

RESEARCH PAPER

PPAR γ agonists regulate bidirectional transport of amyloid- β across the blood–brain barrier and hippocampus plasticity in *db/db* mice

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BACKGROUND AND PURPOSE

There is emerging evidence suggesting that abnormal transport of amyloid- β (A β) across the blood–brain barrier (BBB) is involved in diabetes-associated cognitive decline. We investigated whether PPAR γ agonists restore A β transport across the BBB and hippocampal plasticity in *db/db* mice.

EXPERIMENTAL APPROACH

Efflux and influx of A β across the BBB were determined by stereotaxic intra-cerebral or i.a. infusion of [¹²⁵I]-A β _{1–40} respectively. Receptor for advanced glycation end products (RAGE) and low-density lipoprotein receptor-related protein 1 (LRP1), which are involved in A β influx and efflux, PPAR γ and NF- κ B p65 at the BBB, as well as hippocampal A β , caspase-3, Bax and Bcl-2 were assayed by Western blot, immunohistochemistry and RT-PCR. *In vivo*, hippocampal LTP was recorded, and Morris water maze and Y-maze tasks were performed.

KEY RESULTS

Treatment with PPAR γ agonists, rosiglitazone (0.8 mg·kg⁻¹) and pioglitazone (9.0 mg·kg⁻¹), for 6 weeks significantly increased A β efflux and decreased A β influx across the BBB in *db/db* mice. Concomitantly, they decreased hippocampal A β _{1–40} and A β _{1–42}, suppressed neuronal apoptosis, as indicated by decreased caspase-3 activity and increased ratio of Bcl-2/Bax, and increased hippocampal plasticity, characterized by an enhanced *in vivo* LTP and better performance in behavioural tests. Furthermore, the PPAR γ agonists induced the expression of LRP1 gene by activation of PPAR γ and suppressed RAGE gene expression by inactivation of NF- κ B signalling at the BBB of *db/db* mice.

CONCLUSIONS AND IMPLICATIONS

PPAR γ agonists modify abnormal A β transport across the BBB and this is accompanied by amelioration of β -amyloidosis and an improvement in hippocampal plasticity in diabetic mice.

Abbreviations

AD, Alzheimer's disease; A β , amyloid- β ; AGEs, advanced glycation end products; BBB, blood–brain barrier; ID, injected dose; LRP1, low-density lipoprotein receptor related protein 1; MWM, Morris water maze; OCT, optimal cutting temperature; PS, population spike; RAGE, receptor for advanced glycation end products; STZ, streptozotocin; TCA, trichloroacetic acid

Tables of Links

TARGETS	
Nuclear hormone receptors^a	Other protein targets
PPAR γ	Bax
Enzymes^b	Bcl-2
Caspase 3	FABP4
	RAGE

LIGANDS	
Amyloid β (A β ; β amyloid)	Insulin
BDNF	Pioglitazone
Glucose	Rosiglitazone

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (^{a,b}Alexander *et al.*, 2013a,b).

Introduction

There is a mass of evidence suggesting that diabetes impacts cognitive function (Ott *et al.*, 1999; Roberts *et al.*, 2014), and epidemiological studies have also indicated that patients with diabetes are at a higher risk of developing Alzheimer's disease (AD) (Kopf and Frölich, 2009; Maher and Schubert, 2009; Morris *et al.*, 2014). Cognitive deficits have also been reported in studies on rodent models of diabetes such as the *db/db* mice, which exhibit the characteristics of obesity, insulin resistance, hyperglycaemia and also have increased levels of corticosterone in the circulation due to a mutation that inactivates the leptin receptor (Hummel *et al.*, 1996). These *db/db* mice have decreased levels of brain-derived neurotrophic factor (BDNF) and increased inflammatory cytokines (IL-1 β , IL-6 and TNF- α) in the hippocampus, which cause impairments in hippocampus-dependent spatial recognition memory, perforant path synaptic plasticity and adult neurogenesis (Stranahan *et al.*, 2008; Hong *et al.*, 2009; Diné *et al.*, 2011). Interestingly, there is accumulating evidence suggesting that diabetes affects the transport activity of the blood–brain barrier (BBB), which might be a causative factor in cognitive impairment and AD (Maeng *et al.*, 2007; Huber, 2008; Hong *et al.*, 2009; Liu *et al.*, 2009). The BBB is primarily built up of brain endothelial cells, astrocyte end-feet, pericytes, perivascular macrophages and a basal membrane. It is critical for maintaining brain function through regulating the transport of materials between the CNS and the peripheral circulation. An impairment of the influx and efflux transporters existing at the BBB may contribute to the pathogenesis of AD (Ueno *et al.*, 2010).

Two major proteins, low-density lipoprotein receptor-related protein 1 (LRP1) and the receptor for advanced glycation end products (RAGE), are involved in receptor-mediated amyloid β (A β) trafficking across the BBB (Deane *et al.*, 2004; Zhang and Lee, 2011). RAGE, present at the lumina of the endothelial cytoplasm, is thought to transport blood A β into the brain, while LRP1, located at the ablumina of the endothelial cytoplasm, transports A β out of the brain (Shibata *et al.*, 2000; Deane *et al.*, 2003). These transporters

at the BBB play a critical role in regulating A β homeostasis in the brain. We have previously shown that the up-regulation of RAGE or down-regulation of LRP1 at the BBB contribute to cerebral A β accumulation in streptozotocin (STZ)-induced diabetic mice (Hong *et al.*, 2009; Liu *et al.*, 2009), which initiates the pathological cascades involved in the neuronal and neurovascular dysfunctions. Thus, rectifying the abnormal A β transport across the BBB is an important strategy for protecting neurons from damage caused by intracerebral A β accumulation under diabetic conditions. PPARs are ligand-dependent transcription factors. PPAR γ activation is known to modulate lipid and glucose metabolism and to increase insulin sensitization. Highly selective PPAR γ agonists such as pioglitazone and rosiglitazone are currently approved for a p.o. monotherapy in patients with type 2 diabetes (Sood *et al.*, 2000). Our recent research showed that pioglitazone reverses memory impairments in high-fat diet plus STZ-induced diabetic mice or STZ-induced diabetic mice, and this involved inhibition of cerebral A β accumulation (Jiang *et al.*, 2012; Liu *et al.*, 2013). However, the mechanism by which these PPAR γ agonists reduce cerebral A β is not well understood. Here, we investigated the effects of these PPAR γ agonists on A β influx and efflux and hippocampal plasticity in *db/db* mice.

Methods

Animals and experimental protocol

Male C57BL/KsJ *db/db* (BKS. Cg-m +/+ Lep^{db}/J) mice (approx. 6 weeks old, weighing 30–40 g; Model Animal Research Center of Nanjing University, China), and their age-matched lean controls *db/m* mice were used for the experiments. The former strain of mice is obese and known to develop type 2 diabetes, followed by diabetic kidney disease (Zhang *et al.*, 2013). The experimental procedures are in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), and the experimental protocol was approved by the Institutional Review Committee for the use of Animal Subjects of China Pharmaceutical University. All studies

involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). All animals were maintained in a well-ventilated holding room at constant humidity of $55 \pm 5\%$ and temperature of $24 \pm 1^\circ\text{C}$ under a 12 h light/12 h dark cycle (lights on 07:00–19:00 h) with free access to water and standard laboratory chow.

db/db mice with similar degrees of body weight and hyperglycaemia were randomly divided into *db/db* vehicle (*db/db* + Veh), 0.8 mg·kg⁻¹ rosiglitazone (*db/db* + Rsg 0.8) and 9.0 mg·kg⁻¹ pioglitazone (*db/db* + Pio 9.0) groups. Age-matched normal homozygote mice (*db/m* + Veh, $n = 29$) were administered the vehicle p.o. The total number of animals in each group was 29 to 31 mice. Rosiglitazone or pioglitazone were dissolved in 0.5% CMC-Na. After intragastric administration of the drugs for 6 weeks, some of the mice from each group were used for stereotaxic intra-cerebral infusion ($n = 3$ –4 for each group) or i.a. infusion ($n = 3$ –4 for each group) of [¹²⁵I]-Aβ_{1–40}, others were assigned for *in vivo* LTP recording ($n = 4$ –5 for each group). The rest of the mice were used for the behavioural tests. The expression of RAGE, LRP1, PPARγ and NF-κB p65 was determined in the isolated brain capillaries and hippocampal Aβ, pro- and cleaved caspase-3, Bax, Bcl-2 and advanced glycation end products (AGEs) were also detected ($n = 6$ –8 for each group). We also monitored the fasting blood glucose and insulin levels.

Brain clearance studies

Brain interstitial fluid mediated clearance of [¹²⁵I]-Aβ_{1–40} was determined simultaneously with the reference marker [¹⁴C]-inulin as has been described previously (Shibata *et al.*, 2000; Hong *et al.*, 2009). Briefly, a stainless steel guide cannula was stereotaxically inserted into the right caudate nucleus of anaesthetized mice (i.p. chloral hydrate, 350 mg·kg⁻¹). The coordinates for the cannula were 1.9 mm lateral to bregma and 0.9 mm anterior, and 2.9 mm below the dural surface (Shibata *et al.*, 2000; Cirrito *et al.*, 2005). Mice were allowed to recover for 1 week after surgery. After this period, mice were re-anaesthetized to conduct radiotracer studies. Tracer fluid (0.5 μl) containing 60 nM [¹²⁵I]-Aβ_{1–40} was injected over a period of 5 min, along with 12 nM of [¹⁴C]-inulin, at 30 min after anaesthesia. After injecting these tracers, blood and brain samples were collected within 30 min and their radioactivity was analysed. Trichloroacetic acid (TCA) was added to the plasma and brain samples at a final concentration of 10% and then the samples were centrifuged at 4°C, 24 840 g for 10 min. A γ counter was used to analysis radioactivity in the supernatant and pellet fractions. Based on the TCA analysis, [¹²⁵I]-Aβ_{1–40} injected into the brain was more than 97% intact and several indices including % [¹²⁵I]-Aβ_{1–40} recovered in the brain, the percentage of BBB clearance and the percentage of injected dose (ID) mL⁻¹ plasma were determined. The percentage of radioactivity remaining in the brain after microinjection was determined as

$$\%^{125}\text{I-A}\beta_{1-40}\text{recovery in brain} = N_{\text{b(A}\beta)} / N_{\text{i(A}\beta)} \times 100$$

and the percentage of BBB clearance of [¹²⁵I]-Aβ_{1–40} was calculated as

$$\% \text{ BBB clearance} = \left[\left(1 - N_{\text{b(A}\beta)} / N_{\text{i(A}\beta)} \right) - \left(1 - N_{\text{b(inulin)}} / N_{\text{i(inulin)}} \right) \right] \times 100$$

and then distribution of [¹²⁵I]-Aβ_{1–40} in the blood was expressed as % injected dose mL⁻¹ plasma

$$\% \text{ ID/mL plasma} = \left(^{125}\text{I-A}\beta_{1-40} \text{ in plasma/mL plasma} \right) / \left(N_{\text{i(A}\beta)} \right) \times 100$$

where N_i is the radioactivity injected into the brain, and N_b is the radioactivity remaining in the brain at the end of the experiment. In all calculations, cpm is counts per minute for TCA-precipitated ¹²⁵I radioactivity and dpm is disintegration per minute for [¹⁴C]-inulin. Inulin, is a metabolically inert polar reference marker and is neither transported across the BBB nor retained by the brain.

Brain perfusion technique

This method was conducted to evaluate the influx of radiolabelled ligands across the BBB and has been described previously (Deane *et al.*, 2003; Liu *et al.*, 2009). Briefly, after the mice had been anaesthetized by an i.p. injection of chloral hydrate, the right common carotid artery of mice was connected to an extracorporeal perfusion circuit and perfused with oxygenated medium containing D-glucose, electrolytes, dextran (formula weight 70,000, 48 g·L⁻¹) and sheep red cells at 1 mL·min⁻¹. Then 4.5 nmol·L⁻¹ of [¹²⁵I]-Aβ_{1–40} was infused i.a. simultaneously with [¹⁴C]-inulin at 0.1 mL·min⁻¹. Electrolytes, pH, blood gases, arterial BP and perfusion pressure were kept within physiological range. Mice were killed, and their brains were used to analyse radioactivity at a preconcerted time (10 min). Trichloroacetic acid sedimentation was used to evaluate the integrity of ¹²⁵I-Aβ_{1–40}. The permeability surface area product of the [¹²⁵I]-Aβ_{1–40} was determined with [¹⁴C]-inulin, using the following calculation.

$$\text{Permeability surface area product} = \left\{ \left[\left(^{125}\text{I cpm per g brain} \right) \times \text{TCA} \div \left(^{125}\text{I cpm per mL plasma} \right) \times \text{TCA} \right] - \left(^{14}\text{C dpm per g brain} \right) \div \left(^{14}\text{C dpm per mL plasma} \right) \right\} \div T$$

where T is the infusion time.

Isolation of brain microvessels

Brain microvessels were isolated and prepared as described previously (Singh *et al.*, 2013). Mice were anaesthetized i.p. with chloral hydrate (350 mg·kg⁻¹) and then decapitated, then pial vessels and the cerebral cortex were removed and chopped into pieces in an ice-cold dish. These were then

homogenized in a 5 mL handheld glass dounce tissue grinder with 3.5-fold excess volume of ice-cold buffer solution containing (in mM): sodium pyruvate (1), HEPES (15), glucose (10), NaHCO₃ (25), KH₂PO₄ (1.2), CaCl₂ (2.5), KCl (4.7), NaCl (130) and MgSO₄ (1.2), at pH 7.4. The homogenate was suspended in an equal volume of 26% dextran (MW 64 000–76 000), spun at 5800 *g* for 15 min, and the pellet (brain vessels) was carefully separated from the supernatant. The pellet was then resuspended in an ice-cold buffered solution, filtered using a 100 μ m filter, and the filtrate passed through a 40 μ m filter. The unfiltered microvessels were harvested by washing into a low binding tube and resuspended in an ice-cold buffered solution. The microvessels were then washed three times with 1.5 mL ice-cold buffered solution and then resuspended in an ice-cold lysis buffer, sonicated, centrifuged at 20 000 *g* for 20 min, and supernatant was used for Western blot or RT-PCR analysis.

Behavioural assessments

The Morris water maze (MWM) was used to assess spatial and long-term memory and the Y-maze test for the evaluation of working and short-term memory. These behavioural tests were carried out as described previously (Tang *et al.*, 2013; Tang *et al.*, 2014). Furthermore, the general locomotor activity of the mice in this study was also assessed by use of an open field test, as described previously (Wang *et al.*, 2014). More detailed descriptions of these behavioural tasks can be seen in the Supporting Information.

In vivo hippocampal LTP recording

Mice were anaesthetized with i.p. urethane (1.0 g·kg⁻¹) and then immobilized on a stereotaxic frame (SR-5, Narishige, Japan) as described previously (Tang *et al.*, 2014). A stimulating electrode and a recording glass pipette were inserted into the perforant path and dentate gyrus of the dorsal hippocampus respectively. Coordinates for the stimulating electrodes (tungsten with Teflon coating; Bilaney) were 3.0 mm from the midline, 3.8 mm caudal to bregma and 1.5 mm below the dural surface and for the recording electrode, 1.4 mm from the midline, 2 mm caudal to bregma and 1.5 mm below the dural surface. The responses evoked were filtered at 1/60 Hz and amplified, 100 μ s pulses of 0.3 mA until the appearance of a population spike (PS). Input–output curves were firstly generated so as to confirm the maximal amplitude of the PS, and then the intensity of the stimulus was set at a level that evoked a PS between 55% and 65% of the maximum amplitude. The amplitude of the PS was collected and averaged every 5 min. High-frequency stimulation, eight sessions of 400 μ s stimuli at 400 Hz with a 10 s interval, was applied to induce LTP which was measured as normalized PS amplitude (%). All stimulation and recording were performed by an online computerized oscilloscope–stimulator and data analysis interface system. The percentage of the ratio of absolute PS amplitude to basal value was used to represent the PS amplitude.

Data analysis

Data shown are expressed as mean \pm SEM. In particular, a repeated measure ANOVA with 'group' as the between-subject factor and 'days' as the within-subject factor were used to

analysis group differences in the MWM escape latencies. The other data were analysed by a one-way ANOVA followed by a Dunnett's *post hoc* analysis for multiple comparisons. The level of statistical significance was $P < 0.05$. All analyses were carried out using SPSS 20.0.

Materials

Rosiglitazone and pioglitazone were both purchased from Jiangsu Hengrui Medicine Co. Ltd. (Nanjing, China). Antibodies were obtained from different companies: anti-A β _{1–40}, anti-A β _{1–42} and anti-RAGE from Abcam Technology Co., Ltd. (Hong Kong); anti-nuclear factor κ B (NF- κ B) p65 and anti-PPAR γ from Cell Signaling Technology, Inc. (Boston, MA, USA); Anti-LRP1 from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany); anti-histone H3, anti- β -actin and secondary antibodies from Bioworld Technology Co., Ltd. (Minneapolis, MN, USA). Streptavidin-biotin complex immunohistochemistry kit was purchased from Boster Biotechnology Co., Ltd. (Wuhan, China). Mouse insulin and AGES ELISA kit were purchased from Cusabio Biotech Co., Ltd. (Wilmington, DE, USA). Coomassie (Bradford) protein assay kit and Glucose Oxidase Kit were purchased from Nanjing Jiancheng Biotech Institute (Nanjing, China). Nucleoprotein extraction kit was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Human A β _{1–40} protein fragment was purchased from Sigma-Aldrich (USA). Na^[125I] and [¹⁴C]-inulin (5,000 Da, 1.5 μ Ci·mg⁻¹) were purchased from Chengdu Gaotong Isotope Co., Ltd. (Chengdu, China) and American Radiolabeled Chemicals, Inc. (USA) respectively. Human [¹²⁵I]-A β _{1–40} was synthesized at Jiangnan Institute of Nuclear Medicine (Wuxi, China). More than 99% of [¹²⁵I]-A β _{1–40} (4,330 Da; 70 μ Ci· μ g⁻¹) radioactivity was present in the form of nonoxidized monomeric peptide. Aliquots of radiolabelled A β _{1–40} were kept at –20°C for a maximum of 4 weeks before use. All the other chemicals were of analytical grade and commercially available.

Results

PPAR γ agonists increase [¹²⁵I]-A β _{1–40} efflux across the BBB in *db/db* mice

To observe the changes in A β efflux across the BBB in *db/db* mice, we carried out brain clearance studies. As shown in Figure 1, the efflux of [¹²⁵I]-A β _{1–40} from the brain at 30 min was markedly reduced in *db/db* mice compared with that of the control *db/m* group, as indicated by a significant decrease in the percentage of [¹²⁵I]-A β _{1–40} BBB clearance ($P < 0.01$; Figure 1A) and increase in the percentage of [¹²⁵I]-A β _{1–40} recovery in brain ($P < 0.01$; Figure 1B). Moreover, intact [¹²⁵I]-A β _{1–40} was shown to be present in plasma within 30 min of its administration to the brain and its level was markedly lowered in plasma ($P < 0.01$; Figure 1C) from the *db/db* mice compared with that of the *db/m* mice. Rosiglitazone or pioglitazone administration markedly increased the distribution of [¹²⁵I]-A β _{1–40} in plasma (both $P < 0.05$) and the percentage of [¹²⁵I]-A β _{1–40} BBB clearance (both $P < 0.01$), while the percentage of [¹²⁵I]-A β _{1–40} recovered in the brain significantly decreased (both $P < 0.01$).

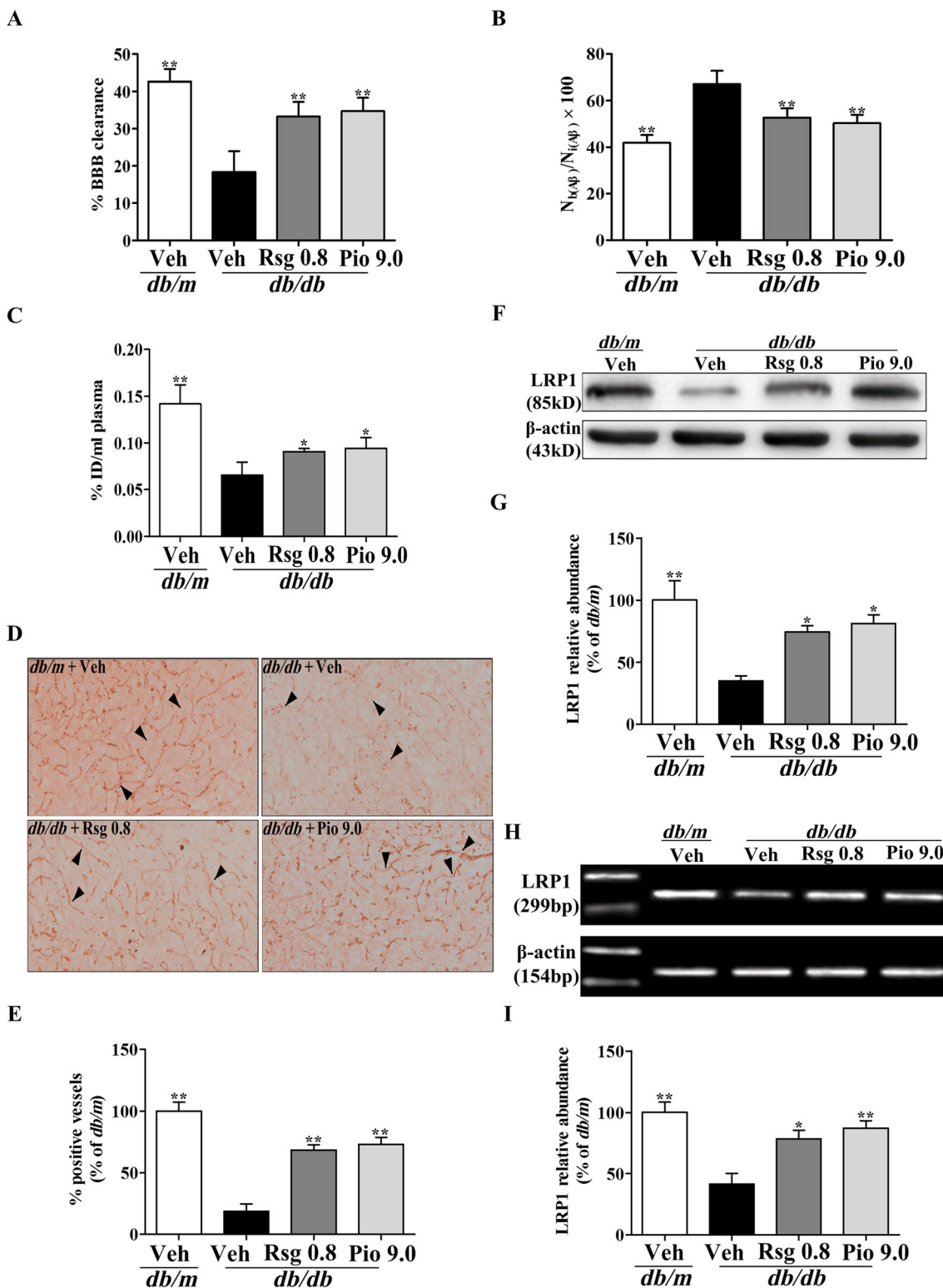


Figure 1

PPAR γ agonists increase [125 I]-A β_{1-40} efflux and LRP1 expression at the BBB in *db/db* mice. [125 I]-A β_{1-40} and [14 C]-inulin were injected simultaneously, the percentage of [125 I]-A β_{1-40} BBB clearance (A) and the levels of [125 I]-A β_{1-40} in the brain (B) and plasma (C) were determined by γ counting after 30 min. (D) Representative immunohistochemical microphotographs of LRP1, (E) analysis for % of LRP1-positive vessels (arrow, brain microvessels positive LRP1), (F) representative immunoblot detected by Western blot, (G) protein quantification, (H) LRP1 mRNA expression and (I) mRNA quantification of expressed as a percentage of the *db/m* are shown. Original magnification is 200 \times . Values shown are expressed as mean \pm SEM; $n = 3-4$. * $P < 0.05$, ** $P < 0.01$ versus *db/db* + Veh group.

PPAR γ agonists increase LRP1 expression at the BBB in *db/db* mice

LRP1 has been shown to mediate the transcytosis of A β out of the brain across the BBB (Shibata *et al.*, 2000; Hong *et al.*, 2009). To further determine whether the increased A β efflux across the BBB induced by the PPAR γ agonists results from the up-regulation of LRP1 expression, we determined LRP1 expression at the BBB in *db/db* mice. Both vascular immunohistochemical staining and Western blot analysis of the isolated brain microvessels showed that LRP1 protein was significantly decreased in *db/db* mice treated with vehicle but markedly increased in *db/db* mice treated repeatedly with

rosiglitazone or pioglitazone (both $P < 0.05$; Figure 1D-G). As shown in Figure 1H, I, RT-PCR analysis further indicated that the PPAR γ agonists increased LRP1 protein by enhancing its mRNA transcription at the BBB in *db/db* mice.

PPAR γ agonists decrease [125 I]-A β_{1-40} influx across the BBB in *db/db* mice

To determine whether PPAR γ agonists affect A β influx across the BBB in *db/db* mice, we carried out brain perfusion experiments with [125 I]-A β_{1-40} , evaluated as cerebrovascular permeability surface area product. As indicated in Figure 2A, the

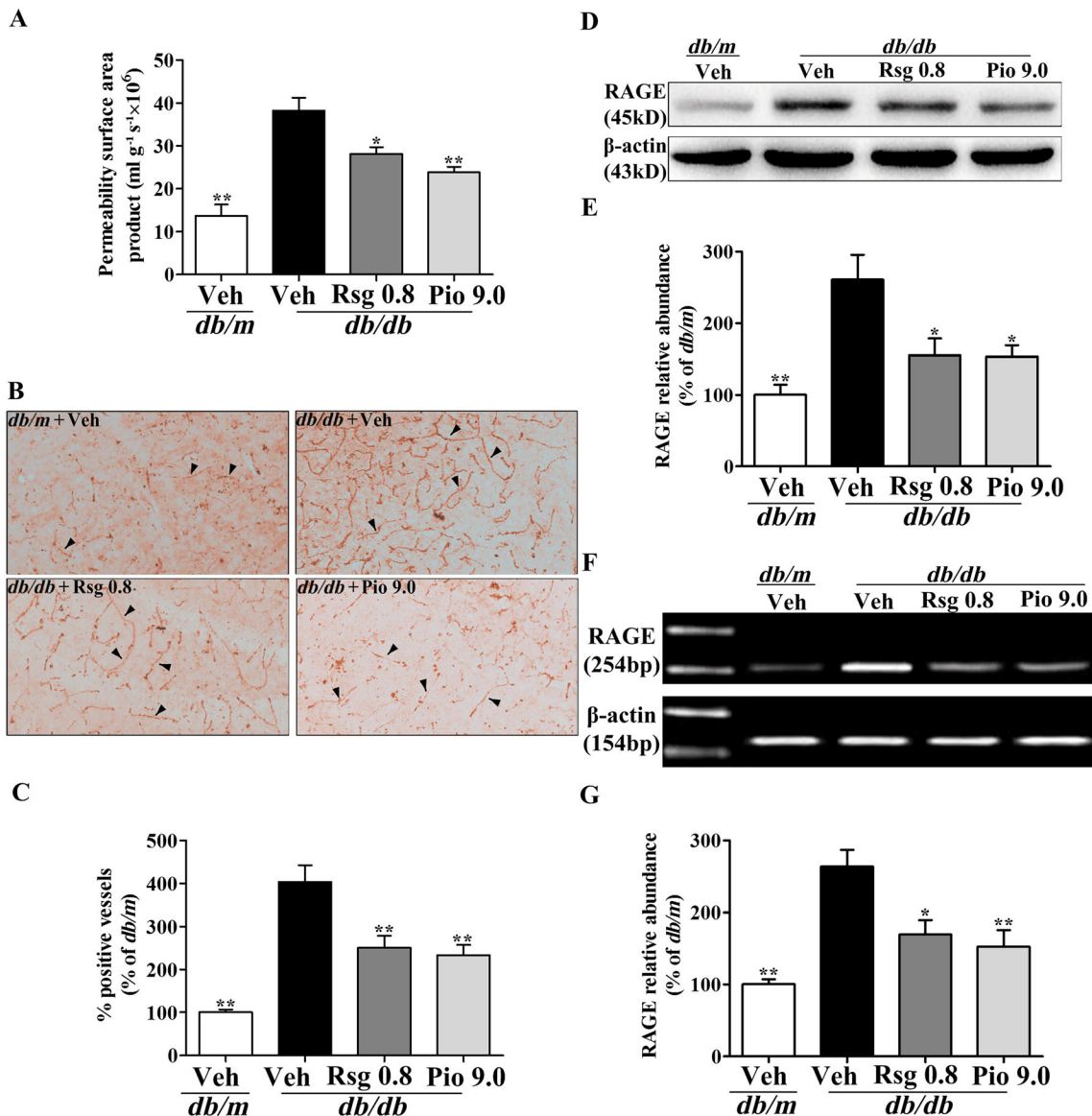


Figure 2

PPAR γ agonists decrease [125 I]-A β_{1-40} influx and RAGE expression at the BBB in *db/db* mice. (A) Cerebrovascular permeability surface area product was determined by γ counting after 10 min of [125 I]-A β_{1-40} infusion. (B) Representative immunohistochemical microphotographs of RAGE, (C) analysis for % of RAGE-positive vessels (arrow, brain microvessels positive RAGE), (D) representative immunoblot detected by Western blot, (E) protein quantification, (F) RAGE mRNA expression and (G) mRNA quantification of RAGE expressed as the percentage of the *db/m* are shown. Original magnification is 200 \times . Values shown are expressed as mean \pm SEM; $n = 3-4$. * $P < 0.05$, ** $P < 0.01$ versus *db/db* + Veh group.

influx of circulating [125 I]-A β_{1-40} across the BBB was significantly increased in *db/db* mice relative to that of *db/m* mice ($P < 0.01$), whereas chronic treatment with rosiglitazone or pioglitazone markedly reduced the influx of peripheral [125 I]-A β_{1-40} across the BBB in *db/db* mice ($P < 0.05$ and $P < 0.01$ respectively).

PPAR γ agonists decrease RAGE expression at the BBB in *db/db* mice

It has been confirmed that RAGE mediates A β influx across the BBB (Deane *et al.*, 2003; Liu *et al.*, 2009). To clarify whether the decreased A β influx across the BBB induced by PPAR γ agonists can be attributed to the down-regulation of RAGE protein, we evaluated the expression of RAGE protein at the BBB in *db/db* mice. Immunohistochemical staining showed that brain microvessels positive for RAGE staining were obviously increased in *db/db* mice treated with vehicle but significantly decreased in *db/db* mice treated with rosiglitazone or pioglitazone (Figure 2B, C). Western blot analysis of isolated brain microvessels also showed a significant reduction in the level of RAGE protein in *db/db* mice treated with rosiglitazone or pioglitazone (both $P < 0.05$; Figure 2D, E). As shown in Figure 2F, G, RT-PCR analysis further revealed that the PPAR γ agonists significantly decreased RAGE mRNA transcription at the BBB in *db/db* mice.

Effects of PPAR γ agonists on PPAR γ and NF- κ B p65 at the BBB in *db/db* mice

The next important question that we addressed is how PPAR γ agonists regulate LRP1 or RAGE expression at the BBB. The binding site of PPAR γ has been shown to be located in the promoter site of the adipocyte LRP1 gene, and the expression of functional LRP1 is promoted by PPAR γ ligand-induced up-regulation of transcription (Gauthier *et al.*, 2003).

Interestingly, we found that PPAR γ protein was markedly down-regulated in the nuclei of brain microvessel, endothelial cells of *db/db* mice ($P < 0.01$; Figure 3A, B), and significantly elevated in those of *db/db* mice treated with PPAR γ agonists ($P < 0.05$ and $P < 0.01$ respectively). Analysis of the putative promoter region of the RAGE gene has revealed the presence of potential NF- κ B binding sites (Li and Schmidt, 1997). Like peripheral tissue, NF- κ B signalling activation was observed at the BBB in *db/db* diabetic mice, as indicated by increased NF- κ B p65 in the nuclei of brain microvessel, endothelial cells (both $P < 0.01$; Figure 3A, C). More importantly, treatment with rosiglitazone or pioglitazone markedly reduced nuclear NF- κ B p65 in brain microvessel, endothelial cells, which suggests that PPAR γ agonists suppress NF- κ B signalling at the BBB in diabetic mice.

PPAR γ agonists reduce A β levels and apoptosis-related molecules in the brain of *db/db* mice

Next, in order to determine whether A β levels in the brain decrease when the A β efflux across the BBB is altered and the effects of the PPAR γ agonists, A β levels in the hippocampus, a key brain region related to memory, were assayed. As expected, treatment with rosiglitazone or pioglitazone effectively and reversibly suppressed the increases in A β_{1-40} and A β_{1-42} ($P < 0.05$ and $P < 0.01$ respectively; Figure 4A-C) in the hippocampus of *db/db* mice. Immunohistochemical staining also demonstrated similar results (Figure 4D-G). As A β accumulation is likely to cause activation of apoptotic pathways in brain (Stadelmann *et al.*, 1999; Awasthi *et al.*, 2005; Rohn *et al.*, 2008), we also investigated the expressions of apoptosis-related molecules, caspase-3, Bcl-2 and Bax, as by Western blot analysis. As shown in the Supporting Information Fig. S1, rosiglitazone and pioglitazone treatment

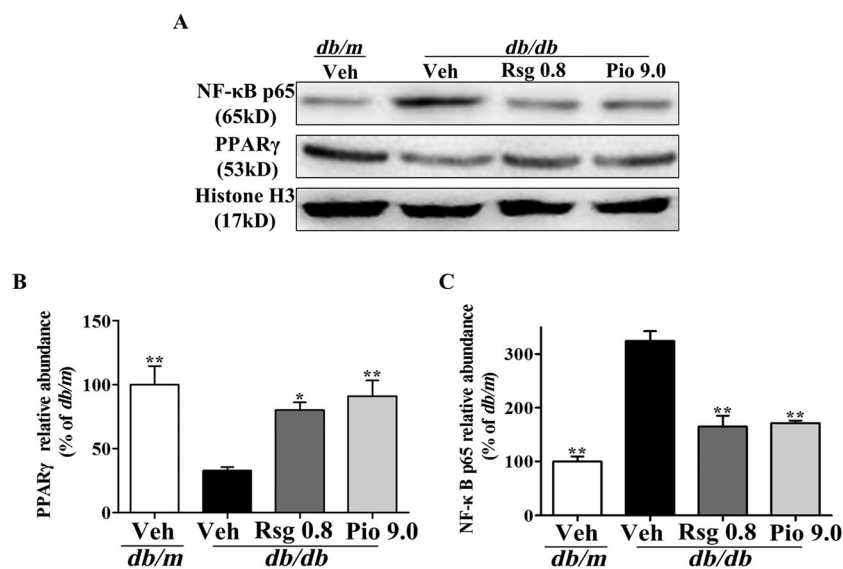
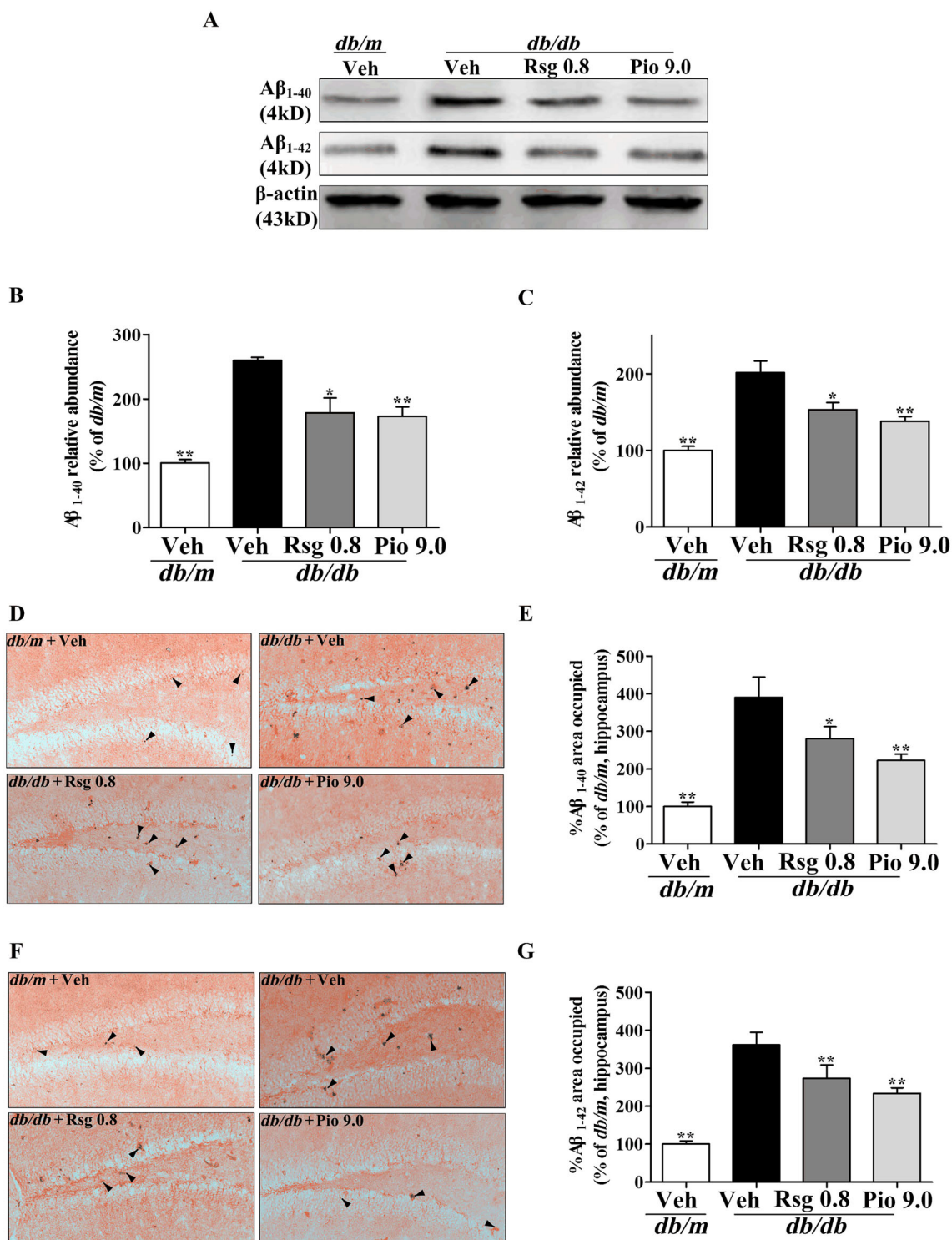


Figure 3

Effects of PPAR γ agonists on PPAR γ and NF- κ B p65 at the BBB in *db/db* mice. NF- κ B p65 or PPAR γ (A) was detected by Western blot. Histone H3 was used as a loading control. Quantification of PPAR γ (B) and NF- κ B p65 (C) are expressed as a percentage of the *db/m*. Values shown are expressed as mean \pm SEM; $n = 3$. * $P < 0.05$, ** $P < 0.01$ versus *db/db* + Veh group.

**Figure 4**

PPAR γ agonists reduce A β levels in the brain of *db/db* mice. (A) Representative immunoblot of A β_{1-40} and A β_{1-42} detected by Western blot, quantification of A β_{1-40} (B) and A β_{1-42} (C) expressed as a percentage of the *db/m* are presented. Representative immunohistochemical microphotographs of A β_{1-40} (D) and A β_{1-42} (F), analysis for % of A β_{1-40} (E) and A β_{1-42} (G) area occupied (arrow, A β_{1-40} or A β_{1-42} positive area respectively) are shown. Original magnification is 200 \times . Values shown are expressed as mean \pm SEM; $n = 3$. * $P < 0.05$, ** $P < 0.01$ versus *db/db* + Veh group.

markedly suppressed caspase-3 activation, Bax up-regulation and Bcl-2 down-regulation (both $P < 0.05$) in the hippocampus.

Effects of PPAR γ agonists on hippocampal LTP in *db/db* mice

A β accumulation can also damage synaptic plasticity; thus, we recorded the hippocampal LTP, a well-established indicator of synaptic plasticity closely related to memory. The LTP recording revealed that hippocampal LTP was modestly inhibited in *db/db* mice ($130.90 \pm 3.17\%$) after the tetanic stimulation persisting for more than 60 min compared with the *db/m* group ($222.22 \pm 7.46\%$, $P < 0.01$; Figure 5A, B), and markedly increased in *db/db* mice treated with rosiglitazone or pioglitazone ($156.97 \pm 6.21\%$ and $173.66 \pm 10.31\%$, $P < 0.05$ and $P < 0.01$ respectively).

PPAR γ agonists ameliorate memory impairment in *db/db* mice

Hippocampal LTP should be in line with the enhanced hippocampus-dependent learning and memory by the behavioural tests. In a non-spatial visible-platform variant of the MWM, mice showed similar escape latency indicating

no significant difference in motivation and vision in each group (four trials per mouse per day for 2 days, effect of day, $P < 0.01$; effect of group, $P > 0.05$; effect of group-by-day interaction, $P > 0.05$; Figure 6A). In the spatial hidden-platform variant, compared with the *db/m* group, *db/db* mice had a significantly increased escape latency ($P < 0.05$; Figure 6B). Interestingly, rosiglitazone or pioglitazone treatment significantly decreased escape latency (four trials per mouse per day for 3 days, effect of day, $P < 0.01$; effect of group, $P < 0.01$; effect of group-by-day interaction, $P > 0.05$). In the probe trial, *db/db* mice showed a significant decrease in the percentage of total time in the target quadrant (one trial per mouse for the last day, $P < 0.01$; Figure 6C, D), the number of target crossings (one trial per mouse for the last day, $P < 0.01$; Figure 6E) compared with the *db/m* group. In contrast, *db/db* mice treated with rosiglitazone or pioglitazone displayed significant increases in these indices compared with those of *db/db* group. The Y-maze test also indicated that rosiglitazone or pioglitazone significantly increased the number of correct choices and decreased the latency to enter the shock-free compartment in *db/db* mice (Figure 6F, G). For the open field test, there was no significant difference in the total distance travelled by each group (Supporting Information Fig. S2A). No significant effect of rosiglitazone or pioglitazone

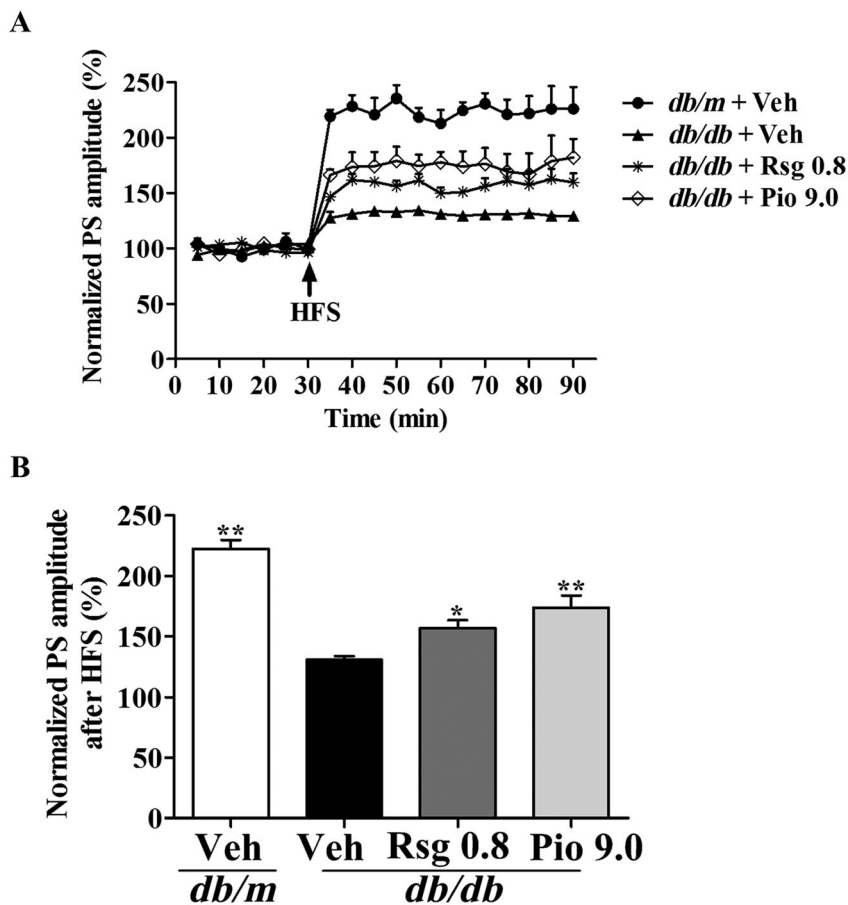


Figure 5

Effects of PPAR γ agonists on hippocampal LTP in *db/db* mice. High-frequency stimulation (HFS) was given at the end of 30 min baseline recording of PS. The time course of PS after HFS (A) and PS at 60 min post-HFS is summarized in (B). Values shown are expressed as mean \pm SEM; $n = 4-5$. * $P < 0.05$, ** $P < 0.01$ versus *db/db* + Veh group.

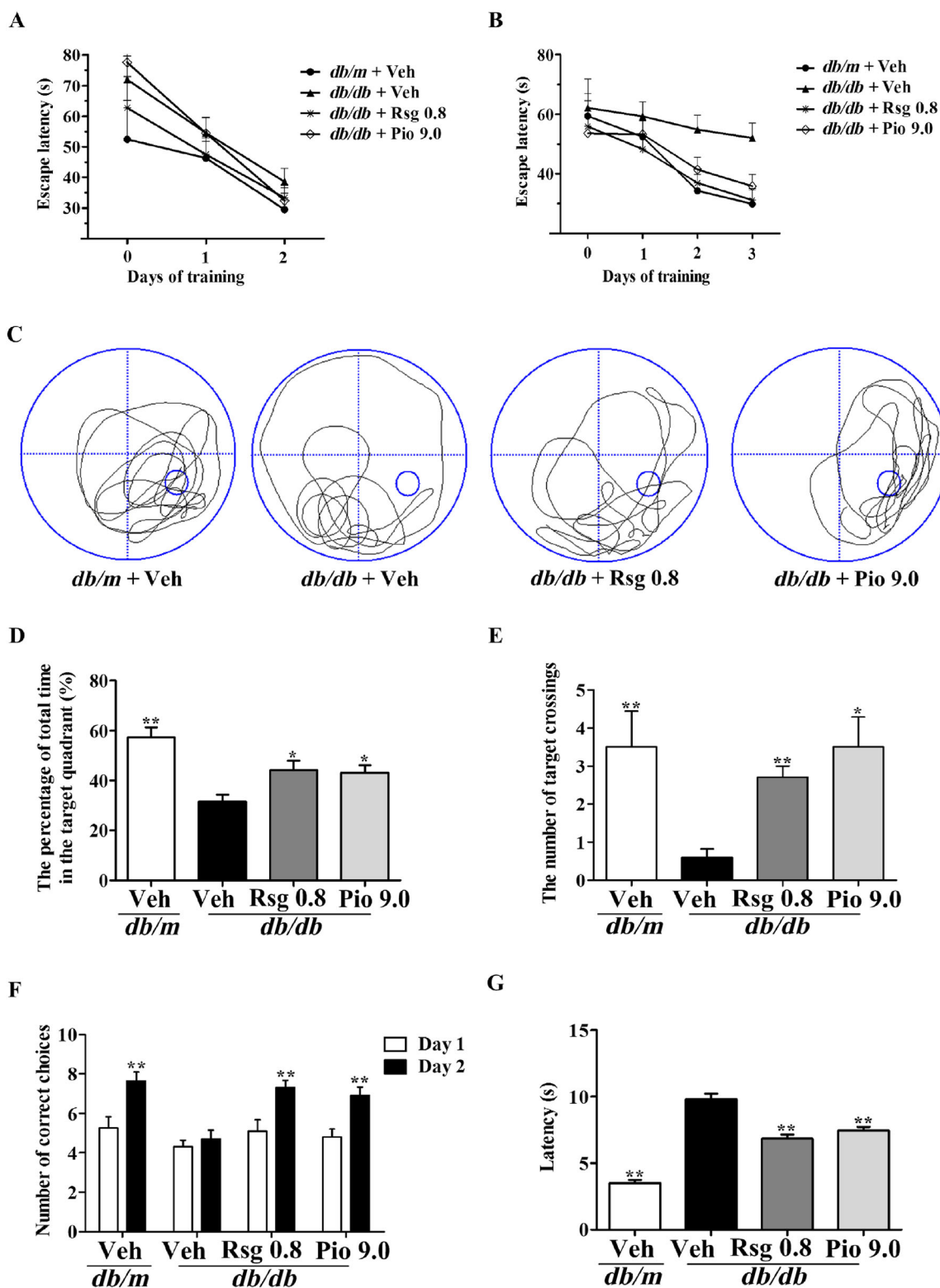


Figure 6

PPAR γ agonists ameliorate memory impairment in *db/db* mice. In the MWM task, day 0 indicates performance on the first trial, and subsequent points represent average of all daily trials. (A) No differences were found in the escape latency among all groups during the 2-day visible platform test. (B) Changes in escape latency to reach the hidden platform during the 3-day acquisition trials, (C) representative swim paths during the spatial probe test, (D) the percentage of total time in the target quadrant, (E) the number of target crossings during the probe trial test, (F) the number of correct choices on days 1–2 and (G) the latency to enter the shock-free compartment on day 2 in the Y-maze test. Values shown are expressed as mean \pm SEM; $n = 8$ –10. * $P < 0.05$, ** $P < 0.01$ versus *db/db* + Veh group.

alone on memory was observed in age-matched normal homozygote mice (data not shown). These data indicate that PPAR γ agonists ameliorate memory impairments in *db/db* mice.

Effects of PPAR γ agonists on brain AGEs, fasting serum glucose and insulin levels in *db/db* mice

As shown in Table 1, Rosiglitazone or pioglitazone pronouncedly ameliorated hyperglycaemia (both $P < 0.05$), hyperinsulinaemia ($P < 0.01$ and $P < 0.05$ respectively) and inhibited the generation of AGEs in both the hippocampus and cortex ($P < 0.01$ and $P < 0.05$ respectively), but did not affect body weights.

Discussion and conclusion

We initially demonstrated that PPAR γ agonists increase LRP1 expression and A β transport from the brain into the bloodstream by stimulating PPAR γ , and significantly decrease RAGE expression and A β transport from the bloodstream into the brain by inhibiting NF- κ B activation at the BBB in *db/db* mice. The bidirectional regulation of A β transport across the BBB was correlated with a decrease in A β accumulation and amelioration of apoptotic responses, as the PPAR γ agonists suppressed caspase-3 activation and increased the ratio of Bcl-2/Bax in the hippocampus, which is associated with short- and long-term memory. More importantly, the PPAR γ agonists effectively improved hippocampus plasticity, as they enhanced LTP and improved learning and memory, reflected by an improved performance in the MWM and Y-maze tasks. In addition, the PPAR γ agonists also significantly inhibited the formation of cerebral AGEs and improved insulin resistance, as they decreased both serum glucose and insulin in the *db/db* mice.

The vascular hypothesis on cognitive dysfunction associated with diabetes proposes that functional or structural changes of the BBB, which result in the accumulation of numerous neurotoxic and vasculotoxic macromolecules in the brain or a reduction in cerebral blood flow and hypoxia, can induce neurodegeneration (Huber, 2008; Kodl and Seaquist,

2008; Bell *et al.*, 2010). Specifically, abnormalities in the BBB decrease A β efflux from the brain into the bloodstream (Deane *et al.*, 2004) and may increase the influx of peripheral A β into the brain (Atwood *et al.*, 2002; Eisele *et al.*, 2010), elevating brain A β levels. These changes occur in insulin-deficient animals (Liu *et al.*, 2006; Liu *et al.*, 2008; Hong *et al.*, 2009; Liu *et al.*, 2009). Interestingly, *db/db* mice with hyperinsulinaemia also showed a similar alteration in A β influx and efflux across the BBB. An increased expression of RAGE expression in cerebral vessels, neurons and glial cells has been shown to be associated with diabetes-related cognitive impairment (Toth *et al.*, 2006), suggesting that RAGE has a crucial role in the development of cerebral dysfunction (Yan *et al.*, 1997). RAGE, a member of the immunoglobulin superfamily, is a multiligand receptor that binds to a range of molecules including AGEs, A β and the S100/calgranulin family of pro-inflammatory mediators (Hofmann *et al.*, 1999; Stern *et al.*, 2002). The most prominent among these is A β , which plays a key role in AD pathology. RAGE is associated with chronic diabetes-related damage in the kidneys, retina, peripheral nerves and blood vessels (Kim *et al.*, 2005). RAGE gene promoter contains NF- κ B transcription elements, which interact between cell-surface RAGE and its ligand setting up a positive feedback mechanism, which can rapidly accelerate disease progression (Li and Schmidt, 1997). In our study in *db/db* mice, AGEs and A β significantly accumulated in the brain, and NF- κ B p65 translocated into the nucleus of brain microvessel endothelial cells, subsequently stimulating the expression of RAGE gene and the transport of peripheral A β into the brain. PPAR γ plays a key role in maintaining cerebrovascular health. Its genetic inactivation in mice causes increased reactive oxygen species generation, endothelial dysfunction and hypertrophy of cerebral arterioles (Beyer *et al.*, 2008; Ketsawatsomkron *et al.*, 2010). Overexpression of PPAR γ or its activation with ligands can decrease the expression and activity of MMP and adhesion molecules in the vasculature (Keen *et al.*, 2004; Ramirez *et al.*, 2008; Huang *et al.*, 2009), thereby maintaining levels of tight junction proteins and preventing leukocyte–endothelial interactions (Fiala *et al.*, 2002). Evidence is emerging suggesting that the PPARs interact with NF- κ B to decrease its activity, which may suppress gene expression (Ricote *et al.*, 1998; Zipser

Table 1

Effects of PPAR γ agonists on brain AGEs, serum glucose and insulin levels in *db/db* mice

Groups	Serum Glucose (mmol·L ⁻¹)	Serum insulin (mIU·L ⁻¹)	AGEs levels (μg·g ⁻¹ protein)	
			Hippocampus	Cortex
<i>db/m</i> + Veh	8.33 ± 1.47**	8.63 ± 1.01**	35.27 ± 3.15**	46.31 ± 4.64**
<i>db/db</i> + Veh	31.67 ± 1.83	45.70 ± 2.44	86.87 ± 6.14	97.61 ± 6.26
<i>db/db</i> + Rsg 0.8	23.99 ± 1.85*	36.50 ± 2.10**	64.15 ± 5.27*	75.96 ± 5.08*
<i>db/db</i> + Pio 9.0	22.88 ± 2.78*	38.15 ± 1.88*	51.69 ± 4.51**	66.27 ± 6.45**

Values shown are expressed as mean ± SEM; $n = 5$ –10.

* $P < 0.05$.

** $P < 0.01$ versus *db/db* + Veh group.

et al., 2007). In the present study, PPAR γ stimulation by its agonists led to decreases in NF- κ B activity, RAGE expression and A β influx at the BBB in *db/db* mice. LRP1 also binds to a broad range of ligands, including α 2-macroglobulin, apoE, amyloid precursor protein (APP) and A β (Zlokovic, 2004). It has been demonstrated that functional LRP1 expression is regulated by PPAR γ ligands, which involves ligand-induced up-regulation of a transcription factor via the activation of PPAR γ -RXR α heterodimers that bind to a newly identified PPRE in the promoter region of the LRP1 gene (Gauthier *et al.*, 2003). As expected, pioglitazone or rosiglitazone stimulated PPAR γ and increased its localization in the nucleus of the isolated brain microvessel cells, consequently, LRP1 expression and its associated A β efflux through the BBB were significantly increased in *db/db* mice. The neurotoxicity of A β , especially A β _{1–42}, a more neurotoxic A β species, has been well documented (Lai *et al.*, 2014). It was observed in *db/db* mice that hippocampal A β accumulation was accompanied by apoptotic responses, and the PPAR γ agonists effectively suppressed apoptosis, as indicated by decreased caspase-3 activity and an increased ratio of Bcl-2/Bax in the hippocampus. Many studies have demonstrated that diabetes induces cognitive impairments. In particular, the hippocampus is susceptible to the negative consequences of the diabetic condition. Stimulating PPAR γ in diabetes can reverse behavioural impairments and increase LTP at the perforant path–dentate gyrus synapses. This restorative effect on hippocampal plasticity is involved in the amelioration of cerebral β -amyloidosis, because there are no differences in the glucose or insulin levels in the hippocampus of insulin-resistant and insulin-deficient animals (Stranahan *et al.*, 2008). In addition, chronic treatment with a PPAR γ agonist also showed significant attenuation of hyperglycaemia and hyperinsulinaemia in *db/db* mice; thus, these peripheral effects should contribute somewhat to the reduction in RAGE-mediated A β influx at the BBB because the formation of AGEs is also reduced. Furthermore, *db/db* mice are also characterized by an increase in body weight, leading to abnormal lipid metabolism and insulin resistance and an increase in the incidence of cognitive impairment or AD (Kanoski and Davidson, 2011). It has been shown that PPAR γ agonists improve lipid utilization by multiple pathways in adipose tissue, inducing an increase in the levels of lipid metabolism-associated genes, such as lipoprotein lipase and fatty acid binding protein 4 (FABP4; Kawai *et al.*, 2010). These changes in gene expression promote the incorporation of free fatty acids into adipose tissue, which lowers serum free fatty acids levels and increases lipid deposits in the liver and skeletal muscle. Thus, PPAR γ agonists improve the memory impairment in *db/db* mice in both a direct and indirect way.

Taken together, our results show that PPAR γ agonists not only increase LRP1-mediated A β efflux but also decrease RAGE-mediated A β influx across the BBB in diabetic mice, and this bidirectional effect resulted in a marked decrease in hippocampal A β and improvements in synaptic plasticity. Our findings suggest that PPAR γ agonists can be promoted for potential therapeutic use for the prevention and treatment of diabetes-associated cognitive decline, which has important implications for patients with type 2 diabetes.

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Author contributions

H. H. designed the experiments and wrote the manuscript. W. H., C. F., Z. K. L. and T. S. S. performed the experiments. L. Y., M. M. X. and L. J. M. performed the isotope experiments. H. M. and S. H. B. analysed the data and revised the manuscript. All the authors agreed that the final approval of the version to be published and ensured questions relating to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

Conflict of interest

The authors declare that there is no duality of interest associated with this manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

<http://dx.doi.org/10.1111/bph.13378>

Figure S1 Effects of PPAR γ agonists on the levels of apoptosis-related molecules including caspase-3, Bcl-2 and Bax in hippocampus of *db/db* mice. (A) Pro-caspase-3, cleaved caspase-3, Bcl-2 and Bax were determined in hippocampus by Western blot using respective antibodies. (B) Caspase-3 activation is expressed as the ratio of caspase-3 fragment to pro-caspase-3, (C) Bcl-2 and (D) Bax were expressed as the ratio (in percentage) of the *db/m*. Values shown are expressed as mean \pm SEM; $n = 3$. * $P < 0.05$, ** $P < 0.01$ versus *db/db+Veh* group.

Figure S2 Effects of PPAR γ agonists on the general locomotor activity in *db/db* mice. (A) The total distance travelled in the open field test is shown. Values shown are expressed as mean \pm SEM; $n = 8–10$.