

## Original Article

## Aging effects on circulating adiponectin levels and the expressions of adiponectin and adiponectin receptor 1 in the brains of male rats

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## SUMMARY

**Background:** At present, many studies address circulating adiponectin (APN) levels in patients with Alzheimer's disease (AD) and moderate cognitive impairment, but the results of these studies are controversial. We assumed that Alzheimer-like changes in the brain may be accompanied by the changes in APN and its receptor expression in aging process. This study sought to investigate the effects of aging on serum APN levels, APN and AdipoR1 expression in the rat brain.

**Methods:** Serum APN levels, Tau protein phosphorylation, APN and its receptor expressions in brain tissue were observed in male Sprague-Dawley rats at the age of 12 weeks (young group), 36 weeks (adult group) and 96 weeks (elder group) ( $n = 12$ ).

**Results:** No significant difference in Tau5 protein expression was detected between those groups ( $P > 0.05$ ). Phosphorylation of Tau at Ser262 and Ser396 was gradually increased with age in the hippocampus ( $P < 0.05$ ), but no significant difference in the cerebral cortex ( $P > 0.05$ ). Serum APN levels gradually reduced with age in rats ( $P < 0.05$ ). Moreover, APN expression gradually diminished in the hippocampus with age ( $P < 0.05$ ). However, no significant difference in APN expression was observed in the cerebral cortex with age ( $P > 0.05$ ). With age, AdipoR1 expression gradually increased both in the hippocampus and cerebral cortex ( $P < 0.05$ ).

**Conclusion:** Aging can simulate Alzheimer's disease-like degeneration of the brain, with reduced circulating APN levels, decreased expression of APN and increased expression of AdipoR1 in the brains of male rats.

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## 1. Introduction

Clinical manifestations of Alzheimer's disease (AD) are mainly characterized by progressive intellectual deterioration, memory impairment, cognitive impairment, and psychiatric symptoms. The pathogenesis of AD remains unclear, mainly containing genetic factors, neurotransmitter disorders, cytoskeletal changes, hypertension, diabetes, hyperlipemia, high serum homocysteine, central obesity, atrial fibrillation, traumatic brain injury, chronic viral infection, low educational level, smoking, and history of exposure to heavy metals.<sup>1,2</sup> Tau protein phosphorylation can reduce the ability of microtubule assembly and damage nerve cells. Aging can cause the phosphorylation of Tau protein at multiple sites.

Recent studies suggested that the disorders of energy metabolism may be one of pathogenesis of AD, mainly because AD patients often suffer from visceral fat accumulation, insulin resistance and abnormal secretion of leptin and APN. APN, an adipokine secreted by adipose tissue, is involved in the regulation of energy metabolism, enhances insulin sensitivity, glucose uptake and fatty acid oxidation, effectively resists inflammation and atherosclerosis, and protects vascular endothelial cells.<sup>3</sup> The decrease in circulating APN levels is correlated with insulin resistance syndrome and visceral fat deposition, and can be found in some clinical diseases, such as obesity, dyslipidemia, diabetes and depression.<sup>4</sup> APN and its receptors are widely expressed in the brain. AdipoR1 is mainly expressed in the hippocampus. Combining with diverse physiological functions of APN, it is indicated that APN signaling system may exert a crucial effect on cognitive function.

Teixeira et al.<sup>3</sup> confirmed that low circulating APN levels were associated with cognitive dysfunction, and the decrease in APN levels could reflect the pathological process of AD. On the

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contrary, Kamogawa et al<sup>5</sup> verified that the increase in plasma APN levels was a protective factor for dementia in males, but did not impact cognitive function in females after the investigation in 517 community members, which was consistent with Une et al's study.<sup>6</sup> Roberts et al<sup>7</sup> suggested that no significant difference in circulating APN levels was visible between mild cognitive impairment (MCI) patients and controls. Interestingly, the results of van Himbergen et al's study<sup>8</sup> are contrary to above results. They believed that the increase in circulating APN levels was an independent risk factor for dementia or AD in females, which was contrary to multiple physiological functions of APN. Another previous study showed that circulating APN levels had obvious sex difference, and the levels were higher in females than in males.<sup>5</sup> Thus, APN levels may be affected by estrogen levels.<sup>9</sup> Therefore, it is presumed that results of van Himbergen et al's study were probably associated with estrogen levels in females. Pakaski et al<sup>10</sup> demonstrated that serum APN levels increased progressively with the time of taking donepezil in AD patients, and also indirectly verified that APN had protective effects on AD patients. Accordingly, current results support the protective effect of on cognitive function.

There is extensive cross-linking on the pathogenesis of AD and type II diabetes, which associates with aging.<sup>11</sup> More and more studies have shown that APN may be the key point of their cross-linking. Age-associated degenerative lesions have been shown to accelerate the pathological process of AD and other neurodegenerative diseases.<sup>12</sup> Taken together, we assumed that Alzheimer-like changes in the brain may be accompanied by the changes in APN and its receptor expression in aging process. This study sought to investigate the effects of aging on serum APN levels, APN and AdipoR1 expression in the rat brain.

## 2. Materials and methods

### 2.1. Experimental animals

Unmated male Sprague-Dawley rats at the age of 12 weeks (young group), 36 weeks (adult group) and 96 weeks (elder group) ( $n = 12$ ) were purchased from Dashuo Biotechnology Co., Ltd. (Chengdu, China; license No. SCXK2008-24). All rats were housed in the Clean Animal Room, and allowed free access to food and water, housed at 24–26 °C and relative humidity of 70% in 12-h light/dark cycles, with regular ultraviolet disinfection and ventilation. After 3 days of acclimation, rats were sacrificed by excessive intraperitoneal injection of phenobarbital 40 mg/kg body weight. Blood from inferior vena cava was obtained from all rats for detection of serum APN concentration. Brains of six rats from each group were collected for western blot assay. Brains of remaining rats were gained for immunohistochemistry. The protocols were conducted in accordance with the Animal Study Outline of Shanghai Jiao Tong University in China, and approved by the Animal Research Committee of Shanghai Jiao Tong University in China.

### 2.2. Enzyme linked immunosorbent assay (ELISA) for serum APN concentration

Mice were fasted overnight before sacrifice. Blood was immediately collected from the inferior vena cava after sacrifice. Blood was placed in a pre-cooling tube for 1 h, centrifuged at 1600 g for 15 min and 4 °C. Serum was obtained and stored at –80 °C for further use (no more than 2 months). Serum APN concentration was detected with a commercial ELISA kit (USCN Life Science Inc., Wuhan, Hubei Province, China). Experiments were performed in triplicate.

### 2.3. Immunohistochemical staining

After paraformaldehyde perfusion in the heart, brain tissue was obtained and fixed in 4% paraformaldehyde for 24 h, dehydrated, permeabilized, and embedded in paraffin. Above sample was then sliced into 4  $\mu\text{m}$ -thick sections with a RM 2255 hand slicer (Leica Microsystems, Wetzlar, Germany). Before staining, sections were dried, dewaxed, dehydrated, rehydrated, and treated with 10 mM citrate buffer (pH 6), boiled in a microwave oven for 3 min, cooled at room temperature for 30 min, blocked with 0.3% H<sub>2</sub>O<sub>2</sub> to inactivate endogenous peroxidase for 10 min, lysed with 0.2% Triton X-100 for 30 min, and blocked with normal goat serum (1:10) for 20 min. Above sections were incubated with mouse monoclonal anti-adiponectin antibody (1:200), mouse polyclonal anti-AdipoR1 antibody (1:200) or rabbit polyclonal anti-GAPDH antibody (1:1000) (diluted in 0.1 M PBS-dissolved 1% bovine serum albumin; Abcam, Cambridge, USA) at 4 °C overnight. After three washings in PBS, the specimens were exposed to a PowerVision™ goat anti-mouse IgG antibody-horseradish peroxidase polymer (Dako, Carpinteria, USA) for 30 min to detect immunoactivity. Antibody binding was visualized by incubation with DAB (Boster, Wuhan, China) for 6 min at room temperature followed by counterstaining with hematoxylin (Baso, Zhuhai, China). Subsequently, sections were dehydrated in a graded series of ethanol, cleared in xylene, and coverslipped. Sections that did not incubated with primary antibody served as negative controls. All sections were observed and photographed under the light microscope (Olympus, Tokyo, Japan).

### 2.4. Western blot assay

After sacrifice, the brain was obtained immediately. Cerebral cortex and hippocampus were isolated and stored in liquid nitrogen separately. When proteins were extracted, radioimmune precipitation assay buffer lysate (1:10) and 0.1 mM phenylmethyl sulfonyl fluoride (Beyotime, Nantong, Jiangsu Province, China) were added, followed by sonication. Samples were lysed on ice for 15 min, and centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatant was obtained. Protein concentration was determined with a Bicinchoninic Acid Protein Assay Kit. Total protein was extracted and 5  $\times$  loading buffer was added at 4:1. After boiling for 5 min, samples were stored at –80 °C for western blot assay. In strict accordance with the instruction of reagent, western blot assay was conducted. Briefly, 8% sodium dodecylsulfate-polyacrylamide gel was prepared. 40  $\mu\text{g}$  protein of each sample was electrophoresed on above gel, and the separated proteins were electrically transferred to polyethylene membrane (Beyotime, Nantong, Jiangsu Province, China). Following three washes with 10 mM Tris Buffered Saline with 1.0% Tween-20 (TBST) for 10 min each, the membrane was blocked with 5% skimmed milk powder (dissolved in 10 mM TBST) for non-specific protein binding. Above membrane was incubated in rabbit monoclonal anti-Tau (phospho S396) antibody dissolved in 1% bovine serum albumin, rabbit polyclonal anti-Tau (phospho S262) antibody, mouse monoclonal anti-Tau 5 antibody, mouse monoclonal anti-adiponectin antibody, mouse polyclonal anti-AdipoR1 antibody (1:1000; Abcam, Cambridge, USA), or rabbit polyclonal anti-GAPDH antibody (1:1000; Bioworld, St. Paul, USA) at 4 °C overnight. On the next day, the membrane was washed three times with TBST for 10 min each. The polyethylene membrane was subsequently incubated with horseradish peroxidase goat anti-rabbit IgG secondary antibody (1:1000; Dako, Carpinteria, USA) at room temperature for 60 min, washed three times with TBST for 10 min each, and visualized with the Super Signal Chemiluminescent Substrate System (Pierce, Rockford, USA). Quantitative analysis was performed with Bio-Rad image lab 2.0 software (Bio-Rad Laboratory, Hercules, USA).

### 2.5. Statistical analysis

Data were expressed as the mean ± standard error of the mean, and analyzed using SPSS 16.0 software package (SPSS Inc., Chicago, USA). One-way analysis of variance was conducted, followed by least significant difference test for pairwise comparison ( $\alpha = 0.05$ ).

## 3. Results

### 3.1. Aging effects on Tau protein phosphorylation in the rat brain

Tau protein hyperphosphorylation is a feature of neuronal degeneration, and plays a crucial effect on the pathological process of AD-like neurodegeneration. To verify the neurodegeneration in the brain of aging rats, we detected phosphorylation of Tau at Ser262 and Ser396, and measured the expression of Tau5 protein in the brain of rats aged 12, 36 and 96 weeks. The choice of above sites is in accordance with a previous study, because Ser262 and Ser396 are involved in the pathological process of AD.<sup>12</sup> Western blot assay results demonstrated that no significant difference in Tau5 protein expression was detectable in the cerebral cortex and hippocampus of aging rats. Phosphorylation of Tau at Ser262 and Ser396 gradually increased in the hippocampus with age, but above changes were not apparent in the cerebral cortex with age (Fig. 1). Results verified that phosphorylation of Tau protein increased with age in the hippocampus, and aging could simulate AD-like degeneration in the brain.

### 3.2. Aging effects on circulating APN concentration and APN expression in the rat brain

ELISA results suggested that significant differences in serum APN levels were visible in rats aged different weeks, and serum APN levels diminished gradually with age (Fig. 2). Serum APN levels decreased approximately 25% in 96-week-old rats compared with 12-week-old rats. To identify the changes in APN expression in the brain, western blot assay was conducted separately in the cerebral cortex and the hippocampus (Fig. 3). Results suggested that APN expression decreased gradually with age in the hippocampus, which showed significant differences. However, no significant difference in APN expression was identified in the cerebral cortex of

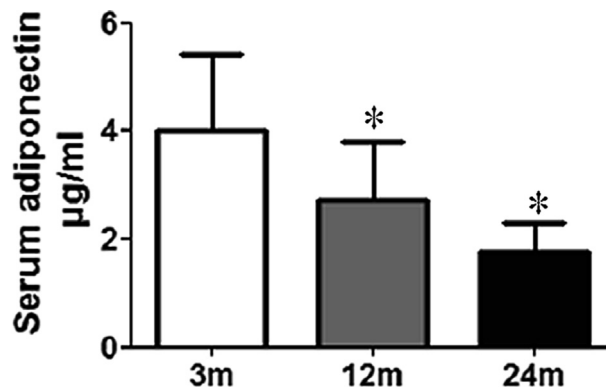


Fig. 2. ELISA results suggested serum APN levels diminished gradually with age. (\*P < 0.05).

rats at different ages. Results suggested that aging could reduce circulating APN levels and APN expression in the cognition-related hippocampus.

### 3.3. Aging effects on AdipoR1 expression in the rat brain

Western blot assay was carried out in the cerebral cortex and hippocampus. Results indicated that the trend for AdipoR1 expression was consistent in the hippocampus and cerebral cortex, and AdipoR1 expression was increased gradually with age, showing significant differences (Fig. 3). Immunohistochemical staining results revealed that increased AdipoR1 was expressed in the cerebral cortex and hippocampus, mainly in neurons of the hippocampus. (Fig. 4). It is concluded that aging could increase the number of neurons expressing AdipoR1 in the cognition-related hippocampus.

## 4. Discussion

Tau protein is a microtubule-associated protein widely expressed in the nervous system, promotes microtubule formation and stabilizes microtubule structure. Hyperphosphorylated Tau protein decreases its binding capacity to microtubules, so Tau

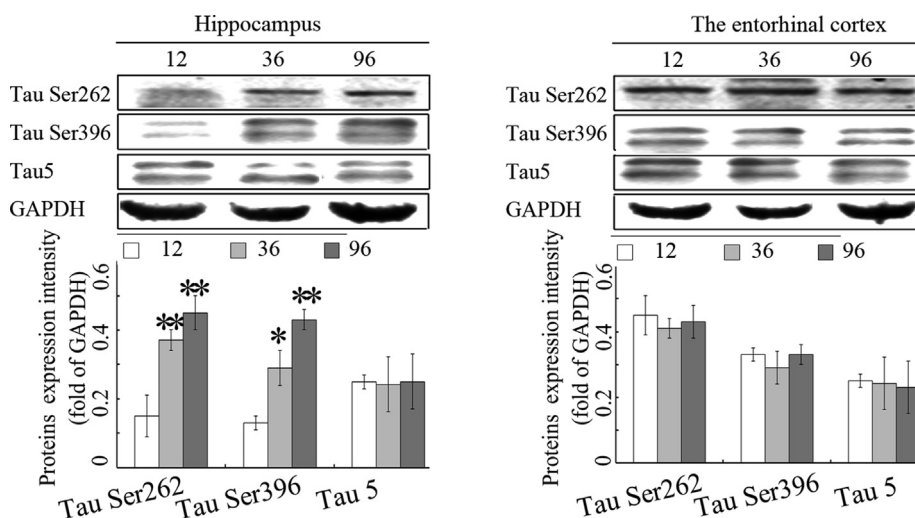
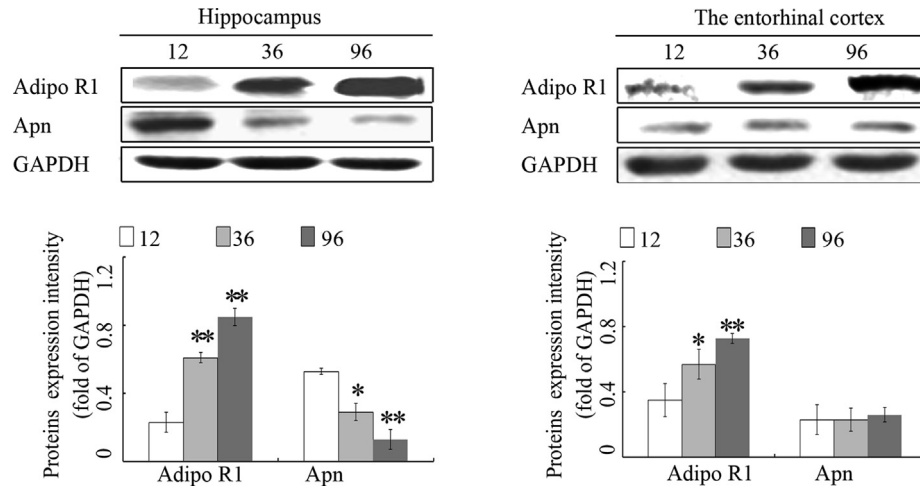
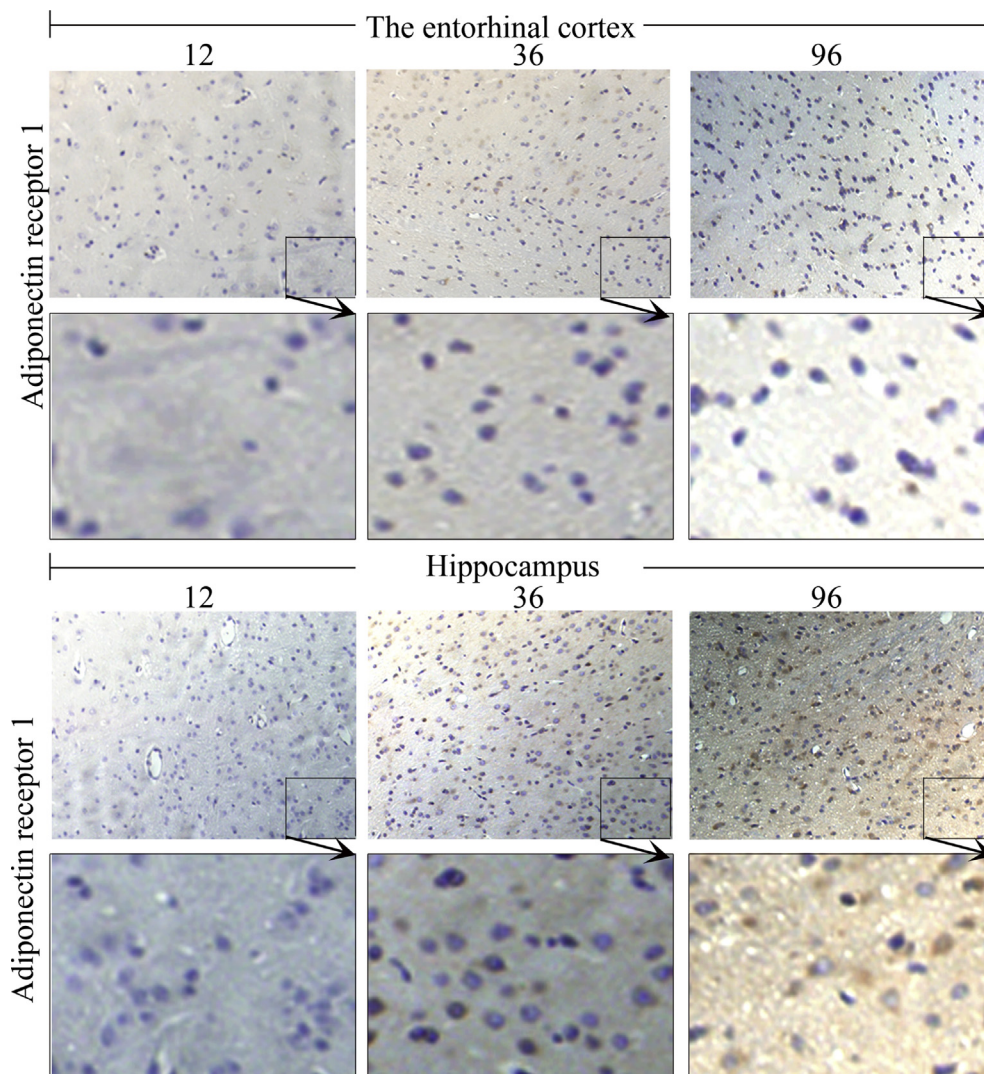


Fig. 1. Western blot assay results demonstrated that no significant difference in Tau5 protein expression was detectable in the cerebral cortex and hippocampus of aging rats. Phosphorylation of Tau at Ser262 and Ser396 gradually increased in the hippocampus with age, but above changes were not apparent in the cerebral cortex with age. (\*P < 0.05; \*\*P < 0.01).





**Fig. 3.** Western blot assay results demonstrated that APN expression decreased gradually with age in the hippocampus, and no significant difference in the cerebral cortex. AdipoR1 expression was increased gradually with age both in the hippocampus and cerebral cortex. (\* $P < 0.05$ ; \*\* $P < 0.01$ ).



**Fig. 4.** Immunohistochemical staining results revealed that AdipoR1 was expressed in the cerebral cortex and hippocampus, mainly in neurons of the hippocampus.

protein cannot play a role in stabilizing microtubules, thereby damaging nerve cells. Hyperphosphorylated Tau protein accumulated in cells is a structural basis for neurofibrillary tangle formation, and neurofibrillary tangle is a marker of pathological changes in AD. Therefore, to verify whether aging rats can simulate the pathological process of AD, the present study identified total Tau protein (Tau5) and phosphorylated Tau protein (phosphorylation at Ser262 and Ser396). Our results demonstrated that no significant difference in Tau5 protein was observed in the cerebral cortex and hippocampus during aging. Moreover, there was no significant difference in the phosphorylation of Tau protein at Ser262 and Ser396 in the cerebral cortex. However, phosphorylation of Tau protein at Ser262 and Ser396 in the cognition-related hippocampus increased with age, which was consistent with Jung et al's study.<sup>12</sup> Taken together, aging is accompanied by AD-like degeneration in the region associated with cognitive function. In this study, APN and its receptor expression during aging could indirectly reflect the relationship of AD-like degeneration with APN and its receptor expression.

To verify whether circulating APN could protect cognitive function during aging, we detected the alterations in circulating adiponectin levels in aging rats. Our results suggested that circulating APN levels reduced with age. Kawasumi et al's study<sup>13</sup> results are consistent with ours. They concluded that circulating APN levels were remarkably lower in dogs aged 8–12 years than dogs aged 0–7 years. Nevertheless, previous results<sup>14–17</sup> are contrary, and other studies<sup>9,18</sup> did not show noticeable difference in APN levels during aging. To find out the reason, Li et al<sup>18</sup> detected circulating APN levels in mice aged 2, 6 and 24 months, although no significant difference was detected in APN levels. Considering the increased fat content induced by aging, Li et al<sup>18</sup> identified the ratio of plasma APN levels to visceral fat content, and confirmed that above ratio was diminished with age. Tomicek et al<sup>19</sup> confirmed that circulating APN levels were higher in 6-month rats than in 24-month rats, and ovariectomy reduced APN levels. These results indicated that circulating APN levels in perimenopause could be impacted by estrogen. Matsui et al<sup>20</sup> verified that circulating APN levels in females showed “U” shape during aging, and were positively correlated with aging in postmenopause, and negatively correlated with age in early menopause. Riestra et al<sup>21</sup> verified that APN levels were positively associated with sex hormone-binding globulin levels. Numerous studies<sup>14–17</sup> demonstrated that circulating APN levels were greater in females than in males, but they did not conduct any analysis in different stages of menopause. Furthermore, during the analysis, they considered the effects of sex hormone-binding globulin levels. Thus, above studies may not precisely reflect the changes in APN expression in aging females. To exclude the significant interference of estrogen on study results, we selected male rats as materials. Our results confirmed that circulating APN levels reduced with age, indicating that APN probably played an important role in aging-related diseases.

To further investigate the correlation of APN and its receptor with age-related cognitive dysfunction, this study investigated APN and its receptor expression in the brain of aging rats. Our results showed that with age, APN expression diminished in the hippocampus, but did not apparently alter in the cerebral cortex, which suggested that APN may be associated with cognitive function. Animal experiments showed that atorvastatin reduced oxidative stress in amyloid precursor protein-Tg mice, and accompanied by the increase in circulating APN levels.<sup>22</sup> *In vivo* and *in vitro* studies further verified that APN lessened A $\beta$ -induced neurotoxicity by antagonizing oxidative stress.<sup>23,24</sup> Miao et al<sup>25</sup> thought that the effects of APN overexpression on improving neurological rehabilitation in elder mice of cerebral ischemia were better than that in young mice, which also confirmed our view. With the aging

process, decreased APN levels possibly played a key role in dementia-like pathological process in the elderly.

Results from this study also demonstrated that a trend for AdipoR1 expression is consistent between cerebral cortex and hippocampus, which is positively associated with aging. AdipoR1 and AdipoR2 were expressed in the brain.<sup>26,27</sup> Coope et al<sup>28</sup> further verified that AdipoR1 and AdipoR2 can simultaneously express in the hypothalamus, but APN role produced by the hypothalamus depends on AdipoR1, but not on AdipoR2. Therefore, this study observed AdipoR1 changes in the brain with age, but did not detect AdipoR2. Sakr et al<sup>29</sup> suggested that memory function reduced in diabetic rats, accompanied by decreased AdipoR1 in the hypothalamus. Sitagliptin can be used to treat diabetes, and improves memory function and upregulates AdipoR1 mRNA expression. It can be seen that AdipoR1 may play a role in pathological process of AD. However, our results are contrary to theirs. AdipoR1 expression increased in aging rats. APN receptor expression in the hypothalamus could be regulated by many factors such as energy metabolism.<sup>30</sup> It remains unclear whether an increase in AdipoR1 expression in the brain with age is a negative feedback regulation induced by decreased APN expression, or a pathological process of aging, which deserves further investigation.

In conclusion, circulating APN and APN expression in the hippocampus reduced with age, which is accompanied by the increased phosphorylation of Tau protein, indicating that APN system may be involved in aging-induced AD-like pathological process. AdipoR1 expression in the hippocampus increased with age, and its precise mechanisms and effects deserve further investigations.

## Conflicts of interest

No conflict of interest exists.

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