BBA - Molecular Basis of Disease 1865 (2019) 371–377

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Evidence of metabolic memory-induced neurodegeneration and the therapeutic effects of glucagon-like peptide-1 receptor agonists *via* Forkhead box class O

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ARTICLE INFO

Keywords:
Metabolic memory
Neurodegeneration
Glucagon-like peptide-1 receptor agonists
Forkhead box class O

ABSTRACT

Metabolic memory, which refers to diabetic stresses that persist after glucose normalization, is considered a major factor in addition to hyperglycaemia for diabetes complications, including dementia. We previously reported that glucagon-like peptide-1 receptor agonist (GLP-1RA) alleviated neuronal injury in diabetes-related dementia models. However, our understanding of the effects and mechanisms of GLP-1RA on metabolic memory-induced neurodegeneration are limited. The present study mainly focuses on the mechanisms of action of GLP-1RA on metabolic memory-induced neurotoxicity *in vivo* and *in vitro*. Thus, in this study, aiming at mimicking metabolic memory phenomena, *in vivo* and *in vitro* models were exposed to high glucose first and then normal glucose. We also used advanced glycation end products, which are key metabolic memory-related factors, to induce neuronal injury *in vitro*. Based on the models, here, we report that GLP-1RA alleviated neurodegeneration in *db/db* mice with normalized blood glucose levels controlled with metformin and neuronal damage induced by high glucose treatment followed by withdrawal. GLP-1RA ameliorated metabolic memory-induced amyloid-β and tau pathologies *in vivo* and *in vitro*. Furthermore, the data suggested that GLP-1RA can protect neurons against metabolic memory *via* Forkhead box class O (FoxO) pathways, and silent information regulator 2 homolog 1-dependent deacetylation and protein kinase B-dependent phosphorylation of FoxO1 were involved in the mechanisms underlying protective effects. This study provides evidence of the beneficial effects of GLP-1RA on neuronal cell metabolic memory, as well as GLP-1 analogues and metformin combination therapy efficiency on cognitive impairment.

1. Introduction

Concern about the relationship between diabetes and cognitive impairment has grown in recent years, and dementia has been referred to as a diabetes complication [1–4]. Hyperglycaemia is a primary pathological factor that is responsible for numerous diabetic complications. Hyperglycaemia may be the primary initiator of diabetic complications and glycaemic control is achieved using medication; however, the progression of diabetic complications persists after complete glucose normalization, which supports a phenomenon called metabolic memory [5]. Metabolic memory refers to diabetic stresses that persist after glucose normalization, and hyperglycaemia and metabolic memory are major factors for diabetes complications [5]. An initial report almost 30 years ago suggested the existence of metabolic memory, which was responsible for the progression of incipient diabetic retinopathy during good glycaemic control in diabetic dog models [6]. Metabolic memory was widely studied in different types of models related to diabetic complications, such as diabetic nephropathy [7] and diabetic retinopathy [8] *in vivo* and vascular smooth muscle cells [9] and endothelial cells [10] *in vitro*. Dementia and neurodegenerative disorders are complications of diabetes, which may be mediated *via* metabolic memory [5]. However, our understanding of the mechanisms

Abbreviations: GLP-1, glucagon-like peptide-1; AGEs, advanced glycation end products; GLP-1R, glucagon-like peptide-1 receptor; GLP-1RA, GLP-1R agonist; FoxO, Forkhead box class O; Aβ, amyloid-β; Akt, protein kinase B; Sirt1, silent information regulator 2 homolog 1; Met, metformin; Ex-4, exendin-4; Lixi, lixisenatide; HG, high glucose

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https://doi.org/10.1016/j.bbadis.2018.11.012
Received 1 June 2018; Received in revised form 14 November 2018; Accepted 16 November 2018
Available online 20 November 2018

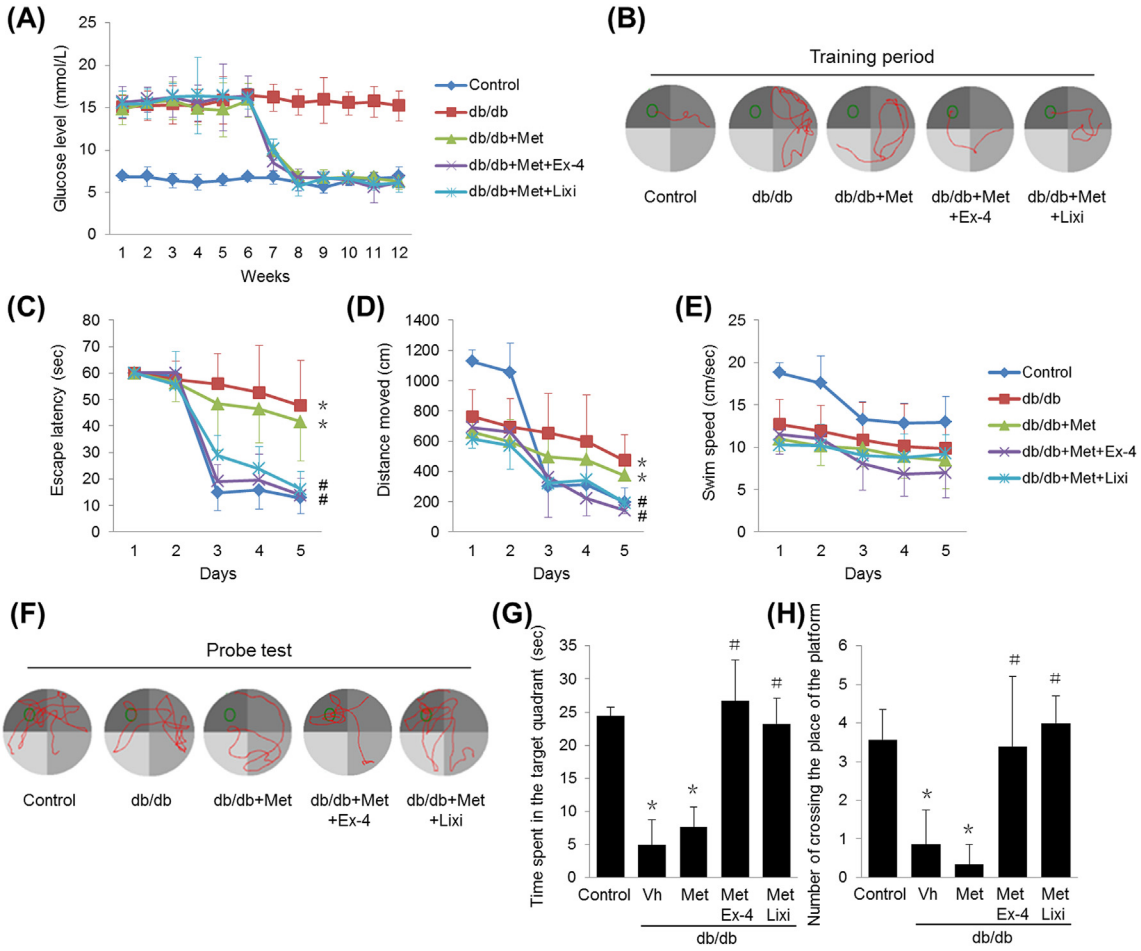

Fig. 1. Effects of GLP-1RA on blood glucose levels (A) and learning and memory (B–H) in metabolic memory mouse models. Representative swim paths (B), escape latency (C), distances moved (D), and swim speed (E) were analysed during the training period in the Morris water maze test. Representative swim paths (F), time spent in the target quadrant (G) and number of crossing place of the platform (H) were shown for the probe test. $n = 10$. Met represented metformin (200 mg/kg i.g. twice - daily); Ex-4 represented exendin-4 (25 nmol/kg s.c. twice - daily); Lixi represented lixisenatide (10 nmol/kg s.c. once - daily). $*P < 0.05$, compared to the Control group; $\#P < 0.05$, compared to the *db/db* + Met group.

underlying the relationship of metabolic memory to central nervous diseases is not complete, and effective therapeutic strategies are urgently needed. Our understanding of the molecular mechanisms underlying metabolic memory is limited, but evidence supports that advanced glycation end products (AGEs) and reactive oxygen species play essential roles [5]. The binding of AGEs to the receptor for AGEs elicits abnormal reactive oxygen species generation, and reactive oxygen species accumulation also excessively activates the AGEs/the receptor for AGEs axis [5]. This activation leads to a self-maintaining vicious loop that is independent of glucose level and results in the target organ damage that is responsible for diabetic complications [5]. Therefore, the addition of potential agents may be beneficial in the treatment of AGEs- and reactive oxygen species-mediated damage and abnormalities, in addition to glucose normalization, to ameliorate diabetic complications, including dementia.

Glucagon-like peptide-1 (GLP-1) is a glucose metabolic regulator, and GLP-1 and its analogues (exendin-4 and lixisenatide) may exert beneficial effects in diabetes and diabetes complications, especially dementia, because of its blood-brain barrier crossing abilities [11–13]. Several studies [4,14–26] demonstrated the potential neuroprotective activities of GLP-1 and its analogues. GLP-1 analogues were neuroprotective in well-characterized animal models of Alzheimer's disease [18–20], Parkinson's disease [21] and Huntington's disease [22]. GLP-1 receptor agonists exhibited promising anti-stroke effects [21,23,24]. For example, preclinical evidence indicated that GLP-1 receptor activation reduced brain damage following stroke in transient middle cerebral artery occlusion models in Sprague-Dawley rats [21] and type 2 diabetic Goto–Kakizaki rats [23]. GLP-1 receptor signaling is especially involved in the crosstalk between diabetes and the brain [25,26]. Our previous studies indicated that GLP-1 and its analogues alleviated brain neuronal injury in different dementia animal and cell models [4,14–17], especially some diabetes-related factors, such as streptozotocin- [4] and AGEs-induced models [14–16]. Activation of the GLP-1 receptor (GLP-1R) in neurons inhibited the receptor for AGEs and reactive oxygen species pathways [16], which play crucial roles in metabolic memory. However, more direct evidence is needed to elucidate the relationship between metabolic memory and cognitive impairment as a diabetes-related disease. Further studies are also needed to examine the related underlying mechanisms of GLP-1R agonist (GLP-1RA). The present study established cell and mouse models to investigate metabolic memory-induced neuronal cell damage and the effects of GLP-1RA on this type of injury.

2. Materials and methods

2.1. Animals and in vivo experimental design

Eight-week-old male B6.BKS(D)-Lepr^{db}/JNju mice (*db/db* mice) and C57BL/6JNju mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). Mice were housed in a

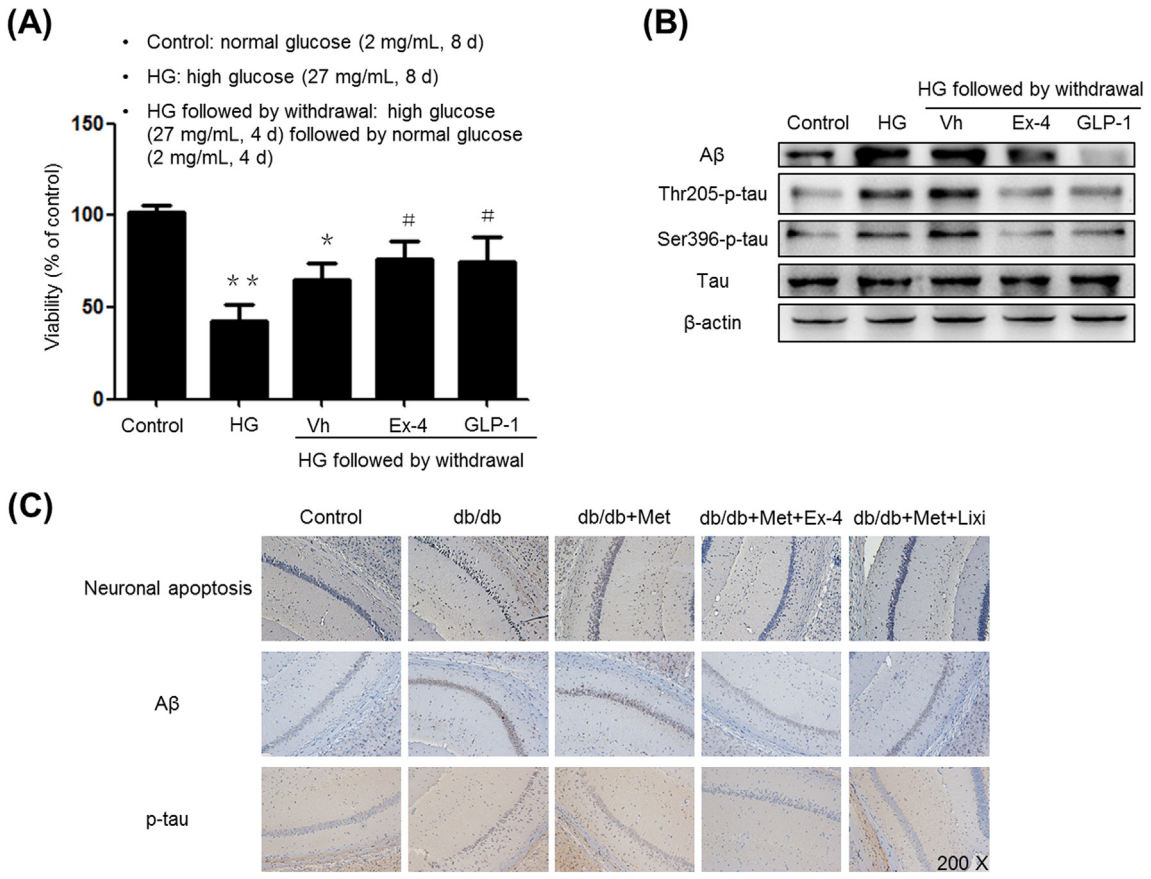

Fig. 2. Effects of GLP-1RA on PC12 cell injury (A) induced by high glucose treatment followed by withdrawal, Aβ and tau pathologies in metabolic memory cellular models (B), and neuronal apoptosis, Aβ and tau pathologies in metabolic memory mouse models (C) (Hippocampus: 200 X). All experiments were repeated at least three times. Control group represented normal glucose (2 mg/mL, 8 d); HG group represented high glucose (27 mg/mL, 8 d); HG followed by withdrawal group represented high glucose (27 mg/mL, 4 d) followed by normal glucose (2 mg/mL, 4 d). $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the Control group; $\#P < 0.05$, compared to the HG followed by withdrawal + Vh group.

specific pathogen-free environment and given food and water *ad libitum*. The *db/db* mice were randomized into four groups (10 per group) after 6 weeks and treated for 6 weeks with vehicle (*db/db* mouse group), metformin (200 mg/kg i.g. twice - daily) (*db/db* mouse with normalized blood glucose level group), exendin-4 (25 nmol/kg s.c. twice - daily) in combination with metformin (exendin-4 intervention on *db/db* mouse with normalized blood glucose level group) or lixisenatide (10 nmol/kg s.c. once - daily) in combination with metformin (lixisenatide intervention on *db/db* mouse with normalized blood glucose level group). The persistent injury to mouse brains after complete normalization of glucose represented a metabolic memory phenomenon. Blood glucose and body weight were monitored weekly. The Institutional Animal Care and Use Committee of China Pharmaceutical University approved the *in vivo* experiments in this study, which were performed in compliance with the rules in the Guide for the Care and Use of Laboratory Animals.

2.2. Cell culture and in vitro experimental design

PC12 cells were cultured in Roswell Park Memorial Institute 1640 medium (Gibco, Grand Island, NY, USA) containing 10% foetal bovine serum (Gibco, Grand Island, NY, USA) and maintained in a 37 °C, 5% carbon dioxide culture incubator (Thermo Scientific, Waltham, MA, USA). Cells in the control group were incubated with normal glucose (2 mg/mL) containing media (mimic normal blood glucose environment *in vitro*) for 8 days, and cells in the high glucose group were incubated with high glucose (27 mg/mL) containing media (mimic high blood glucose environment *in vitro*) for 8 days. With respect to the metabolic memory mimic group, after incubation with high glucose (27 mg/mL) containing media (mimic high blood glucose environment *in vitro*) for 4 days, cells were incubated with normal glucose (2 mg/mL) containing media (to mimic the complete normalization of glucose after a high blood glucose environment *in vitro*) for 4 days, and the persisting injury on cells after complete glucose normalization represented metabolic memory phenomenon. Cells in drug intervention groups were subjected to high glucose (27 mg/mL) containing media (to mimic high blood glucose environment *in vitro*) for 4 days and then treated with exendin-4/GLP-1 (100 nM, GL Biochem, Shanghai, China) in normal glucose (2 mg/mL) containing media (to mimic the complete normalization of glucose after a high blood glucose environment *in vitro*) for 4 days. The metabolic memory-related key factor AGEs-induced neuron injury model was described previously [15]. Cell viability was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT, Sigma, St. Louis, MO, USA) assay [4]. Forkhead box class O (FoxO) 1-siRNA was obtained from Biomics Biotechnologies Co., Ltd. (Nantong, China). The sense strand of FoxO1 was 5′-GCACCGAC UUUAUGAGCAAdTdT-3′, and the antisense strand of FoxO1 was 5′-UUGCUCAUAAAGUCGGUGCdTdT-3′. FoxO1-siRNA was transfected into cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guidelines. Sirtinol (15 μM, Sigma, St. Louis, MO, USA) was added to cells 30 min prior to other treatments.

2.3. Behavioural assessment

Spatial learning and memory function was evaluated using the Morris water maze, as previously described [17]. The time required to reach the platform (escape latency), the distance swam to the platform,

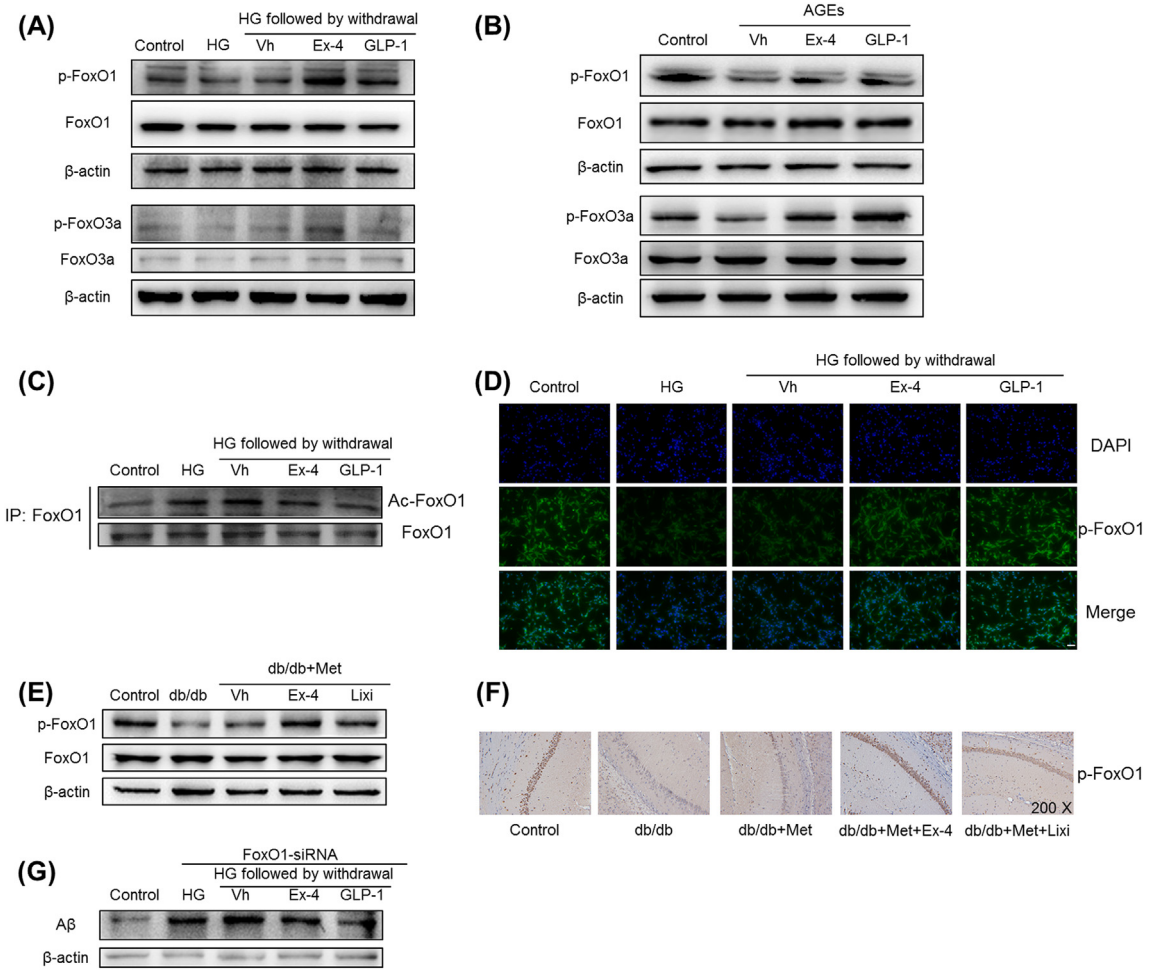

Fig. 3. Effects of GLP-1RA on FoxO (A–F) in metabolic memory and AGEs-induced neuron models and the role of FoxO1 in GLP-1RA function in neuronal degeneration (G). The effects of GLP-1RA on p-FoxO1, FoxO1, p-FoxO3a, and FoxO3a were examined using Western blot in metabolic memory (A) and AGEs-induced (B) neuron models. The deacetylation of FoxO1 was analysed using immunoprecipitation (C), and the phosphorylation level change of FoxO1 was confirmed using immunofluorescence (D) (Scale bar = 50 μm) in metabolic memory neuron models. The phosphorylation level change of FoxO1 was analysed in metabolic memory animal models using Western blot (E) and immunostaining (F) (Hippocampus: 200 X). The role of FoxO1 in the GLP-1RA effect on neuronal degeneration was confirmed using RNA interference (G). All experiments were repeated at least three times.

the swimming speed in the training period, the time spent in the target quadrant and the frequency of crossing through the place of the platform in the probe test were analysed.

2.4. Western immunoblotting

Western blotting was performed as previously described [4,14–17]. The following primary antibodies were used: anti- amyloid-β (Aβ), Tau, protein kinase B (Akt), Ser473-p-Akt, Ser256-p-FoxO1, Ser253-p-FoxO3a, silent information regulator 2 homolog 1 (Sirt1), FoxO1, FoxO3, acetylated-lysine, and β-actin (Cell Signaling Technology, Beverly, MA, USA); and anti-Thr205-p-tau and Ser396-p-tau (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

2.5. Histology

Brains were fixed in a 4% paraformaldehyde solution for 24 h at 4 °C, and sections were cut at a 7-μm thickness. Terminal deoxynucleotidyl transferase dUTP nick end-labelling (TUNEL) staining was performed to analyse neuronal apoptosis. Immunostaining was performed using primary antibodies against Aβ, p-tau, p-FoxO1 and Sirt1. The immunoreaction was detected using the appropriate species HRP-conjugated antibodies and diaminobenzidine tetrahydrochloride. Images were captured using a microscope (Olympus, Tokyo, Japan).

2.6. Immunoprecipitation

Briefly, whole-cell protein extracts were incubated with primary antibody against FoxO1 at 4 °C for 1 h followed by incubation with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight. Western immunoblotting analysis was performed after the immunoprecipitates were washed three times with lysis buffer.

2.7. Immunofluorescence

Cells were fixed in 4% paraformaldehyde, permeabilized with 1% Triton X-100 and incubated with primary antibody, followed by incubation with FITC-conjugated secondary antibody (Sigma, St. Louis, MO, USA). Images were captured using a fluorescence microscope (Olympus, Tokyo, Japan).

2.8. Statistical analysis

The mean ± standard error of the mean was determined for each group in all experiments. Comparisons between groups were performed using analysis of variance followed by Tukey's test. A P value < 0.05

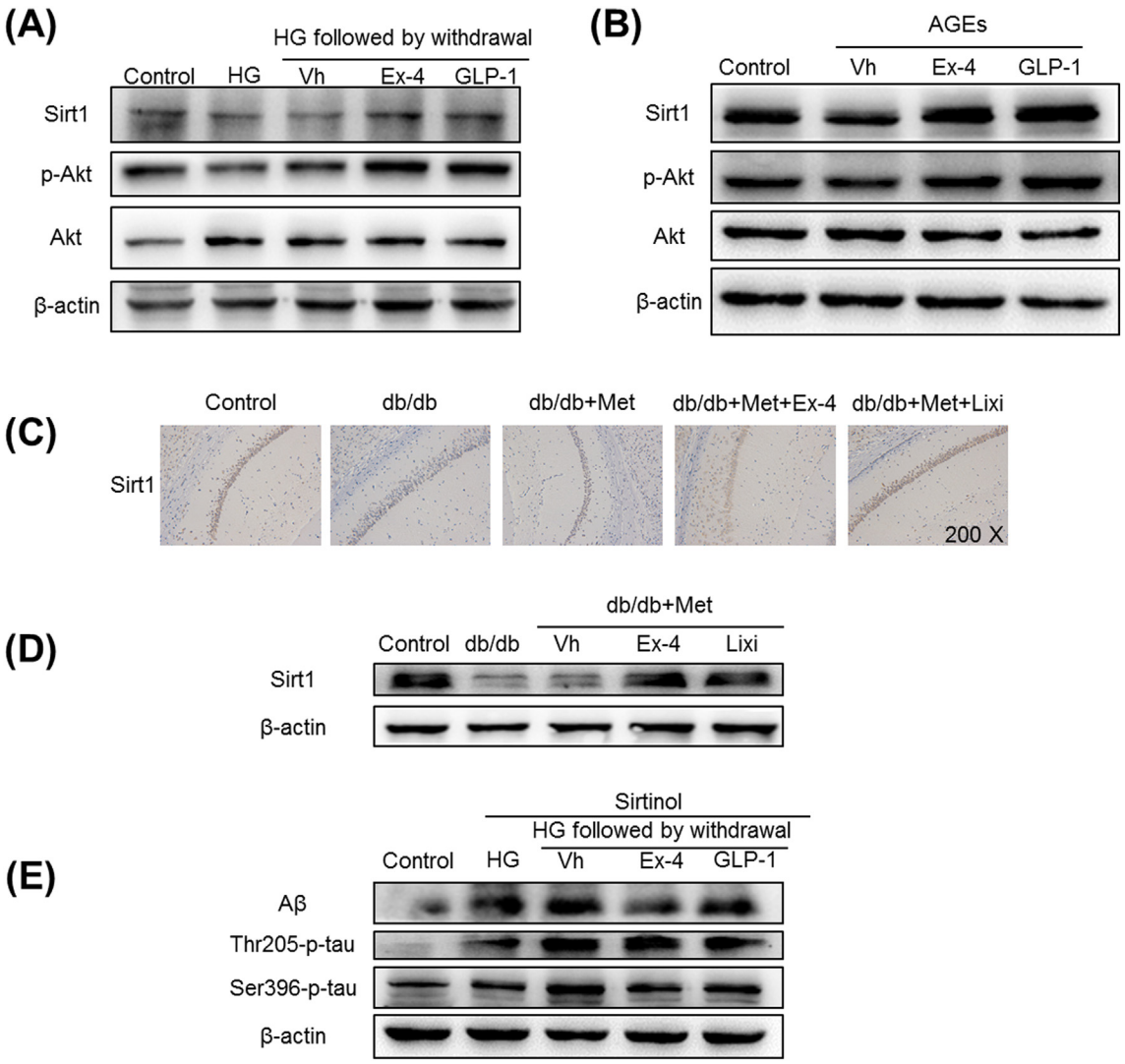

Fig. 4. The mechanisms underlying the effects of GLP-1/FoxO1 in metabolic memory models. The effects of GLP-1 analogues on Sirt1, p-Akt and Akt were examined using Western blot in metabolic memory (A) and AGEs-induced (B) neuron models. The changes in Sirt1 were further analysed in metabolic memory animal models using immunostaining (C) (Hippocampus: 200 X) and Western blot (D). The role of Sirt1 in GLP-1/FoxO1 effect on neuronal degeneration was confirmed using the Sirt1 inhibitor Sirtinol (E). All experiments were repeated at least three times.

was considered significant.

3. Results

3.1. The beneficial effects of GLP-1RA on learning and memory in db/db mice with normalized blood glucose levels

We investigated the effects of GLP-1RA *in vivo* using *db/db* mice with normalized blood glucose levels (Fig. 1A). Metformin was used to normalize blood glucose levels in this study. The mean escape latency and mean distance moved in the hidden platform tasks decreased in the exendin-4 and lixisenatide groups compared to the model group, in which the blood glucose levels of *db/db* mice were normalized with metformin treatment (Fig. 1B, C, D, E). The mean percent time spent in the target quadrant and the number of crossings of the original platform location in probe trials increased in the exendin-4 and lixisenatide groups compared to the model group (Fig. 1F, G, H).

3.2. High glucose treatment followed by withdrawal produced injury in PC12 cells and GLP-1RA relieved this injury

Cells in the present study were incubated with high glucose (27 mg/mL)-containing media for 4 days followed by treatment with normal glucose-containing media for 4 days. High glucose treatment followed by withdrawal injured PC12 cells *in vitro*, and GLP-1RA (100 nM) relieved this injury in the cell models (Fig. 2A).

3.3. GLP-1RA ameliorated Aβ and tau pathologies in metabolic memory cellular and mouse models

Amyloid plaques formed by Aβ and neurofibrillary tangles composed of abnormally hyperphosphorylated tau are the major pathological features of Alzheimer's disease and dementia. Aβ and tau pathologies were evaluated in *in vitro* (Fig. 2B) and *in vivo* (Fig. 2C) models in this study. The amelioration of Aβ and tau pathologies was involved in the beneficial effects of GLP-1RA in metabolic memory cellular and mouse models in the present study.

3.4. FoxO mediated the beneficial effects of GLP-1RA on neuronal degeneration in metabolic memory models

FoxO activity played an important role in the effects of GLP-1RA in cellular models in the present study (Fig. 3A, B). We investigated FoxO1 and FoxO3a in the metabolic memory neuron model and the metabolic

memory-related key factor AGEs-induced neuron injury model described previously [15]. GLP-1 and exendin-4 ameliorated the abnormal levels of p-FoxO1 in these two neuronal models (Fig. 3A, B). A similar change in the phosphorylation level of FoxO3a was observed in the AGEs-induced neuron model (Fig. 3B); in the metabolic memory neuron model, although exendin-4 and GLP-1 upregulated p-FoxO3a level, the difference between the control and model groups was not significant (Fig. 3A). We primarily focused on p-FoxO1 in the metabolic memory neuron model and found that the regulation of the deacetylation (Fig. 3C) and phosphorylation (Fig. 3A, D) levels of FoxO1 by GLP-1RA was related to the underlying mechanisms. We also confirmed the change in p-FoxO1 in the mouse model in this study (Fig. 3E, F). FoxO1-siRNA partially attenuated the beneficial effects of GLP-1 and exendin-4 on the metabolic memory neuron model (Fig. 3G).

3.5. Sirt1-dependent deacetylation in neurons was involved in the GLP-1/FoxO1 effects in metabolic memory models

Sirt1 is an important molecule that is responsible for FoxO1 deacetylation [27], and Akt is a key kinase that is responsible for FoxO1 phosphorylation [28]. We assessed the effects of GLP-1RA on Sirt1 expression and Akt activation in the cellular and mouse models in this study. The results indicated that Sirt1-dependent deacetylation and Akt-dependent phosphorylation signaling pathways were involved in the effects of GLP-1RA in cellular and mouse models (Fig. 4A, B, C, D). The change in p-Akt was consistent with p-FoxO1 in the metabolic memory neuron model (Fig. 4A) and AGEs-induced neuron model (Fig. 4B). Interestingly, Sirt1 was also downregulated in the neuron models and upregulated by GLP-1 and exendin-4 (Fig. 4A, B). These changes in Sirt1 was consistent with the changes in FoxO1 acetylation levels in this study, but FoxO acetylation may promote the phosphorylation of FoxO [29] and in this aspect the Sirt1-mediated effects of GLP-1 and its analogues on p-FoxO1 may conflict with the Akt-mediated effects. Therefore, we further confirmed the changes in Sirt1 in the mouse model, and GLP-1RA regulated the abnormal levels of Sirt1 in the metabolic memory mouse model (Fig. 4C, D). The Sirt1 inhibitor Sirtinol partially attenuated the beneficial effects of GLP-1 and exendin-4 on the metabolic memory neuron model (Fig. 4E).

4. Discussion

The risks of diabetes and dementia increase with ageing. Epidemiological and pathological data suggest that the risk of developing dementia is higher in patients with diabetes and diabetes mellitus contributes to cognitive impairment [1,2]. Diabetes complications, including cognitive impairment, persist and progress despite the achievement of glycaemic control using antidiabetic drugs because of metabolic memory [5]. Our previous studies indicated that GLP-1 and analogues played important roles in the treatment of dementia, especially diabetes-related dementia [4,14–17]. We reported that GLP-1 and its analogue prevented neuron toxicity in the intracerebroventricular-streptozotocin rat model [4], AGEs-induced mouse model [14–16] and APP/PS1 mouse model [17]. However, the specific effects of GLP-1RA on neuronal injury related to metabolic memory and the underlying mechanisms are not known, and further investigation is urgently needed. Therefore, the present study primarily focused on metabolic memory-induced neuronal damage and the effects of GLP-1 and its analogues.

In vivo and *in vitro* models were established to mimic metabolic memory phenomenon and investigate the effects and mechanisms of GLP-1RA on metabolic memory-induced neurodegeneration. *In vivo* studies used *db/db* mice to investigate metabolic memory-induced neuronal cell damage, and the FDA-approved anti-diabetic drug metformin was used to normalize blood glucose levels in the present study. Several diabetes treatment drugs other than, in addition to GLP-1 and analogues were extensively examined in anti-dementia drug development because of the links between diabetes and dementia. Most of the data for insulin [30–32] and peroxisome proliferator-activated receptor-γ agonists rosiglitazone [33–35] and pioglitazone [36–38] in preclinical and clinical studies concerning dementia revealed beneficial effects on cognition. The effects of metformin on cognitive impairment were also investigated [39–42]. However, the function of metformin on cognitive impairment remains controversial, and many of these studies did not demonstrate direct beneficial effects on dementia. An increased risk of dementia was attributed to the different adverse effects of metformin in these studies [39–41]. A previous study also indicated that different metformin doses may account for the different results [42]. Metformin is recommended as the first-line glycaemic treatment [38,43], and a recent study investigated the protective effects of several anti-diabetes drugs on dementia risk in individuals with diabetes who were stable metformin users [38]. We also used metformin to normalize blood glucose levels and evaluate the ameliorative effect of GLP-1RA on learning and memory decline in *db/db* mice with normalized blood glucose levels. Exendin-4 and lixisenatide produced significantly beneficial effects on learning and memory in the exendin-4/lixisenatide groups compared to the model group.

We previously found that GLP-1 and exendin-4 exerted protective effects against high glucose-induced toxicity in PC12 cells [4,14]. However, the mechanisms of action of GLP-1RA on metabolic memory-induced neurotoxicity are largely unknown. Our *in vitro* experiments indicated that incubation of cells with GLP-1 or exendin-4 protected against neuronal cell injury induced by high glucose treatment followed by withdrawal. Detailed investigations of the underlying molecular mechanisms were also performed to evaluate the beneficial effects of GLP-1 and exendin-4 on neuronal cells as a suitable *in vitro* model for metabolic memory.

The FoxO family of transcription factors plays key roles in diabetic complications and diabetes-induced oxidative stress [44], which is related to metabolic memory. Several modifications of FoxO, including phosphorylation and deacetylation, affect its activation [27–29]. *In vitro* results demonstrated that FoxO activity, especially FoxO1, played a crucial role, and Sirt1-dependent FoxO1 deacetylation and Akt-dependent FoxO1 phosphorylation were involved in the effects of GLP-1. The *in vivo* experiments also confirmed that the GLP-1R/FoxO1 axis played an important role in the attenuating effect of exendin-4/lixisenatide in high glucose-induced metabolic memory in neuronal cells. The regulatory trend of p-Akt was consistent with p-FoxO1. The role of Sirt1 was much more complicated. Previous studies suggested that Sirt1 exerted dual effects on FoxO1 [27,29]. Therefore, we confirmed the roles of Sirt1/FoxO1 in metabolic memory and GLP-1 analogue effects *in vivo*, and further examined Sirt1/FoxO1 roles using a Sirt1 inhibitor and FoxO1 siRNA *in vitro*.

In conclusion, the present study demonstrated that GLP-1 and its analogues exerted beneficial effects on neuronal cell damage produced by high glucose treatment followed by withdrawal and learning and memory in *db/db* mice with normalized blood glucose levels. The most likely mechanisms of these beneficial effects involved Sirt1-dependent deacetylation and Akt-dependent phosphorylation of FoxO1. This study provides evidence for the beneficial effects of GLP-1RA on metabolic memory in neurons and the GLP-1 analogue and metformin combination therapy efficiency on dementia.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (Nos. 81473216, 81673435, 81872850 and 81430082), China; the Open Project of State Key Laboratory of Natural

Medicines (No. SKLNMZZCX201822), China; the “Double First-Class” University Project (CPU2018GF08), China; the “111 Project” from the Ministry of Education of China and the State Administration of Foreign Expert Affairs of China (No. 111-2-07), China; PAPD (A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions), China.

Conflict of interest

The authors declare no conflicts of interest.

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