

# Folate/vitamin-B12 Prevents Chronic Hyperhomocysteinemia-Induced Tau Hyperphosphorylation and Memory Deficits in Aged Rats

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**Abstract.** Hyperhomocysteinemia is associated with an increased risk of Alzheimer's disease (AD). Our previous work has demonstrated that combined folate and vitamin B12 (vit-B12) supplementation prevents tau hyperphosphorylation and memory deficits induced by acute administration of homocysteine in young rats. Here, we further investigated whether folate/vit-B12 supplementation is also effective in aged rats with a chronically high level of homocysteine. 18-month-old rats were injected with homocysteine via the vena caudalis with or without a concurrent folate/vit-B12 supplementation for 28 weeks. We found that hyperhomocysteinemia induced tau hyperphosphorylation and accumulation in hippocampus and cortex. Concurrent signaling changes included the activation of glycogen synthase kinases-3 $\beta$ , cyclin-dependent kinase-5, c-Jun N-terminal kinase, extracellular signal-regulated kinase, and p38MAPK, and inhibition of protein phosphatase 2A. Although the ability to learn was not affected, the aged rats exhibited significant memory deficits. Folate/vit-B12 supplementation attenuated these biochemical and behavioral correlates. These data demonstrate that folate/vit-B12 supplementation is also effective in a chronic hyperhomocysteinemia model in reversing the AD-like tau pathologies and memory deficits.

**Keywords:** Aged rat, Alzheimer's disease, folate, hyperhomocysteinemia, vitamin-B12

## INTRODUCTION

Homocysteine is a major intermediate in sulfur-containing amino acid metabolism. At the intersection

of the remethylation and transsulfuration pathways, homocysteine can be converted into either methionine or cysteine. Deficiency of either folate or vit-B12, which is required for one-carbon-transfer reactions and remethylation [1], is one of the major causes of hyperhomocysteinemia. In rare cases, hyperhomocysteinemia has also been observed as a result of inborn errors of metabolism, e.g., such as when transsulfuration is impeded by cystathionine  $\beta$ -synthase deficiency [2]. Hyperhomocysteinemia is associated with neurological deficits including stroke, mental retardation,

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age-associated dementia, and Alzheimer's disease (AD) [3–7].

AD is the most common type of dementia in the aged population and is characterized pathologically by the presence of neurofibrillary tangles and senile plaque [8]. The tangles are mainly composed of the hyperphosphorylated conformation of the tau protein [9, 10], which is, in this state, unable to bind to, and stabilize, the microtubules. This eventually leads to the disruption of neuronal transport and degeneration of the affected neurons [11, 12]. The actual events leading to the formation of neurofibrillary tangles are still not fully understood.

Accumulating clinical evidence has demonstrated that there is a positive correlation between elevated plasma homocysteine and the occurrence of AD, and thus hyperhomocysteinemia has been forwarded as an important and independent risk factor of AD [13, 14, 6]. In contrast, other studies have been unable to demonstrate an association between plasma homocysteine levels and AD-related dementia [15] or between plasma homocysteine levels in AD dementia patients with and without evidence of behavioral and psychological impairment [16]. Yet, experimentally hyperhomocysteinemia can clearly induce brain amyloid- $\beta$  burden [17, 18], and homocysteine impairs DNA repair in hippocampal neurons, thus rendering the neurons more vulnerable to a subsequent challenge with the amyloid- $\beta$  peptide [19]. Several signaling events have been implicated in homocysteinemia-induced phenotypes including, but not limited to, the activation of ERK and CREB [20], p38 MAP kinase [21], calcium influx associated with activation of the glutamate NMDA receptor [22], and the inactivation of the protein phosphatase 2A (PP2A) [23]. Acute exposure of young rats to high levels of homocysteine induces AD-like tau hyperphosphorylation and memory deficits, both of which can be prevented with the simultaneous supplementation with folate/vit-B12 [23, 24]. Given that the prevalence of AD is significantly increasing [25], aging is now recognized as the predominant risk factor for developing AD. With aging, plasma homocysteine levels increase and this is exacerbated in the AD patient [1]. Therefore, a clearer understanding of both the biochemical and behavioral correlates of chronically elevated levels of homocysteine in aged rats is needed. It is equally as important to determine whether folate/vit-B12 can exert any effect(s) on these correlates

In the present study, we used 18-month-old rats to investigate the effects of chronic hyperhomocysteinemia on tau phosphorylation and solubility,

signaling events and spatial memory, and to determine whether supplementation with folate/vit-B12 is beneficial under these conditions. We demonstrate that hyperhomocysteinemia leads to memory deficits and tau hyperphosphorylation, the latter being evident in several regions normally associated with AD-related pathology in the clinical setting. We also demonstrate that hyperhomocysteinemia activates several protein kinases, including glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), cyclin-dependent kinase-5 (Cdk-5), c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38MAPK, and inhibits protein phosphatase 2A (PP2A). These effects of chronic homocysteinemia in the aged rat can all be reversed by supplementation with folate/vit-B12.

## MATERIALS AND METHODS

### *Antibodies and chemicals*

The primary antibodies used in this study are listed in Table 1. DL-homocysteine was obtained from Sigma Chemical Co. (St Louis, MO). Folate was from Yabang Aipusen Co. (Jiangsu, China) and vit-B12 was from Yunpeng Co. (Shanxi, China). The Bicinchoninic acid (BCA) protein detection kit, chemiluminescent substrate kit, goat anti-rabbit and anti-mouse peroxidase-conjugated secondary antibodies were purchased from Pierce Chemical Co. (Rockford, IL).

### *Animal treatment and Morris water maze test*

Aged male Sprague-Dawley rats (18 months-old,  $500 \pm 20$  g), supplied by the Experimental Animal Center of Tongji Medical College (Huazhong University of Science and Technology), were housed with *ad libitum* access to food and water. All animal experiments were performed according to the "Policies on the Use of Animals and Humans in Neuroscience Research" (the Society for Neuroscience, 1995). Rats were kept in cages under a 12:12 light-dark (L/D) cycle with the light on from 7:00 am to 7:00 pm. To establish the chronic model of hyperhomocysteinemia in these rats, we injected the rats with homocysteine (dissolved in normal saline, 1.6 mg/kg, every Tuesday and Friday,  $n=20$  per group) via the vena caudalis [23]. Normal saline with the same volume was injected in the control group ( $n=20$ ). The rats were grouped according to whether they received, or not, a dietary supplement of folate (4 mg/kg/day) and vit-B12 (250  $\mu$ g/kg/day) through their drinking water [26] for 28 weeks. Injections were routinely carried out

Table 1  
Information of the primary antibodies used in this study

Antibody	Specificity <sup>a</sup>	Type <sup>b</sup>	Dilution <sup>c</sup>	Source
Tau-5	Total tau	Mono-	1 : 1000 WB	Neo Markers
PHF-1	p-tau: Ser396/404	Mono-	1 : 1000 WB	Dr.Davies (Albert Einstein College of Medicine)
pS396	p-tau at Ser396	Poly-	1 : 1000 WB 1 : 200 IHC	Biosource
pS214	p-tau at Ser214	Poly-	1 : 1000 WB	Biosource
pS262	p-tau at Ser262	Poly-	1 : 1000 WB	Biosource
pT231	p-tau at Thr231	Poly-	1 : 1000 WB	Biosource
Tau-1	un-p-tau at Ser198/199/202	Mono-	1 : 30,000 WB 1 : 200 IHC	Chemicon
DMIA	a-tubulin	Mono-	1 : 5,000 WB	Sigma
PP2AC	Total PP2AC	Poly-	1 : 1000 WB	Cell Signaling
P-PP2AC	P-PP2AC at Tyr307	Poly-	1 : 1000 WB	Santa Cruz
GSK-3p	Total GSK-3 $\beta$	Poly-	1 : 1000 WB	Cell Signaling
p-GSK-3p	p-GSK-3 $\beta$ at Ser9	Poly-	1 : 1000 WB	Cell Signaling
Cdk-5	CDK5 C-8	Poly-	1 : 500 WB	Santa Cruz
p35	p35 C-19	Poly-	1 : 500 WB	Santa Cruz
PKA-Ca	Catalytic subunit ofPKA	Poly-	1 : 500 WB	Santa Cruz
PKA-RP	Regulatory subunit of PKA	Poly-	1 : 500 WB	Santa Cruz
p38	Total p38	Poly-	1 : 1000 WB	Santa Cruz
p-p38	p- p 38	Poly-	1 : 1000 WB	Santa Cruz
JNK	Total JNK	Mono-	1 : 1000 WB	Cell Singaling
p-JNK	p-JNK	Poly-	1 : 1000 WB	Biosource
ERK	Total ERK	Poly-	1 : 1000 WB	Santa Cruz
p-ERK	p-ERK	Poly-	1 : 1000 WB	Santa Cruz

a) p, phosphorylated. b) Mono-, monoclonal; Poly-, polyclonal. c) WB, Western-blot; IHC, immunohistochemistry.

between 9 : 00 am and 2 : 00 pm. In 29th week, the rats were trained for 6 consecutive days (4 trials per day, between 2 : 00–8 : 00 pm) in the Morris water maze [27, 28]. The swimming pathways and latencies of each rat to find the hidden platform were recorded during each test period. On the 7th day, the platform was removed and the rats were put in the opposite quadrant for 1-min test periods. All trials were monitored using a computerized tracking system. The mean target quadrant occupancy of all four test periods was calculated. The rats were sacrificed and tissues were harvested immediately after the Morris water maze test for further experiments.

#### Analysis of plasma homocysteine

Rats in all groups were anesthetized by intraperitoneal injection of 6% chloral hydrate (400 mg/kg) after Morris water maze test. Blood samples were collected via cardiac puncture and promptly centrifuged at 2,000 g for 15 min at 4°C, and the supernatant was stored at –80°C. Plasma homocysteine levels

were analyzed by fluorescence high-performance liquid chromatography (HPLC) as described previously [29]. Briefly, 60  $\mu$ l plasma sample was added to 30  $\mu$ l of 10% trichloroethyl phosphate (TCEP, Sigma-Aldrich Co., AL) in 0.25 M borate buffer (pH 10.5). Then, 200  $\mu$ l of 0.5 M perchloric acid containing 0.5 mM EDTA- $\text{Na}_2$  was added to the reaction. The mixture was centrifuged at 13,000 g for 10 min. 30  $\mu$ l of the supernatant was mixed with 20  $\mu$ l of 0.5 M NaOH and 30  $\mu$ l of 7-fluorbenzo-2-oxa-1,3-diazole-4-sulfonic acid (ABD-F, Sigma-Aldrich Co., 4.25 mM in 0.1 M borate buffer, pH 10.5) containing 2 mM of EDTA. A 10  $\mu$ l sample of this solution was analyzed by fluorescence-HPLC, at an excitation/emission maxima of 385/515 nm. Separation was carried out using a reversed-phase column (C<sub>18</sub> ODS Hyper-sil 125  $\times$  4.6 mm). Analysis was performed under isocratic conditions (0.1 M  $\text{KH}_2\text{PO}_4$ : methanol was 95 : 5, pH 2.7) at a flow rate of 1.2 ml/min for 10 min. A standard curve in a linear range of 0.0  $\mu$ M to 45.0  $\mu$ M of homocysteine ( $r^2 = 0.9993$ ) was used for quantitative analysis of homocysteine.

### Western blot

Rats were decapitated immediately following the spatial memory retention test (Morris Water Maze, described above). The hippocampi were rapidly removed and homogenized at 4°C using a Teflon glass homogenizer in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 5 mM EDTA, 2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride. The extract was mixed with sample buffer (3 : 1, v/v) containing 200 mM Tris-HCl (pH 7.6), 8% SDS, 40% glycerol, 40 mM DTT, boiled for 10 min and then centrifuged at 12,000 × g for 10 min at 25°C. The supernatant was stored at -80°C for western blot analysis. The protein concentration in the supernatant was determined using the BCA kit according to manufacturer's instructions. Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The primary antibody at 4°C overnight and then with a corresponding anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (1 : 5000) at 37°C for 1 h. Protein expression was then visualized with enhanced chemiluminescence. The blots were quantitatively analyzed by Kodak Digital Science 1D software (Eastman Kodak Co., CT).

### Immunohistochemistry

Rats were anesthetized and perfused via aortic puncture with 100 ml 0.9% NaCl followed by 400 ml phosphate buffer containing 4% paraformaldehyde. Brains were removed and post-fixed in perfusate overnight and then cut into sections (20 μm) with a vibratome (Leica, VT1000 S, Germany). The sections of rat brain were collected consecutively in PBS. Immunohistochemistry was performed as described [30]. Briefly, free floating sections were blocked with 0.3% H<sub>2</sub>O<sub>2</sub> in absolute methanol for 30 min and non-specific sites were blocked with bovine serum albumin (BSA) for 30 min at room temperature. Sections were then incubated overnight at 4°C with primary antibodies (Table 1). After thorough washes in PBS, sections were incubated with biotin-conjugated secondary antibodies at 37°C for 1 h. The immunoreaction was detected using horseradish peroxidase-conjugated antibody at 37°C for 1 h and visualized with the diaminobenzidine tetrachloride chromogenic (staining brown) system. For each primary antibody, 3–5 consecutive sections from each brain were used. The images were observed using a microscope (Olympus BX60, Tokyo, Japan).

### Biochemical analysis of tau protein solubility

Biochemical fractionation of tau protein was basically performed as described previously [31]. Briefly, the rat hippocampi (20 mg) representing each treatment group, were homogenized in 100 μl RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20 mM EDTA, 1% Nonidet-P40, 50 mM sodium fluoride, 0.25% Na-deoxycholate, and 1 mM PMSF). The homogenates were agitated at 4°C for 1 h and then centrifuged at 11,300 × g for 20 min at 4°C. The supernatant was collected as the RIPA fraction. The pellet was washed once in RIPA, and then homogenized in 50 μl (or half volume of the RIPA buffer) of 70% formic acid (FA) and centrifuged again at 11,300 × g for 20 min at 4°C. The supernatant was collected, and after evaporation of the formic acid (using a Speed Vac), the pellet was in 50 μl SDS-PAGE sample buffer (240 mM Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, 0.06% bromophenol blue). The RIPA fraction contains relatively soluble tau protein and the FA fraction contains RIPA-insoluble tau protein that can be extracted with 70% formic acid. Equal volumes of the extracts were resolved and applied to SDS-PAGE (10%) for western blot analysis. RIPA and FA fractions were loaded in proportion of 1/2.2.

### Assay for the microtubule-binding activity of tau protein

Microtubule-binding activity of tau protein was measured by the method described previously [32, 33]. In brief, the hippocampi were rapidly removed and homogenized at 4°C using a high salt reassembly buffer (100 mM Tris, 0.5 mM MgSO<sub>4</sub>, 1 mM EGTA, 2 mM dithiothreitol, and 750 mM NaCl, pH 6.8) supplemented with 0.1% Triton X-100, 20 μM taxol, 2 mM GTP, and a mixture of protease inhibitors (1 μg/ml for each of the following inhibitors: 2 mM PMSF, L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone, L-1-chloro-3-(4-tosylamido)-7-Amino-2-heptanone-hydrochloride, leupeptin, pepstatin A, and soy bean trypsin inhibitor). The homogenates were centrifuged at 50,000 g for 20 min at 25°C. The supernatant (S) containing unbound tau protein was removed and the remained pellet (P) was resuspended in sample buffer. After measuring the protein concentration, samples were subjected to western blot analysis as described above. The ratio of tau protein bound to microtubules (P) versus unbound (S) was assessed by comparing the immunoreactivity of tau protein in these two fractions.

### Statistic analysis

Data were expressed as mean  $\pm$  S.D. and analyzed using SPSS 12.0 statistical software (SPSS Inc., Illinois). The one-way analysis of variance procedure followed by least significant difference post hoc tests was used to determine the statistical significance of differences of the means.

## RESULTS

### *Folate/vit-B12 prevents tau protein hyperphosphorylation and accumulation induced by chronic hyperhomocysteinemia in aged rats*

A significant elevation of plasma homocysteine was detected in 18-month-old rats injected with homocysteine through vena caudalis. This was attenuated by supplementation with folate acid plus vitamin B12 (folate/Vit-B12) (Fig. 1A). Body weights were similar in the three treatment groups over the test period (Fig. 1B). To investigate whether chronic hyperhomocysteinemia affects tau phosphorylation, we measured

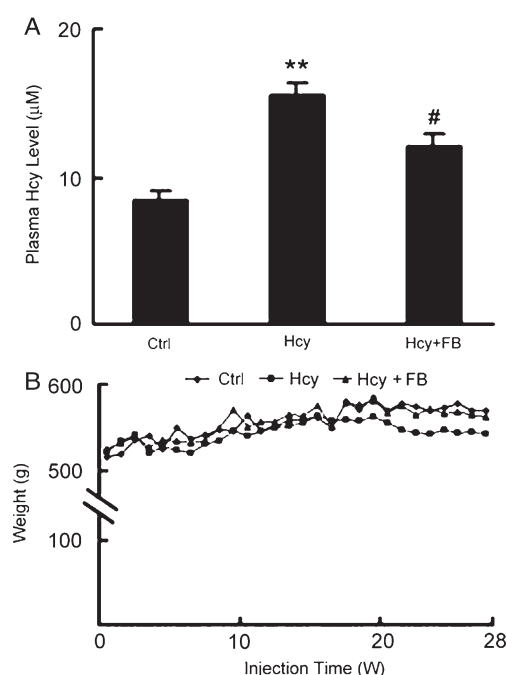


Fig. 1. Chronic exposure of the aged rats to homocysteine (Hcy) increased the plasma Hcy level. The 18-month-old rats accepted injection of Hcy twice per week through vena caudalis and were provided drinking water with or without folate and vitamin B12 (FB) for 28 weeks, then plasma homocysteine levels were measured by HPLC (A). Body weights were recorded once a week (B). Results were expressed as mean  $\pm$  S.D. \*\* $p < 0.01$  versus control; # $p < 0.05$  vs Hcy.

the expression of the hippocampal tau protein phosphorylation on several residues (i.e., pS214, pT231, pS396, and pS396/pS404 (PHF-1)). Levels of total tau protein were also determined. We observed significant increases of phosphorylation of the S214, S396, and PHF-1 epitopes, whereas phosphorylation of pT231 remained unchanged. We also used Tau-1, which recognizes tau protein when it is not phosphorylated at S198, S199 and S202, and Tau-5, which is a phospho-independent antibody that recognizes total amounts of tau protein. We observed a decrease of Tau-1 immunoreactivity in the hyperhomocysteinemic rats that was reversed by folate/vit-B12 supplementation (Fig. 2A). There was no difference in the immunodetection of Tau-5 (Fig. 2A). The increase in pS396 immunoreactivity and the concurrent decrease in Tau-1 immunoreactivity in the hyperhomocysteinemic rats, and their sensitivity to folate/vit-B12 supplementation were confirmed by immunohistochemistry of hippocampal subfields (the effect being particularly noticeable in, although not limited to, the CA3 region) (Fig. 2B). These combined data clearly indicate that tau protein phosphorylation occurs in discreet brain regions, particularly regions with a relevance to AD, as a consequence of hyperhomocysteinemia. As importantly, these data also reveal that these events are sensitive to folate/vit-B12 supplementation.

We next chose to characterize the solubility of the phosphorylated tau protein in corresponding rat hippocampal extracts. We dissociated soluble tau protein with RIPA buffer and extracted the RIPA-insoluble tau protein with 70% FA, and analyzed the two fractions by western blot. In hyperhomocysteinemic group, the immunoreactivity of pS396 and pS214 increased in both the RIPA soluble and insoluble (Fig. 3A, C). These increases were attenuated by folate/vit-B12 supplementation (Fig. 3A, C). In contrast, Tau-1 immunodetection was decreased (Fig. 3B), whereas pS231 immunodetection was increased (Fig. 3D), specifically in the RIPA-insoluble fraction and both of these changes subsequently normalized in the supplementation group (Fig. 3B, D). These results suggest that the hyperhomocysteinemia might impart different solubilities to the different phosphorylated conformations of tau protein.

### *Folate/vit-B12 preserves the chronic hyperhomocysteinemia-suppressed biological activity of tau*

To study whether the high homocysteine affects the biological activity of tau, we measured the

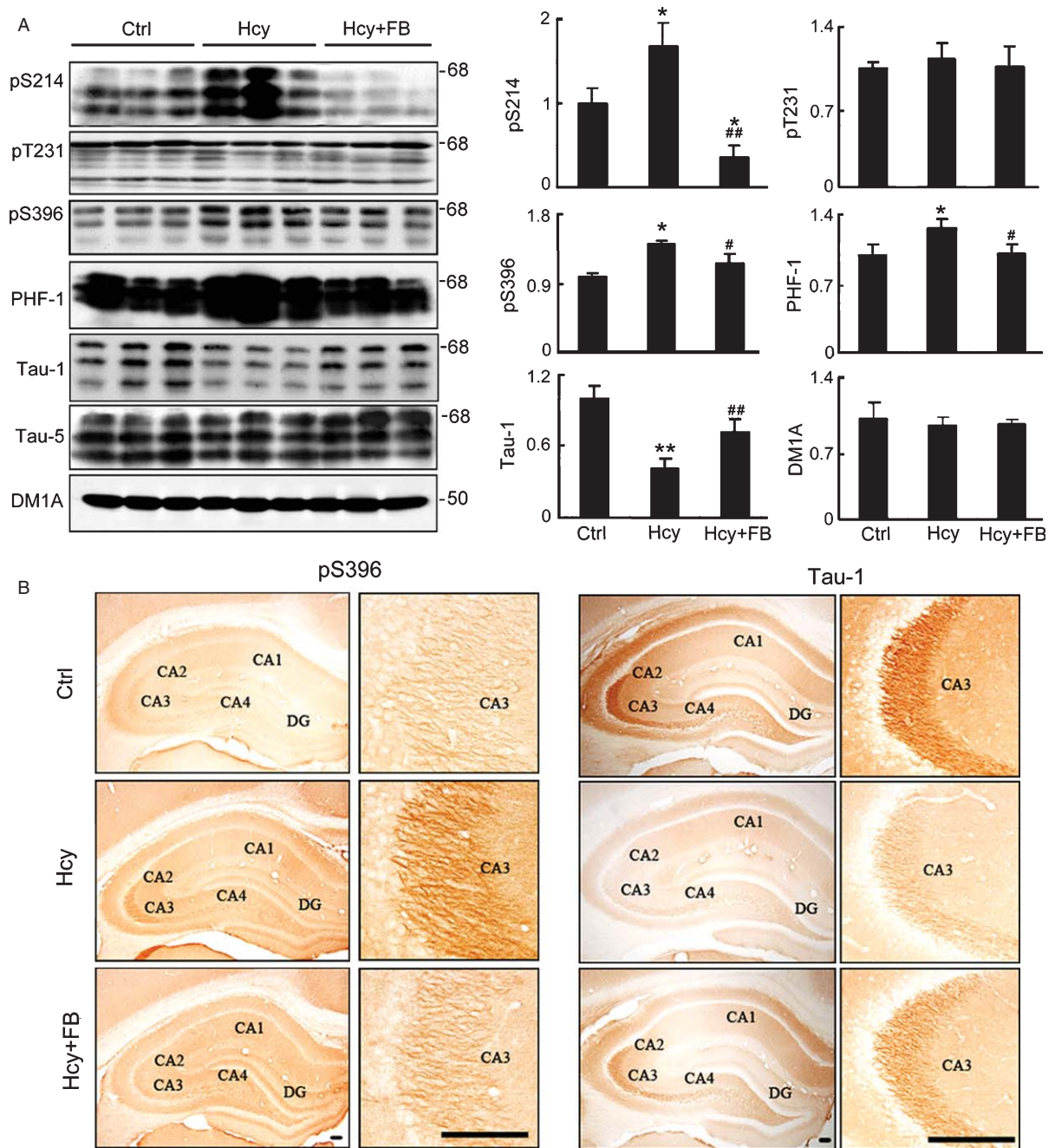


Fig. 2. FB prevents the chronic hyperhomocysteinemia-induced tau hyperphosphorylation in aged rats. Equal amount of hippocampal extracts was loaded in each lane for western blot (A). The relative level of total tau probed by Tau-5 was presented as percentage of the control after normalized to DM1A. The relative levels of the non-phosphorylated tau probed by Tau-1 and the phosphorylated tau probed by pS214, pT231, pS396, and PHF-1 were presented as percentages of corresponding controls after normalized to DM1A. The results were expressed as the mean  $\pm$  S.D. \* $p < 0.05$ , \*\* $p < 0.01$  versus control; # $p < 0.05$ , ## $p < 0.01$  versus Hcy. The phosphorylated and non-phosphorylated taus were analyzed by immunohistochemistry using pS396 and Tau-1 antibodies respectively (B). Right panels showed higher magnifications of CA3 region. Scale bar: 100  $\mu$ m.

microtubule-binding capacity of tau protein. We found that the level of tau protein bound to the taxol-stabilized microtubules (pellet fraction) decreased

while the unbound tau protein (supernatant fraction) increased after chronic exposure to homocysteine. The microtubule-binding capacity of tau protein was also

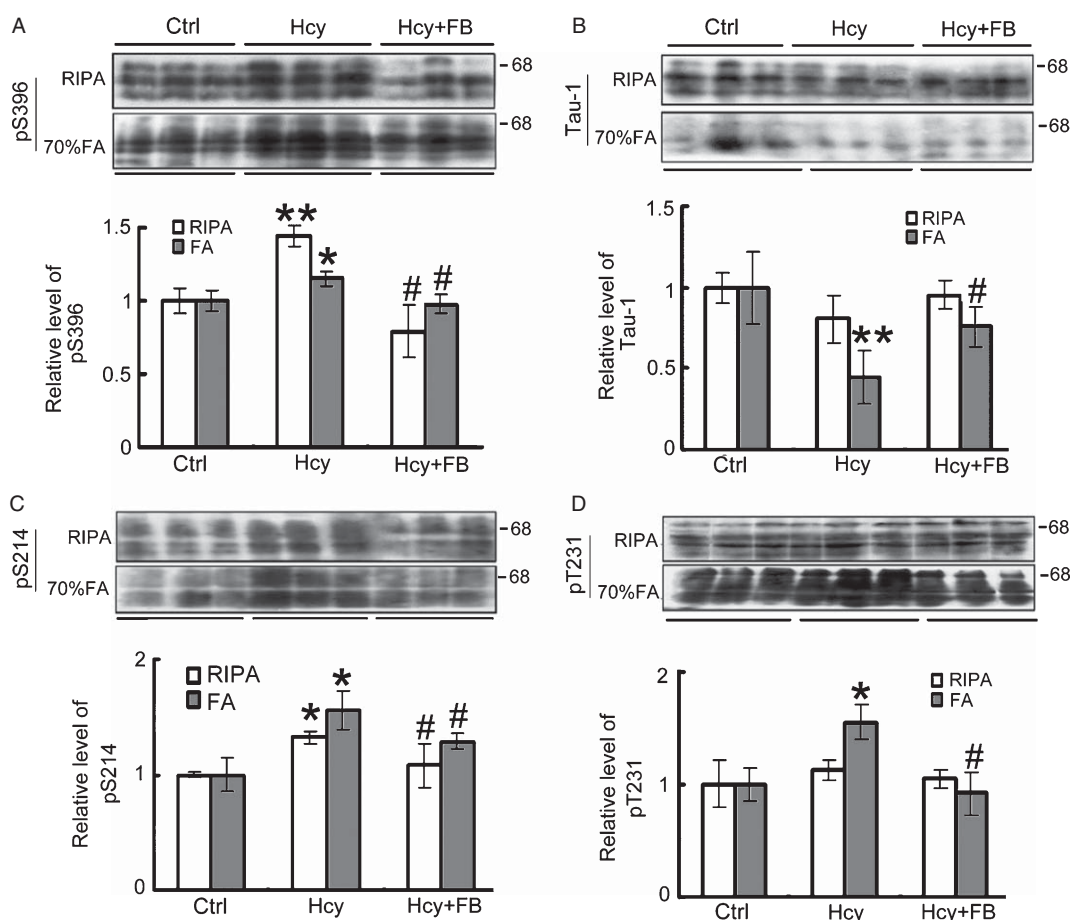


Fig. 3. FB decreases the chronic hyperhomocysteinemia-induced precipitation of tau proteins. Hippocampus proteins were sequentially extracted with RIPA buffer and 70% formic acid (FA), and levels of tau in each fraction were evaluated by western blot (upper). The relative levels of phosphorylated tau (pS214, pT231 and pS396) and non-phosphorylated tau (Tau-1) from two fractions were quantitatively analyzed (below). The results were expressed as the mean  $\pm$  S.D. \* $p < 0.05$ , \*\* $p < 0.01$  versus control; # $p < 0.05$  versus Hcy.

ameliorated by supplementation with folate/vit-B12 (Fig. 4). These results suggest that elevation of homocysteine suppresses the microtubule-binding activity of tau and folate/vit-B12 could preserve the biological function of tau.

#### *Folate/vit-B12 rectifies the chronic hyperhomocysteinemia-induced imbalance of protein kinases and phosphatase*

To determine the signaling mechanisms that potentially underlie the hyperhomocysteinemia-induced hyperphosphorylation of tau protein, we examined the activity-dependent posttranslational modifications of GSK-3 $\beta$ , Cdk-5, MAPK kinases (p38, JNK, and ERK), and PP2A. These signaling molecules were examined because they have been implicated in tau protein phosphorylation at Ser-214, Thr-231,

Ser-396/404, and Ser-199/202 residues. We observed that the Ser-9-phosphorylated GSK-3 $\beta$  (inactive form) decreased, whereas p35 (activator of Cdk-5) and the activated/phosphorylated forms of p38, JNK and ERK (i.e., p-p38, p-JNK, and p-ERK, respectively) increased with no obvious change of the total levels after homocysteine treatment (Fig. 5). The Tyr-307-phosphorylated PP2A<sub>C</sub> (p-PP2A<sub>C</sub>, an inactive form), which is associated with AD neurofibrillary pathology [34], increased after homocysteine treatment (Fig. 5). We also noticed that the level of PKA $\alpha$ -cat (catalytic subunits of PKA) decreased while the level of PKA-R $\beta$  (regulatory subunits of PKA) increased after homocysteine treatment (Fig. 5), implying inactivation of PKA that ruled out the involvement of PKA in hyperhomocysteine-induced tau hyperphosphorylation. The activation of GSK-3 $\beta$ , Cdk-5, JNK, and ERK but not p38 and inhibition of PP2A was

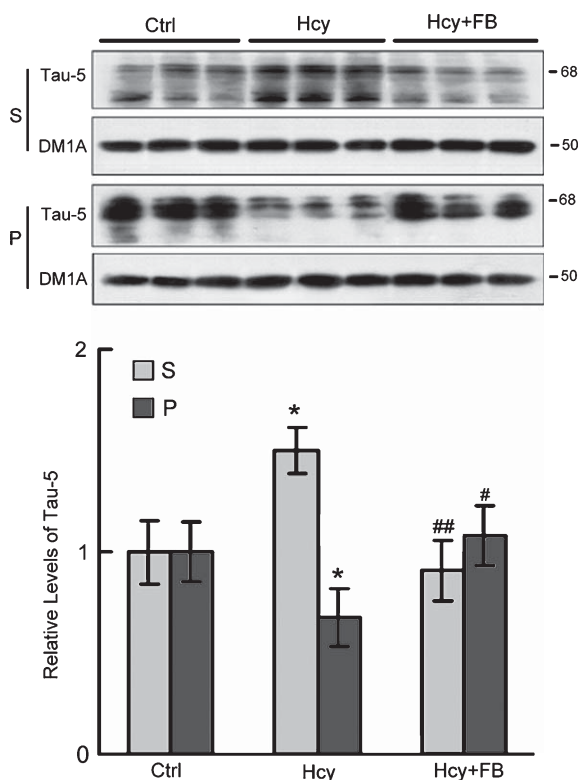


Fig. 4. FB preserves the chronic hyperhomocysteinemia-disrupted microtubule binding ability of tau. The alteration of the microtubule binding activity of tau was assessed by measuring tau and tubulin in the supernatant (S) and the pellet (P) respectively probed with Tau-5 and DM1A. The microtubule-binding activity of tau was decreased by Hcy and was recovered by FB. \* $p < 0.05$  versus control; # $p < 0.05$ , ## $p < 0.01$  versus Hcy.

fully or partially reversed by supplementation with folate/vit-B12 (Fig. 5). These data suggest that high plasma homocysteine may increase tau phosphorylation through activating multiple kinases, including GSK-3 $\beta$ , Cdk-5, JNK, and ERK, and inhibiting PP2A.

#### *Folate/vit-B12 rescues the chronic hyperhomocysteinemia-induced deficit in spatial memory in aged rats*

To determine whether hyperhomocysteinemia can affect spatial memory in the aged rats, we tested the rats using the Morris water maze. During the 6-day learning process, we did not observe any significant difference between groups in the mean latency to find the submerged platform (Fig. 6A), which means that hyperhomocysteinemia did not affect the learning ability. On the 7th day, the platform was removed and the rats were tested for spatial memory. We found

that the occupancy in the target quadrant was significantly decreased in rats receiving only homocysteine, whereas the occupancy in the target quadrant was significantly prolonged in the rats that received the concurrent supplementation with folate/vit-B12 (Fig. 6B). These results associate high plasma homocysteine with a deficit in the spatial memory in the aged rats, and demonstrates that this deficit can be reversed by supplementation with folate/vit-B12.

## DISCUSSION

AD is the most common cause of senile dementia characterized with formation of neurofibrillary tangles [8] and amyloid plaques [35]. Mean homocysteine levels tend to be higher in AD patients compared with controls [36, 37]. In fact, homocysteine levels correlate with region-dependent atrophy in pre-demented patients [38]. Given that, hyperhomocysteinemia is an early event in AD progression, it has been proposed to be a strong and independent risk factor for developing AD [39, 6]. The changes in homocysteine levels are often associated with changes in other molecules associated with one-carbon transfer (methylation) reactions, such as folate and vitamin B12 (vit-B12), and it is therefore not surprising that lower folate and/or vit-B12 levels and hyperhomocysteinemia are associated with dementia and/or cognitive decline [13, 40, 41]. Recently, we have reported that acute hyperhomocysteinemia could induce tau protein hyperphosphorylation and amyloid- $\beta$  overproduction with associated memory deficits in young rats and that simultaneous supplementation with folate/vit-B12 could attenuate the hyperhomocysteinemia-induced pathological alterations in this model [23, 24].

The process of neurodegeneration often takes years and, as such, aging is now recognized as the most important precipitating factor of AD. We chose to investigate in the present study whether a chronically elevated level of plasma homocysteine in aged rats could also induce tau protein hyperphosphorylation and memory deficits. We included a group receiving supplementation with folate/vit-B12 to help us to characterize the pathological processes in the aged rat. Our model of chronic hyperhomocysteinemia in aged rats (18 month-old rats receiving homocysteine for 28 weeks) clearly resulted in hyperhomocysteinemia. These rats all displayed changes in tau protein phosphorylation and tau protein accumulation, and exhibited significant spatial memory deficits. The simultaneous supplementation with folate/vit-B12 not



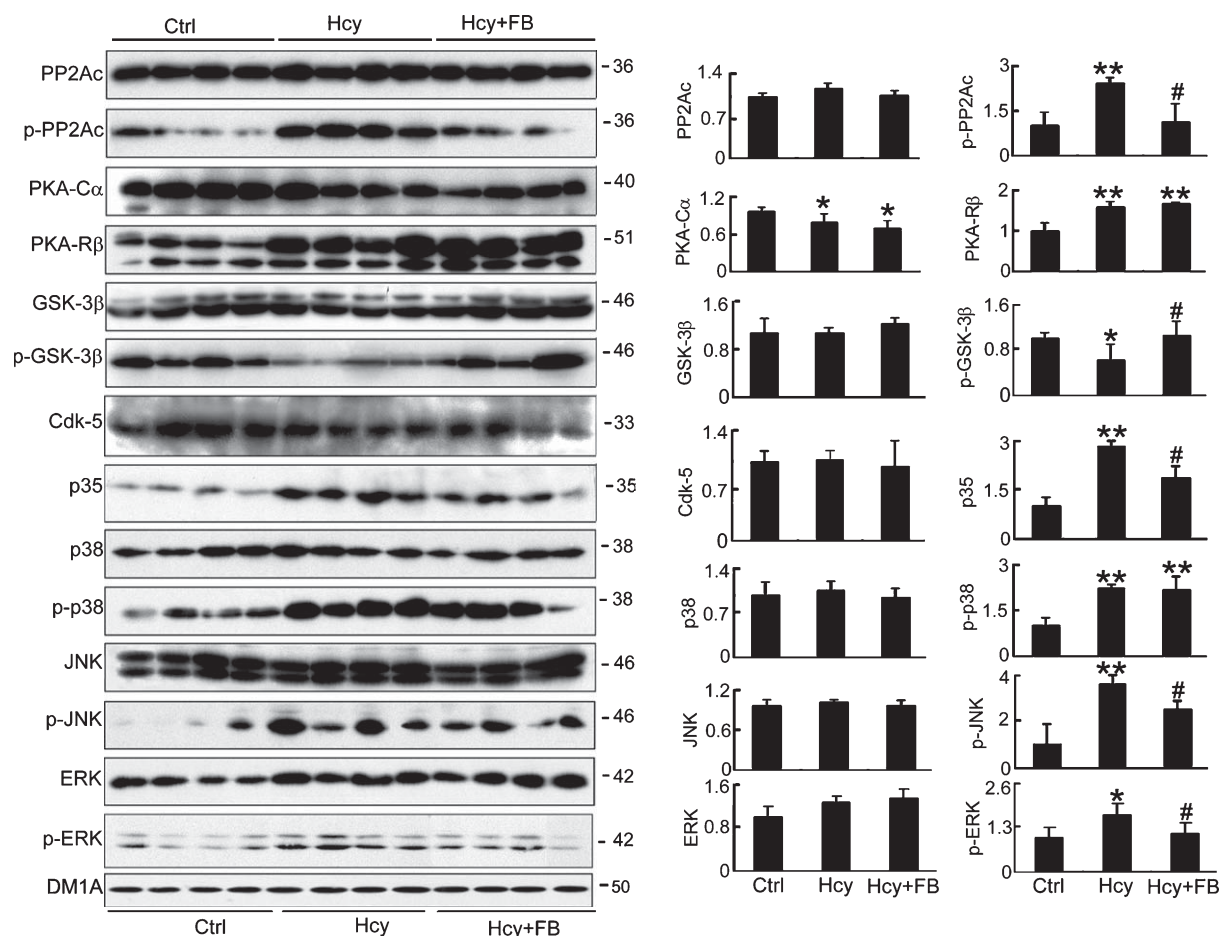


Fig. 5. FB restores the chronic hyperhomocysteinemia-induced imbalance of protein kinases and PP2A. PP2Ac and p-PP2Ac, The PKA-Cα and PKA-Rβ, GSK-3β and p-GSK-3β, Cdk-5 and p35, p38 and p-p38, and JNK and p-JNK, ERK and p-ERK were estimated by Western blotting and quantitatively analyzed as indicated in each panel. The results were expressed as the mean  $\pm$  S.D. \*  $p < 0.05$ , \*\*  $p < 0.01$  versus control; #  $p < 0.05$ .

only reversed the tau pathology and the memory deficit, but also restored plasma homocysteine levels.

To explore the possible mechanism underlying the tau protein phosphorylation associated with hyperhomocysteinemia, we examined the activities of protein kinases and phosphatase, including GSK-3β, PKA, Cdk-5, MAPKs and PP2A, all of which are involved in tau phosphorylation [42–47]. We found that the activity of PP2A decreased and the activities of GSK-3β, Cdk-5, and MAPKs increased. These observations indicate that the chronic high homocysteine exposure of the aged rats can cause the imbalance of multiple protein kinases and PP2A. Previous studies have demonstrated that homocysteine decreased PP2A activity by increasing p-PP2Ac level and decreasing methy-PP2Ac [48], and also affects the activities of GSK-3β, Cdk-5, and MAPKs [21, 24, 49]. However, the mechanisms by which

hyperhomocysteinemia disturbs the balance of protein kinases and phosphatase still remain unclear. We also found that supplementation with folate/vit-B12 did not attenuate PKA-Cα, PKA-Rβ, and p-p38 levels induced by homocysteine, this suggests that they were not sensitive to folate/vit-B12 in these aged rats, or long treatment of Hcy made their activity keep in high level and cannot be rescued to control-like conditions.

Tau proteins isolated from the neurofibrillary tangles of the AD brains are mainly in the form of insoluble twisted filaments [50, 51]. To analyze the solubility of the accumulated tau proteins induced by chronic high plasma homocysteine, we extracted tau proteins in RIPA buffer as well as in formic acid. We found that the high plasma homocysteine level increased most prominently the insolubility of the Ser214- and Thr231-phosphorylated tau and supplementation of folate/vit-B12 could restore the solubility of tau proteins.

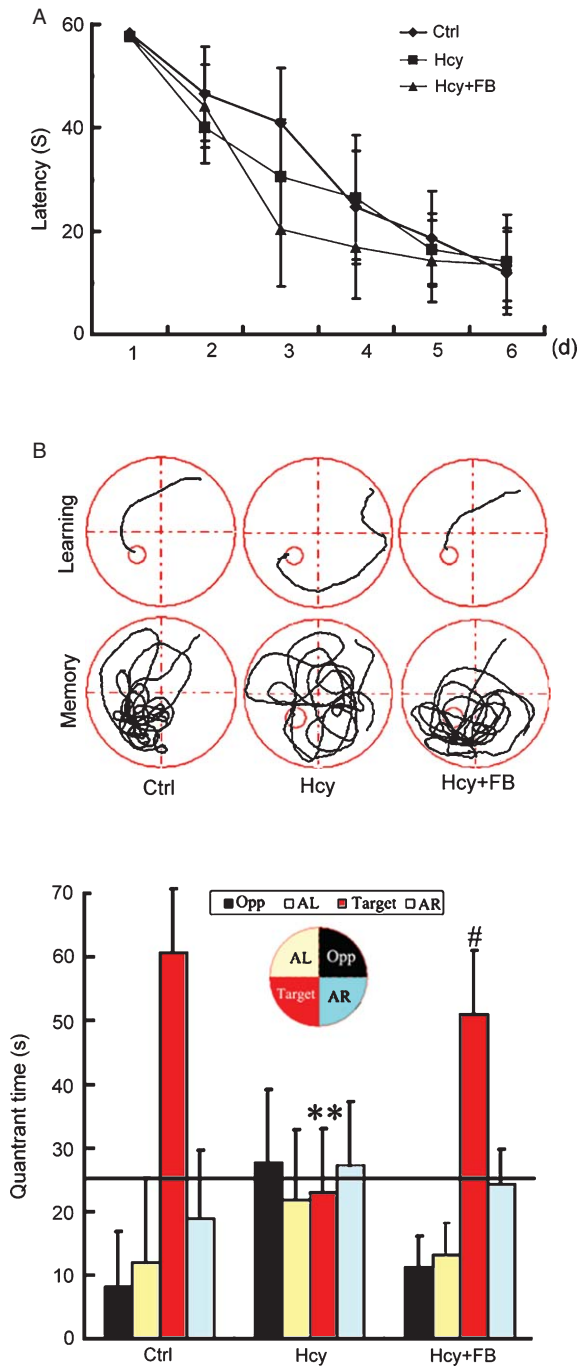


Fig. 6. FB rescues the chronic hyperhomocysteinemia-induced memory deficits in aged rats. After 28-weeks injection of Hcy and supplementation of FB, the rats were trained for 6 days, and the swimming pathways and latencies of the rats to find the hidden platform were recorded each day (A). At the 7th day, the rats' memory abilities were detected by Morris water maze without platform. The swimming pathways and quadrant occupancy during probe trials (means of four trials) of the water maze was calculated (B). The results were expressed as the mean  $\pm$  S.D.  $**p < 0.01$  versus control;  $\#p < 0.05$  versus Hcy.

A previous study has demonstrated that high plasma homocysteine level precedes the development of dementia [52]. Therefore, homocysteine is proposed to be a modest independent predictor of cognitive function in community-dwelling elderly Latinos, and reducing plasma homocysteine by administering B-vitamin supplements has been recommended to protect against the cognitive decline in this elderly populations [53]. We found that hyperhomocysteinemia was able to induce tau hyperphosphorylation and accumulation with formation of tangle-like structures in the hippocampus and cortex neurons. As a major protein component of the neurofibrillary tangles that is positively correlated with the dementia in AD patients [54], the hyperphosphorylated tau protein is a recognized factor contributing to the memory deficits in the context of AD. Therefore, we also examined the effects of hyperhomocysteinemia on spatial learning and memory in the aged rats and found that high plasma homocysteine impaired memory. These results are in good accordance with the previous observation that insoluble tau protein is seen in neurofibrillary tangles of the affected neurons in AD brains, and that these neurons have been damaged by insoluble tau proteins that interfere with axonal transport and eventually lead to memory deficits [55]. More importantly, we also found that supplement of folate/vit-B12 could also rescue the memory in our model of chronic exposure to homocysteine. But there are no significant changes in the learning ability of the aged rats, the mechanism of hyperhomocysteinemia on spatial learning and memory is not clear and needs further investigation. In our previous work using young rats, we observed that hyperhomocysteinemia did not affect GSK-3 $\beta$  activity [23]. We speculate that this difference may be caused by the differences in age between the two test populations.

Homocysteine is produced from the essential amino acid, methionine. This reaction is catalyzed by methionine synthase, which requires respectively vitamin B12 and methyltetrahydrofolate acid as cofactor and cosubstrate. An alternative route of homocysteine disposal is degradation to cysteine through the transsulfuration pathway by two sequential vitamin B6-dependent reactions [56]. B-vitamin deficiency induced homocysteinemia and selectively impaired Morris Water Maze performance in apolipoprotein-E deficient mice [57] and folate deficiency induces leucine carboxyl methyltransferase-1 and protein phosphatase 2A B $\alpha$  subunit protein levels decrease that correlate with enhanced tau phosphorylation [58]. In this study, we found that folate and vit-B12 supplementation can reduce chronically-induced hyperhomocysteinemia in

aged rats, our data suggest that simple addition to a normal diet of large doses of folate and vit-B12 may be a reasonable strategy to prevent senile dementia in aged rats, which give a new evidence for future studies to test the effect of folate and B vitamins on AD patients [6].

In summary, we have found in the present study that chronic exposure of aged rats to homocysteine increases tau protein phosphorylation with elevations in relevant protein kinases as well as a decrease in PP2A activities. These biochemical changes correlate well with changes in spatial memory. Perhaps most importantly, simultaneous supplementation with folate and vit-B12 attenuates the hyperhomocysteinemia and the tau pathologies and memory deficits in chronically hyperhomocysteinemic aged rats.

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