Published online: December 30, 2019

Original Research

Effects of cellular prion protein on rapid eye movement sleep deprivation-induced spatial memory impairment

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DOI:10.31083/j.jin.2019.04.1163

The effects of cellular prion protein on rapid eye movement sleep deprivation-induced spatial memory impairment were investigated, and the related mechanisms explored. Male C57BL/6 mice were randomly divided into four groups: environment control, sleep deprivation control, sleep-deprived-plasmid adeno-associated virus-green fluorescent protein group, and sleep-deprived-plasmid adeno-associated virus-cellular prion protein-green fluorescent protein group. Overexpression of cellular prion protein was induced by stereotaxic injection of adeno-associated viral plasmids-CAG-enhanced green fluorescent protein-cellular prion protein-Flag (a small label, which can be detected with corresponding tagged antibodies) into the hippocampus. Sleep-deprived mice were allowed no rapid eye movement sleep for 72 hours. Morris water maze was used to assess the effects of cellular prion protein on spatial learning and memory. The expression of amyloid-β was also investigated in all groups. The sleep-deprived- plasmid adeno-associated virus- cellular prion protein-green fluorescent protein group spent significantly more time in a goal quadrant compared with the sleep-deprived- plasmid adeno-associated virus-green fluorescent protein group. Sleep deprivation resulted in increased amyloid-β in the hippocampus, which was reversed by the overexpression of hippocampus cellular prion protein. Overexpression of cellular prion protein in the hippocampus rescues rapid eye movement sleep deprivation-induced spatial memory impairment in mice. It is shown that amyloid-β in the hippocampus might be one of the mechanisms.

Keywords

Cellular prion protein; sleep deprivation; Morris water maze; amyloid-β; memory; mice

1. Introduction

The cellular prion protein (PrP^C) is a glycoprotein localized on neuronal plasma membranes by a C-terminal glycosylphosphatidyl (GPI) anchor and is expressed by neurons, glial cells and other cells (Brown et al., 1998; Kretzschmar et al., 1986; Moser et al., 1995). Anomalous conformer of this protein is a scrapie isoform of the prion protein (PrP^{SC}), which can result in severe neurodegeneration and death in humans and animals, gives rise to the pathogen that causes prion diseases such as kuru, Creutzfeldt-Jakob disease (Kitamoto et al., 1986) and fatal family insomnia (Gallassi et al., 1992). However, the major physiological effect of PrP^C remains obscure.

Recent studies have shown that PrP^C is widely expressed in the central nervous system and plays pleiotropic roles, such as for cell signaling cell adhesion/differentiation and memory (Luckenbill-Edds, 1997; Martins et al., 1997). Previous studies have shown that the PrP^C--deficient mouse has compromised spatial learning and memory that is rescued by neuronal expression of PrP^C (Criado et al., 2005). Interestingly, a role for PrP^C on circadian rhythmicity and sleep regulation (Huber et al., 1999; Tobler et al., 1996, 1997) has also been suggested. The deficit this function for PrP^C would be clinically significant and is consistent with the disruption of circadian rhythmicity and sleep in some prion protein (PrP) related diseases such as fatal familial insomnia (Lugaresi et al., 1998). Both mixed B6129 PrnpZrchl/Zrchl and co-isogenic 129/Ola PrnpEdbg/Edbg background mice have been found to show disordered circadian rhythms, increased sleep fragmentation, and slow-wave activity after sleep deprivation (SD) (Tobler et al., 1997). Rapid eye movement (REM) sleep phase is a critical phase in sleep, and that is closely related to long-term potentiation and synaptic plasticity (Davis et al., 2003; Siegel, 2001). To date, no research has reported the potential role of PrP^C on memory impairment induced by SD.

Alzheimer's disease (AD) is the most common dementia and one of the common neurodegenerative diseases (Ballard et al., 2011). Its clinical characteristics are a progressive decline in memory and cognitive dysfunction. The neuropathological of AD is

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deposition of amyloid-β (Aβ) peptides in extracellular to generate senile plaques and Tau protein phosphorylation in intracellular to forms neurofibrillary tangles. These two pathological hallmarks are considered to be sequentially correlative (Larson et al., 2012; Querfurth and LaFerla, 2010). Currently, there are no available drugs to delay or stop the progression of AD. Nevertheless, the pathogenesis of AD remains uncertain, cerebral accumulation of Aβ peptides is considered the central role of AD pathogenesis (amyloid hypothesis) (Hardy and Allsop, 1991; Hardy and Selkoe, 2002; Selkoe, 2001; Yankner and Lu, 2009).

Aβ is derived from the proteolytic processing of the amyloid precursor protein (APP). APP is cleaved by β-secretase (β-site APP cleaving enzyme-1, BACE1) and γ-secretase (containing the presenilin) to product Aβ, (Vardy et al., 2005). The initial cleavage of APP by BACE1 is the rate-limiting step in the amyloidogenic pathway of APP processing, and BACE1 is the key enzyme (Cole and Vassar, 2007). Some studies show that several cellular proteins can influence BACE1 activity and thereby Aβ production, including PrP^C (Parkin et al., 2007). The levels of Aβ and BACE1 activity were significantly increased in PrP$^{-/-}$ mice (Griffiths et al., 2011; Parkin et al., 2007). AD pathogenesis is characterized by cerebral accumulation of Aβ peptides. In the present study, Aβ levels increased after SD (Kang et al., 2009).

These reports prompted an investigation of whether PrP^C mediates cognitive impairment associated with sleep deprivation and, further, whether Aβ is involved in this process. Therefore, the role of PrP^C was investigated in hippocampus-dependent spatial memory impairment after SD using the Morris water maze (MWM) method to evaluate Aβ changes in the mouse hippocampus of the PrP^C treated group.

2. Materials and methods

2.1 Animals

Male C57BL/6 mice, six weeks old, were used in the present study. Mice were housed in Plexiglass cages (five per cage), with food and water available freely, under an automatic 12 hour: 12-hour light-dark cycle (lights on at 08: 00 a.m.). The room environment was maintained at 22-24 °C and humidity at 50-60%. This study was approved by the Institutional Animal Care and Use Committee at Second Military Medical University, P. R. China (NO.SYXK(HU)2012-0003).

2.2 REM sleep deprivation

Mice in the SD group were subjected to REM sleep deprivation (REMSD) for 72 hours employing the modified multiple platform method (Sá-Nunes et al., 2016; Zager et al., 2012). The deprivation started and ended towards the beginning of the light phase, and the room remained in the standard light-dark cycle. The apparatus (41 cm $\times$ 34 cm $\times$ 16.5 cm) contained 12 columns (3 cm diameter, 5 cm high, and located 1 cm up water surface), the platforms arranged in three lines and spaced 3 cm from each other (edge to edge), from which the mice could freely move around by jumping from one platform to other. When mice reached the REM sleep phase, they fell into the water because of muscle atonia and woke up. Environment control (EC) group mice were kept in their home cages in the same room, placed on large platforms to give that other types of controls for SD, and also allowed the regular sleep to occur.

2.3 Stereotaxic injections

Mice were anesthetized with 10% chloral hydrate and placed in a three-dimensional stereotaxic solid positioned brain frame that allowed for real-time adjustment of micro-injector (Hamilton, Bonaduz, Switzerland) placement. The scalp was opened using a midline incision to expose the skull and using a skull drill drilled a small hole. The micro-injector was then mounted in the stereotaxic frame and positioned for hippocampal injection at lambda coordinates: AP -2.3 mm, ML 0 mm, DV 2 mm, for CA1 injection at A/P: -2 mm, ML: 1.3 mm, DV: 1.4 mm from Bregma. Virus particles of pAAV (adeno-associated viral plasmids)-CAG (the promoter that initiate subsequent expression of proteins)-eGFP (enhanced green fluorescent protein)-PrP^C(-cellular prion protein)-Flag (i.e, a small label, which can be detected with corresponding tagged antibodies) or pAAV-CAG-eGFP (2 μL) were injected over 10 minutes at a flow rate of 0.5 μL/minute. After the virus was completely injected, the needle must stay in place for three minutes. The surgical wounds were disinfected using iodophor and sutured (Reimann et al., 2015).

2.4 Western blot analysis

After the completion of SD, mice were decapitated, and the hippocampus was obtained. The hippocampus was then homogenized, and the supernatant collected after centrifugation. Protein levels were determined by the use of a bicinchoninic acid protein assay kit (Wuhan Guge Biotechnology Pty Ltd, Wuhan, P. R. China) (Smith et al., 1985). A 20 μL protein sample was added to 5 μL of protein loading buffer, and the solution boiled for 15 minutes at 95 °C. A 40 μg protein sample was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose filter (Wuhan Guge Biotechnology Pty Ltd) by electroblotting. The membranes were incubated on a horizontal shaker overnight at 4 °C with prediluted primary antibodies, anti-PrP^C (Abcam, 1 : 10000), anti-Flag (Wuhan Guge Biotechnology Pty Ltd, 1 : 10000), GAPDH (Wuhan Guge Biotechnology Pty Ltd, 1 : 10000) and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam, 1 : 10000) on a horizontal shaker for one hour at room temperature. Finally, the blots were visualized by using enhanced chemiluminescence reagents (Shanghai, Biyuntian, Biotechnology Pty Ltd, Shanghai, P. R. China). Densitometry measurements were quantified by ImageJ analysis software (version 2x).

2.5 Morris water maze

We used the MWM test to assay spatial learning and memory. The MWM test was conducted in a circular pool (120 cm diameter, 50 cm depth), used as the testing chamber, filled 24 $\pm$ 1°C water (30 cm depth). The test chamber was separated into four equal quadrants of equal area, and a hidden platform (10 cm diameter) submerged 1.5 cm beneath the water surface was placed in the middle of the southwest quadrant. The maze release signs were placed at each quadrant around the tank. To learn the location of the hidden platform, mice accepted four tests each day for five days. Each test was terminated immediately the mouse found the platform and stayed for at least five seconds. If the mouse did not find the platform within 120 s, it was guided onto the platform for 30 s. It was then removed from the pool, and the next trial was

started after 20 s. The time is taken for each mouse to discover the hidden platform (escape latency), and the distance traveled by each animal in reaching the platform was recorded automatically by a smart video tracing software (Ji Liang Technology, Shanghai, P. R. China). After REMSD, a probe trial was employed. The platform was taken away, and each mouse was required to swim freely for one minute. Spatial memory was assessed by measuring the percent time spent in the target quadrant.

2.6 Enzyme-linked immunosorbent assay

The PrP^C and Aβ levels in the mouse hippocampus samples were examined following the instructions offered by the manufacturer using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Cloud-Clone Corp, Wuhan, P. R. China). Hippocampal tissues were lysed, homogenized, and applied to microplates that were precoated with a mouse anti-Aβ1-42 polyclonal antibody using an Aβ1-42 ELISA kit (CEA946 Mu). A biotinylated antigen and standards were added and incubated for one hour at 37 °C. The plates were washed three times, blotted with HRP-labeled avidin, and then incubated for 30 minutes at 37 °C. After five washes, a TMB/E substrate was placed on the plates, and they were incubated for 10-20 minutes at 37 °C, then added the stop solution to halt the reaction. Absorbance at 450 nm was quantified using a microplate reader, and the concentration of Aβ1-42 in the hippocampal tissue was assessed as the ratio of Aβ1-42 to the total soluble protein content. The PrP^C levels were measured using a PrP ELISA kit (SEB680 Mu). Tissue homogenate and standards were added to the microplates that were pre-coated with mouse anti-PrP polyclonal antibody and incubated for two hours at 37 °C; a biotinylated antigen was then added into microplates, and the plates were incubated for one hour at 37 °C. After three washes, the next process was ditto the Aβ1-42 ELISA kit test described previously.

2.7 Immunofluorescence

Hemisections of the mouse brain containing the hippocampus were fixed for 24 hours using fresh 4% (v/v) paraformaldehyde. After the brains were fixed, they were embedded in paraffin for 24 hours. Parasagittal sections (5 μm) were obtained using a Leica RM2016 microtome (Leica, Shanghai, P. R. China). For immunofluorescence, the sections were blocked in 5% bovine serum albumin for 20 minutes and then incubated with diluted in phosphate buffer saline (PBS) with 0.2% Triton X-100 primary antibody overnight at 4 °C. (Guge Biotechnology Pty Ltd, Wuhan, P. R. China).

The following antibodies were used: anti-PrP^C (1 : 100, Abcam EP1802Y) and anti-GFP (1 : 100), Sanying Biotechnology limited company, Wuhan, P. R. China). After being rinsed three times in PBS, sections were sequentially incubated for one hour with the appropriate biotinylated anti-rabbit and anti-mouse IgGs (50-100 μL) and peroxidase-labeled streptavidin. Following incubated with a freshly prepared substrate chromogen solution containing 3% 4, 6-diamidino-2-phenylindole and hydrogen peroxide for 10 minutes, and performed further staining. Finally, the sections were counterstained with hematoxylin, washed with water, and then viewed with an inverted fluorescence microscope (Olympus BX51/U-RFLT50, Japan).

2.8 Statistical analysis

Statistical analysis was processed by SPSS 21.0 software. Experimental results were presented as the mean ± S.E.M. The data were analyzed using analysis of variance (ANOVA) with repeated measures for multi-group comparisons or two-way ANOVA, followed by Tukey's post hoc test. Statistical significance was accepted for P value of less than 0.05.

3. Result

3.1 Intracerebral administration of pAAV-eGFP-PrP^C--Flag induces the expression levels of PrP^C in the CA1 region of the hippocampus

We used immunofluorescent co-localization analysis to verify the successful induction of the expression levels of PrP^C in the CA1 region of the hippocampus after the intracerebral injection of plasmid adeno-associated virus (pAAV) -enhanced green fluorescent protein (eGFP)-PrP^C--Flag. For fluorescent microscopy, the expression levels of PrP^C in the CA1 region of hippocampus in the mouse brain revealed increased co-expression with GFP (Fig. 1A-D). Also, PrP^C--Flag expression was also evaluated by Western blot after 293T cell infection of pAAV-EGFP- PrP^C--Flag. The PrP^C--Flag expression in the cells significantly decreased in the pAAV-eGFP- PrP^C--Flag group compared with the control groups (Fig. 1E-F).

3.2 Spatial learning and memory

Before SD, all four groups of mice were tested in the MWM to assess spatial learning. This consisted of assessing the time mice spent on finding the hidden platform (escape latency). As Fig. 2A shows, the escape latency of all groups decreased throughout the training. The differences in escape latency of all groups from the first to the fifth day of training were significant (F = 84.927, $P < 0.01$). But, there was no significant difference in the escape latencies among the four groups. This indicated that the overexpression of PrP^C did not affect spatial learning.

3.3 Overexpression of PrP^C rescues REMSD-induced spatial memory impairment

A probe trial was performed to evaluate spatial memory after SD. The results showed that the percent time spent in the target quadrant was different for the four groups (F = 6.196, $P < 0.01$; Fig. 2B). During the probe trial, the mice from the SD-control and the SD-pAVV-GFP groups spent significantly less time in the target quadrant than did those from the EC group ($P < 0.01$). The SD-pAVV-GFP group spent significantly less time in the target quadrant than did the SD-pAVV- PrP^C-GFP group ($P < 0.05$). Fig. 2C shows mice in the four groups performed the different swim trajectories in the probe trial. Before SD, the swimming trajectory of mice in all four groups was similar, and the mice swam mostly in the goal quadrant. After SD, the mice in the EC and SD-pAVV-PrP^C-GFP group swam mostly in the goal quadrant, while mice in the SD-control and SD-pAVV-GFP groups swam in relatively diffuse patterns.

3.4 Decreased Aβ expression after intracerebral administration of pAAV-eGFP- PrP^C--Flag

An ELISA assay was employed to examine the difference in the expression levels of Aβ and PrP^C in the mouse hippocampus after SD. Our results showed a significant difference in Aβ expression among the four groups ($P < 0.05$) (Fig. 3). After 72 hours of

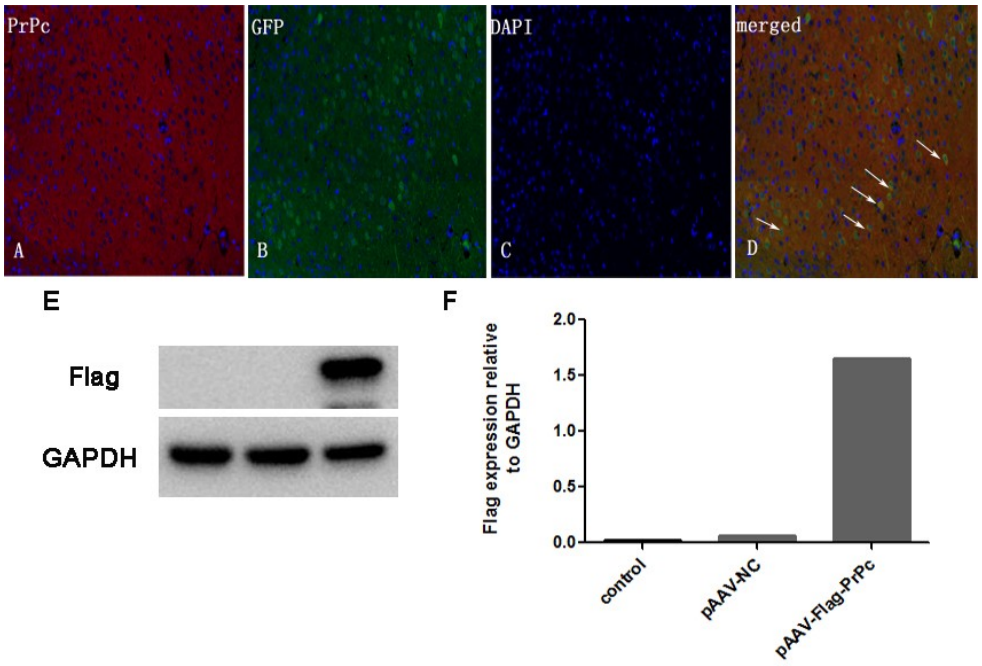

Figure 1. Immunofluorescence and Western blot analysis of the expression of PrP^C conducted to identify PrP^C levels three weeks after intracerebral injection of pAAV-eGFP- PrP^C–Flag. A-D, Hippocampus CA1 region of mouse brain showed an increase in PrP^C co-expressed with GFP used to label the virus vehicle. E, Western blot result. F, Histogram of the Flag / GAPDH ratio among the three groups. Scale bar, 100 μm. DAPI, 4',6'-diamidino-2-phenylindole dihydrochloride.

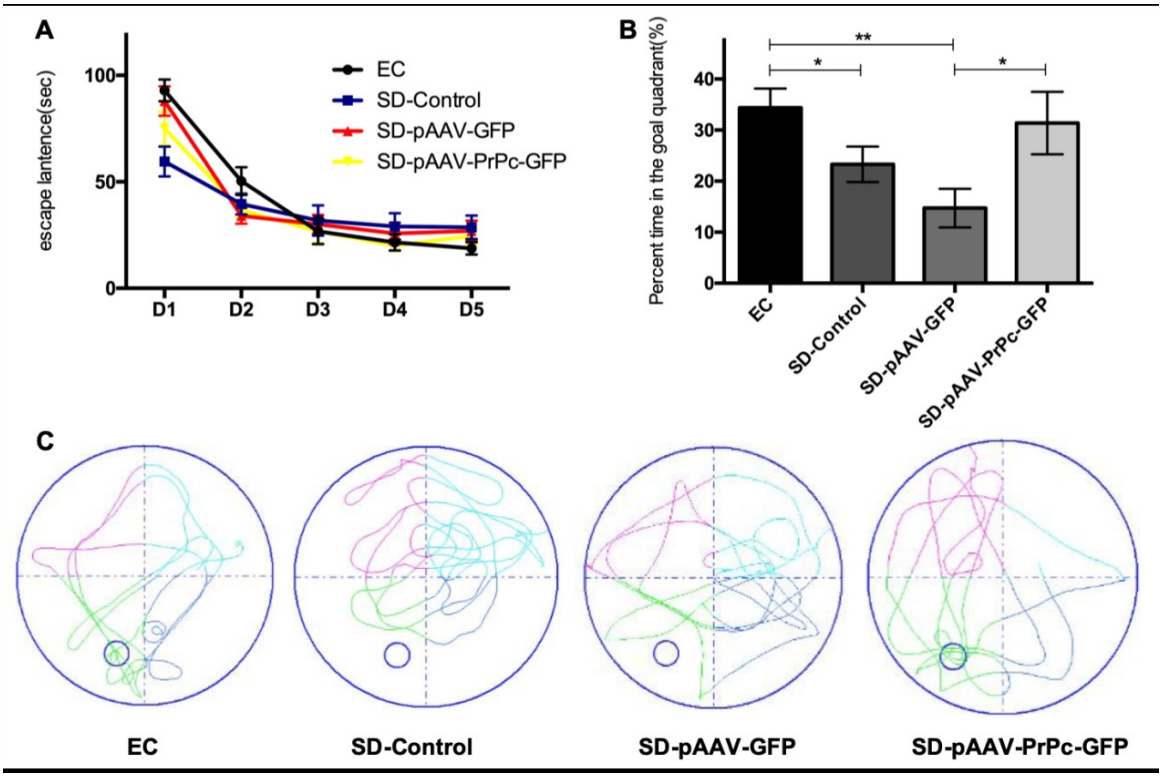

Figure 2. Performance of mice in a Morris water maze during a spatial learning and memory task. (A) Mean escape latency of mice in finding the hidden platform in the spatial learning test. (B) Percent time mice spent swimming in the goal quadrant during the probe test. (C) Representative swim trajectories in the probe test. $*P < 0.05$, $**P < 0.01$. EC: environment control; SD: sleep deprivation; escape latency: latency to find the hidden platform.

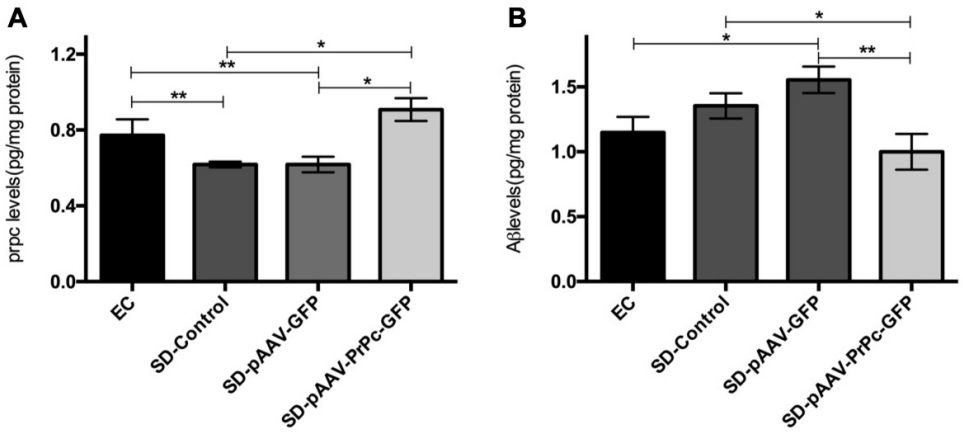

Figure 3. ELISA analysis of PrP^C and Aβ expression in the mouse hippocampus after sleep deprivation. (A) Histogram of PrP^C levels among four groups. (B) Histogram of Aβ levels among four groups. $*P < 0.05$, $**P < 0.01$. EC: environment control; SD: sleep deprivation.

REMSD, the Aβ expression levels (pg/mg protein) significantly decreased in the SD-pAVV- PrP^C-GFP group compared with the SD-control group and SD-pAVV-GFP group (6.28 ± 1.94 vs. 8.51 ± 1.64 and 9.77 ± 1.44, $P < 0.05$). Meanwhile, there was no significant difference in Aβ expression (pg/mg protein) between the SD-pAVV- PrP^C-GFP group and EC group (6.28 ± 1.94 vs. 7.21 ± 2.01, $P > 0.05$). After 72 hours of REMSD, the PrP^C expression levels (pg/mg protein) significantly increased in the SD-pAVV- PrP^C-GFP group compared with the SD-control group and SD-pAVV-GFP group (0.91 ± 0.06 vs. 0.62 ± 0.02 and 0.62 ± 0.04, $P < 0.05$). Additionally, there were no significant difference in PrP^C expression (pg/mg protein) between the SD-pAVV- PrP^C-GFP group and the EC group (0.91 ± 0.06 vs. 0.78 ± 0.08, $P > 0.05$).

4. Discussion

PrP^C is a depth conservative membrane protein; in other words, a glycoprotein localized on neuronal plasma membranes by a GPI anchor. Although the major physiological function of PrP^C remains obscure, a variety of roles have been suggested for the biological function of PrP^Cincluding, the regulation of sleep and the formation and consolidation of memory (Linden et al., 2008; Martins and Brentani, 2002).

Tobler et al. (1996) and Sánchez-Alavez et al. (2007) have shown that PrP^C plays a role in sleep homeostasis and sleep continuity, and there is an alteration in both circadian rhythmicity and sleep patterns in mice devoid of PrP. Through an ELISA analysis of hippocampal tissue, here it has been demonstrated that PrP^C expression significantly decreases after 72 hours of REMSD compared with environment control.

Graves et al. (2003) and Hendricks et al. (2001) have found that the REM sleep phase is closely associated with cognition and that its absence impairs cognitive function, particularly hippocampus-dependent spatial learning and memory. The present data demonstrate that spatial learning and memory are impaired after 72 hours of REMSD (Guan et al., 2004; Li et al., 2009; Wang et al., 2009).

It has been found that lack of neuronal PrP^C cause disturbance in hippocampus-dependent spatial learning and hippocampus short- and long-term synaptic plasticity in transgenic mPrP-/- mice, deficits that could be rescued by expressing PrP^C in neurons under control of the neuron-specific enolase promoter (Criado et al., 2005). Data has previously indicated that overexpression of PrP^C by adenovirus increases the percentage of time spent in the target quadrant; however, the time spent in the target quadrant decreased significantly after 72 hours of REMSD, without a significant decrease in mean latency to platform discovery. As Fig. 2B shows, overexpression of PrP^C rescues REMSD-induced spatial memory impairment, whereas, this has no impact on spatial learning (Fig. 2A).

Aβ is the cleavage and proteolytic processing product of APP. APP is cleaved by β-secretase (β-site APP cleaving enzyme-1, BACE1) and γ-secretase (containing the presenilin) to product Aβ (Vardy et al., 2005). The initial cleavage of APP by BACE1 is the rate-limiting step in the amyloidogenic pathway of APP processing, and BACE1 is the key enzyme (Cole and Vassar, 2007). It has been found that various cellular proteins influence this step, including PrP^C (Parkin et al., 2007). The present research shows that the expression levels of endogenous Aβ were significantly increased in the transgenic $mPrP^{-/-}$ mouse brain (Griffiths et al., 2011).

Aβ expression significantly increased after sleep deprivation, indicating that under this condition, Aβ plays an important role in learning and memory. However, overexpression of $PrP^C$by the administration of an adenovirus-associated complex to the hippocampus decreased Aβ expression after SD. Thus, it is proposed that this might be associated with the mechanisms of a PrP^C protective function in the hippocampus that mitigates memory impairment. More experiments are required to elucidate the causal effects of Aβ in the hippocampus and spatial memory impairment.

In conclusion, it was found that overexpression of $PrP^C$by adenovirus-mediated gene targeting in mice could rescue REMSD-induced spatial memory impairment. This finding could be employed as a therapeutic strategy for the treatment of patients with cognitive impairment in the future. Currently, there are no available drugs to delay or stop the progression of AD. PrP^Cmay provide treatment for AD patients. It was found that overexpression of PrP^C decreased the production of Aβ in the hippocampus, suggesting a possible mechanism relating to Aβ.

Authors' contributions

Li Hu and Peng Li performed the research. Peng Li analyzed the data. Li Hu wrote the manuscript. Zhendong You, Zhaohuan Zhang, and Hongyang Jin provided help and advice on the experiments. Liuqing Huang designed the research study.

Acknowledgment

This work was supported by a grant from the National Natural Science Foundation of China (No. 81371459).

Conflict of interest

The authors declare no competing interests.

Submitted: August 21, 2019
Accepted: October 30, 2019
Published: December 30, 2019

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