Exendin-4, a glucagon-like peptide 1 receptor agonist, protects against amyloid- β peptide-induced impairment of spatial learning and memory in rats

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Abstract: Type 2 diabetes mellitus (T2DM) and Alzheimer's disease (AD) share specific molecular mechanisms, and agents with proven efficacy in one may be useful against the other. The glucagon-like peptide-1 (GLP-1) receptor agonist exendin-4 has similar properties to GLP-1 and is currently in clinical use for T2DM treatment. Thus, this study was designed to characterize the effects of exendin-4 on the impairment of learning and memory induced by amyloid protein (A β) and **its probable molecular underlying mechanisms**. The results showed that (1) intracerebroventricular (*i.c.v.*) injection of A β 1-42 resulted in a significant decline of spatial learning and memory of rats in water maze tests; (2) pretreatment with exendin-4 effectively and dose-dependently protected against the A β 1-42-induced impairment of spatial learning and memory; (3) Exendin-4 treatment significantly decreased the expression of Bax and cleaved caspase-3 and increased the expression of Bcl2 in A β 1-42-induced Alzheimer's rats. The vision and swimming speed of the rats among all groups in the visible platform tests did not show any difference. These findings indicate that systemic pretreatment with exendin-4 can effectively prevent the behavioral impairment induced by neurotoxic A β 1-42, and the underlying protective mechanism of exendin-4 may be involved in the Bcl2, Bax and caspase-3 pathways. Thus, the application of exendin-4 or the activation of its signaling pathways may be a promising strategy to ameliorate the degenerative processes observed in AD.

Keywords: Exendin-4; Amyloid- β Peptide; spatial learning and memory; Bcl2/Bax, caspase3

Introduction

Alzheimer's disease (AD), which affects more than 40 million people worldwide, is a chronic, age-related and irreversible neurodegenerative disorder characterized by progressive cognitive dysfunction and the inability to perform complex daily activities (Anand et al., 2014). One of the hallmarks of AD is the presence of a high density of senile plaques composed of amyloid protein (A β) in the hippocampus and cerebral cortex (Zetterberg et al., 2010). A β is a proteolytic cleavage product of the sequential cleavages of amyloid precursor proteins (APPs) by the β -site of the APP-cleaving enzyme (BACE), followed by γ -secretase, to generate a

1 family of A β peptides of 40 and 42 amino acids that aggregate to form oligomers
2 capable of causing synaptic dysfunction and neuronal degeneration (Hardy and Selkoe,
3 2002; Sambamurti et al., 2006). The neurotoxicity of A β peptides, including different
4 A β fragments such as A β 1-42, A β 1-40, A β 25-35 and A β 31-35, have been widely
5 reported *in vivo* and *in vitro* (He et al., 2012; Luo et al., 2012; Wang et al., 2014).
6 Consequently, it is important to clear the aggregated A β or block the A β toxicity in the
7 brain for the prevention and clinical treatment of AD.

8 Interestingly, it has recently been reported that AD and type 2 diabetes mellitus
9 (T2DM), another age-related degenerative disorder in the elderly, share several
10 common clinical, pathological and biochemical characteristics (Gotz et al., 2009;
11 Jones et al., 2009; Luchsinger and Gustafson, 2009). For example, some risk factors
12 implicated in the development of T2DM, such as genetic predisposition, oxidative
13 stress, age, diet, obesity, and physical inactivity, appear to be involved in AD,
14 implicating T2DM as a risk factor for the development of AD (Gotz et al., 2009; Jin
15 and Patti, 2009). Moreover, AD is associated with the peripheral and central insulin
16 abnormalities, including the desensitization of insulin receptors in the brain (Craft,
17 2007; Jones et al., 2009; Li and Holscher, 2007). Therefore, an effective treatment
18 strategy against T2DM could also have potential value in AD.

19 Emerging evidence indicates that glucagon-like peptide 1(GLP-1), an incretin
20 hormone, can cross the blood–brain barrier (BBB) on its receptor (GLP-1R) and
21 facilitate insulin signaling (Gengler et al., 2012; Lovshin and Drucker, 2009). GLP-1R
22 is a member of the secretin receptor family of the G-protein-coupled receptor and is
23 expressed in the brain. It has been reported that GLP-1 binds to its receptor and
24 triggers cellular signaling pathways, which has shown a protective effect on neuronal
25 cells against apoptosis (Perry et al., 2002a; Perry et al., 2002b). However, due to the
26 shorter half-life of GLP-1 by the rapid inactivation of the enzyme dipeptidyl
27 peptidase-4 (Luchsinger and Gustafson, 2009), **its potential therapeutic application**
28 **is greatly limited**. Exendin-4 is a glucagon-like peptide-1 (GLP-1) receptor agonist
29 with a longer plasma half-life than GLP-1 and has been developed as a first-in-class
30 diabetes therapy. It has been reported that exendin-4 could readily cross the

1 blood-brain barrier (Kastin and Akerstrom, 2003). In the brain, GLP-1R is expressed
2 widely (Hamilton and Holscher, 2009), and its activation produces multiple biological
3 responses (Li et al., 2009; Perry and Greig, 2003). Some studies showed that
4 exendin-4 could reduce endogenous levels of A β in AD transgenic mice (Li et al.,
5 2010) and enhance neuronal progenitor proliferation in the brains of diabetic mice
6 (Hamilton et al., 2011). Therefore, it is of interest to further investigate whether the
7 A β -induced impairment in the central nervous system can be effectively alleviated by
8 exendin-4. In this study, we first observed the effects of intracerebroventricular (*i.c.v.*)
9 injection of exendin-4 on A β 1-42-induced impairment of spatial learning and memory
10 of rats in the Morris water maze (MWM) test. We also revealed the possible
11 molecular mechanism of exendin-4 against the A β 1-42-induced impairments in the
12 spatial cognition of rats.

13 **Materials and methods**

14 *Animal and Surgical Procedure*

15 With approval of the Shaanxi Animal Research Ethics Committee, adult male
16 Sprague–Dawley (SD) rats (230–250 g) supplied by the Research Animal Center of
17 Xi'an Jiaotong University were divided randomly into six groups: control, A β 1-42,
18 Exendin-4 0.02 nmol, 0.2 nmol, 2 nmol and Exendin-4 plus A β 1-42. The surgical
19 preparation procedure was similar to our previous report (Zhang et al., 2006). Briefly,
20 SD rats were anesthetized (chloral hydrate, 0.3 g/kg, *i.p.*) and placed in a stereotaxic
21 apparatus (Narishige, Tokyo, Japan), and a hole was drilled in the right side of the
22 skull (0.8 mm posterior to bregma and 1.3 mm lateral to the midline) to introduce a
23 guide cannula for *i.c.v.* injection of drugs/vehicle.

24 *Chemicals and drug application*

25 Both Exendin-4 and A β 1-42 were purchased from Sigma (St. Louis, MO, USA)
26 and dissolved in normal saline, and A β 1-42 was “aged” before surgery by incubation
27 at 37°C for 4 days (Wang et al., 2014; Yenkovyan et al., 2009). A β 1-42 (5 nmol per rat),
28 Exendin-4 (0.02, 0.2, 2 nmol per rat) and vehicle solutions were injected *i.c.v.* using a
29 Hamilton microsyringe over 10 min in a total volume of 5 μ l. All rats in the
30 co-application group were pretreated with Exendin-4 15 min before the A β 1-42

1 injection. Two weeks later, the Morris water maze (MWM) test was performed.

2 *Morris Water Maze (MWM) Test*

3 Rats' spatial learning and memory were evaluated using a classic MWM, a
4 device commonly used for studying cognitive deficits in APP transgenic mice (Perry
5 et al., 2007). The Morris water maze consisted of a large circular pool (diameter, 150
6 cm; height, 50 cm) and a small circular platform (diameter, 14 cm; height, 29 cm).
7 The inner surface of the pool and the platform were painted black, and the platform
8 was placed at the midpoint of one quadrant and submerged approximately 1.0 cm
9 below the surface of the water at a temperature of 23±2°C. Prominent visual cues
10 were provided in the testing area of the room. The swimming path of the rats during
11 each trial was monitored via a camera mounted overhead above the center of the pool
12 and connected to a video tracking system (EthoVision, VA, USA). Tracking was
13 achieved using a white rat on a black background. The hidden platform test was
14 performed 2 weeks after drug injection. The tests were performed four times per day
15 for 5 consecutive days. In each trial, the rats were released into the water facing the
16 pool wall in one of the four quadrants (Zone 1, 2, 3, and 4) designated by computer
17 software and were allowed to swim freely to the escape platform. After climbing onto
18 the platform, the rats were allowed to rest on it for 20 sec. In probe trials on the sixth
19 day, the platform was removed from the pool, and rats were given 120 sec to swim in
20 the pool to measure the spatial bias. The percentages of total time and distance spent
21 in the different quadrants **were** measured. After the probe trials, motor skill ability and
22 visual acuity were also tested using a visible platform located opposite the original
23 position within the pool. The test comprised four trials with the visible platform
24 elevated above the water level (approximately 2 cm), and the latency and swimming
25 speed at which the animal arrived at the platform were recorded. These data were
26 recorded and analyzed by a behavioral software system (Ethovision 3.0, Noldus
27 Information Technology, Wageningen, the Netherlands).

28 *Real-Time PCR*

29
30 After finishing the MWM test, the rats were rapidly decapitated, and the bilateral

1 hippocampi were collected. Total RNA from the hippocampus was isolated using
2 Trizol reagent (Life Technologies, Grand Island, NY, USA), and the concentration of
3 RNA was determined by NanoDrop 2000 Spectrophotometer (THERMO, USA). Prior
4 to RT-PCR, RNA was initially treated with DNase I (Ambion Inc., Austin, TX) to
5 degrade genomic DNA. Nearly 2 µg of total RNA was reverse transcribed with the
6 RevertAid First Strand cDNA Synthesis Kit (THERMO, USA). Quantitative real time
7 qPCR was processed using the DNA Engine Opticon 2 system (Bio-Rad, USA)
8 with the protocol of denaturation at 94°C for 5 min, followed by 34 cycles of
9 denaturation at 94°C for 1 min and annealing/extension at 52°C or 30 s. Data analysis
10 was performed by Mx3005P QPCR Systems (STRATAGENE, USA). The mRNA
11 content was determined as a relative value normalized to GAPDH mRNA level. The
12 sequences of the primers used were as follows: For GAPDH, forward:
13 5'-AAGAAGGTGGTGAAGCAGGC-3' and reverse:
14 5'-TCCACCACCCTGTTGCTGTA-3'; for Bcl2, forward:
15 5'-ACTGAGTACCTGAACCGGCATC-3' and reverse: 5'-
16 GGAGAAATCAAACAGAGGTCGC-3'; for Bax, forward:
17 5'-AGTGTCTCAGGCGAATTGGC-3' and reverse:
18 5'-CACGGAAGAAGACCTCTCGG-3'. All experiments were repeated three times.

19 *Western Blot Analysis*

20 Proteins were extracted from the collected whole hippocampus of the rats (n = 6
21 per group). The protein concentration was determined using a bicinchoninic acid
22 (BCA) protein assay kit (Pierce, USA). Proteins (50µg) were separated by
23 SDS-PAGE and transferred to membranes. The protocol for western blotting was
24 described previously (Chen et al., 2008). The primary antibodies used were rabbit
25 anti-cleaved caspase-3 (#9661, Cell Signal Tech) and mouse anti-actin (A5441,
26 Sigma). The films were scanned, and the images were quantitatively analyzed using
27 ImageJ software (NIH, USA).

28 **Data analysis**

29

30 All data were presented as the mean±standard error (SEM). In MWM, the

latency and distance swum in four trials for each rat on each day were averaged. A two-repeated measures analysis of variance (ANOVA) with Tukey's post hoc analysis for multiple comparisons was used with day as the repeated measure and latency or distance. For probe trials, the two trials for each rat were averaged, and the means were compared between groups via one-way ANOVA. For the visible platform test, the second trial for each rat was recorded and compared between groups using one-way ANOVA. Real-time PCR and western blot were analyzed using one-way ANOVA, and all statistical analyses were performed using the statistical software package SPSS13.0. $P < 0.05$ was considered statistically significant.

Results

I.c.v. injection of A β 1-42 impairs spatial learning and memory of rats

To explore the effect of A β 1-42 on spatial learning and memory, we first assessed the learning ability of rats by performing the hidden platform test on 5 consecutive days of training. As expected, **there was a significant effect of *i.c.v.* injection on the mean escape latency and mean escape distance between the A β 1-42 treatment and control group.** As shown in Fig. 1, compared with the control group (Fig. 1, ●, $n=10$), an *i.c.v.* injection of 5 nmol A β 1-42 (Fig. 1, ○, $n=10$) resulted in a significant decrease with the increase in training days in spatial learning, with longer latency (Fig. 1A) and distance (Fig. 1B) in searching for the underwater platform ($P < 0.01$ for each training day). In the control group, the mean escape latencies as reported on training days 1, 2, 3, 4, and 5 as 81.8 ± 4.6 s, 52.4 ± 4.5 s, 34.2 ± 3.1 s, 19.5 ± 2.6 s and 14.2 ± 2.2 s, respectively, show progress in the learning ability of the rats. However, 5 nmol of A β 1-42 treatment resulted in more time to find the hidden platform than the control group. The mean escape latencies (Fig. 1A) on the same training days increased to 84.4 ± 5.3 s ($P > 0.05$), 67.4 ± 4.6 s ($P < 0.01$), 49.1 ± 3.7 s ($P < 0.01$), 35.9 ± 2.8 s ($P < 0.01$) and 26.1 ± 2.2 s ($P < 0.01$). Similarly, as shown in Fig. 1B, the mean escape distances in the hidden platform tests on training days 1, 2, 3, 4, and 5 in the A β 1-42 group **were** 1933.7 ± 216.2 cm ($P > 0.05$), 1548.4 ± 151.4 cm ($P < 0.01$), 980.3 ± 151.7 cm ($P < 0.01$), **593.8 $\pm$ 96.8 cm** ($P < 0.01$) and 381.9 ± 60.5 cm ($P < 0.01$), respectively, which were significantly larger than the values of

1 1856.1±120.2, 1158.4±169.7 cm, 739.8±104 cm, 408.5±69.6 cm and 222.8±67.1 cm,
2 respectively, in the control group. These results indicate that Aβ1-42 impaired the
3 spatial learning of rats. In order to further investigate the spatial memory ability of
4 rats in the MWM, probe trials without the platform were tested on day 6. The
5 percentages of total time (Fig. 1C) and distance swum (Fig. 1D) in the target quadrant
6 were significantly decreased in the Aβ1-42 group, being 25.89± 2.62% and
7 25.78±2.35%, respectively, smaller than the values of 45.69 ±2.91% and 44.88±
8 2.56%, respectively, in the control group ($P<0.01$). These results show that Aβ1-42
9 seriously impaired the spatial memory function of rats.

10 *Exendin-4 alone did not affect normal cognitive function but prevented*
11 *Aβ1-42-induced impairment of spatial learning and memory in a dose-dependent*
12 *manner*

13 To verify the neuroprotective effect of exendin-4 against Aβ1-42-induced
14 cognitive impairment, we first observed the effect of exendin-4 alone on spatial
15 learning and memory. As shown in Fig. 1, *i.c.v.* administration of 2 nmol exendin-4
16 (Fig. 1, ▼, n=10) alone did not affect the average escape latency and distance to the
17 hidden platform during the acquisition test when compared with those of the control
18 group (Fig. 1, ●, n=10) ($P>0.05$). The mean escape latencies in the 2 nmol exendin-4
19 group were 79.7±4.4 s, 50.41±3.4 s, 33.1±2.5 s, 18.2±2.5 s and 12.4±1.5 s, and the
20 mean escape distances were 1835.7±167.5 cm, 1135.7±167.5 cm, 728.3± 89.6 cm,
21 387.3 ±77.6 cm and 230.4 ±33.9 cm on training session days 1, 2, 3, 4, and 5,
22 respectively. Compared to the values in the control group, there was no significant
23 difference at any training day ($P>0.05$). In the probe trial, the percentage of total time
24 elapsed (Fig. 1C) and distance (Fig. 1D) in the target quadrant in the exendin-4 group
25 were 43.94±2.04% and 44.12±1.33%, respectively, without any significant difference
26 compared with those of the control group ($P>0.05$).

27 Furthermore, we investigated the effects of pretreatment with different dosages
28 (0.02–2 nmol) of exendin-4 on the Aβ1-42-induced impairment of spatial learning and
29 memory of rats. Our results depicted that pretreatment with exendin-4 (0.2–2 nmol)
30 could obviously prevent against Aβ1-42-induced impairment in the spatial cognition

of rats in a dose-dependent manner. In a hidden platform experiment, both the escape latency and escape distance in the co-application of exendin-4 and A β 1-42 groups were improved compared to those in the A β 1-42 alone group. As shown in Fig. 2, the impairment of learning was not significantly improved in the 0.02 nmol exendin-4 plus A β 1-42 group (Fig. 2, $\circ$, $n=10$), with similar escape latency and similar escape distance compared to the A β 1-42 alone group (Fig. 2, $\bullet$, $n=10$) ($P>0.05$). In the 0.02 nmol exendin-4 plus A β 1-42 group, the escape latencies were 82.3 ± 4.1 s, 66.4 ± 4.9 s, 48.9 ± 3.7 s, 35.1 ± 2.9 s and 24.3 ± 2.3 s on training days 1, 2, 3, 4 and 5, respectively, and the corresponding swim distances were 1922.2 ± 245.7 cm, 1472.2 ± 223.1 cm, 901.8 ± 113.3 cm, 536.7 ± 73.2 cm and 365.6 ± 48.6 cm. However, pretreatment with 0.2 nmol or 2 nmol exendin-4 significantly reversed the spatial learning impairments induced by A β 1-42 ($P < 0.05$) on training days. For example, when compared with those of the A β 1-42 alone group, the mean escape latencies in the 0.2 nmol exendin-4 plus A β 1-42 group (Fig. 2, $\blacktriangledown$, $n=10$) decreased to 81.1 ± 4.1 s ($P>0.05$), 61.2 ± 3.1 s ($P<0.05$), 44.7 ± 2.8 s ($P<0.05$), 30.9 ± 3.1 s ($P<0.05$) and 20.4 ± 2.5 s ($P<0.05$) on training days 1, 2, 3, 4 and 5, respectively; corresponding swim distances were also decreased to 1890.8 ± 134.6 cm ($P>0.05$), 1319.2 ± 206.1 cm ($P<0.05$), 850.1 ± 93.8 cm ($P<0.05$), 488.7 ± 65.7 cm ($P<0.05$) and 305.6 ± 45.1 cm ($P<0.05$). In the 2 nmol exendin-4 plus A β 1-42 group, these values were further decreased. As shown in Fig. 2 (Δ , $n=10$), the mean escape latencies were 80.5 ± 3.5 s ($P>0.05$), 55.7 ± 3.4 s ($P<0.05$), 37.9 ± 2.9 s ($P<0.05$), 25.9 ± 3.7 s ($P<0.05$), and 16.9 ± 2.8 s ($P<0.05$), and the mean escape distances were 1871.4 ± 157.1 cm ($P>0.05$), 1236.3 ± 58.4 cm ($P<0.05$), 749.1 ± 92.7 cm ($P<0.05$), 386.2 ± 89.4 cm ($P<0.05$), and 235.6 ± 59.7 cm ($P<0.05$) on training days 1, 2, 3, 4, and 5, respectively, which were significantly shorter than those of the A β 1-42 alone group. Compared with the group injected with the lower concentration (0.02 nmol) of exendin-4, higher concentrations (0.2 nmol and 2 nmol) of exendin-4 show a faster decrease in mean escape latency and mean escape distance in the co-application groups. In the probe trials (Fig. 2), we found that 0.02 nmol exendin-4 alone did not affect memory, with a similar degree of swimming preference in the target quadrant as the control group, but 5 nmol A β 1-42 markedly decreased the

percentage of mean time elapsed and the distance swum in the target quadrant. Interestingly, pretreatment with different dosages of exendin-4 prevented the A β 1-42-induced memory deficit in a dose-dependent manner. As shown in Fig. 2, the percentage of total time (Fig. 2E) elapsed and the distance (Fig. 2F) swum in the target quadrant after removing the platform significantly increased in both higher concentrations of exendin-4 groups compared to the A β 1-42 alone group. In the 0.2 nmol and 2 nmol exendin-4 plus A β 1-42 groups, the time percentages in the target quadrant increased to $35.42 \pm 3.09\%$ and $32.12 \pm 3.05\%$, respectively, which were significantly larger than the values in the A β 1-42 alone group ($P < 0.05$); the distance swum in the target quadrant increased to $42.08 \pm 3.01\%$ and $40.63 \pm 2.71\%$, respectively, which were also significantly larger than in the A β 1-42 alone group ($P < 0.05$). Overall, these results indicate that exendin-4 could dose-dependently protect against A β 1-42-induced spatial learning and memory impairment.

Both Exendin-4 and A β 1-42 did not affect the vision and swimming speed of rats

To exclude the possibility that the results above were due to the impairments of visual and motor function of rats, the escape latencies of rats were examined with a visible platform after finishing the probe trials, and the mean swimming speeds in probe trials were also compared. As shown in Fig. 3, there was no significant statistical difference ($P > 0.05$) in swimming speeds among all groups in 5 successive days of learning acquisition, with a mean swim speed of approximately 20 cm/s. In addition, the mean time for rats to reach the visible platform was approximately 17 s, without any significant difference among all groups in the visible platform test. These results confirm that none of the drugs used in the present study affected the vision and motor ability of rats.

Exendin-4 Effectively Prevented the A β 1-42-Induced Change in the Levels of Bax /Bcl2 and caspase-3 in the Hippocampi of Rats

Apoptosis induced by A β plays an important role in the pathogenesis of AD. To elucidate the probable molecular mechanism underlying the protection of exendin-4 against A β 1-42-mediated deficit in spatial cognition, we assessed the changes of three factors involved in apoptosis pathways. Because Bcl-2 is an important anti-apoptotic

protein and Bax is a major apoptotic protein, we first investigated the levels of both factors in the hippocampi of rats after the MWM test using real-time PCR. As expected, A β 1-42 and exendin-4 treatments had significant effects on the expression of *Bcl2* and *Bax*. Fig. 4A showed that A β 1-42 injection resulted in a significant decrease in the expression of *Bcl-2*, and this decrease was reversed by exendin-4 treatments (0.2 nmol or 2 nmol) ($P < 0.01$). Meanwhile, as shown in Fig. 4B, A β 1-42 injection produced a significant increase the expression of *Bax* mRNA, which was dramatically inhibited by adding exendin-4 (0.2 nmol or 2 nmol) ($P < 0.01$). Caspase-3 is the last effector in the apoptotic pathway, so we examined the effects of exendin-4 on caspase-3 modulation. As shown in Fig. 4C, A β 1-42 caused a significant increase in cleaved-caspase-3 expression compared with controls using western blotting. However, pretreatment with different dosages of exendin-4 dose-dependently prevented the up-regulation of cleaved-caspase-3 ($P < 0.01$). These results indicate that exendin-4 acts as a neuroprotective factor potentially by modulating the expression of *Bcl2*, *Bax* and caspase-3.

Discussion

The major finding of this study was that exendin-4 dose-dependently prevented the A β 1-42-induced deficits in spatial learning and memory of rats by modulating the expression of *Bcl2*, *Bax* and caspase-3. The accumulation of A β 1-42 oligomers in the brain is thought to be responsible for the decline of learning and memory in AD (Bao et al., 2012; Esparza et al., 2013; Herskovits et al., 2013; Yang et al., 2013). It has been shown that soluble A β oligomers damaged synaptic plasticity and memory formation (Selkoe, 2008). A β 1-42 oligomers can combine to form plasma membrane proteins (Kam et al., 2013; Kim et al., 2013; Lesne et al., 2013), enhance intracellular calcium levels (Zempel et al., 2013; Zempel and Mandelkow, 2012), render tau mislocalization, disrupt microtubules, change membrane trafficking processes and surface expression levels of critical synaptic ion channels (Lacor et al., 2007), induce reversible spine loss in neurons (Shankar et al., 2007; Wei et al., 2010) and finally, result in reversible defects of spatial memory (Cleary et al., 2005; Klyubin et al., 2011). The neurotoxicity of A β *in vivo* and *in vitro* has been widely reported (Alkam

et al., 2007; Ferreira et al., 2007; Luo et al., 2012; Mattson, 2004). For example, A β 1-42 induced deficits in learning and memory have been reported in different kinds of animal behavioral tests, including Y-maze (Alkam et al., 2007), T-maze (Kunesova et al., 2008) and MWM (Wang et al., 2010). In the present study, we also found that *i.c.v.* injection of synthetic A β 1-42 in rats resulted in marked deficits in spatial learning and memory. These results were very recently given strong support by Takeda's observations in which the cognitive function of mice was significantly impaired by *i.c.v.* injection of A β 1-40 (Takeda et al., 2009). However, the effective neuroprotective treatments against A β neurotoxicity are still controversial.

Exendin-4, which has 53% amino acid sequence homology with mammalian GLP-1, is a potential ligand for the GLP-1R and is more useful for treating diabetes because of its profound resistance to degradation by DPP-IV and greater biological activity than natural GLP-1 (Idris et al., 2002). One of the main interests in the present study is the neuroprotective properties of exendin-4. It has been reported that exendin-4 has important biological effects in the pancreas, including decreased glucagon levels, elevated insulin synthesis, stimulation of glucose-dependent insulin secretion and inhibition of β -cell apoptosis (Xu et al., 1999). The function of exendin-4 is mainly mediated through the G protein-coupled GLP-1R. GLP-1R was widely expressed throughout the brain, including the hippocampus, a brain area involved in memory formation (Cork et al., 2015; During et al., 2003; Hamilton and Holscher, 2009). GLP-1 and exendin-4 mediate differentiation in PC12 cells and protect neurons from damage *in vitro* through the activation of the GLP-1 receptor and a cascade involving cAMP (Perry et al., 2002b). Moreover, overexpressing GLP-1R in the hippocampus promoted neurite growth and enhanced learning and memory (During et al., 2003), whereas GLP-1R knockout resulted in deficiencies of spatial learning and memory and the development of hippocampal LTP (long-term potentiation) (Abbas et al., 2009). Some studies indicate that *i.c.v.* injection of GLP-1 and infusion of exendin-4 attenuate the neuronal apoptosis and nerve injury induced by kainite and pyridoxine (Perry et al., 2007) *in vivo*. In the present study, we first confirmed the neuroprotective effect of exendin-4 in the cognitive behavior

experiment. Interestingly, exendin-4 alone did not affect the mean escape latency and the percentage of total time elapsed in the target quadrant, but *i.c.v.* injection of 0.2 nmol and 2 nmol, but not 0.02 nmol, of exendin-4 dose-dependently prevented the A β 1-42-induced deficits in spatial learning and memory of rats. The mean escape latencies and distances of rats in the exendin-4 plus A β 1-42 group were significantly decreased in searching for the hidden platform of the MWM, while the swimming time and distance elapsed in the target quadrant after removing the platform significantly increased in the probe trials compared to those in the A β 1-42 alone group. Moreover, some GLP-1R agonists, such as Val8-glucagon-like peptide-1, Liraglutide and Lixisenatide, have also been proven to rescue spatial memory from amyloid beta protein-induced impairments in rats (Cai et al., 2014; Han et al., 2013; Holscher, 2014; Wang et al., 2010). Similar to these results, our results indicate that exendin-4 can effectively prevent the A β 1-42-induced impairment of spatial learning and memory in rats, suggesting that exendin-4 could play an important role in the prevention or treatment of memory loss in AD.

The molecular mechanisms by which exendin-4 could ameliorate A β 1-42-induced cognitive impairments are not well understood. It is generally accepted that A β -induced apoptosis is a critical factor in the pathogenesis of AD (De Felice et al., 2007; Kudo et al., 2012). The balance between pro- and anti-apoptotic proteins affects the initiation or the lack of apoptosis. It has been shown that the anti-apoptotic protein Bcl-2 has a critical role in regulating apoptosis and various neurodegenerative disorders, including AD (Desagher and Martinou, 2000). For example, the overexpression of Bcl2 could decrease the processing of amyloid precursor protein and tau and reduce extracellular deposits of A β (Rohn et al., 2008). In the present study, we found that A β 1-42 significantly reduced the levels of Bcl-2, but this effect was apparently prevented by pretreatment with exendin-4. On the other hand, Bax also plays a central role in oligomeric A β -induced neuronal cell death (Kudo et al., 2012). Bax is a member of the Bcl-2 family of proteins that acts as a regulator of the mitochondrial-dependent apoptosis pathway. Bax is primarily located in the cytosol in normal tissues and is translocated to the mitochondria to regulate cell death after

1 apoptotic stress (Antonsson, 2001). Thus far, the effects of exendin-4 on the
2 expression of Bax have not been reported. We found that A β 1-42 increased the
3 expression of *Bax* mRNA, indicating that mitochondrial membrane dysfunction
4 contributes to the apoptosis process induced by A β 1-42. However, the up-regulation
5 of *Bax* mRNA was potentially reduced by exendin-4 treatment. In accordance with a
6 recent report, oligomeric A β decreased the level of Bcl-2 and increased the expression
7 of Bim, resulting in the activation of Bax and neuronal death in hippocampal slice
8 culture and *in vivo* (Kudo et al., 2012). Furthermore, the inhibition of Bax activity by
9 either Bax-inhibiting peptide or gene knockout remarkably protected neurons against
10 A β -induced apoptosis (Kudo et al., 2012). It is likely that exendin-4 exerted an
11 anti-apoptosis effect in an AD model by regulating the balance of Bcl-2 family
12 proteins.

13 The loss of neurons in AD has been closely associated with the over-activation of
14 caspases (D'Amelio et al., 2011; Zhang et al., 2011). Caspase-3 is a key downstream
15 effector of the activation of intracellular apoptotic pathways and has been shown to be
16 elevated in neurons and astrocytes of AD patients (Bader Lange et al., 2010; Ghobrial
17 et al., 2005). Thus, in the present study, we further observed the activity of caspase-3
18 using western blot. As expected, our results showed that A β 1-42 significantly
19 increased the levels of active caspase-3 in the hippocampus of rats, while exendin-4
20 pretreatment effectively prevented the increase of cleaved caspase-3. The evidence
21 suggests that Bcl2, Bax and/or caspase-3 could be common targets of A β and
22 exendin-4; the decrease of the anti-apoptotic Bcl-2 and the increase in the
23 pro-apoptotic Bax and the active caspase-3 in the brain may be involved in the
24 neuroprotective mechanism of exendin-4 in improving spatial memory. In addition,
25 the cAMP/PKA signaling pathway may play an essential role in the mechanism by
26 which exendin-4 prevents A β 1-42-induced impairments in spatial memory. It has
27 been reported that A β suppresses the protein kinase A/cAMP response element, while
28 GLP-1 and a GLP-1 receptor agonist could enhance cAMP concentration in astrocytes
29 (Iwai et al., 2006) and hypothalamic neurons (Dalvi et al., 2012); GLP-1 treatment
30 induces a maximal 1200-fold increase of cAMP levels in PC12 cells (Perry et al.,

2002b) and in cultured hippocampal neurons (Perry et al., 2003). The up-regulation of cAMP/PKA activity can drive CREB phosphorylation and gene transcription in the nucleus and ultimately may improve learning and memory behavior via this pathway (Sharma et al., 2014).

In summary, the present study showed for the first time that exendin-4 can protect against A β 1-42-induced impairments of cognitive behavior in a dose-dependent manner. The neuroprotective action of exendin-4 against A β 1-42 may be, at least in part, mediated by the up-regulation of anti-apoptotic proteins and down-regulation of pro-apoptotic pathways. Further studies are necessary to clarify a more comprehensive understanding of the cellular mechanisms both *in vitro* and *in vivo*. The effective neuroprotective action of exendin-4 in preventing neurotoxicity strongly affirms that the up-regulation of GLP-1 signaling with a long-acting agonist in the brain, such as exendin-4, may be a novel and promising strategy in the early treatment of AD, particularly when associated with T2DM or glucose intolerance.

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Figure legends:

Fig. 1 The effect of A β 1-42 and exendin-4 on the spatial learning and memory of rats

1 in the hidden platform test and in the probe trial in the Morris water maze. The
2 latencies (in seconds, A) and distances (in centimeters, B) to search the hidden
3 platform over five consecutive training days are presented for the control, A β 1-42 and
4 exendin-4 groups (n = 10 rats per group). (C and D) A β 1-42 decreased the time and
5 distance in the target quadrant in the probe trial. Histograms showing the percentage
6 of time (C) and distance (D) in the target quadrant. Each point represents the
7 mean $\pm$ SEM of four trials per day. ** $P < 0.01$ compared to the control group.

8 **Fig. 2** Exendin-4 treatments reversed A β 1-42-induced impairment in spatial learning
9 and memory in a dose-dependent manner. The latencies (in seconds, A) and distances
10 (in centimeters, C) to find the hidden platform over five consecutive training days are
11 shown for the A β 1-42 alone group and the co-application of different dosages of
12 exendin-4 (0.02 nmol, 0.2 nmol, 2 nmol) (n = 10 rats per group) plus A β 1-42 group.
13 The histograms (B, D) summarize the effects of the co-application of different
14 dosages of exendin-4 and A β 1-42 on the escape latencies and distances. Probe trials
15 were performed at the 6th training day. The percentages of total time (in seconds, E)
16 and total distance (in centimeters, F) of rats spent in the previous target quadrant are
17 shown. Each column is the mean $\pm$ SEM of four trials per day; a, b, and c represent
18 $P < 0.05$ compared with the A β 1-42 alone group, the 0.02 nmol exendin-4 plus
19 A β 1-42 group, and the 0.2 nmol exendin-4 plus A β 1-42 group, respectively.

20 **Fig. 3** Both A β 1-42 and exendin-4 did not affect the vision and swimming speed of
21 rats. The latencies (in seconds, A) to find the visible platform and swim speeds (B) for
22 the A β 1-42 alone group (n=10), the exendin-4 alone group and the co-application of
23 different dosages of exendin-4 (0.02 nmol, 0.2 nmol, 2 nmol, n = 10 rats per group)
24 plus A β 1-42 group. Each point represents the mean $\pm$ SEM. No significant difference
25 between groups were found ($P > 0.05$).

26 **Fig. 4** Effects of A β 1-42 and exendin-4 on the expression of Bax/Bcl2 and caspase-3
27 were determined in the hippocampus of rats. Plots show that 5 nmol A β 1-42 resulted
28 in a significant decline in the Bcl2 mRNA levels (A) and an increase in Bax mRNA
29 levels (B) by real-time PCR, but this down-regulation of Bcl2 and up-regulation of
30 Bax were reversed by exendin-4 treatments (0.2 nmol, and 2 nmol). (C) Western blot

1 shows that A β 1-42 increased cleaved-caspase-3 protein expression, but this
2 up-regulation was dose-dependently prevented by exendin-4 treatments. The results
3 are presented as the mean $\pm$ SD (n = 6/group). ** P <0.01 compared with the control
4 group, [#] P <0.05 and ^{##} P <0.01 compared with the A β 1-42 group, respectively.

5

Figure(1)

Figure(2)

Figure(3)

Figure4)

A

C

B