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·Original Article·

Humanin attenuates Alzheimer-like cognitive deficits and pathological changes induced by amyloid β-peptide in rats

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ABSTRACT

Amyloid β-peptide (Aβ) has been implicated as a key molecule in the neurodegenerative cascades of Alzheimer's disease (AD). Humanin (HN) is a secretory peptide that inhibits the neurotoxicity of Aβ. However, the mechanism(s) by which HN exerts its neuroprotection against Aβ-induced AD-like pathological changes and memory deficits are yet to be completely defined. In the present study, we provided evidence that treatment of rats with HN increases the number of dendritic branches and the density of dendritic spines, and upregulates pre- and post-synaptic protein levels; these effects lead to enhanced long-term potentiation and amelioration of the memory deficits induced by $A\beta_{1-42}$. HN also attenuated $A\beta_{1-42}$-induced tau hyperphosphorylation, apparently by inhibiting the phosphorylation of Tyr307 on the inhibitory protein phosphatase-2A (PP2A) catalytic subunit and thereby activating PP2A. HN also inhibited apoptosis and reduced the oxidative stress induced by $A\beta_{1-42}$. These findings provide novel mechanisms of action for the ability of HN to protect against $A\beta_{1-42}$-induced AD-like pathological changes and memory deficits.

Keywords: Humanin; amyloid-beta; Alzheimer's disease; tau; apoptosis

INTRODUCTION

Alzheimer's disease (AD) is the most prevalent age-related neurodegenerative disorder and the most common form of senile dementia. It is characterized by deposition of amyloid β (Aβ) plaques, accumulation of intracellular neurofibrillary tangles, and regionalized neuronal dysfunction and dendritic degeneration[1-4]. The clinical manifestations include a progressive deterioration of memory and cognitive disorders[5]. There is currently no specific treatment to stop or reverse the progression of AD.

The Aβ peptides, ranging from 39 to 43 amino-

acids in length, are generated by enzymatic cleavage of the transmembrane amyloid precursor protein[6-7]. These peptides are generally believed to be responsible for the cognitive impairment and mental decline in AD patients. It has been postulated that different soluble or insoluble higher-molecular-weight forms of Aβ trigger a complex pathological cascade that causes synaptic dysfunction, inflammatory processes, neuronal loss, cognitive impairment, and finally the onset of the disease[8-10]. Apoptotic cell death is also believed to be involved in Aβ-induced cytotoxicity[11-13]. Therefore, developing therapeutic strategies that aim to suppress Aβ-induced cytotoxicity has been the main focus of research[14-16].

Humanin (HN) is a 24-amino-acid peptide that was identified in the occipital lobe of AD brains[17]. HN suppresses the neurotoxicity of various causative genes for familial AD (FAD) including FAD-related mutant PS1 and PS2 genes[17-19], and inhibits the neuronal cell death caused by Aβ[20,21]. HN is also effective against cell death caused by non-AD-related insults, such as serum deprivation, prion peptide 118-135, and insulin-like growth factor binding protein 3[22-24]. However, the neuroprotective effects and mechanism(s) of action of HN on Aβ-induced AD-like pathological changes and memory deficits *in vivo* are still not fully described. This study therefore examined the effects of HN against $A\beta_{42}$ *in vivo* and explored the underlying mechanisms in rats.

MATERIALS AND METHODS

Antibodies and Chemicals

The antibodies used are listed in Table 1. Secondary antibodies for western blotting were from Amersham Pharmacia Biotech (Little Chalfort, Buckinghamshire, UK). $A\beta_{1-42}$ and HN were from Sigma Chemical Co. (St. Louis, MO) and were dissolved in normal saline to a final concentration of 5 μg/μL and 100 μmol/L, respectively. The kits for detecting superoxide dismutase (SOD),

Table 1. Antibodies used in Western blotting and their properties

Antibody	Specificity	Type	Dilution for WB	Source
Tau-5	Total tau	Mono-	1:500	SAB
Tau-1	Unphosphorylated tau at Ser-198/199/202	Mono-	1:30000	SAB
pT205	Phosphorylated tau at Thr205	Poly-	1:1000	SAB
pS262	Phosphorylated tau at Ser262	Poly-	1:1000	SAB
pS396	Phosphorylated tau at Ser396	Poly-	1:1000	SAB
pS404	Phosphorylated tau at Ser404	Poly-	1:1000	Cell Signaling
$PP2A_C$	PP2A catalytic subunit	Poly-	1:500	Cell Signaling
p-$PP2A_C$	Phosphorylated $PP2A_C$ at Tyr307	Mono-	1:200	Abcam
Synaptophysin	Total Synaptophysin	Mono-	1:1000	Chemicon
Synapsin-1	Total synapsin 1	Poly-	1:1000	Chemicon
p-synapsin-1	Phosphorylated synapsin 1 at Ser 9	Poly-	1:1000	Abcam
NR2A	NMDAR2A C-term	Poly-	1:1000	Abcam
NR2B	NMDAR2B C-term	Poly-	1:1000	Abcam
PSD-95	Total PSD-95	Mono-	1:1000	Biosource
Bax	Bax N-term	Poly-	1:500	Chemicon
Bcl-2	Total Bcl-2	Poly-	1:500	Chemicon
DM1A	α-tubulin	Mono-	1:1000	Sigma
β-actin	Total actin	Mono-	1:1000	Cell Signaling

Mono-, monoclonal; poly-, polyclonal.

malondialdehyde (MDA) and glutathione peroxidase (GSH-PX) were from Nanjin Jian-Cheng Biological Engineering Institute (Nanjing, China). Other high-quality reagents were from commercial sources.

Animals and Hippocampal CA1 Injection

Male Wistar rats (3–4 months old, 280 ± 20 g, *n* = 60) were supplied by the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology. They were raised in cages under a 12/12 h light-dark cycle (lights on at 07:00), with food and water *ad libitum*. All animal experiments were performed according to the "Policies on the Use of Animals and Humans in Neuroscience Research" revised and approved by the Society for Neuroscience in 1995. The rats were randomly allocated to three groups: control, $A\beta_{1-42}$ treatment, and $A\beta_{1-42}$ + HN treatment. Rats were deeply anesthetized by intraperitoneal injection (i.p.) of chloral hydrate (300 mg/kg) and placed in a stereotaxic instrument (SR-6N, Narishige, Tokyo, Japan). After exposure of the occipital bone, a hole was drilled at the coordinates 4.0 mm anterior-posterior relative to bregma, 2.0 mm medial-lateral, and 3.0 mm dorsal-ventral according to the rat brain atlas[25]. Two microliters of $A\beta_{1-42}$ (5 μg/μL, 0.2 μL/min) was injected into the hippocampal CA1 region of rats in the $A\beta_{1-42}$ treatment group[26]. In the $A\beta_{1-42}$ + HN treatment group, rats were given 2 μL of HN (100 μmol/L) at the same site after injection of $A\beta_{1-42}$. Rats in the control group received normal saline injection only. The injection cannula was kept *in situ* for an additional 10 min following drug infusion to avoid spread of the solution along the pipette track. All surgical procedures were completed under sterile conditions and penicillin (80 000 U, i.p.) was used to prevent infection. All the animal experiments were approved by the Institutional Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology.

Morris Water Maze Test

Spatial memory was assessed using the Morris water maze test on day 7 after injection[27, 28]. Before each experiment (2 h), the rats were brought to the site for habituation. For spatial learning, rats were trained in the maze to find a submerged platform in four trials per day at 15-min intervals from 14:00 to 20:00 for six consecutive days. Each training session began by placing the rat in the water in the center of a quadrant and ended when it climbed onto the submerged platform. Rats were left to swim for a fixed time (60 s), after which they were guided to the platform if necessary. During these training sessions, rats acquired learning and spatial memory about the location of the platform. On day 7, the platform was removed and the testing session was performed. The swimming patterns were recorded on each of the 7 days by a video camera connected to a computerized analysis system (Ethovision, Noldus Information Technology, Holland). Using this software, the time taken to reach the hidden platform (latency), the pathway and the length that the rats passed through the target quadrant, and the total times crossing the place where platform was located were acquired. After this spatial memory retention test, rats were euthanized and the hippocampus or the whole brain was harvested for further studies.

Electrophysiological Recording

Rats were anesthetized with urethane (i.p., 1.2–1.5 g/kg) and fixed in the SR-6N stereotaxic instrument with a bath circulator to maintain the body temperature at 37 ± 0.5°C. A small hole was drilled in the skull and a parallel stimulating/recording electrode was inserted into the right hippocampus. As described previously[29], the tip of the stimulating electrode was placed in the CA3 area (3.5 mm posterior to bregma, 3.5 mm lateral to the midline, and 3.5 mm below the surface of the skull) and the tip of the recording electrode was positioned in CA1 (4.0 mm posterior to bregma, 2.0 mm lateral to the midline, and 3.0 mm below the surface of the skull). Detailed electrophysiological recordings were as described previously[30]. Briefly, initial baseline responses were obtained by delivering a single pulse of stimulation once every 30 s. In each recording experiment, a stable baseline (<10% change) for at least 30 min was required before application of conditioning stimuli. Long-term potentiation (LTP) at the hippocampal CA3-CA1 synapses was elicited using high-frequency stimulation (ten trains of 20 pulses, 200 Hz, and inter-train interval of 2 s) at the test stimulus intensity.

Western Blotting

Rats were decapitated after the spatial memory retention test. The hippocampi were rapidly removed and homogenized at 4°C using a Teflon pestle and a glass homogenizer in (in

mmol/L) 50 Tris-HCl (pH 7.4), 150 NaCl, 10 NaF, 1 Na_3VO_4, 5 EDTA, 2 benzamidine, and 1 phenylmethylsulfonyl fluoride. The protein concentration in the extracts was determined using a bicinchoninic acid kit according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). The extracts were diluted with sample buffer (1:9, *m*/*v*) containing 200 mmol/L Tris-HCl (pH 7.6), 8% sodium dodecyl sulfate, 40% glycerol, and 40 mmol/L dithiothreitol, boiled for 10 min, and then used for western blotting[31]. Equal amounts of protein were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk dissolved in TBST (50 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 0.2% Tween-20) for 1 h and probed with primary antibody at 4°C overnight. Finally, the blots were incubated with anti-rabbit or anti-mouse IgG conjugated to IRDye™ 800CW for 1 h at 15–25°C and the target protein bands were visualized using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

Golgi Staining

Golgi staining was performed according to methods established in our laboratory[29]. Rats (3 per group) were euthanized by overdose with chloral hydrate (500 mg/kg), and perfused through the aorta with 200 mL 0.9% NaCl containing 0.5% sodium nitrite followed by 500 mL 0.9% NaCl containing 5% formaldehyde. The brain was fixed *in situ* by perfusion with 400 mL Golgi fixative (0.9% NaCl, 5% formaldehyde, 5% potassium dichromate, and 5% chloral hydrate) under reduced light, then was removed and rapidly processed for Golgi staining under reduced light. Briefly, the brain was postfixed for 3 days in the same fixative, and impregnated with 1.0% aqueous silver nitrate for 3 days. Coronal sections of the hippocampus were imaged using a fluorescence microscope (Olympus BX60, Tokyo, Japan).

Nissl Staining

Apoptotic events in the CA1 region were monitored using Nissl staining. Unilateral hippocampal specimens (5 animals per group) were immediately fixed in 4% paraformaldehyde for 24 h, routinely dehydrated, embedded in paraffin, and cut into 5-μm sections. The coronal sections were stained with 1% toluidine blue (4 sections per animal). The number of surviving pyramidal cells in each millimeter of the CA1 area was counted under a light microscope (Olympus Optical Co., Ltd, Japan) at high magnification (400×) in a blinded manner. Only cells with evident nuclei and nucleoli were included. Image-Pro Plus software (Media Cybernetics, Bethesda, MD) was used to analyze cell numbers.

Oxidative Stress Assays

The anti-oxidative stress effect of HN in rat hippocampal extracts was determined by measuring the MDA level, and SOD or GSH-PX activity. Rats were sacrificed and the hippocampus was immediately removed and homogenized at 4°C using a Teflon™ glass homogenizer with buffer containing 10 mmol/L Tris-HCl, 0.1 mmol/L EDTA-2Na, 10 mmol/L sucrose, and 0.8% NaCl (pH 7.4). The homogenates were agitated at 4°C for 6 min and then centrifuged at 12 000 g for 15 min at 4°C. The supernatant was used to determine the MDA level and antioxidant enzyme activity according the manufacturer's instructions.

Electron Microscopy

Sections for electron microscopy were prepared as previously described[32]. In brief, rats were anesthetized and perfused with normal saline followed by 0.5% glutaraldehyde in 4% paraformaldehyde. The brain was removed and placed in 2.5% glutaraldehyde for 12 h at 4°C. The CA1 region was removed from the brain slices. After osmication for 30 min in 0.1 mol/L phosphate buffer containing 1% OsO_4, the samples were dehydrated in ethanol and embedded in Epon 812. Finally, thin sections, cut with a diamond knife on an ultramicrotome (UCT, Leica, Germany), were collected on mesh grids coated with a thin Formvar film, and were stained in uranyl acetate and lead citrate for 25 min, and viewed in an electron microscope (FEI Tecnai G2 12, Holland).

Statistical Analysis

Data are presented as mean ± SD and were analyzed using SPSS 12.0 (SPSS Inc., Chicago, IL). One-way analysis of variance, followed by the least significant difference *post hoc* test, was used to determine significant differences between groups.

RESULTS

HN Attenuates $A\beta_{1\text{-}42}$-Induced Cognitive Abnormality

During the training session, the time to find the hidden platform (latency) was significantly increased in rats treated

with $A\beta_{1-42}$ compared with control rats; however, this was reversed in rats treated with HN after $A\beta_{1-42}$ treatment (Fig. 1A). In the testing session on day 7, $A\beta_{1-42}$-treated rats were less likely to remain in the quadrant where the platform had been located and, again, rats treated with HN tended to revert to the control behavioral phenotype (Fig. 1B–D). These findings demonstrated that HN supplementation attenuates the cognitive deficits induced by $A\beta_{1-42}$.

HN Attenuates $A\beta_{1-42}$-Induced LTP Impairment *In Vivo*

Synaptic plasticity is the functional basis of learning and memory. We found that the population spike amplitude and slope of the field excitatory post-synaptic potential (fEPSP) were much lower in $A\beta_{1-42}$-treated rats than in control rats (Fig. 2). Supplementation with HN restored the amplitude and slope of the fEPSP (Fig. 2). These data suggested that HN mitigates the synaptic transmission deficits induced by $A\beta_{1-42}$.

HN Increases the $A\beta_{1-42}$-Induced Reduction in Dendritic Branches and Spine Density in the CA1 Region

To investigate the mechanisms by which HN attenuated the cognitive deficits induced by $A\beta_{1-42}$, the dendritic morphology and dendritic spine density of the pyramidal neurons in the CA1 region were analyzed by Golgi staining. The average number of dendritic branches was remarkably reduced by $A\beta_{1-42}$ (Fig. 3A, E); however, HN increased their number. We also found that $A\beta_{1-42}$ treatment reduced the spine density and the percentage of mushroom spines on tertiary branches, while HN ameliorated these spine deficits (Fig. 3C, F, G). There were no significant differences in the average length of each branch between the $A\beta_{1-42}$ treatment and control groups (Fig. 3A, D).

HN Inhibits the Decrease of Synaptic Proteins Induced by $A\beta_{1-42}$

To further explore the molecular mechanisms involved in the HN-sensitive amelioration of $A\beta_{1-42}$-induced memory deficits, we examined the expression of several memory-associated proteins in presynaptic neurons. These were synaptophysin, synapsin 1, and synapsin 1 phosphorylated at Ser 9 (p-synapsin 1). Western blot analysis revealed that $A\beta_{1-42}$ significantly decreased the levels of all three

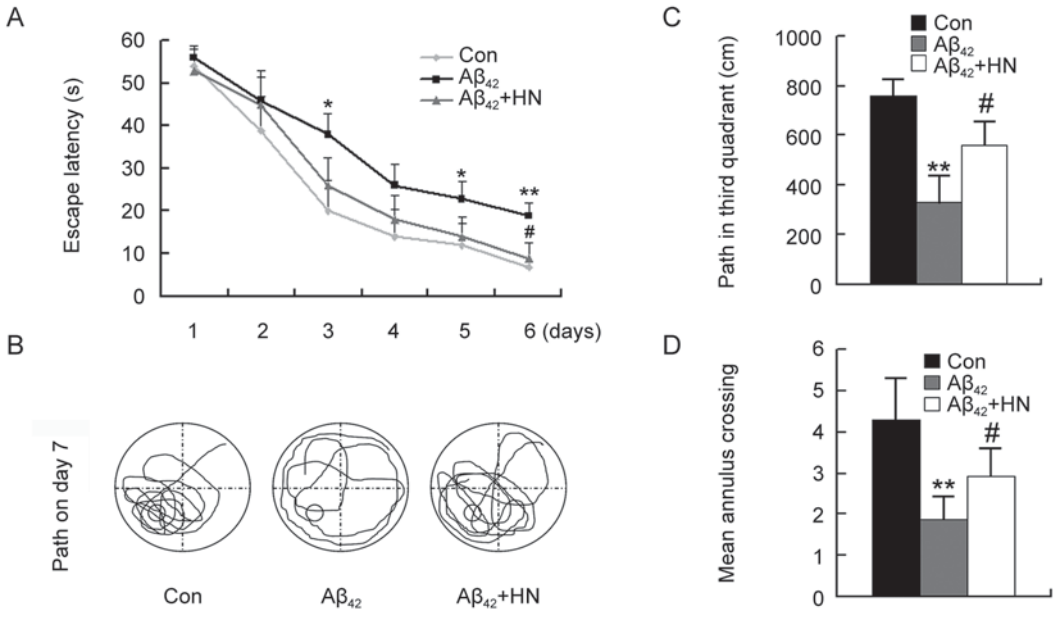

Fig. 1. HN ameliorates memory deficits induced by $A\beta_{1-42}$ in rats. The spatial memory test was performed on day 7 after injection of $A\beta_{1-42}$ (5 μg/μL, 2μL) into the CA1 region with or without subsequent HN supplementation (100 μmol/L, 2 μL). A: Escape latency (time to find the submerged platform) during the first six days of training session in control, $A\beta_{1-42}$ treatment, and $A\beta_{1-42}$ + HN treatment groups. B: Representative swim-paths in the test session on day 7 (when the platform had been removed). C and D: Swimming distance within the target quadrant (C), and number of times the rat crossed the target quadrant (D) within 1 min on day 7. Data are expressed as mean ± SD (n = 20; *P <0.05, **P <0.01 *vs* control (Con); $^{\#}P$ <0.05 *vs* $A\beta_{42}$).

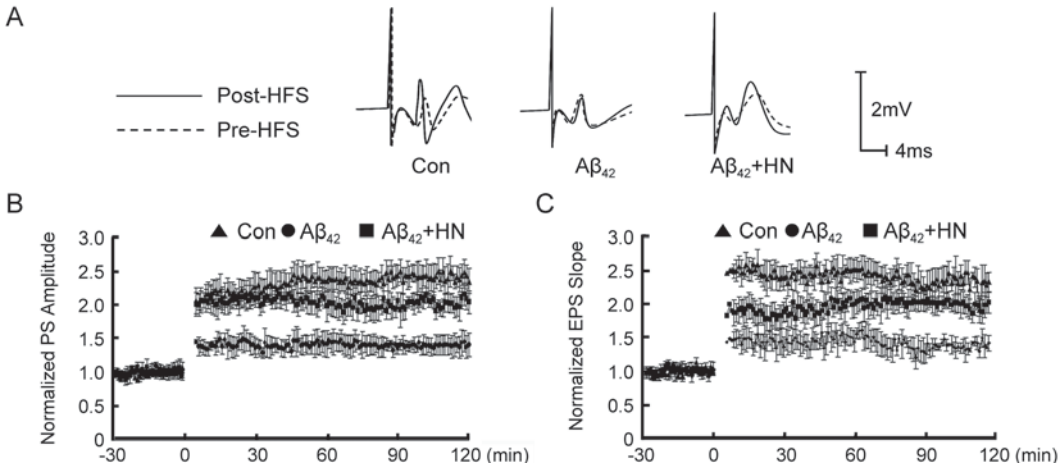

Fig. 2. HN attenuates $A\beta_{1-42}$-induced suppression of LTP *in vivo*. After $A\beta_{1-42}$ (5 μg/μL, 2μL) was injected into the CA1 region with or without subsequent HN (100 μmol/L, 2μL), LTP was monitored (5 per group). A: Representative traces of evoked potentials before (broken line) and after (solid line) high-frequency stimulation (HFS). B and C: Alterations of LTP represented by normalized amplitude of the population spike (PS) (B) and slope of the field excitatory postsynaptic potential (EPSP) (C). The recordings were started 30 min before HFS (-30).

proteins, while HN reversed all the changes (Fig. 4A, B). In addition, supplementation with HN significantly attenuated the $A\beta_{1-42}$-induced decrease of the postsynaptic proteins NR2A, NR2B, and PSD95 (Fig. 4C, D).

HN Attenuates $A\beta_{1-42}$-Induced Tau Hyperphosphorylation by Activation of Protein Phosphatase-2A (PP2A)

Tau hyperphosphorylation is a characteristic feature in the brain of AD patients[33] and it contributes to learning and memory deficits[2]. We measured the effects of HN on the tau phosphorylation induced by $A\beta_{1-42}$ and found a significant increase in the pT205, pS262, pS396, and pS404 residues (Fig. 5A, B). Decreased immunoreactivity of Tau-1 (the antibody reacts with the epitope of tau non-phosphorylated at Ser198/202) (Fig. 5A, B) was found in $A\beta_{1-42}$-treated rats, while in rats treated with HN, the $A\beta_{1-42}$-induced changes were significantly reversed (Fig. 5A, B). No evident changes were found in total tau probed by Tau-5. These results indicated that HN attenuates $A\beta_{1-42}$-induced tau phosphorylation at multiple AD-related sites *in vivo*.

We next examined the activity of PP2A, which is most involved in the AD-related changes in tau phosphorylation[34]. We found that the level of $PP2A_C$ phosphorylated at Tyr307 (p-$PP2A_C$, inactive form) was increased after $A\beta_{1-42}$ injection (Fig. 5C, D) and this was attenuated by HN. The levels of total $PP2A_C$ remained unaffected by either treatment. These data support a role for activation of $PP2A_c$ in the attenuation of $A\beta_{1-42}$-induced tau hyperphosphorylation by HN.

HN Prevents $A\beta_{1-42}$-Induced Neuron Apoptosis

$A\beta_{1-42}$ is neurotoxic to neurons[6,35-38]. Using Nissl staining, we found a significant reduction in the number of surviving neurons after $A\beta_{1-42}$ treatment, while HN treatment attenuated this neurotoxicity (Fig. 6A, B). In addition, we found that treatment with $A\beta_{1-42}$ caused an increase in the expression of pro-apoptotic Bax and a decrease in the expression of the anti-apoptotic Bcl-2 protein (Fig. 6C, D). These changes in expression were both sensitive to HN treatment (Fig. 6C, D). These data support a neuroprotective role for HN.

HN Reduces $A\beta_{1-42}$-Induced Oxidative Stress

Oxidative stress plays an important role in mediating $A\beta_{1-42}$-induced neurotoxicity[35,36,38,39]. Recent studies have confirmed that HN has a cytoprotective effect against oxidative stress in atherosclerosis[40]. To determine whether HN is also protective against $A\beta_{1-42}$-induced oxidative stress *in vivo*, the level of MDA, a lipid peroxidation product, and the GSH-PX and SOD activity in the hippocampus were measured. We found that $A\beta_{1-42}$ significantly increased the MDA level and decreased the GSH-PX activity, while

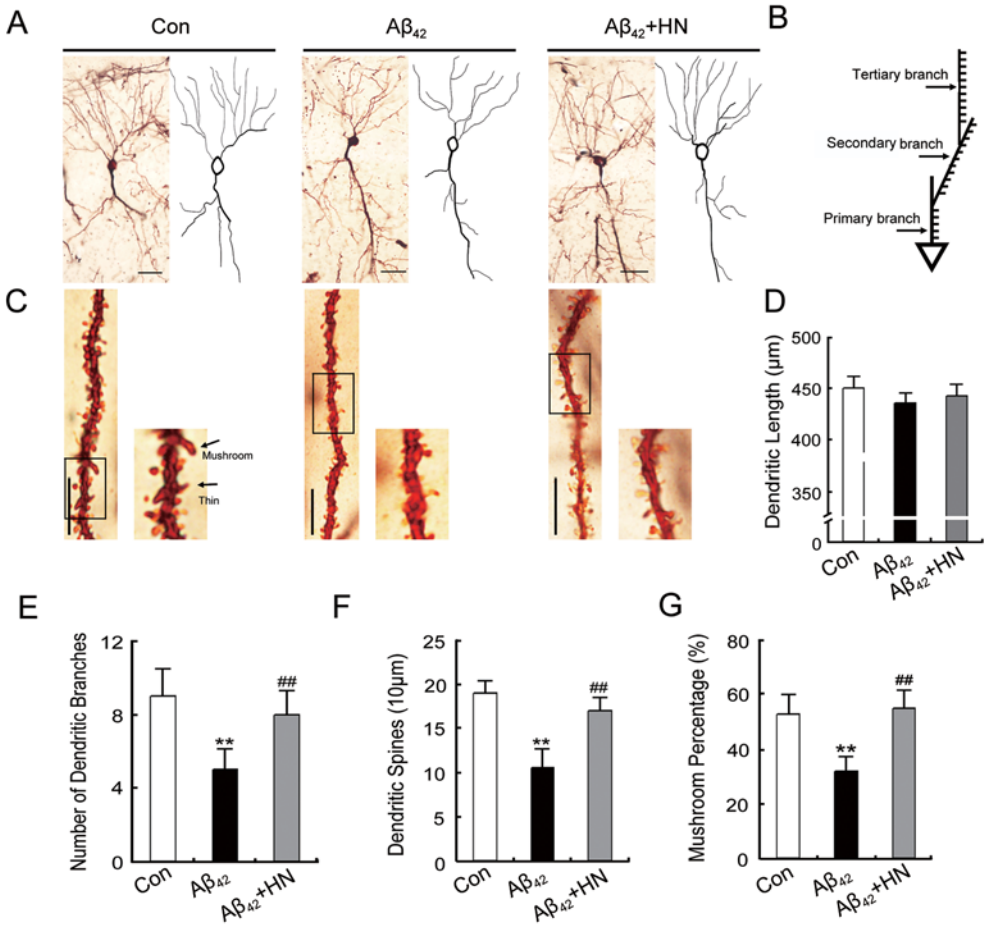

Fig. 3. HN increases $A\beta_{1-42}$-induced reduction of dendritic branching and spine density in the CA1 region. Rats were treated as in Figure 1 and Golgi staining was used to examine the dendrite branching and spine densities in CA1. A: Representative Golgi staining of hippocampal CA1 pyramidal neurons and the corresponding drawings in the three groups (scale bars, 10 μm). B: Diagram showing the apical dendrite branches of a CA1 neuron. C: Representative dendrites of neurons from the three groups in panel (A) (scale bars, 10 μm). D: Total dendrite length of randomly-selected neurons in the three groups. E: Number of dendritic branches in randomly-selected neurons. F: Number of dendritic spines in randomly-selected dendritic segments. G: Percentage of mushroom spines. The data are expressed as mean ± SD (n = 3; $^{**}P$ <0.01 *vs* control (Con); $^{\#\#}P$ <0.01 *vs* $A\beta_{42}$).

HN reversed both changes (Fig. 7). In contrast, the SOD activity was significantly changed only with combined HN and $A\beta_{1-42}$ treatment (Fig. 7). These results implicated the attenuation of oxidative stress in the ability of HN to inhibit $A\beta_{1-42}$-induced neurotoxicity.

HN Attenuates $A\beta_{1-42}$-Induced Ultrastructural Damage in Hippocampal Neurons

Transmission electron microscopy showed uniformly-distributed rough endoplasmic reticulum (ER) and ribosomes with the presence of large numbers of mitochondria and synapses in control rats (Fig. 8). In contrast, in hippocampal neurons of rats treated with $A\beta_{1-42}$, the nucleus was irregular in shape, nuclear material was condensed, and the nuclear envelope was invaginated. Furthermore, the structure of the rough ER was fractured and the number of free ribosomes was significantly decreased in the cytoplasm. Mitochondria were degenerating and vacuolated. Demyelinated fibers and decreased numbers of synapses with flimsy post-synaptic densities were also observed in $A\beta_{1-42}$-treated samples. HN was able to partly reverse these alterations.

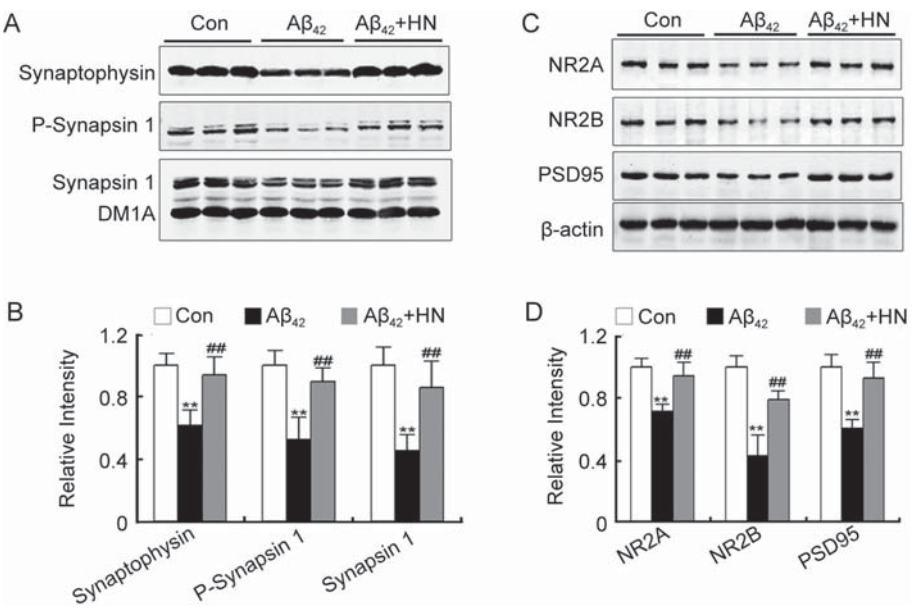

Fig. 4. HN attenuates the reduction of synapse-associated protein levels induced by $A\beta_{1\text{-}42}$. Rats were treated as in Figure 1 and (A) the levels of synaptophysin, synapsin I, and synapsin I phosphorylated at Ser 9 (p-synapsin I), and (C) the NR2A and NR2B subunits and PSD95 in the hippocampus from the three groups were detected by western blotting. Corresponding quantitative analysis is shown in (B) and (D). The data are expressed as mean ± SD (n = 4; $^{**}P$ <0.01 *vs* control (Con); $^{\#\#}P$ <0.01*vs* $A\beta_{1\text{-}42}$).

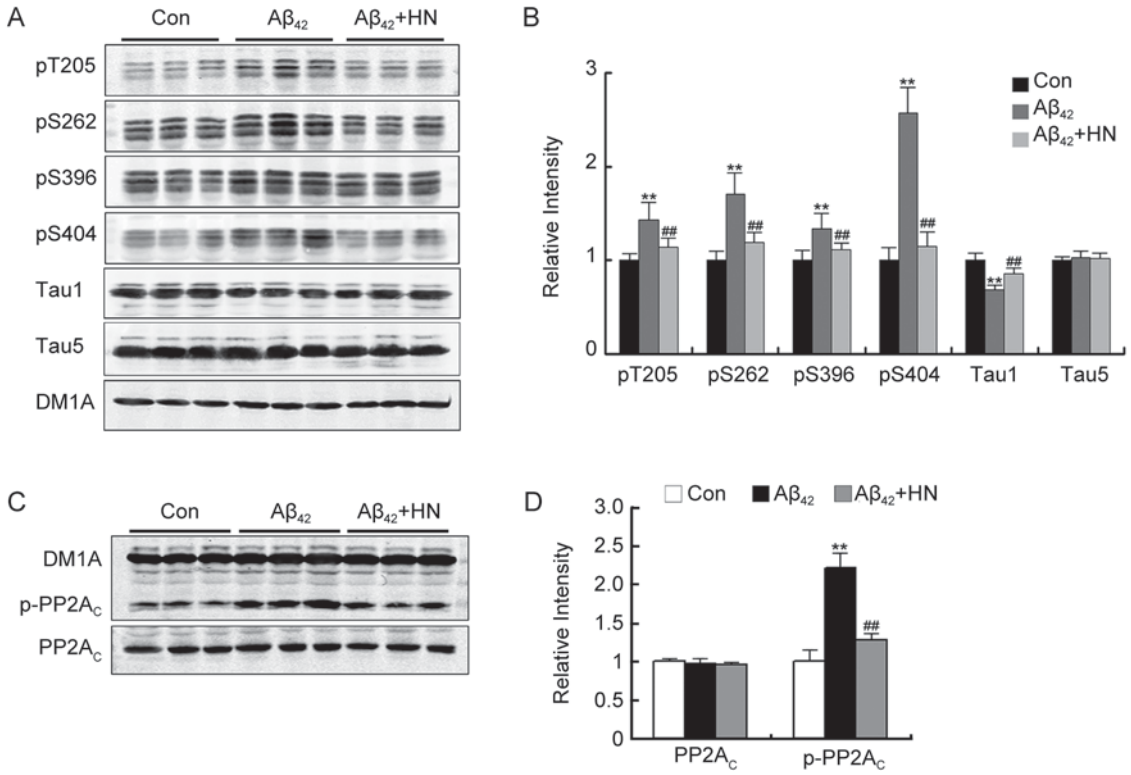

Fig. 5. HN attenuates $A\beta_{1\text{-}42}$-induced tau hyperphosphorylation by activation of PP2A. A: The levels of phosphorylation of tau at multiple epitopes, and C: total $PP2A_C$, and $PP2A_C$ phosphorylated at Tyr307, were detected by western blotting. Quantitative analyses are depicted in B and D. The data are expressed as mean ± SD (n = 5; $^{**}P$ <0.01 *vs* control (Con); $^{\#\#}P$ <0.01 *vs* $A\beta_{42}$).

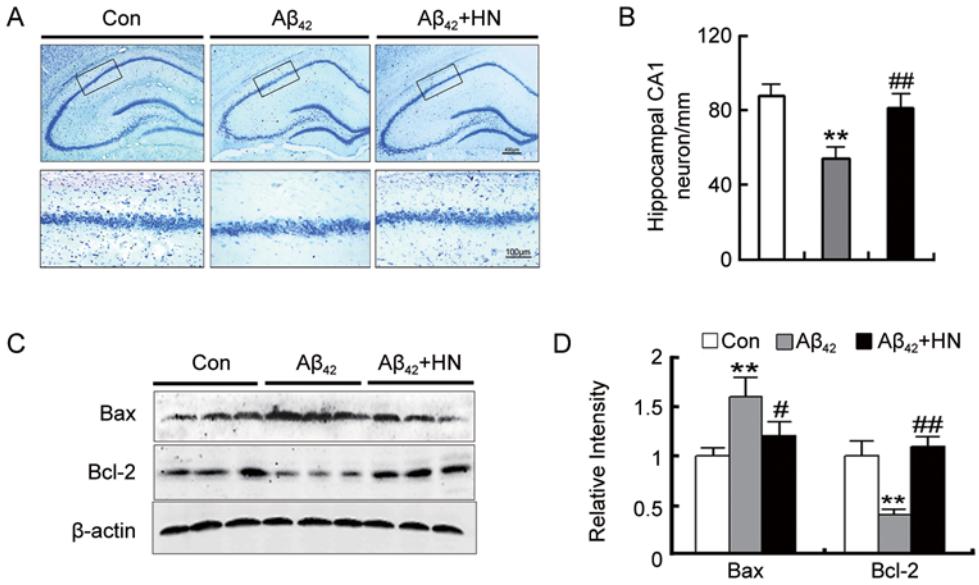

Fig. 6. HN prevents $A\beta_{1-42}$-induced neuronal apoptosis. (A) Nissl staining and (B) cell numbers revealed that HN prevented neuronal death in the CA1 region after $A\beta_{1-42}$ injection. C: The levels of Bax and Bcl-2 were altered by the treatments. Relative intensities are shown in D. Data are expressed as mean ± SD (n = 5; $^{**}P$ <0.01 *vs* control (Con); $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 *vs* $A\beta_{1-42}$).

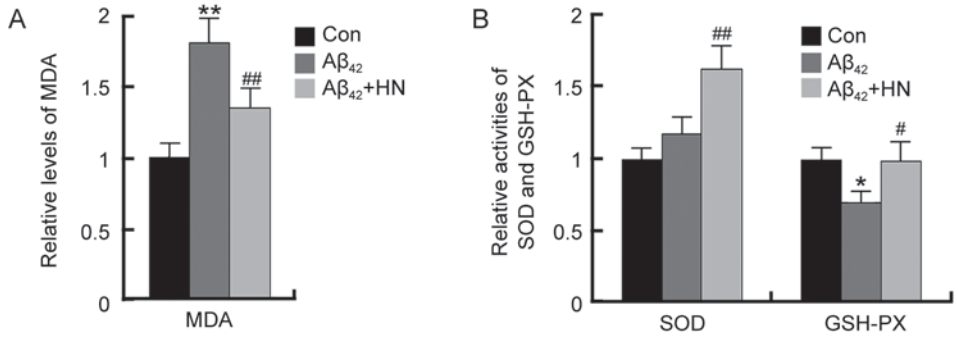

Fig. 7. HN reverses the effect of $A\beta_{1-42}$-induced oxidative stress. Rats were treated as in Figure 1. (A) The relative MDA level and (B) SOD and GSH-PX activity in the hippocampus from the three groups. The data are expressed as mean ± SD (n = 5; $^{*}P$ <0.05, $^{**}P$ <0.01 *vs* control (Con); $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 *vs* $A\beta_{42}$).

DISCUSSION

HN is a small molecular-weight peptide that potently functions against AD-related insults by promoting cell survival *in vitro*[17]. Although the neuroprotective effects of HN are now well known[21,41] and have been confirmed in transgenic AD mice[42], the intrinsic mechanisms by which it suppresses AD-like pathological changes and memory deficits *in vivo* are still unclear. In the present study, we showed that HN ameliorated the $A\beta_{1-42}$-induced spatial memory deficiency by reversing LTP impairment, synaptic dysfunction, dendritic degeneration, neuronal apoptosis, oxidative stress, and tau hyperphosphorylation. HN also attenuated the ultrastructural pathological changes induced by $A\beta_{1-42}$.

It is well recognized that synaptic plasticity, which can be assessed by LTP and/or the morphology of synapses, is an important biological foundation of learning and memory[43]. By measuring the amplitude and slope of the fEPSP, we found that $A\beta_{1-42}$ inhibited the induction of LTP in the hippocampus, whereas simultaneous supplementation

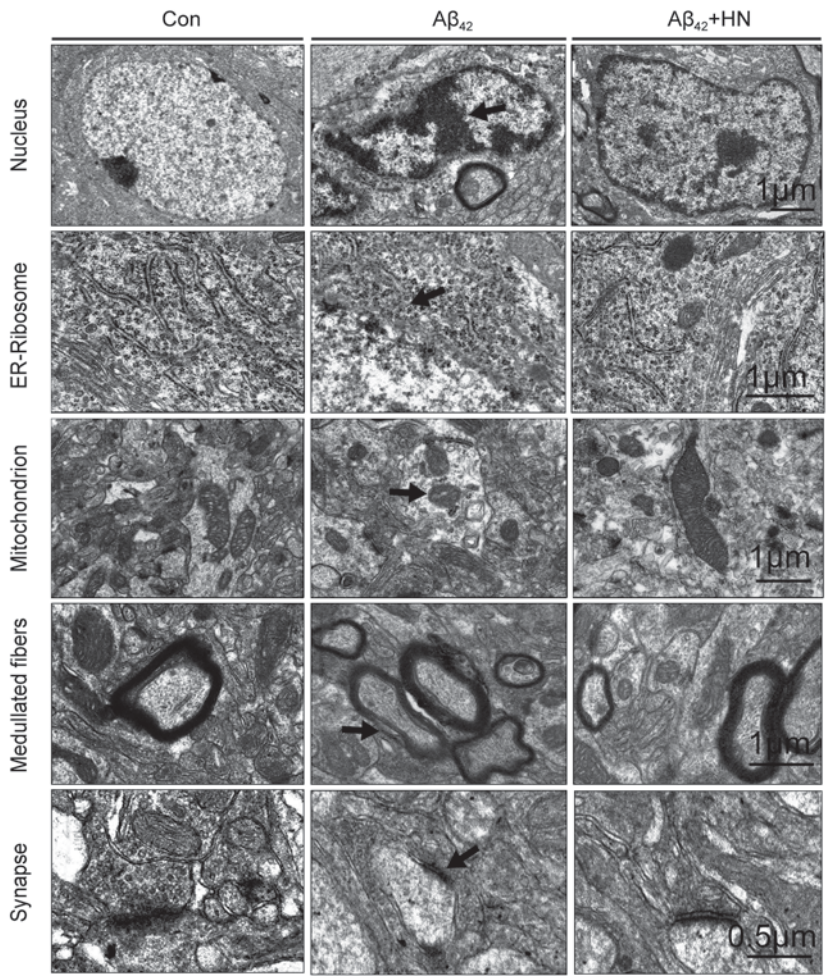

Fig. 8. HN attenuates $A\beta_{42}$-induced ultrastructural damage of neurons in the CA1 region. In $A\beta_{42}$-treated rat hippocampi, concentrated nuclei, fractured rough ER and mitochondria, decreased ribosomes, demyelinated fibers, and decreased synapses with flimsy density were observed (arrows in each corresponding $A\beta_{42}$ group). This was partially reversed by treatment with HN (*n* = 3).

with HN ameliorated this inhibition. The synaptic vesicle-associated proteins synaptophysin and synapsin I, putative markers of synaptic plasticity in neuronal networks[44-46], are involved in the release of neurotransmitters from presynaptic neurons. The decreased levels of several postsynaptic-associated proteins, NR2A, NR2B, and PSD95, in $A\beta_{1-42}$-treated hippocampi were also sensitive to HN, suggesting that functional changes underlie the $A\beta_{1-42}$-induced memory deficits. Similarly, the reversal of $A\beta_{1-42}$-induced decreases in the number of dendritic branches and density of dendritic spines and mushroom spines revealed a structural component of the neuroprotective profile of HN. Results of ultrastructural studies in the CA1 region also showed that HN has a significant neuroprotective effect. Therefore, HN exerts its neuroprotective effects by remodeling dendritic structural complexity.

Hyperphosphorylation of tau is one of the characteristic features of the AD brain[33,47]. We confirmed that $A\beta_{1-42}$ significantly increases tau phosphorylation at multiple AD-sites *in vivo*: Thr-205 (pT205), Ser-262 (pS262), Ser-396 (pS396), Ser-404 (pS404), and Ser-199/202 (Tau-1). While some studies have demonstrated that both total and phosphorylated forms of tau are unaffected by Gly^{14}-HN treatment in transgenic-AD mice[42], the present data demonstrated that $A\beta_{1-42}$-induced tau hyperphosphorylation was efficiently suppressed by simultaneous administration

of HN. This effect appeared to be largely due to HN's ability to activate PP2A, by decreasing the phosphorylation of Tyr307 on the $PP2A_c$ subunit. We have previously reported that the activity of PP2A is a central candidate in AD-related tau hyperphosphorylation[48,49]. These data indicate that HN attenuates $A\beta_{1-42}$-induced tau pathology by balancing the effects of upstream protein kinases and phosphatases.

HN influences a variety of survival-promoting features: it protects cerebrovascular smooth muscle cells from Aβ-induced toxicity[50], and HN or HN-like peptides such as rattin, a rat homologue of HN, are protective against serum withdrawal[51] and N-methyl-D-aspartate-induced excitotoxicity in primary cortical neuronal and glial co-cultures[52]. HN negates Aβ-induced neuronal death *in vitro*[50]. It has been reported that HN inhibits Bax-mediated apoptosis by binding to, and sequestering Bax[53]. To our knowledge, the present *in vivo* data are the first evidence of HN-mediated neuroprotection against $A\beta_{1-42}$-induced apoptosis that results from the pro-survival expression of Bcl-2 and Bax proteins.

There is an increasing awareness of the ubiquitous role of oxidative stress in neuropathology[54] and the literature based on animal models and studies of the human brain suggest that oxidative stress plays an important role in neuronal apoptosis in AD[55]. Recently, HN was found to attenuate oxidative stress in atherosclerosis by reducing reactive oxygen species production[40]. We now report that MDA, a marker of oxidative stress, increased, whereas GSH-Px activity decreased in hippocampal extract after $A\beta_{1-42}$ injection *in vivo*, and that HN treatment counteracted these indices of oxidative stress. This would certainly contribute to its neuroprotective profile in the hippocampus. Combined with the reported studies, the molecular signaling mechanisms of HN-mediated neuroprotection include stimulation of the PI3 kinase/AKt pathway[56], inhibition of pro-apoptotic Bcl-2 family members[57], and modulation of the JAK/STAT pathways[58] by binding to a complex or complexes involving ciliary neurotrophic factor receptor, the IL-27receptor WSX-1, and gp130[59].

In summary, we have found that HN attenuates the $A\beta_{1-42}$-induced memory deficits, tau phosphorylation, neuronal dysfunction and dendritic degeneration, and neuronal apoptosis, all of which could ameliorate the neuropsychopathology associated with senile dementia.

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