A novel dual GLP-1 / GIP receptor agonist alleviates cognitive decline by re-sensitizing insulin signaling in the Alzheimer icv. STZ rat model

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, accompanied by memory loss and cognitive impairments, and there is no effective treatment for it at present. Since type 2 diabetes (T2DM) has been identified as a risk factor for AD, the incretins glucagon-like peptide 1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP), promising antidiabetic agents for the treatment of type 2 diabetes, have been tested in models of neurodegenerative disease including AD and achieved good results. Here we show for the first time the potential neuroprotective effect of a novel dual GLP-1/GIP receptor agonist (DA-JC4) in the icv. streptozotocin (STZ)-induced AD rat model. Treatment with DA-JC4 (10 nmol/kg ip.) once-daily for 14 days after STZ intracerebroventricular (ICV) administration significantly prevented spatial learning deficits in a Y- maze test and Morris water maze tests, and decreased phosphorylated tau levels in the rat cerebral cortex and hippocampus. DA-JC4 also alleviated the chronic inflammation response in the brain (GFAP-positive astrocytes, IBA1-positive microglia). Apoptosis was reduced as shown in the reduced ratio of pro-apoptotic BAX to anti- apoptotic Bcl-2 levels. Importantly, insulin signaling was re-sensitized as evidenced by a reduction of phospho-IRS1^{Ser1101} levels and phospho-Akt^{Ser473} up-regulation. In conclusion, the novel dual agonist DA-JC4 shows promise as a novel treatment for sporadic AD, and reactivating insulin signaling pathways may be a key mechanism that prevents disease progression in AD.

Keywords: Alzheimer's disease; Incretin; Insulin signaling; Apoptosis; Inflammation; tau phosphorylation; Type 2 diabetes mellitus

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder and it is estimated that the population of dementia patient worldwide may reach 131.5 million in 2050 due to longer life-expectancy in the industrialized nations. The pathology of AD is characterized by the presence of neurofibrillary tangles composed of hyperphosphorylated tau protein (p-tau) and amyloid-β peptide accumulation, as well as neuronal loss in different brain regions which are associated with progressive memory loss and cognitive decline [1-3]. Drug therapies for AD are currently restricted and mainly depend on three cholinesterase inhibitors and the receptor inhibitor memantine, but these drugs cannot fundamentally halt or delay the progression of the disease at present [4-7].

Type 2 diabetes mellitus (T2DM) is a risk factor for developing AD [8-10]. Patients with T2DM present with learning and memory deficits [11] and there is an increase an approximately twofold risk of AD [8, 12]. Also, AD shares many pathophysiological features with T2DM, including insulin resistance, inflammatory stress and amyloid-β peptide accumulation [13]. Based on these shared features, insulin resistance is one of the key underlying mechanisms [12, 14, 15].

Incretin hormones, glucagon-like peptide 1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP) which can treat T2DM have been proposed as a novel therapeutic schedule for AD. Extensive preclinical studies show good effects in animal models of AD by reducing memory loss, synapse loss and amyloid plaque load [16-19], decrease the hyperphosphorylation of τ protein [20-23], exert anti-inflammatory function [24-26] and reduce neuronal loss [22, 27, 28]. A pilot study testing the GLP-1 analogue liraglutide in AD patients showed good protective effects in FDG-PET brain scans [29]. Other clinical trials in patients with AD or Parkinson's disease (PD) are currently ongoing [30]. Dual GLP-1 and GIP receptor agonists have been developed to treat T2DM and have shown first positive results in patients with diabetes [31]. We have tested one of these dual agonists named DA-JC1 in the MPTP mouse model of PD with good results [32, 33]. Here, we are testing the newer dual agonist DA-JC4 that has been optimized to cross the blood-brain barrier in the icv. streptozotocin (STZ) rat model of AD. STZ desensitizes insulin signaling in the brain [34, 35] and produces a range of pathological changes that are also found in the brains of AD patients [13, 36, 37], such as inducing cognitive impairment [21, 38-40], chronic inflammation in the brain [41] and enhanced tau protein phosphorylation [22, 42].

We tested the novel dual agonist DA-JC4 in this model and evaluated memory formation, tau

phosphorylation, chronic inflammation, insulin re-sensitization, growth factor and apoptosis cell signaling.

2. Materials and methods

2.1 Drugs

Streptozotocin (STZ) was purchased from Sigma-Aldrich (St Louis, MO, USA). The dual agonist DA-JC4 was obtained from ChinaPeptides (Shanghai, China). The purity of the peptide was 95% which was confirmed by reversed-phase high performance liquid chromatography (HPLC) and the peptide was identified using matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry.

2.2 Animals and experimental groups

Male Sprague-Dawley rats (210-230g) were procured from the Animal Center of Shanxi Medical University. The animals were maintained in plastic cages (5 rats per cage) with the temperature of 25 ± 2◦C and the humidity of 50 ± 10% and a 12 h light-dark cycle. Animals were allowed free access to food and water. All experimental procedures were conducted in accordance with guidelines for Care and Use of Laboratory Animals and approval of the Research and Ethics Committee of the University. At the first stage of experimental design, rats were randomly divided into four groups: 1) Control group: artificial cerebral spinal fluid (aCSF) intra-cerebral ventricular (ICV) injection plus saline intraperitoneal (IP) injection; 2) DA-JC4: aCSF ICV injection plus dual agonists IP injection; 3) STZ group: STZ ICV injection plus saline IP injection; 4) STZ + DA-JC4 group: STZ ICV injection plus dual agonist IP injection.

2.3. Drug administration

Rats in the STZ group received an ICV injection of STZ (3 mg/kg body weight) dissolved in 10 µl aCSF (NaCl 140mM; KCl 3.0mM; $CaCl_2$ 2.5 mM; $MgCl_2$ 1.2 mM; NaH2PO4 1.2 mM, PH 7.4), and the rats in the sham-operation received the same volume of aCSF. After stereotaxic surgery, rats were treated with a daily IP injection of DA-JC4 (10 nmol/kg body weight) or saline for 2 weeks.

2.4. Injection of STZ

We followed the protocol published previously [43]. For stereotaxic surgery, anesthesia was induced with 10% Chloral hydrate (0.3 ml/100g, ip). Animals were placed on stereotaxic frame with the following coordinates (0.8 mm posterior to bregma; 1.5mm lateral to the sagittal suture; 3.6 mm ventral) for the left side of lateral

ventricle injection. STZ injection to the lateral ventricle was carried out with a Hamilton syringe in a 10 microliter volume over 15 s. The needle remained in position for additional 10 minutes to prevent reflux. The rats in the sham-operation group were given the ICV injection of aCSF in the same manner. After surgery, the rats were put in cage individually and allowed to access to food and water freely.

2.5 Behavioral procedure

At the nineteenth day after intracerebroventricular injection of STZ, behavioral estimations were tested in time sequence. The behavioral tests were conducted between 09:00 a.m. and 01:00 p.m. Experiments were carried out in a sound-proof and air-regulated experimental room. Rats were habituated at least 30 min before each test.

2.5.1 Y-maze test

Spatial working memory was investigated by recording spontaneous alternation behavior in a Y-maze apparatus [44]. The maze consisted of three arms (50 × 10 × 20 cm^3 and 120° apart). Each rat was placed at the terminus of one arm and allowed to move freely through the maze for 8 min. When the rat's tail was entirely within the arm, entry was considered to be complete. The apparatus was cleaned with a 10% ethanol solution and then dried with a paper towel after each trial. The total number of arm entries (N) and the sequence of entries were recorded visually. Alternation behavior was defined as successive entries into all three arms on consecutive occasions. The alternation rate (%) = [the total number of alternations/ (N-2) *100] .

2.5.2 Morris water maze test

MWM task was performed to evaluate spatial learning and memory [45]. A black circular water tank with the 150 cm diameter and 60 cm height was used. The pool surrounded by a blue curtain was filled with tap water to a depth of 30 cm, in which the temperature of water was controlled to 28 ± 1 °C. A transparent platform (14 cm in diameter) was located in the midpoint of the target quadrant and submerged 1.5 cm beneath the surface of the water. Movements of rats were monitored and recorded via a video camera located above the pool. Rats were placed randomly in the water facing the wall in one of the four quadrants and allowed to swim freely to find the hidden platform. If a rat failed to find the platform within 120 s at the first day, it would be guided onto the platform for 15 s. A behavior software system (Ethovision 11.5, Noldus Information Technology, Wageningen, Netherlands) was used to record the escape latency of five

consecutive days, the length of time to reach the platform. To control the influence of different animal's athletic ability, the swim speed was also recorded and analyzed. On day 6, the hidden platform was removed and probe trial was performed. The percentage of swimming time spent in the target quadrant was record in this phase (n = 8–10 each group).

2.6 Immunohistochemistry

After the behavior tests, rats (n=4-5) were transcardially perfused with saline and 4% paraformaldehyde. The brains were removed and fixed in 4% paraformaldehyde for 24 h. After fixation, the tissues were dehydrated and embedded in paraffin. 5μm sections were cut with a microtome and stored at 20 ◦C until immunohistochemistry was performed.

Sections were treated with xylene and graded ethanol solutions in turn. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min. Then the sections were pretreated using heat mediated antigen retrieval with citrate buffer. After blocked with 5% BSA, the sections were incubated with p-Tau (Ser396) (rabbit anti- p-Tau (Ser396); 1:1000, Abcam, Cambridge, MA, USA), GFAP (rabbit anti-GFAP; 1:100; Boster Biotechnology Co., Ltd. Wuhan, China) and IBA1 (rabbit anti-IBA1; 1:100; Boster Biotechnology Co., Ltd. Wuhan, China) at 37 ◦C for 2 h. Then they were incubated with biotinylated goat anti-rabbit IgG at 37 ◦C for 30 min and followed by the avidin-biotin peroxidase complex reagent (Boster Biotechnology Co., Ltd. Wuhan, China) at 37 ◦C for 30 min. The peroxidase was visualized with 3, 3-diaminobenzidine (DAB) (Zhongshan Golden Bridge Biotechnology Co., Ltd. Beijing, China). The photomicrographs of all stained sections were captured with a digital camera (Motic BA210) under a Zeiss light microscope and quantitatively analyzed using Image J software (Version 6.0, developed by National Institutes of Health). Stereological rules were applied [46] and analysis was blind to treatment.

2.7 Western blot

The hippocampus and cortex tissue (n=4-5) were dissected and stored at -80 ◦C for immunoblot analysis. These tissues were cut into pieces in cold RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). After 2 h, tissue lysates added with phenylmethanesulfonyl fluoride (PMSF) and phosphotransferase inhibitor were fully homogenized. Then protein supernatant was taken after centrifugation (14 000 × g for 20minutes at 4°C). Protein concentration was measured using BCA protein assay (Boster Biotechnology Co., Ltd. Wuhan, China). Samples mixed with loading buffer to the same concentration were boiled for 5 min.

Samples with equivalent amounts of protein were run on 8%, 10% or 12% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membrane. After blocking the membrane with 5% bovine serum albumin (Boster Biotechnology Co., Ltd. Wuhan, China) in TBST (Tris-buffered saline contains 0.05% Tween-20) for 1 h, the membranes were probed overnight at 4°C with primary antibodies that specifically detect IRS-1 (1:500; Abcam, Cambridge, UK), p-IRS-1 (Ser1101) (1:750; Cell Signaling Technology, Danvers, MA), Akt (1:1000; Cell Signaling Technology, Danvers, MA), p-Akt (Ser473, 1:2000; Cell Signaling Technology, Danvers, MA), Bcl-2 (1:500; Bioworld Technology Co., Ltd. Nanjing, China), Bax (1: 500; Bioworld Technology Co., Ltd. Nanjing, China), and β-Actin (1:5000; Abcam, Cambridge, UK), followed by labeled with secondary antibodies (goat-anti-rabbit IgG-horseradish peroxidase, HRP), 1:5000; (Abcam, Cambridge, UK) at 4 ◦C for 2h. The relative immunoreactive bands were captured with a chemiluminescent imaging system (Sagecreation, Beijing, China), visualized by using ECL-enhanced chemilluminescence (Boster Biotechnology Co., Ltd. Wuhan, China), and digitalized by the image system of Quantity One 4.31 (Bio-Rad, Hercules, CA, USA).

2.8. Statistical analysis

All experiments data were presented as means ± standard error (SEM). Statistical analysis was conducted using GraphPad Prism 5 (Graph-Pad software Inc., San Diego, CA, USA). Statistical significance was considered at $P < 0.05$, the result of acquisition task in MWM was evaluated by two-way ANOVA with repeated measures, and other data were analyzed by one-way ANOVA and Student-Newman-Keuls post-hoc tests.

3. Results

3.1 DA-JC4 improved STZ-induced learning and memory impairment

In the Y-maze test, there was an overall difference between groups (one-way ANOVA, $F=3.931$, $P < 0.05$). As shown in Fig.1A, rats that received STZ alone (0.4708 ± 0.04312) exhibited significant impairments in spontaneous alternation compared with the control rats (0.6811 ± 0.03200) ($P < 0.05$). Moreover, treatment with DA-JC4 (0.6283 ± 0.06798) partially reversed the impairment induced by STZ treatment ($P < 0.05$) as shown in Newman-Keuls post-hoc tests, n=8-10 per group (Fig.1A).

In the Morris water maze test, the escape latency was gradually shortened during the training period. However, there is virtually no difference until on the fourth and fifth day (Figure 1.B). Based on the

two-way ANOVA analysis, the effect of DA-JC4 treatment on escape latency was significant (F=15.20, P<0.0001 for groups). There was no interaction between groups and time (F=0.5319, P=0.8918). The escape latency time in the STZ group (48.61 ± 2.056) was longer than in the control rats (36.67 ± 1.548) and the major difference began to emerge on day 2. (F=6.459, P<0.01). An outstanding difference was found between the control group (22.81 ± 1.498, 20.02 ± 1.387) and the STZ group on day 4 (F=9.438, P<0.001, 22.81 ± 1.498 vs 39.65 ± 2.793) and on day 5 (F=3.618, P<0.05, 20.02 ± 1.387 vs 26.43 ± 2.086). There was a marked decrease in the escape latency in the STZ group and STZ + DA-JC4 group (30.65 ± 3.372, 21.91 ± 1.243). Figure 1.C shows the exploration time of the probe trial that rats spent in the target quadrant. A one-way ANOVA analysis showed an overall difference between groups (F=7.818, P<0.001). Contrary to the escape latency, rats in the STZ group (0.2800 ± 0.01630) resulted in a decrease in spatial memory compared to the control rats (0.4217 ± 0.01696) (P<0.001). Rats that received both STZ and DA-JC4 (0.3920 ± 0.02713) performed better than STZ saline treated rats (P<0.01). In addition, there was no significant difference in swim speed among all four groups (F=1.151, P=0.3422).

3.2 DA-JC4 reduced the levels of phosphorylated tau protein

Overall differences between groups in the cortex and hippocampus were found via one-way ANOVA analysis (F=8.497, p<0.01; F=10.38, P<0.001). Fig 2. I, II show that levels of p-tau in the STZ group (0.2774 ± 0.02585, 0.2794 ± 0.02579) were higher compared to the control group (0.1610 ± 0.01080, 0.167 ± 0.01308) (P<0.01, P<0.01). However, the STZ + DA-JC4 group (0.1941 ± 0.01825, 0.1902 + 0.008825) attenuated the levels of phospho-tau compared with the control group (P<0.01, P<0.01).

3.3 DA-JC4 normalized the STZ -induced increase of the chronic inflammation response

As to the activation of astrocytes, significant differences between groups in the cortex and hippocampus were found (F=30.83, P<0.0001; F=9.867, P<0.01). There was an increase of GFAP-positive astrocytes in the STZ group (8.340 ± 0.4890, 15.32 ± 2.348) than the control group (2.808 ± 0.3406, 6.761 ± 0.2818) (P<0.001; P<0.01), and injection with DA-JC4 alone (2.881 ± 0.4943, 7.174 ± 0.7706) has no effect. Rats that received both STZ and DA-JC4 (5.010 ± 0.5244, 8.820 ± 0.7110) had reduced levels of activated astrocytes. See Fig. 3.

The immunoreactivity of IBA1-positive microglia was observed in the cortex and hippocampus as shown in Fig. 4. I and Ⅱ**.** There was no difference between control group (0.8879 ± 0.04772, 1.244 ± 0.1184) and

DA-JC4 (0.9417 ± 0.04789, 1.217 ± 0.1018) group. The activation of microglia in the STZ group (4.895 ± 0.2610, 4.103 ± 0.4160) was significantly higher than in the control group (P<0.001, P<0.001), and DA-JC4 administration (1.839 ± 0.2617, 2.586 ± 0.2038) decreased the number of IBA1-positive microglia compared to the STZ only group (P<0.001, P<0.01). See Fig. 4.

3.4 DA-JC4 reversed the STZ-induced increase in apoptotic signalling

The results show that the BAX/Bcl-2 ratio did differ significantly among the four different treatment groups in the rat cortex and hippocampus (one-way ANOVA F=26.78, P<0.0001; F=120.1, P<0.0001). Compared with the controls (0.9915 ± 0.01694, 0.8145 ±0.03596), STZ administration (2.387 ± 0.2384, 2.276 ± 0.1191) markedly increased this ratio (P<0.001, P<0.001). In comparison, the increases in BAX/Bcl-2 ratios induced by STZ were reversed when treated with DA-JC4 (1.192 ± 0.06932, 0.8966 ± 0.02022). Also, injection with DA-JC4 alone (1.034 ± 0.1056, 0.7970 ± 0.03804) did not change the level of apoptotic signaling in the brain. See Fig. 5

3.5 DA-JC4 promoted re-sensitization of insulin signaling in the brain

Total protein of Akt and IRS1 did not differ significantly in any of the groups. A one-way ANOVA analysis showed differences between groups (p<0.001). The levels of phosphorylated Akt and IRS1 was changed by the treatment. As seen in Fig. 6, the western blot assay revealed that the levels of phospho-IRS1^{Ser1101} in the STZ treated group (0.04414 ± 0.001197, 0.03244 ± 0.001044) were significantly increased in both the cortex and the hippocampus, compared with the controls (0.03712 ± 0.001724, 0.02815 ± 0.0008825) (P<0.01, P<0.05). However, rats that had received both DA-JC4 and STZ (0.04034 ± 0.0006476, 0.02869 ± 0.0009052) exhibited lower levels of phospho-IRS1^{Ser1101} in the brain than the STZ only rats (P<0.05, P<0.05).

In contrast with the change of phospho-IRS1^{Ser1101}, STZ treatment markedly suppressed the expression of phospho-Akt^{Ser473} (0.1872 ± 0.02649, 0.1540 ± 0.02694) in both the cortex and the hippocampus as compared to the control group (0.3356 ± 0.02061, 0.2929 ± 0.01382) (P<0.01, P<0.01). However, DA-JC4 (0.2940 ± 0.005428, 0.2114 ± 0.01248) moderately attenuated the STZ-induced inactivation of phospho-Akt^{Ser473} (P<0.05, P<0.05).

4. Discussion

In the present study, we investigated the effect of the novel GLP-1/GIP dual receptor agonist DA-JC4 for the first time in the rat model of STZ- induced cognitive impairment. ICV. administration of STZ at low dosage (3 mg/kg body weight) induces learning and memory deficits [21, 38-40, 43]. Consistent with previous findings, we found that learning and memory impairment were induced by the STZ treatment. We also found that DA-JC4 effectively attenuated the spatial working memory deficit induced by STZ. Consistent with previous findings, the phosphorylation of tau protein was enhanced by icv. administration of STZ [42]. DA-JC4 treatment prevented or reversed the phosphorylation of tau. The chronic inflammation response in the brain is a critical element of AD progression [47, 48]. Chronic inflammation generates free radicals and increases the release of pro-inflammatory cytokines that are detrimental to neurons [49, 50]. Consistent with previous findings, the chronic inflammation response was clearly triggered by the treatment with STZ [41]. DA-JC4 effectively reduced the chronic inflammation response induced by STZ, and the ratio of BAX/Bcl-2 was much improved, demonstrating that cellular apoptotic signaling had been reduced by the novel drug. In addition, insulin signaling as shown in IRS-1 phosphorylation levels and Akt phosphorylation furthermore was re-sensitized by DA-JC4. Levels of pIRS-1 were reduced and of pAkt were enhanced to normalise second messenger cell signalling. The novel dual agonist showed good effects in improving these key biomarkers of neuropathological processes.

Insulin de-sensitization has been observed in the brains of people with AD [51-53] and we previously demonstrated that the GLP-1 analogue liraglutide was able to reverse this [54]. Importantly, brain insulin de-sensitization correlates well with cognitive decline [15, 55] and with tau protein phosphorylation levels [56], and the application of insulin via nasal spray showed good improvements in AD patients [57, 58].

The chronic inflammation response in the brain plays a key role in the progression of neurodegenerative disorders [59], and a reduction of this process most likely also plays a part in the overall improvement of the pathophysiology. GLP-1 has anti-inflammatory properties [60], and we have shown previously that single GLP-1 or GIP receptor agonists can reduce the chronic inflammation response in the brain [16, 17, 26].

Novel dual GLP-1/GIP receptor agonists have the advantage of activating two signaling pathways, and have been shown to be superior to single GLP-1 receptor agonists [31, 61]. We previously reported that another dual agonists named DA-JC1 showed good neuroprotective effects in the MPTP mouse model of Parkinson's disease [33, 62]. That dual agonist was not as effective as a single GLP-1 receptor agonist [63] and had to be injected at a higher dose (50nmol/kg ip). In this study, we used the much lower dose of DA-JC4 (10 nmol/kg ip) for the evaluation of the neuroprotective effects of DA-JC4 in the Alzheimer Rat Model induced by STZ, which compares well to the effective dose of liraglutide of 25nmol/kg ip. [26]. This

first study shows promise that such dual agonists may be superior to single incretin analogues, but further dose response tests need to be conducted in order to find the most potent drug dose for this dual agonist. This is of importance as liraglutide has shown first neuroprotective effects in a pilot clinical study of AD patients. Brain activity and energy turnover was assessed by ^{18}FDG-PET brain imaging, and after 6 months of treatment, brain scans revealed that there was significant deterioration in cortical activity in the placebo group. In contrast, the liraglutide treated group showed no deterioration at all [29]. A larger phase II trial is currently ongoing (clinical trial ID NCT01843075). Furthermore, another GLP-1 receptor agonist, exendin-4, has shown good protective effects in a pilot study in patients with Parkinson's disease [64]. It is therefore of interest to develop more efficient drugs that can activate not just GLP-1 but also the sister incretin signaling pathway GIP, which has shown neuroprotective effects on its own in preclinical studies of AD (Duffy and Holscher, 2013; Faivre and Holscher, 2013) and PD [65, 66]. Novel dual agonists therefore may be superior in the clinic for treating chronic neurodegenerative disorders such as AD or PD.

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Figures

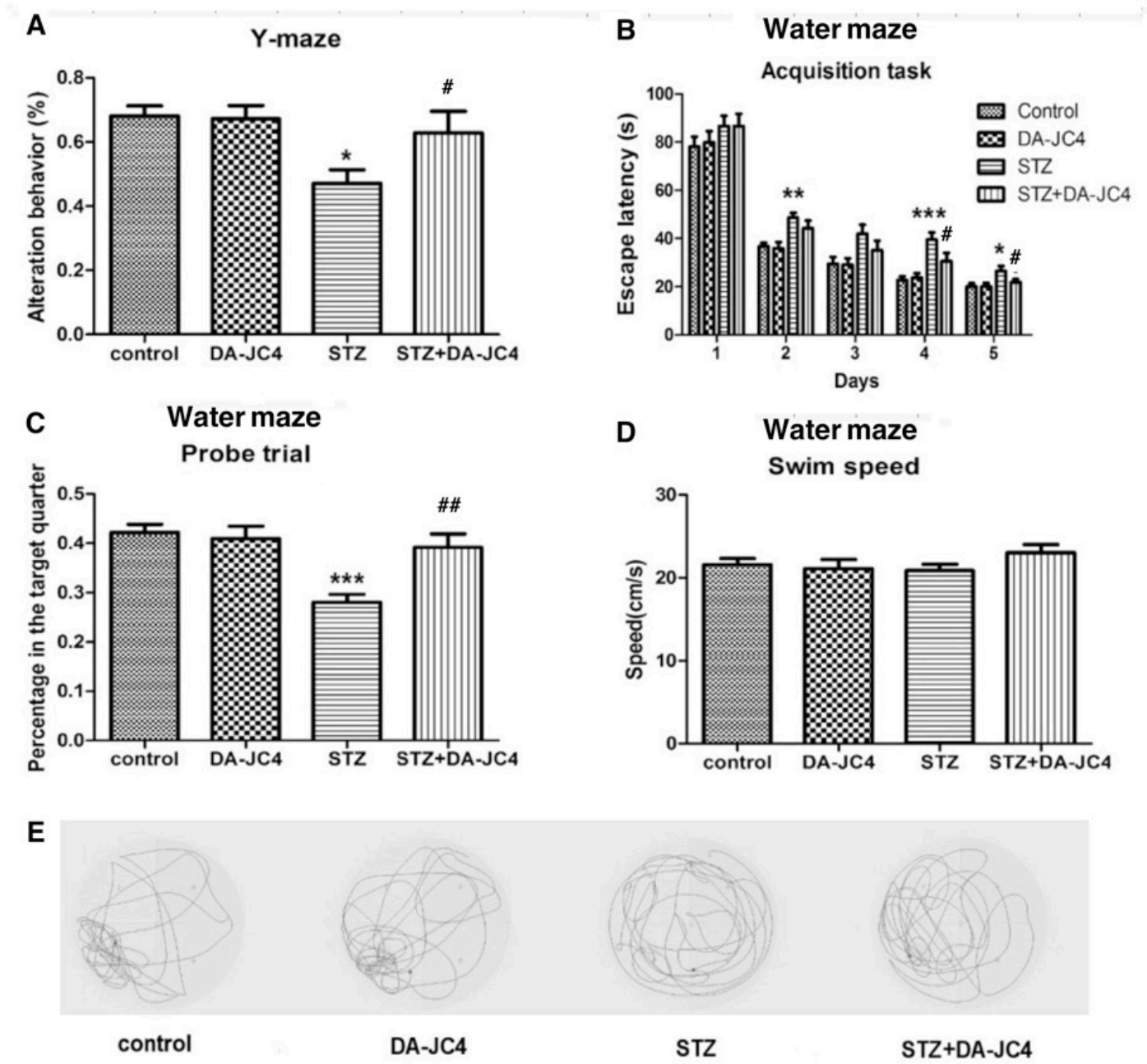

Figure 1. DA-JC4 improved STZ-induced learning and memory impairments. **A.** Spontaneous alternation behavior was significantly decreased in the STZ group compared with the control group (compared with controls, *=P<0.05), and DA-JC4 treatment prevented the STZ-induced learning deficit (compared with STZ group, #=P < 0.05). **B.** Escape latency was significantly increased in the STZ group compared to the control group on day 2, 4, 5 (compared with control, *=P < 0.05; **=P < 0.01; ***=P<0.001), and DA-JC4 treatment partially attenuated the impairment induced by STZ. **C.** STZ treatment significantly decreased the percent of time each rat spent in the target quadrant (P < 0.001), and DA-JC4 treatment availably prevented the STZ-induced memory deficit (P < 0.01). **D.** Swim speed did not differ between groups. **E.** Sample tracks in the probe test.

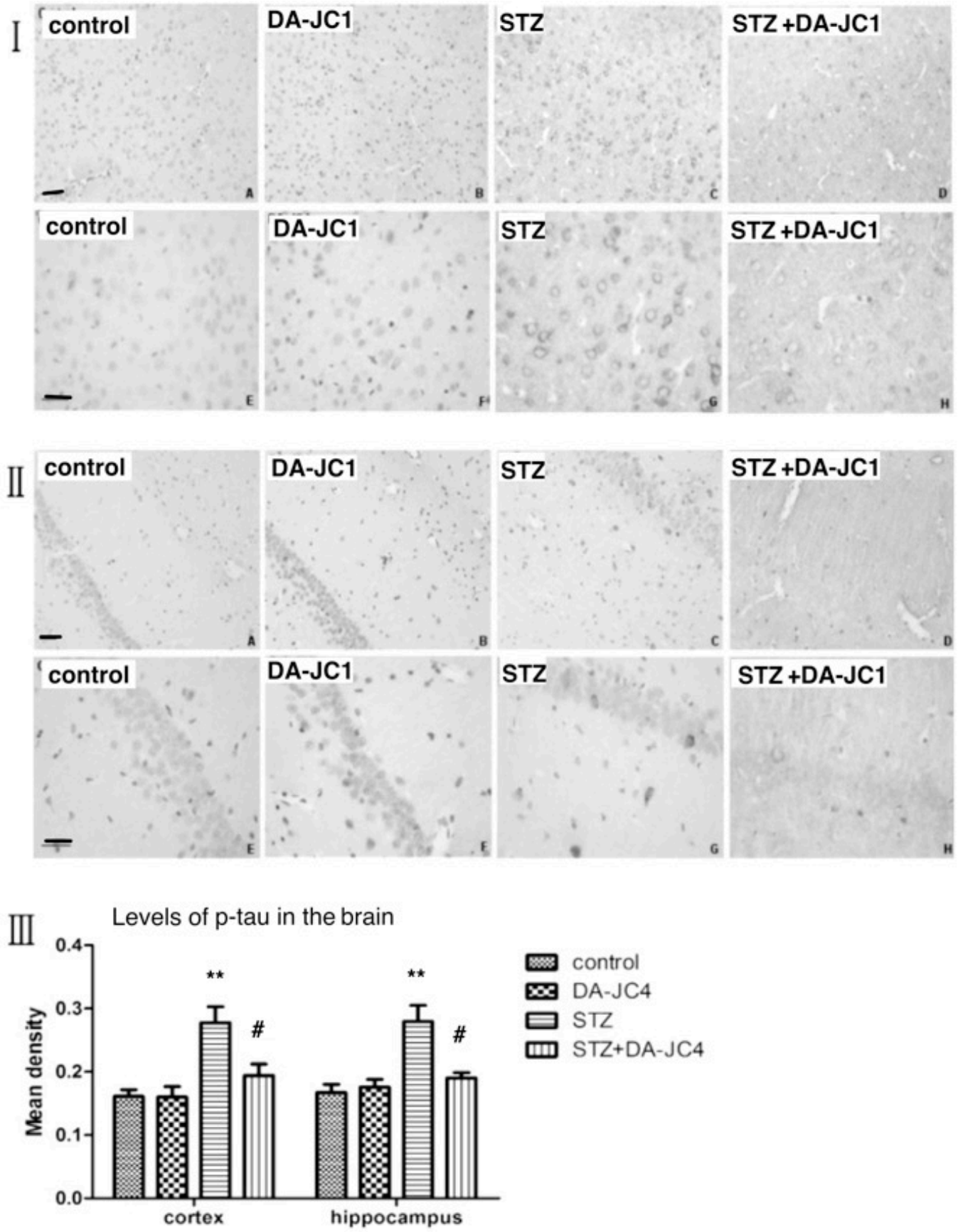

Figure 2. DA-JC4 reduced the levels of phospho-tau protein in the rat cerebral cortex (Ⅰ) and hippocampus (Ⅱ) (**A–D**, scale bar = 100 μm; **E-H**, scale bar = 50 μm). The expression of phospho-tau was increased following treatment with STZ (**=P < 0.01; compared with control), and reduced by DA-JC4 (##=P < 0.01, compared with STZ group).

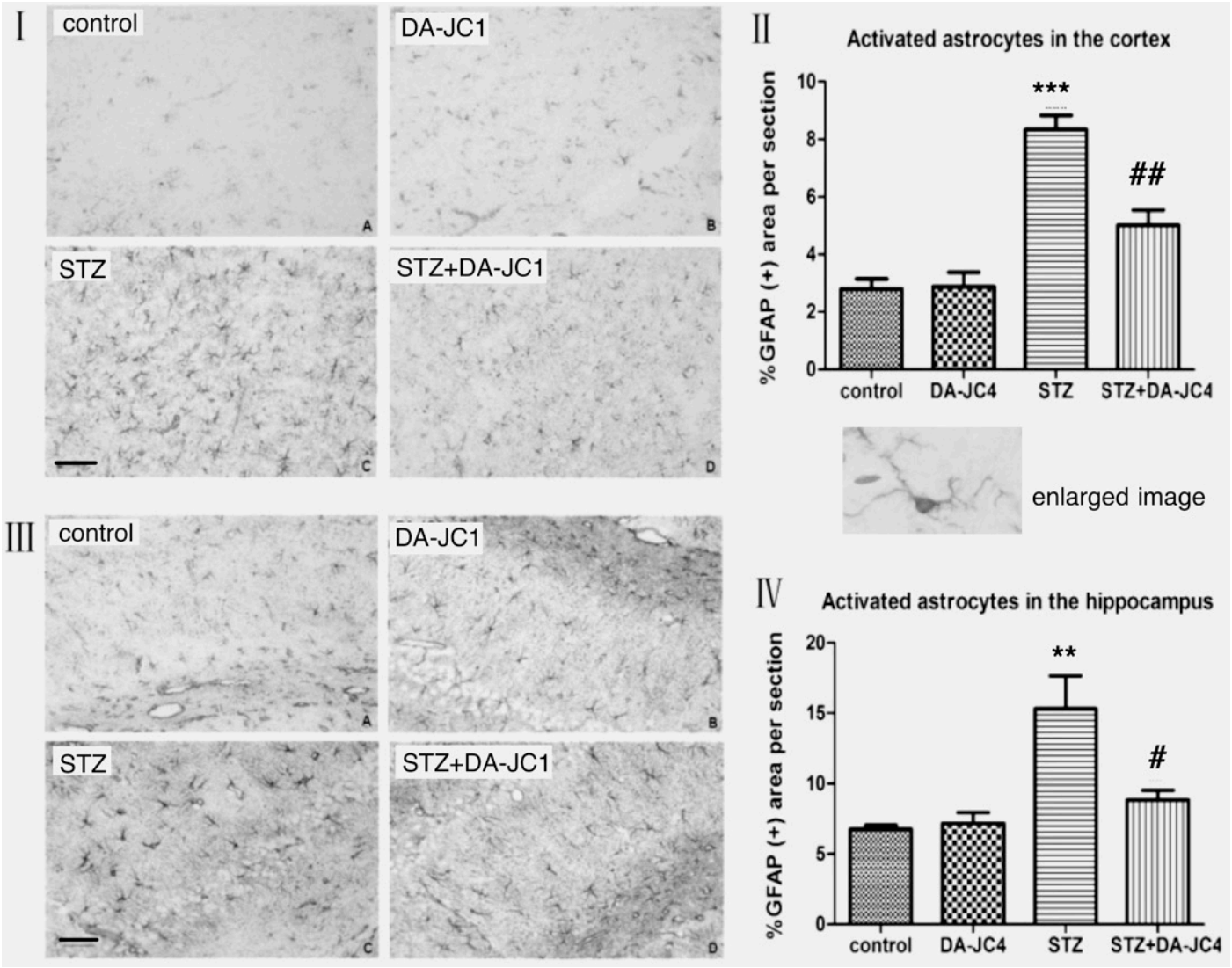

Figure 3. DA-JC4 attenuated the STZ - induced the activation of astrocytes in the rat cerebral cortex (Ⅰ) and hippocampus (Ⅲ) (A-D, scale bar = 100 μm). Quantification of the area of GFAP positive stain in the rat cerebral cortex (Ⅱ) and hippocampus (Ⅳ) demonstrated that STZ injection induced astrogliosis (*** =P < 0.001; ** = P < 0.01 compared to controls). DA-JC4 partially reversed this (### =P < 0.001; ## =P < 0.01 compared with STZ group).

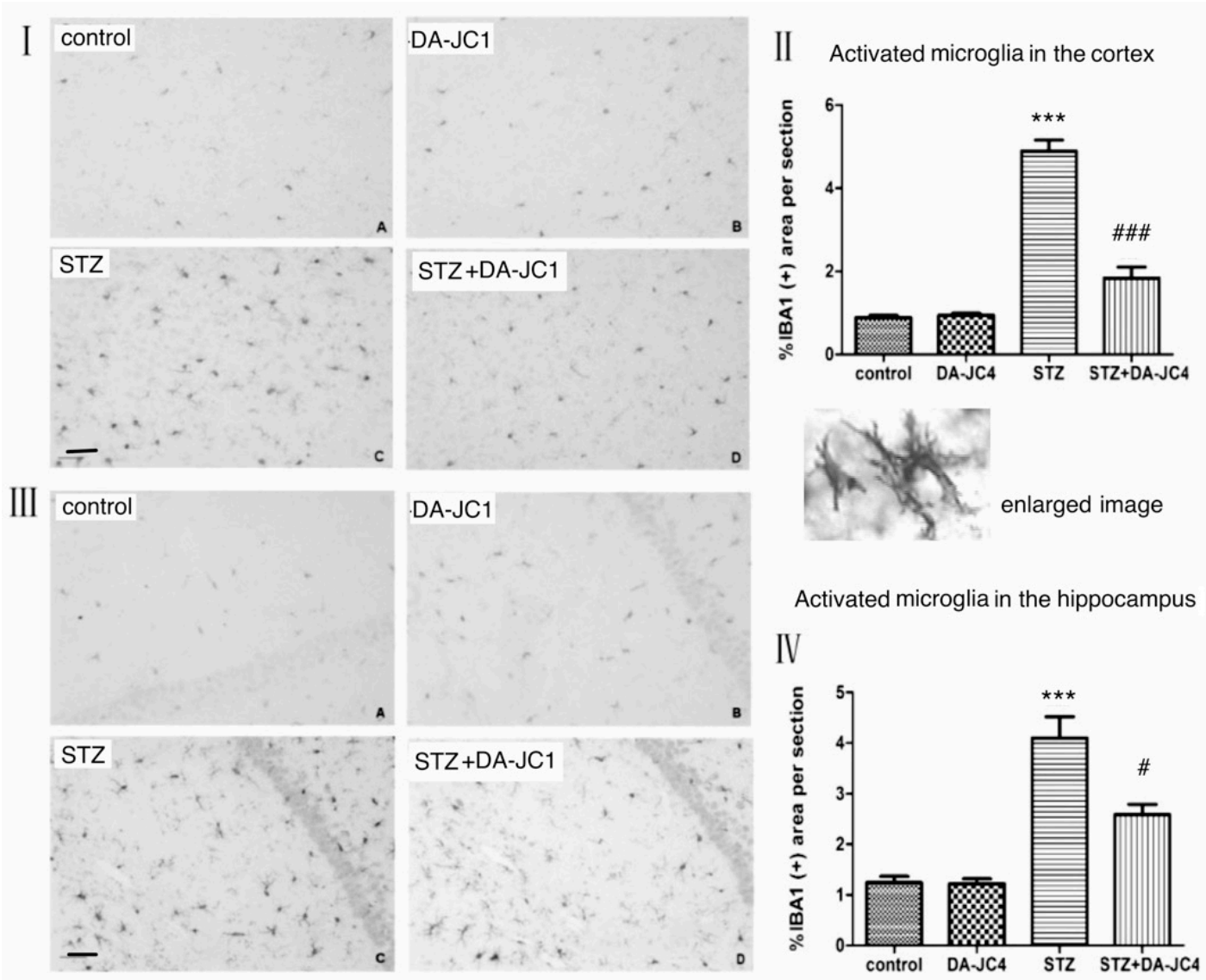

Figure 4. DA-JC4 attenuated the STZ- induced activation of microglia in the rat cortex (Ⅰ) and hippocampus (Ⅲ) (**A-D**, scale bar = 100 μm). Quantification of the area of IBA-1 positive stain in the cerebral cortex (Ⅱ) and hippocampus (Ⅳ) revealed that STZ injection induced microgliosis (*** =P < 0.001 compared to controls). DA-JC4 partially reversed this (### =P < 0.001; ## =P < 0.01 compared with the STZ group).

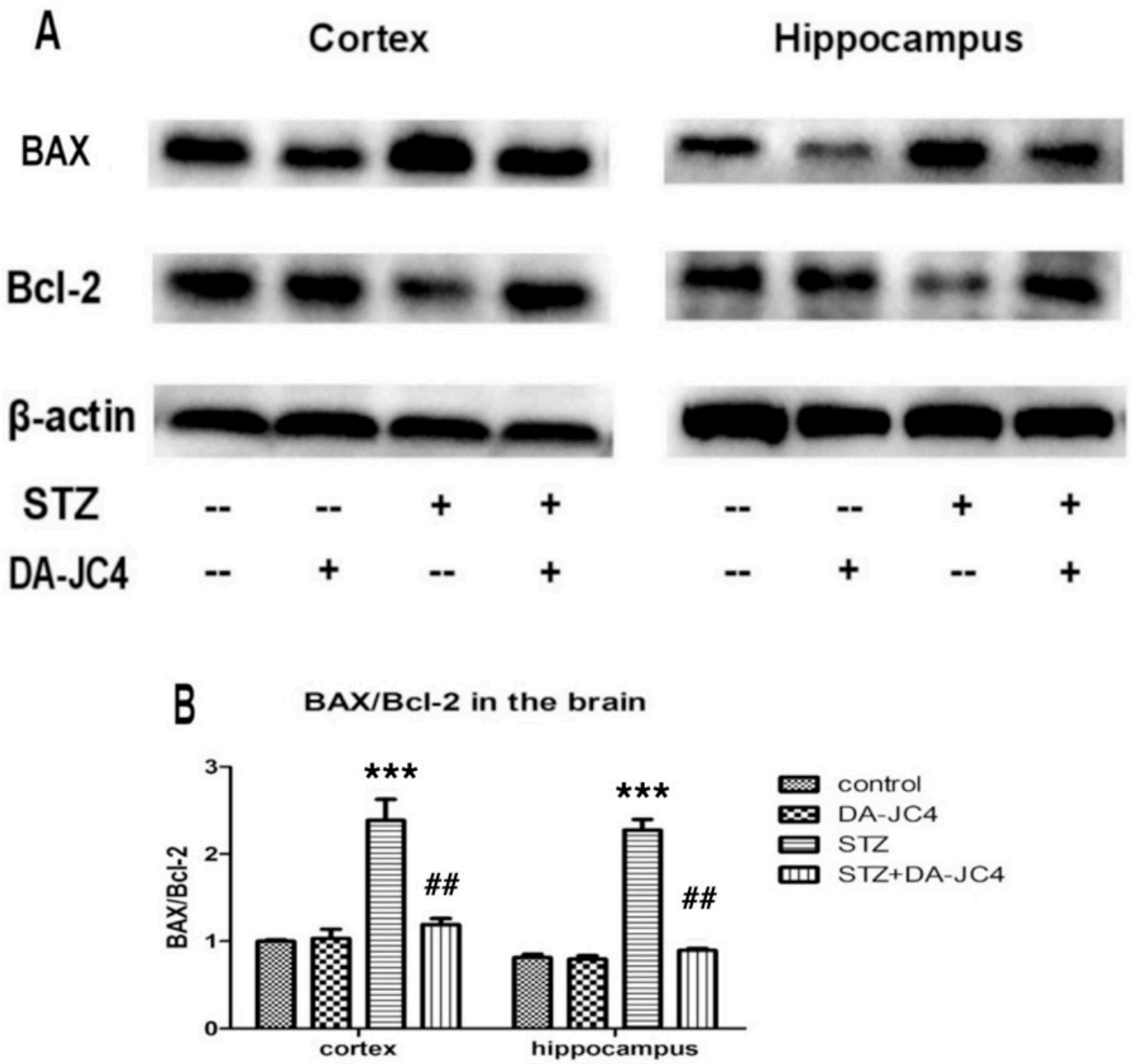

Figure 5. DA-JC4 reduced the STZ-induced apoptotic signaling in the cortex and hippocampus. **A.** Western blot assay of BAX and Bcl-2 in response to STZ and DA-JC4. **B**. Western blot quantification of protein levels of BAX and Bcl-2. A one-way ANOVA found overall differences between groups. Post-hoc analyses showed differences compared to controls (***=P<0.001 compared to controls; ###-=P<0.001 compared to STZ group). Data are represented as mean ± SEM, and show data of 4 blotting repetitions.

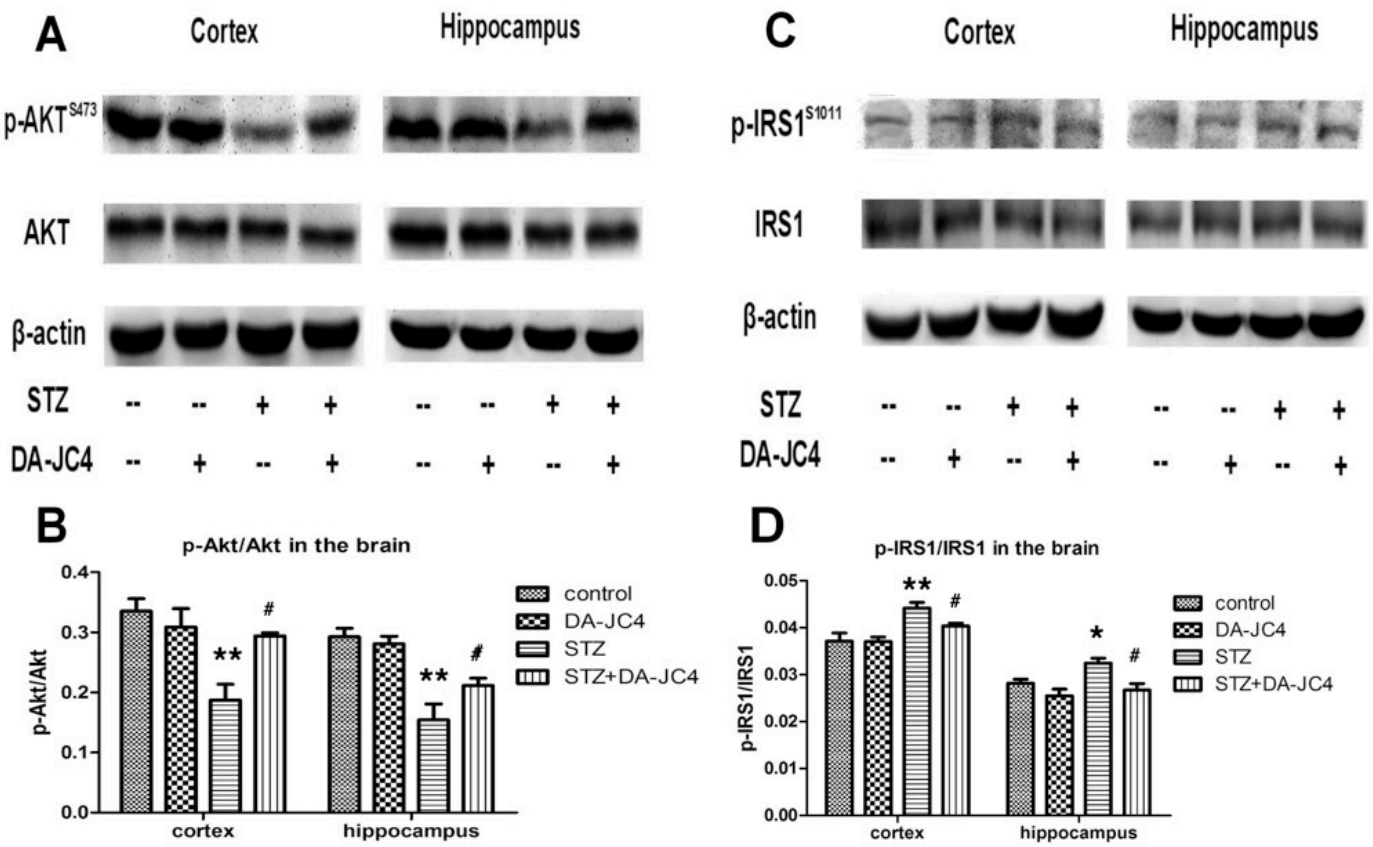

Figure 6. DA-JC4 re-sensitized insulin signaling in the brain. The effects of DA-JC4 on protein expression levels of p-Akt and p-IRS-1 in the cortex and the hippocampus as measured by western blot assay (**A**, **C**). Quantification of p-Akt and p-IRS-1 in the cortex and the hippocampus (**B**, **D**). A one-way ANOVA found overall differences for B ($F= 8.020$, $P<0.01$; $F=13.69$, $P<0.01$) and D ($F= 7.724$, $P<0.01$; $F= 6.841$, $P<0.01$). Post-hoc analyses showed differences compared to controls (*=$P<0.05$; **=$P<0.01$). Data are represented as mean ± SEM, and show data of 4 blotting repetitions.