of littermate gender differences, one-way ANOVA for multiple groups in the same gender, two-way ANOVA for multiple groups of Tg model groups and treated APP/V717I mice groups. Statistical analysis was performed using GraphPad Prism software (version 6.00; GraphPad Software Inc. CA, USA). Statistical significance was defined as $P < 0.05$, compared to APP/V717I Tg mice.

Results

Locomotor activity and anxiety of APP/V717I Tg mice were not disturbed by TLJN treatment

The open field test measures locomotor activity. We conducted an initial drug-screening test to determine if TLJN could stimulate the motor ability of APP/V717I Tg mice. The most commonly used paradigms for this behavioral test are total distance travelled, average speed, and time spent moving. Furthermore, time spent in the center is used to examine the changing patterns of anxiety-related behavior. The more time spent centrally indicates the less anxiety exhibited by the animal.

In the pretreatment strategy, total distance travelled and time spent moving did not significantly differ between APP/V717I Tg and wild-type mice (data not shown). Furthermore, TLJN treatments did not affect locomotor activity in both genders (data not shown). However, comparisons between genders after pretreatments indicated gender differences. Under a high-dose of TLJN, locomoter activity in Tg male mice was significantly ($P < 0.05$ for total distance and time spent moving) higher than that of female mice (Figure 2). In terms of anxiety-like behavior, no significant change in the time spent in the center was found for Tg male or female mice.

In the early treatment experiment, the total distance, average speed, and time spent moving did not significant change between APP/V717I Tg mice and wild-type mice. Locomotor activity in male or female groups was not affected by all three different doses of TLJN. However, a better tendency in the total distance and time spent moving was found in male with a low dose of TLJN compared with the same treatment in female mice (data not shown). No significant changes in the central time were found between the different groups and different genders.

Taken together, the open field tests provided a status of locomotor activity and anxiety-like behavior. Under both treatment strategies, no significant changes were observed between Tg and wide-type mice; however, gender differences were evident for locomotor activity in Tg mice after TLJN treatments.

Spatial learning and retrieval processes in only male mice were improved by TLJN treatment in the pretreatment strategy

We assessed the spatial learning and memory of APP/V717I Tg mice via the MWM task.

Swimming distance and the latency to find the hidden platform were not significantly different in male APP/V717I Tg compared with male wild-type mice. Interestingly, different starting points, which were divided into two distinct regions (region A and region B) of the pool, were observed on the first day of training (Figure 3a1 and a2). In region A, a high or middle dose of TLJN in male Tg mice showed significantly (high dose, $P < 0.001$; middle dose, $P < 0.05$) shorter swimming distances and reduced escape latencies (high dose, $P < 0.001$; middle dose, $P < 0.05$). However, despite no significant differences

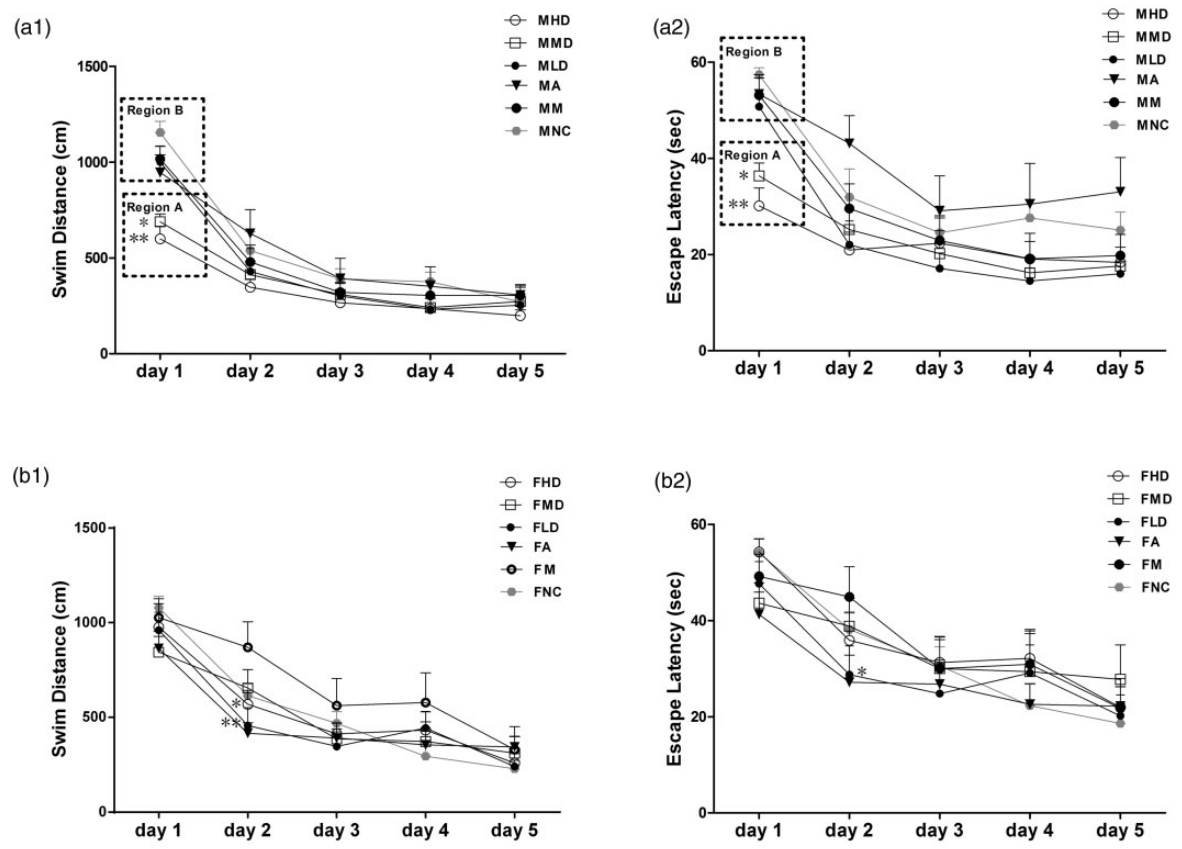

Figure 3 Spatial learning performance of APP/V717I Tg mice in the Morris Water Maze training test under pretreatment strategy. The swim distance (a1) and escape latency (a2) of 6-month-old male mice in five consecutive days in the hidden platform training test. The swim distance (b1) and escape latency (b2) of 6-month-old female mice in five consecutive days in the hidden platform training tests. Each point represents the mean distance or latency of three trials per day. Results are expressed as mean ± SEM of the mice in each group (n = 8), by two-way ANOVA. *P < 0.05, **P < 0.01 vs the Tg model group. Swim distance (in cm), distance to the hidden platform; escape latency (in seconds), time to the hidden platform; cut-off time is 60 s

among treated mice from 1–5 training days in region B, the low-dose-treated Tg mice were similar to wild-type mice in this region (Table 1). Therefore, these results suggested that treatment as early as three months of age enhanced spatial learning in APP/V717I Tg male mice.

Swimming distance was significantly ($P < 0.05$) longer in female Tg mice on days 2 and 4 of training. A high or low dose of TLJN in female Tg mice showed a significant ($P < 0.05$ and $P < 0.01$, respectively) reduction in swimming distance on day 2 of training. A significant ($P < 0.05$) reduction by the low-dose treatment was also observed in the escape latency on day 2 (Figure 3b2). As the positive control, Aricept, also markedly reversed the swimming distance ($P < 0.01$) and escape latency ($P < 0.05$) in female Tg mice (Figure 3b1 and b2). Furthermore, results from a high- or low-dose treatment of TLJ were similar to wild-type female mice (Table 1).

In the probe test, the percentage of distance and time spent in target quadrant (both at approximately 35%) of male Tg mice was slightly reduced compared with wild-type mice (Figure 4), indicating that retrieval ability was still maintained at approximately similar levels to wild-type in these mice at the age of 6 months. However, male Tg mice exposed to all three doses of TLJN showed a significantly better performance in the percentage of distance (high-dose, $P < 0.01$; middle-dose, $P < 0.05$; low-dose, $P < 0.01$) and time spent in the target quadrant (high-dose, $P < 0.05$; middle-dose, $P < 0.05$; low-dose, $P < 0.01$) (Figure 4a1 and a2). Furthermore, Aricept significantly ($P < 0.01$) increased the percentage of time spent in the target quadrant compared with male Tg mice (Figure 4a2). However, retrieval capability was promoted in female Tg mice treated with a middle or low dose of TLJN to a similar level seen in the wild-type group (Figure 4b1 and b2).

Spatial learning and retrieval processes were improved in both genders by early TLJN treatment

In male mice, swimming distance was not significant among treated mice groups. The escape latency was significantly shortened with a high or low dose of TLJN (both $P < 0.05$) on day 4 of training as well as on day 5 with a high dose ($P < 0.01$) or middle dose ($P < 0.05$) (Figure 5a2). Moreover, spatial learning was not evident in male Tg mice. A significant ($P < 0.05$) difference in swimming distance in female Tg mice at day 5 of training was seen with a low dose of TLJN (Figure 5b1). In addition, under early treatment, Aricept did not improve these paradigms in both genders. In the probe test, Tg male mice showed

significantly ($P < 0.01$) worse performance compared with wild-type mice in percentage of distance (Figure 6a1) and percentage of time spent in the target quadrant (Figure 6a2). Male Tg mice treated with a middle dose of TLJN performed significantly ($P < 0.01$) better in the distance percentage (Figure 6a1) and percentage of time spent in the target quadrant (Figure 6a2). Male Tg mice treated with a high or low dose of TLJN spent a significantly ($P < 0.05$) greater portion of time in the target quadrant (Figure 6a1) with an increased swimming distance in the target area (Figure 6a2). Compared with Tg mice, Aricept did not significantly reverse the percentage of distance (Figure 6a1) and percentage of time spent in the target quadrant (Figure 6A2). Compared with female wild-type mice, female Tg mice showed significantly ($P < 0.05$) worse performance for the percentage of distance (Figure 6b1), and a tendency of a reduced percentage of time spent in the target quadrant (Figure 6b2). A significantly ($P < 0.05$) better performance was found for the percentage of distance (Figure 6b1) and percentage of time spent in the target quadrant (Figure 6b2) in female Tg mice exposed to a low dose of TLJN. Aricept did not significantly reverse the percentage of distance (Figure 6b1) and the percentage of time spent in the target quadrant (Figure 6b2) compare with Tg mice.

Table 1 Relative value improved from day 5 to day 1 in pretreatment strategy

		Swim distance		Escape latency	
Gender	Group	Difference between means (cm)	Relative value (%)	Difference between means (s)	Relative value (%)
Male	MNC	883.33	100.00	32.32	100.00
	MHD	401.30	45.43	11.73	36.29
	MMD	416.10	47.11	18.73	57.95
	MLD	746.96	84.56	34.72	107.40
	MA	639.06	72.35	20.31	62.65
	MM	711.60	80.56	33.32	103.10
Female	FNC	850.71	100.00	35.44	100.00
	FHD	717.11	84.30	32.30	91.14
	FMD	532.82	62.63	15.85	44.71
	FLD	719.26	84.55	27.47	77.49
	FA	519.75	61.10	19.11	53.91
	FM	696.36	81.86	27.44	77.41

Note: The difference between means (from day 5 to day 1) of Negative Control group is considered as 100%, and the Relative Value of other groups are compared with Negative Control group. Each group: $n = 8$.

TLJN increased passive avoidance learning in Tg mice

Learning and long-term retention (at 24 h) by measuring inhibitory avoidance were evaluated in mice given early treatment (Figure 7). The escape latency was significantly ($P < 0.01$) different between Tg mice mice and wild type mice in the male (Figure 7a) and female (Figure 7b). Under early treatment in male Tg mice, the three different doses of TLJN significantly ($P < 0.01$) decreased the latency

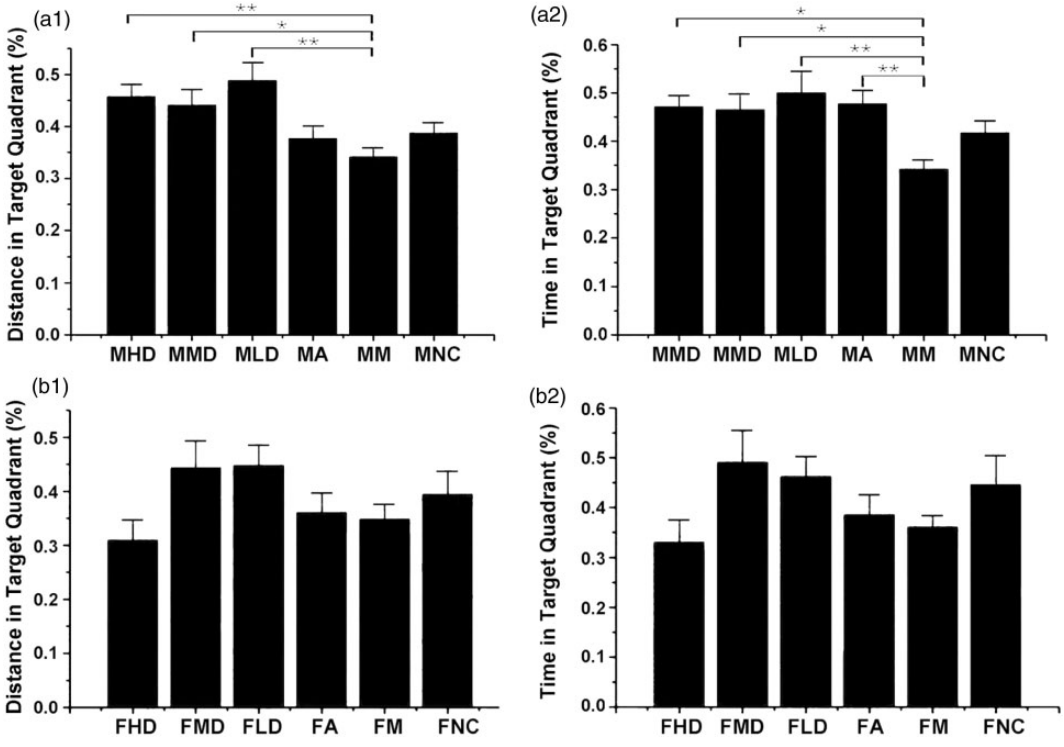

Figure 4 Memory performance of APP/V717I Tg mice in the Morris Water Maze probe test under pretreatment strategy. The percentage of distance in target quadrant (a1) and time in target quadrant (a2) of 6-month-old male mice in probe test. The percentage of distance in target quadrant (b1) and time in target quadrant (b2) of 6-month-old female mice in probe tests. Results are expressed as mean ± SEM of the mice in each group (n = 8), by one-way ANOVA. *P < 0.05, **P < 0.01 vs the model group. Cut-off time is 60 s

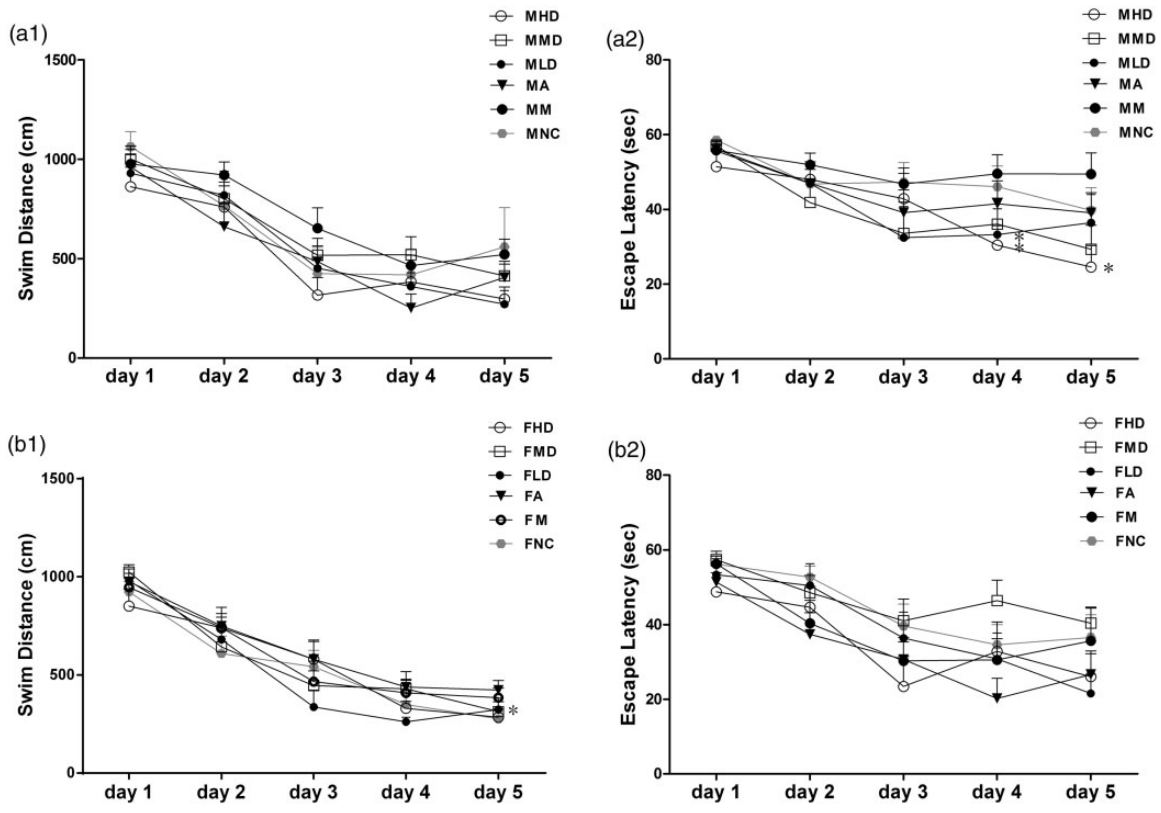

Figure 5 Spatial learning performance of APP/V717I Tg mice in the Morris Water Maze training test under early treatment strategy. The swim distance (a1) and escape latency (a2) of 10-month-old male mice in five consecutive days in the hidden platform training tests. The swim distance (b1) and escape latency (b2) of 10-month-old female mice in five consecutive days in the hidden platform training tests. Each point represents the mean distance or latency of three trials per day. Results are expressed as mean ± SEM of the mice in each group (n = 8), by two-way ANOVA. *P < 0.05, **P < 0.01 vs. the Tg model group. Swim Distance (in cm), distance to the hidden platform; Escape Latency (in seconds), time to the hidden platform; cut-off time is 60 s

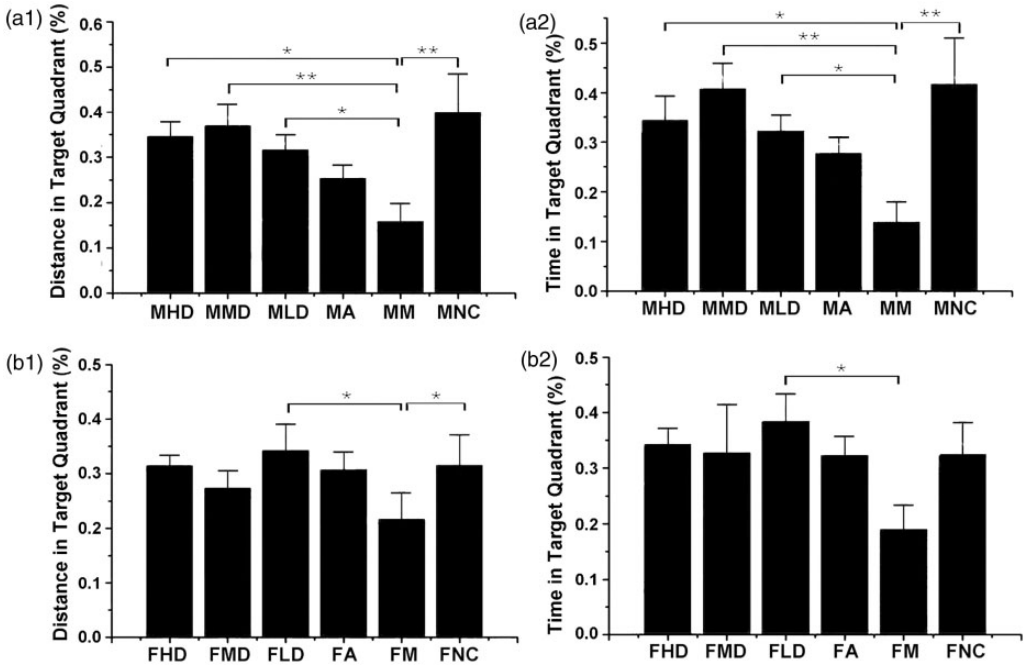

Figure 6 Memory performance of APP/V717I Tg mice in the Morris Water Maze probe test under early treatment strategy. The percentage of distance in target quadrant (a1) and time in target quadrant (a2) of 10-month-old male mice in probe test. The percentage of distance in target quadrant (b1) and time in target quadrant (b2) of 10-month-old female mice in probe test. Results are expressed as mean ± SEM of the mice in each group (n = 8), by one-way ANOVA. *P < 0.05, **P < 0.01 vs the model group. Cut-off time is 60 s

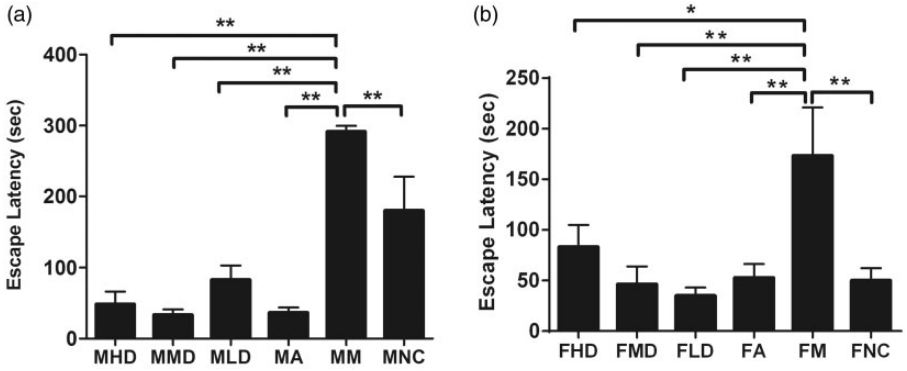

Figure 7 Inhibitory avoidance performances on the APP/V717I Tg mice in step-down test under early treatment strategy. (a) The escape latency of 10-month-old male mice in the training process of step-down test. (b) The escape latency of 10-month-old female mice in the training process of step-down test. Results are expressed as mean ± SEM of the mice in each group (n = 8), by one-way ANOVA.*P < 0.05, **P < 0.01 vs the model group. Escape latency (in seconds), time step up to the platform; cut-off time is 300 s

(Figure 7a). In female Tg mice, latency was also markedly reduced by all three doses (high, $P < 0.05$, and middle and low both $P < 0.01$) (Figure 7b). Therefore, these results indicated that TLJN increased inhibitory avoidance learning in APP/V717I Tg mice. The same behavior was observed for Aricept treatment ($P < 0.01$). Memory deficits, which were only observed in female Tg mice, were not significantly improved by TLJN treatment (data not shown). Moreover, the pretreatment strategy did not significantly improve these deficits (data not shown).

Plaque were not detected in the brain

At the age of 10 months, different levels of APP expression have been found among groups (Supplementary Figure 1). However, although we found senile plaques in hippocampus at the age of 12 months, absence of plaques occurred in both earlier 6-month-old and 10-month-old mice. Also, damage and atrophy were not evident (Figure 8(a) to (d)).

Discussion

In the present study, APP/V717I Tg mice developed behavioral deficits as early as 3 months of age, and thus represented a model of early onset familial AD. Aβ is absent in young APP/V717I Tg mice; however, levels of soluble Aβ in the brain are significantly increased.[14] Under *platelet-derived growth factor beta polypeptide* promoter, senile plaques become evident in mainly the cortex and hippocampus of 12-month-old APP/V717I Tg mice.[15] Furthermore, extensive amyloid peptides are deposited between 12 and 14 months,[14] with Aβ42 as the peptide with the most pronounced increase.[6] This increase is likely to be due to the close γ-cleavage site of London V717I mutation, resulting in increased production of Aβ42.[16] In some studies, amyloid plaques have also been observed at 10 months of age.[17] However, in the present study, amyloid depositions was not seen in Tg mice at this age. This difference may due to the use of a different Aβ antibody.[18]

Although no amyloid deposition occurred in APP/V717I Tg mice in the current study, behavioral impairments were evident as early as 6 months of age. Furthermore, at 10 months, APP/V717I Tg mice performed significantly worse than wild-type mice. Interestingly, gender differences occurred after TLJN treatments. Under the pretreatment strategy, the high or middle dose of TLJN significantly improved spatial learning behavior in Tg male mice, and the high or low dose (particularly the low dose) significantly improved spatial learning behavior in Tg female groups. In contrast, under the early treatment strategy, male mice performed significantly better with all three different doses of TLJN, whereas only a low dose of TLJN significantly improved behavioral performance in female mice (Figure 9). Therefore, we hypothesize that a high dose and middle dose of TLJN are more effective in males, whereas a low dose of TLJN is more efficacious in females. Furthermore, male Tg mice performed significantly better on the first day of training in the MWM task under the pretreatment strategy of a high or middle dose, thus suggesting a preventive role of TLJN. There is a clear gender difference in brain disease patients, where in the USA the prevalence for AD in women has doubled that of men, and in contrast more men than women are diagnosed with Parkinson's disease (PD).[19] Furthermore, neurological diseases, such as AD, PD, Tourette's, and attention-deficit hyperactivity disorder, which involve the dopamine transporter, are more prevalent in women after menopause.[20] Moreover, reduced testosterone levels have been found in men AD patients. Testosterone is metabolized to dihydrotestosterone, which is then converted to 5α-androstane-3α, 5α-androstane-3β,17β-diol, and 17β-diol.[21–23] The latter two hormones have greater affinity for estrogen receptor beta.[21,24] Therefore, sex hormones may play an important role in brain diseases, despite most studies on rodent models for brain disorders favoring the use of male animals due to the argument of the estrous cycle and fluctuating gonadal hormones in female rodents.[25]

One component of TLJN, GRg1, is neuroprotective in the Spraque Dawley rat model of middle cerebral artery occlusion[26] as well as exhibiting estrogen-like protective effects on neurons.[27] The other component, GP, prevents cell damage in rat hippocampal slice culture[28] and exhibits anti-inflammatory effects. In animal models of AD, Aβ42

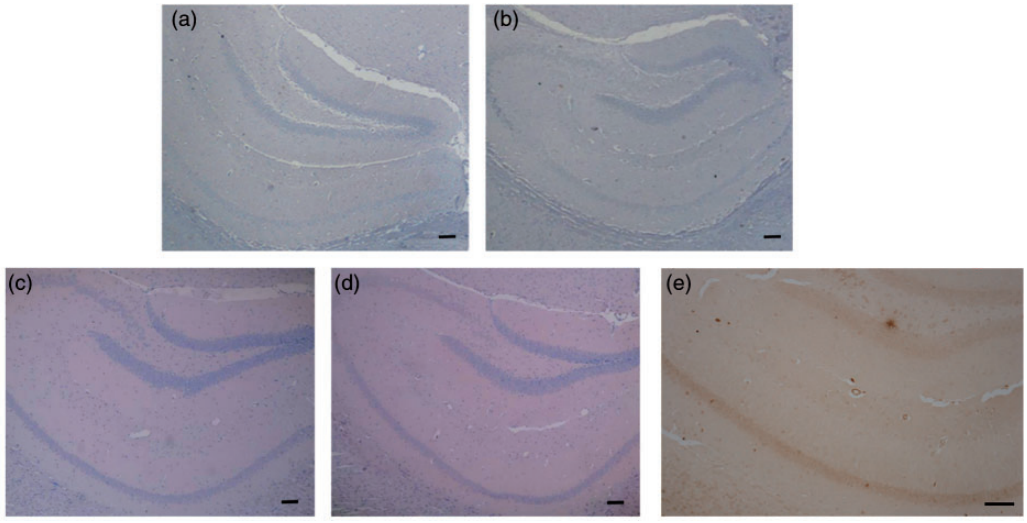

Figure 8 Immunohistochemical analysis of Aβ plaque deposition in brain sections. (a) Image of the hippocampus from representative brains of 6-month-old male mice (scale bar: 100 μm). (b) Image of the hippocampus from representative brains of 6-month-old female mice (scale bar: 100 μm). (c) Image of the hippocampus from representative brains of 10-month-old male mice (scale bar: 100 μm). (d) Image of the hippocampus from representative brains of 10-month-old female mice (scale bar: 100 μm). (e) Image of the hippocampus from representative brains of 12-month-old Tg mice (scale bar: 100 μm). The brains were examined visually and no quantitative analysis was conducted. (A color version of this figure is available in the online journal.)

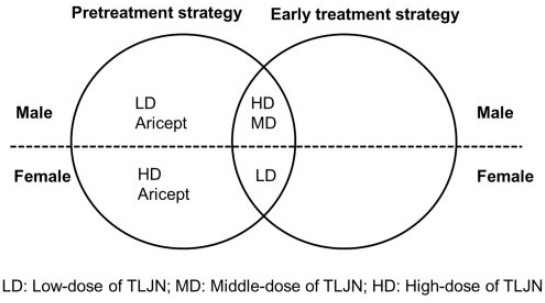

Figure 9 A summary illustration of significantly improved performance under different doses of TLJN and Aricept treated on APP/V717I Tg mice in Morris Water Maze tests

is considered as the first peptide to be deposited in vessels, further entrapping massive amounts of soluble Aβ40 peptide, and ultimately occurring extensive amyloid plaques in vascular plaques.[27] In addition, our previous findings have shown that TLJN reduces amyloidogenic processing of amyloid precursor protein in APP23 Tg mice.[29] At 3 months of age (prior to amyloid deposition), APP23 Tg mice exhibit activated microglia and astroglia, with a heightened inflammatory profile consisting of increased mRNA levels of interleukin (IL)-1β, IL-6, major histocompatibility complex II, and macrophage-colony stimulating factors.[3] Therefore, the combination of accumulated Aβ, vascular plaques, and the inflammatory response may further contribute to neuronal dysfunction, creating a vicious cycle that may ultimately play a role in behavioral impairment and the pathological progression of AD.[3] Although the mechanisms underlying AD still remain elusive, findings from the present study have demonstrated a preventive effect of TLJN, by reversing the behavioral deficits on APP/V717 Tg mice and illustrated the dose-dependent effect on gender differences.

Male Tg mice at 10 months showed less preference for the target quadrant in the probe test of the MWM task. First, this effect may be due to the tendency for a reduced average speed of male Tg mice compared with female Tg mice, suggesting that male mice could not swim through the entire field within the period of 60 s. Second, for the escape latency (within 60 s time-frame), the mean value of female Tg mice was approximately 35 s, whereas that of male Tg mice was approximately 49 s and more than 60% of these did not reach the target quadrant within the 60 s period (hence assigned an escape latency of 60 s). Third, we compared the data to 0.25 by Student's *t*-test, which resulted in no significant difference between percentage of distance in the target quadrant or percentage of time spent in the target quadrant. Therefore, if enough time was given for swimming time, the preference of the target quadrant may occur. The positive control, Aricept, is a member of the acetylcholinesterase inhibitor family, and has been approved by the Food and Drug Administration for ameliorating cognitive and behavioral impairments in AD patients.[30] We observed a positive effect of Aricept on pretreatment strategy rather than the early treatment. This result is consistent with an earlier report in which Aricept improves the outcome from the MWM task of APP/V717I Tg mice treated as early as 3 months.[31] However, under the early treatment strategy, we did not observe a significant improvement in Aricept group. This effect may have been due to the experimental conditions of behavioral tests, the age of mice, sample size, and starting time of drug administration.[30] These factors may have resulted in undetectable behavioral changes in response to Aricept treatment. In addition, the dosage of Aricept in this study was 0.65 mg/kg/day, which is much less than other studies (0.92 mg/kg/

day).[17,18,32] Furthermore, clinical studies have shown that cognitive effects are better at a higher concentration of Aricept (23 mg/day) compared with a lower concentration (5 mg/day or 10 mg/day).[33] Therefore, we suspect that the lack of effect of Aricept on the early treatment strategy was due to its dosage. In summary, TLJN prevented age-related spatial learning and memory deficits in the APP/V717I Tg mouse model, which were gender-dependent. Under pretreatment, APP/V717I Tg mice that exhibited a preventive effect indicated that TLJN delayed AD-like pathogenesis. Furthermore, gender differences were evident after TLJN treatments, thus providing new light on further studies to explore the effect and mechanisms of TLJN on the estrogen pathway.

Author contributions: KY, YT, and FW contributed equally to this paper. KY and YT mainly contributed to the experimental operation and paper writing. FW and QZ mainly contributed to the data analysis and figure/table organization. PS, YZ, NY, YZ, XW, and AF took part in the experimental operation. QH was the designer of this experiment and did the experimental guidance.

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REFERENCES

1. Dewachter I, Dorpe JV, Smeijers L, Gilis M, Kuipéri C, Laenen I, Caluwaerts N, Moechars D, Checler F, Vanderstichele H, Leuven FV. Aging increased amyloid peptide and caused amyloid plaques in brain of old APP/V717I transgenic mice by a different mechanism than mutant presenilin1. *J Neurosci* 2000;**20**:6452–58
2. Hardy J. Amyloid, the presenilins and Alzheimer's disease. *Trends Neurosci* 1997;**20**:154–9
3. Heneka MT, Sastre M, Dumitrescu-Ozimek L, Dewachter I, Walter J, Klockgether T, Leuven FV. Focal glial activation coincides with increased BACE1 activation and precedes amyloid plaque deposition in APP[V717I] transgenic mice. *J Neuroinflam* 2005;**2**:22
4. Hua Q, QingX, Li P, Li W, Hou J, Hu J, Hong Q, Sun P, Zhu X. Brain microvascular endothelial cells mediate neuroprotective effects on ischemia/reperfusion neurons. *J Ethnopharmacol* 2010;**129**:306–13
5. Liu J, Zheng X, Yin F, Hu Y, Guo L, Deng X, Chen G, Jiajia J, Zhang H. Neurotrophic property of geniposide for inducing the neuronal differentiation of PC12 cells. *Int J Devl Neurosci* 2006;**24**:419–24
6. Shi YQ, Huang TW, Chen LM, Pan XD, Zhang J, Zhu YG, Chen XC. Ginsenoside Rg1 attenuates amyloid-β content, regulates PKA/CREB activity, and improves cognitive performance in SAMP8 mice. *J Alzheimer Dis* 2010;**19**:977–89
7. Chen F, Eckman EA, Eckman CB. Reductions in levels of the Alzheimer's amyloid _peptide after oral administration of ginsenosides. *FASEB J* 2006;**20**:1269–71
8. Choi SJ, Kim MJ, Heo HJ, Hong B, Cho HY, Kim YJ, Kim HK, Lim ST, Jun WJ, Kim EK, Shin DH. Ameliorating effect of gardenia jasminoides extract on amyloid beta peptide-induced neuronal cell deficit. *Mol Cell* 2007;**24**:113–8
9. Li X-J, Hou J-C, Sun P, Li P-T, He R-Q, Liu Y, Zhao L-Y, Hua Q. Neuroprotective effects of TongLuoJiuNao in neurons exposed to oxygen and glucose deprivation. *J Ethnopharmacol* 2012;**141**:927–33
10. Zhu W-C, Xiong J-P. *Clinical study of acute cerebral infarction by TLJN injection* [D]. MSc dissertation. Hubei College of Traditional Chinese Medicine, 2007
11. Gu Z-W, Zhao J-J. *Clinical study of stroke by TLJN injection*. MSc dissertation. Changchun University of Chinese Medicine
12. Feng Z, Qin C, Chang Y, Zhang J. Early melatonin supplementation alleviates oxidative stress in a transgenic mouse model of Alzheimer's disease. *J Pineal Res* 2004;**37**:129–36
13. Feng Z, Qin C, Chang Y, Zhang J. Early melatonin supplementation alleviates oxidative stress in a transgenic mouse model of Alzheimer's disease. *Free Radic Biol Med* 2006;**40**:101–9
14. Van der Auwera I, Wera S, Van Leuven F, Henderson ST. A ketogenic diet reduces amyloid beta 40 and 42 in a mouse model of Alzheimer's disease. *Nutr Metab* 2005;**2**:28
15. Feng Z, Chang Y, Cheng Y, Zhang B, Qu Z-W, Qin C, Zhang J-T. Melatonin alleviates behavioural deficits associated with apoptosis and cholinergic system dysfunction in the APP 695 transgenic mouse model of Alzheimer's disease. *J Pineal Res* 2004;**37**:129–36
16. Choi JHK, Kaur G, Mazzella MJ, Morales-Corraliza J, Levy E, Mathews PM. Early endosomal abnormalities and cholinergic neuron degeneration in amyloid-β protein precursor transgenic mice. *J Alzheimers Dis* 2003;**34**:691–700
17. Zhang L, Tian J, Yin J, Shi J, Wang P, Wang R, Hu Q, Zhao Z, Ji Z, Ren Y. Effect of GEPT extracts on spatial learning ability of APPV717I transgenic mice at early stage of dementia and its possible mechanism. *China J Chinese Materia Medica* 2009;**34**:428–32
18. Tian J, Shi J, Zhang L, Yin J, Hu Q, Xu Y, Sheng S, Wang P, Ren Y, Wang R, Wang Y. GEPT extract reduces Abeta deposition by regulating the balance between production and degradation of Abeta in APPV717I transgenic mice. *Curr Alzheimer Res* 2009;**6**:118–31
19. Miller IN, Cronin-Golomb A. Gender differences in Parkinson's disease: clinical characteristics and cognition. *Mov Disord* 2010;**25**:2695–703
20. Watson CS, Alyea RA, Cunningham KA, Jeng Y-J. Estrogens of multiple classes and their role in mental health disease mechanisms. *Int J Women Health* 2010;**2**:153–66
21. Danielle M, Frye CA. Chronic administration of androgens with actions at estrogen receptor beta have anti-anxiety and cognitive-enhancing effects in male rats. *Age* 2009;**31**:191–8
22. Brown TJ, Adler GH, Sharma M, Hochberg RB, MacLusky NJ. Androgen treatment decreases estrogen receptor binding in the ventromedial mucleus of the rat brain: a quantitative in vitro autoradiographic analysis. *Mol Cell Neurosci* 1994;**5**:549–55
23. Edinger KL, Frye CA. Androgens effects to enhance learning and memory may be mediated in part by actions at estrogen receptor-beta in the hippocampus. *Neurobiol Learn Mem* 2007;**87**:78–85
24. Roselli CE, Horton LE, Resko J. Time-course and steroid specificity of aromatase induction in rat hypothalamus-preoptic area. *Biol Reprod* 1987;**37**:628–33
25. ter Horst JP, Oitzl MS. Relevance of stress and female sex hormones for emotion and cognition. *Cell Mol Neurobiol* 2012;**32**:725–35
26. Deacona RMJ, Cholertona LL, Talbota K, Nair-Robertsa RG, Sandersona DJ, Romberga C, Korosb E, Bornemannb KD, Rawlinsa JNP. Age-dependent and -independent behavioural deficits in Tg2576 mice. *Behav Brain Res* 2008;**189**:126–38
27. Tanghe A, Termont A, Merchiers P, Schilling S, Demuth H-U, Scrocchi L, Van Leuven F, Griffioen G, Van Dooren T. Pathological hallmarks, clinical parallels, and value for drug testing in Alzheimer's disease of the APP[V717I] London transgenic mouse model. *Int J Alzheimers Dis* 2010;**pii**:417314
28. Deacon RM, Koros E, Bornemann KD, Rawlins JN. Aged Tg2576 mice are impaired on social memory and open field habituation tests. *Behav Brain Res* 2009;**197**:466–8
29. He P, Li P, Hua Q, Liu Y, Staufenbiel M, Li R, Shen Y. Chronic administration of anti-stroke herbal medicine TongLuoJiuNao reduces amyloidogenic processing of amyloid precursor protein in a mouse model of Alzheimer's disease. *PLoS One* 2013;**8**: e58181

30. Mielke MM, Leoutsakos JM, Corcoran CD, Green RC, Norton MC, Welsh-Bohmer KA, Tschanz JT, Lyketsos CG. Effects of FDA approved medications for Alzheimer's disease on clinical progression. *Alzheimers Dement* 2012;**8**:180–7
31. Cai L-L, Li H, Liu J-G, Liu L-T, Guan J, Liu M-F, Hu J, Wei Y. Effects of early intervention with Huannao Yicong formula effective components on behaviour and cholinergic system of β-amyloid precursor protein transgenic mice. *J Chinese Integr Med* 2011;**9**:292–8
32. Shi J, Tian J, Zhang X, Wei M, Yin L, Wang P, Wang Y. A combination extract of ginseng, epimedium, polygala, and tuber curcumae increases synaptophysin expression in APPV717I transgenic mice. *Chinese Med* 2012;**7**:13
33. Sabbagh M, Cummings J, Christensen D, Doody R, Farlow M, Liu L, Mackell J, Fain R. Evaluating the cognitive effects of donepezil 23 mg/d in moderate and severe Alzheimer's disease: analysis of effects of baseline features on treatment response. *BMC Geriatrics* 2013;**13**:56

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