

Linagliptin, a Dipeptidyl Peptidase-4 Inhibitor, Mitigates Cognitive Deficits and Pathology in the 3xTg-AD Mouse Model of Alzheimer's Disease

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Abstract Glucagon-like peptide-1 (GLP-1) is an incretin hormone shown to be active in the treatment of type-2 diabetes (T2D) and has also been shown as efficacious in Alzheimer's disease (AD). Dipeptidyl peptidase-4 (DPP-4), an enzyme that is expressed in numerous cells, rapidly inactivates endogenous GLP-1. Therefore, DPP-4 inhibition is employed as a therapeutic avenue to increase GLP-1 levels in the management of T2D. The effectiveness of DPP-4 inhibitors in the treatment of AD has been reported in various animal models of AD. With this background, the present study was designed to examine the effectiveness of linagliptin, a DPP-4 inhibitor in the 3xTg-AD mouse model of Alzheimer's disease. Nine-month-old 3xTg-AD mice were administered linagliptin orally (5, 10, and 20 mg/kg) for 8 weeks. At the end of the linagliptin treatment, mice were evaluated for cognitive ability on the Morris Water Maze and Y-maze. Following cognitive evaluation, mice were sacrificed to determine the effect of the linagliptin on brain incretin levels, amyloid burden, tau

phosphorylation, and neuroinflammation. We confirm that linagliptin treatment for 8 weeks mitigates the cognitive deficits present in 3xTg-AD mice. Moreover, linagliptin also improves brain incretin levels and attenuates amyloid beta, tau phosphorylation as well as neuroinflammation. In conclusion, linagliptin possesses neuroprotective properties that may be attributed to the improvement of incretin levels in the brain.

Keywords Glucagon-like peptide-1 · Glucose-dependent insulinotropic polypeptide · Amyloid beta · Tau phosphorylation · Neuroinflammation

Introduction

Alzheimer's disease (AD) is a common neurodegenerative disorder, accounting for 80 % of global dementia cases [1] and leads to many cognitive and behavioral deficits. Acetylcholine esterase inhibitors such as rivastigmine, tacrine, galantamine, and donepezil and memantine (N-methyl-D-aspartate receptor antagonist) are the only drugs approved by the US Food and Drug Administration (FDA) for the symptomatic relief of AD [2]. The precise etiology of AD is still unknown and involves numerous mechanisms owing to its diverse and multifactorial nature [3]. Several disease-modifying strategies aimed at rescuing the progression of neurodegeneration including amyloid beta (A β) and tau aggregation inhibitors and immunotherapies have failed to alter the disease. Therefore, there is an urgent need to develop safe therapeutics that is effective in reversing the disease. Incretin-based drugs including glucagon-like peptide-1 (GLP-1) analogs are one such strategy that has recently been shown to be therapeutically effective in the treatment of AD. Recent studies have confirmed that GLP-1 analogs reverse the

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cognitive deficits and ameliorate neurodegeneration in AD [4, 5]. For instance, administration of liraglutide reduced plaques and improved cognitive performance in AD mouse models [6–8]. Other incretins and their analogs including exendin-4, lixisenatide, and Val(8) GLP-1, D-Ala2GIP have also been shown to be therapeutically beneficial in AD models [9–11].

GLP-1 is an incretin hormone released from the L cells of the gastrointestinal tract in response to food intake and regulates insulin secretion from beta cells of the pancreas. This incretin hormone crosses the blood-brain barrier (BBB) and acts as a neurotransmitter in the brain. However, dipeptidyl peptidase-4 (DPP-4), a serine peptidase, inactivates GLP-1 leading to a very short half-life of the intact hormone (<2 min) [12]. Inhibition of DPP-4 is expected to show neuroprotection by increasing circulating GLP-1 levels. DPP-4 inhibition also improves glucose-dependent insulinotropic polypeptide (GIP), neuropeptide Y, brain natriuretic peptide, and stromal derived factor-1 [13]. The neuroprotective effects of GIP [14, 15], neuropeptide Y [16, 17], and stromal-derived factor-1 [18] are well documented. This further supports our hypothesis of DPP-4 inhibition as a novel therapeutic avenue for AD.

Commercially available DPP-4 inhibitors for human treatment include sitagliptin (Januvia), vildagliptin (Galvus), saxagliptin (Onglyza), alogliptin (Vipidia), and linagliptin (Tradjenta). Sitagliptin [19], vildagliptin [20] and saxagliptin [21] were previously reported as neuroprotective agents in animal models. Linagliptin is a moderately selective DPP-4 inhibitor approved by the FDA in 2011. Linagliptin does not pass through the BBB but peripheral DPP-4 inhibition increases incretin levels threefold, which does cross the BBB to afford neuroprotection [13, 22]. A recent study by Kornelius and colleagues [23] verified the neuroprotective activity of linagliptin on amyloid beta ($A\beta$)-induced cytotoxicity, and Darsalia and colleagues [24] demonstrated similar neuroprotective activity against stroke in normal and diabetic mice. In addition, linagliptin was also proven to be neuroprotective against $A\beta$ -induced neurotoxicity in a *Caenorhabditis elegans* model of neurodegeneration and improved cognitive ability in a mouse model of ischemia [25, 26]. With respect to these findings, we believe linagliptin could be a possible therapeutic for mitigating the symptoms of AD. Hence, the current project was designed to test the therapeutic activity of linagliptin in the 3xTg-AD mouse model of AD.

Methods

Animals and Treatment

Female 3xTg-AD mice (9–10 months old) ($n = 32$) were procured from the animal facility of the University of Macau with prior approval from the Institutional Animal Ethical

Committee. Reports indicate that female 3xTg-AD mice show greater pathological features than males [27, 28], and therefore, we only used female mice in our experiments. Mice were randomized and divided into four groups (eight mice in each), one control and three experimental groups. Mice from experimental groups received 5, 10, and 20 mg/kg of linagliptin orally while the control mice received 0.5 % sodium carboxymethyl cellulose (CMC-Na) once daily for 8 weeks. All mice were housed in individual ventilated cages in a room maintained with optimum temperature and humidity. An illustrative representation of the experimental scheme is shown in Fig. 1.

Morris Water Maze (MWM)

Following 8 weeks of linagliptin treatment, learning and memory skills of animals were tested using the Morris Water Maze (MWM) according to Branca et al. [29]. Briefly, the animals were freely allowed to locate a submerged escape platform in opaque water of a circular pool and the latency to reach the platform was measured from days 1 to 4. Throughout the experiment, the temperature of the pool (25 °C) and the location of the platform (14 in. from the south-west (SW) wall) were maintained constant. Mice were trained for 4 days with four trials per day and were assigned four pseudorandom starting points (E, SE, NW, and N) each day. A total of 16 trials were performed with each mouse in the acquisition phase. The animals were positioned facing the wall at one of the four starting positions and allowed to locate the submerged platform, 1.5 cm beneath the surface of the water. A successful trial was recorded when a mouse found the platform within 60 s. The mouse was allowed to stay on the platform for 5 s prior to being returned to its home cage. If a mouse was unable to locate the platform within 60 s, it was gently guided to the platform and allowed to stay on it for 15 s. The time required (latency) to find the escape platform was measured. A 60-s probe trial was performed without the escape platform on day 5. Time spent in the target quadrant where the escape platform was positioned and the number of platform crossings were recorded during this probe trial.

Y-Maze

The Y-maze apparatus consists of three arms of equal-length prepared with white PVC. Arms of the Y-maze are labeled A, B, and C and are positioned at an equal angle of 120° from each other and extend from the central platform. The test was performed as described by Sarnyai and colleagues [30]. Briefly, one arm (C) was closed during the training phase and kept constant for all the groups. The animal was positioned in one of the open arms (A) and allowed 15 min to explore the accessible arms (A and B) of the Y-maze. The maze was cleaned with 75 % alcohol between trials to avoid olfactory clues. All mice were returned to their home cages

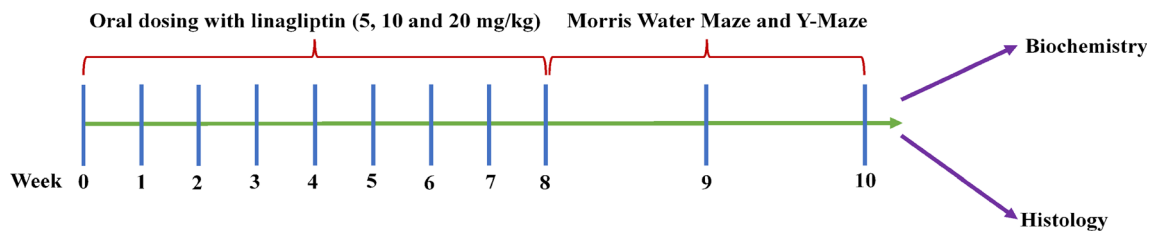


Fig. 1 The schematic representation of the experiment

and given an hour intertrial interval before commencing the next phase. During the test phase, mice were allowed to explore the maze for 5 min with all arms open and the time spent in the novel arm (C) was recorded.

Spontaneous Alternation A 6-min test was performed to measure spatial memory in the Y-maze by placing the mouse in the center of the maze and allowing the animal to freely explore. The maze was cleaned with 75 % alcohol between each trial to prevent olfactory clues. The arm entry sequence of each mouse was manually recorded. A successful alternation is represented by the animal visiting all three arms consecutively (ABC, ACB, BCA, BAC, CAB, and CBA). Percentage of alternation was calculated according to the formula: spontaneous alternation/total possible alternations \times 100.

Biochemical Analysis

For biochemical characterization, 3xTg-AD mice were administered linagliptin or vehicle daily for 8 weeks. Upon completion of treatment, animals were anesthetized and perfused with phosphate buffer saline (PBS). Brains were immediately isolated and divided along the sagittal line into two hemispheres; the right hemisphere was stored at -80°C for ELISA and Western blot analysis, and the left hemisphere was post-fixed in 4 % paraformaldehyde for histological analysis.

Enzyme-Linked Immunosorbent Assay (ELISA) and Glucose Assay

GLP-1, GIP, A β 42, and A β 40 ELISA kits were obtained from Millipore (EGLP-35K and EZRMGIP-55K) and Invitrogen (KMB3441 and KMB3481). Plasma glucose level was measured using an assay kit provided by Shanghai Rongsheng Biotech Co., Ltd. (361500) according to manufacturer instructions. All plates were analyzed on a multimode microplate reader (Molecular Devices, Berkshire, UK).

Western Blotting

Brains were homogenized with RIPA buffer containing protease inhibitor cocktail (05892791001, Roche) and phosphatase inhibitor cocktail (04906845001, Roche). The homogenate

was centrifuged for 10 min at $21,000\times g$ (4°C), the resultant supernatant was separated and centrifuged again for 10 min at $21,000\times g$ (4°C), and the final supernatant was stored at -80°C until use. All samples were mixed with sample buffer (sodium dodecyl sulfate), boiled for 5 min at 95°C , loaded on a bisacrylamide gel (10 %), and electrophoresed. Proteins were transferred onto polyvinylidene difluoride (PVDF) membrane and incubated with the following primary antibodies: anti-tau antibody (MAB361, Millipore), phosphor-PHF-tau antibody (MN1020, Thermofisher), anti-GFAP antibody (MAB360, Millipore), anti-APP antibody (2452, Cell Signaling Technology), and anti-GAPDH antibody (MAB374, Millipore). Membranes were blocked with nonfat milk in TBS-T (tris-buffered saline with Tween 20) for 1 h. Primary antibodies were added in nonfat milk and incubated overnight at 4°C , followed by washing with TBS-T (three washes, 5 min each) and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Membranes were washed with TBS-T (three washes, 5 min each) and immunoreactivity was detected by ECL (6883S, Cell Signaling Technology). Immunoreactive species were visualized on a Bio-Rad system using Imagemag version 5.1 software and quantified using ImageJ software.

Immunohistochemistry

Left hemispheres were post-fixed in paraformaldehyde (4 %) for 48 h and transferred into a sucrose solution (30 %) for 72 h before sectioning. Paraffin-embedded sections (20 μm thick) were obtained using a microtome. Sections were washed with TBS (100 mM Tris pH 7.5, 150 mM NaCl) twice for 5 min each. Quenching was performed by incubating the sections with 90 % formic acid for 7 min followed by 30 min incubation with hydrogen peroxide (3 %). Sections were blocked by incubating in TBS-A (100 mM Tris pH 7.5, 150 mM NaCl, 0.1 % Triton X-100) for 15 min and TBS-B (100 mM Tris pH 7.5, 150 mM NaCl, 0.1 % Triton X-100, 2 % bovine serum albumin) for 30 min. Primary antibody A β 42 (1:200, Biologend, 805502) was diluted with TBS-B and sections were incubated overnight at 4°C [29]. Sections were washed twice in TBS and incubated with biotinylated secondary antibody (31800, Thermofisher) for 30 min. Sections were detected using an ABC kit (32020,

ThermoFisher) and visualized with DAB quanto (TA-060-QHDX, ThermoFisher).

Thioflavin S Staining and Microscopy

Immunofluorescence staining was performed according to Yousefi et al. [31]. Briefly, sections were incubated in Thioflavin S solution for 30 min followed by washing in water, three times (2 min each) and finally by 6 min incubation in 80 % ethanol. Sections were mounted with medium containing DAPI. Fluorescence images were obtained using a fluorescence microscope (Mirax Scan, Carl Zeiss MicroImaging GmbH, Jena, Germany).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism (version 6.01, San Diego, CA, USA) and data expressed as mean \pm standard error mean (SEM). One-way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis was used for all the data analysis except MWM, which was analyzed using an unpaired *t* test. Data equal to or lesser than $p < 0.05$ was considered significant.

Results

Linagliptin Improves Cognitive Performance in 3xTg-AD Mice

To determine the effect of linagliptin treatment on learning and memory, we treated 3xTg-AD mice for 8 weeks and then tested their performance on the MWM and Y-maze. The MWM test was used to investigate the spatial cognitive behavior of animals treated with linagliptin. Initially, we observed no statistically significant difference (the first 2 days) in performance between the linagliptin-treated and control animals as is expected during the acquisition phase of the task. However, mice administered 10 and 20 mg/kg linagliptin demonstrated a gradual improvement in learning during acquisition trials (days 1–4) and revealed a significant decrease in escape latency. On day 3, latency to reach the platform was significantly reduced ($p < 0.01$) following 10 and 20 mg/kg linagliptin treatment as compared to control mice. Latency on day 4 was also significantly reduced with $p < 0.01$ and $p < 0.001$ at 10 and 20 mg/kg respectively as compared to control mice (Fig. 2). On day 5, during the probe trial, mice treated with 20 mg/kg linagliptin demonstrated a significant improvement in time spent in the platform quadrant ($p < 0.01$) and number of platform crossings ($p < 0.05$) compared to control mice (Fig. 2). Path length was also significantly reduced ($p < 0.05$) on days 3 and 4 by linagliptin treatment at 10 and 20 mg/kg. The outcomes from the behavioral tests indicate that linagliptin treatment facilitates

learning and memory in 3xTg-AD mice. Supporting the MWM test, we also evaluated 3xTg-AD mice on a Y-maze to determine the effects of linagliptin treatment. Time spent in the novel arm and the percentage of alterations were measured using the Y-maze test. Mice treated with 20 mg/kg linagliptin displayed a 17.54 % increase in spontaneous alteration and on average spent 66.83 s more time ($p < 0.05$) in the novel arm compared to the control mice (Fig. 3). The spatial and working memory assessed by MWM and Y-maze indicates that linagliptin improved learning and memory in 3xTg-AD mice. Cognitive deficits displayed at 10 months of age in 3xTg-AD mice compared with wild-type mice (C57BL/6) on MWM and Y-maze performance is shown in the supplementary figures (Fig. S1 and Fig. S2).

Linagliptin Increases GLP-1 and GIP in the Brain but Has no Effect on Plasma Glucose Level

DPP-4 inhibition elevates incretin levels, which might be responsible for the neuroprotective activity observed in the current study. To confirm this hypothesis, we measured brain GLP-1 and GIP levels using commercially available ELISA kits. We observed a significant increase in both GLP-1 ($p < 0.01$) and GIP ($p < 0.05$) levels in a dose-dependent manner following linagliptin treatment (Fig. 4). However, statistically significant increases in brain GIP and GLP-1 were only observed at medium and high doses of linagliptin (10 and 20 mg/kg). Low-dose linagliptin (5 mg/kg) did not show significant changes in incretin levels. We also evaluated the plasma glucose levels in 3xTg-AD mice following linagliptin treatment using a glucose assay kit. This was performed to evaluate the effect of linagliptin in non-diabetic mice. No significant changes were observed in plasma glucose levels following linagliptin treatment in the 3xTg-AD mice (Fig. 5).

Linagliptin Reduces A β 42 but not A β 40

Amyloid burden is one of the cardinal pathological features in AD and it increases with age in 3xTg-AD mice [32]. Increases in plaque load positively correlate with cognitive deficits in the mice. Following behavioral testing, animals were sacrificed to determine the mechanisms underlying the alleviation of cognitive deficits following linagliptin treatment. To define the influence of linagliptin on plaque burden, we performed ELISA of brain homogenates and quantified the levels of A β 40 and A β 42. Linagliptin administration for 8 weeks significantly ($p < 0.01$) reduced the A β 42 level at a dose of 20 mg/kg as compared to control mice (Fig. 6). There was no significant change in A β 40 level between the control and treatment groups, demonstrating that linagliptin only affects A β 42 levels.

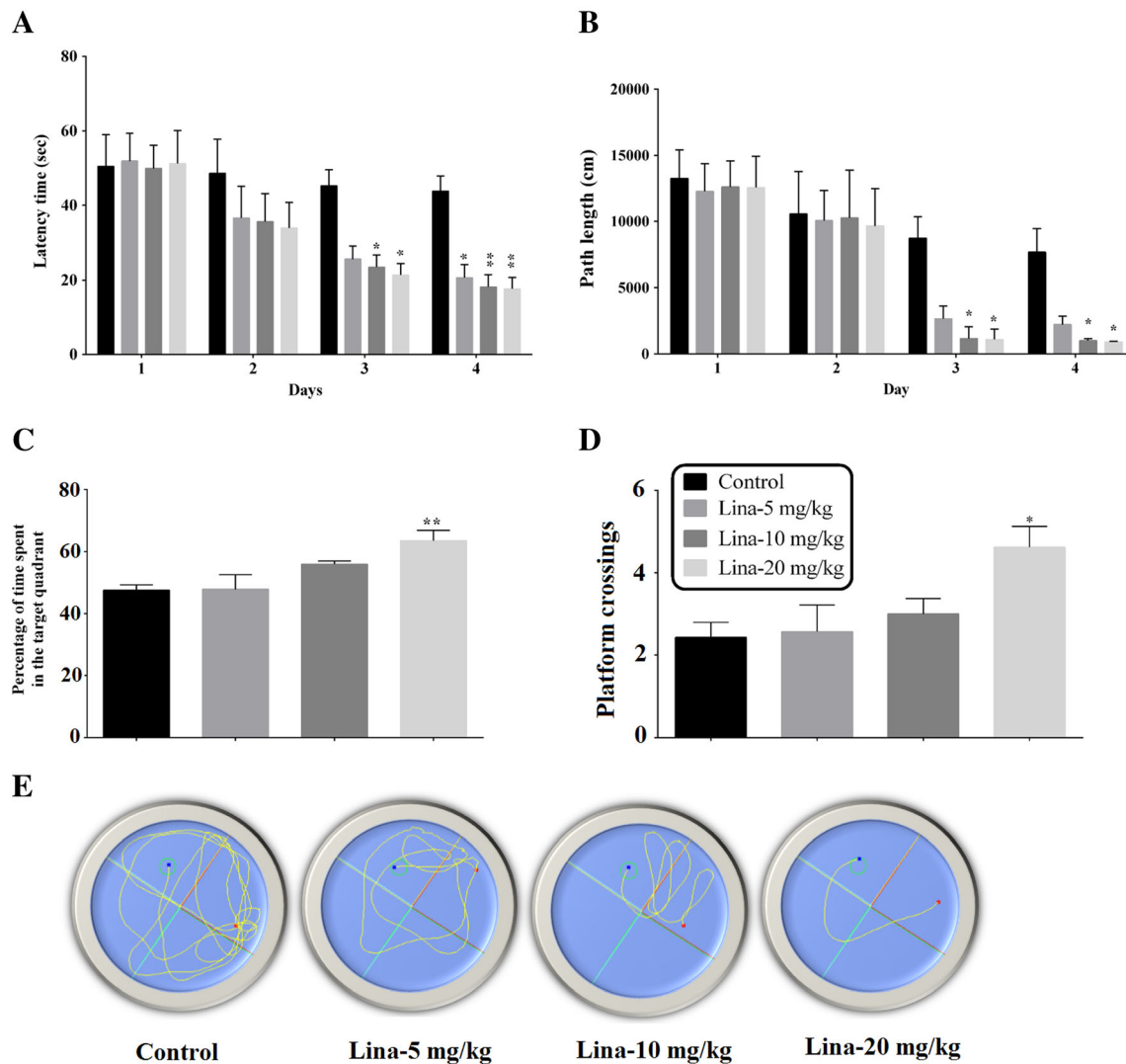


Fig. 2 Linagliptin reversed cognitive deficits in the Morris Water Maze. **a** Latency to reach the escape platform and **b** path length taken to reach the platform during acquisition trials. **c** Percentage of time spent in the platform quadrant and **d** number of platform crossings during the probe

trial. **e** Tracking details on day 4 trial 4 of corresponding groups. Significance was analyzed by unpaired *t* test ($n = 8$, mean \pm SEM) using Graphpad Prism. * $p < 0.05$ and ** $p < 0.01$, compared with control mice

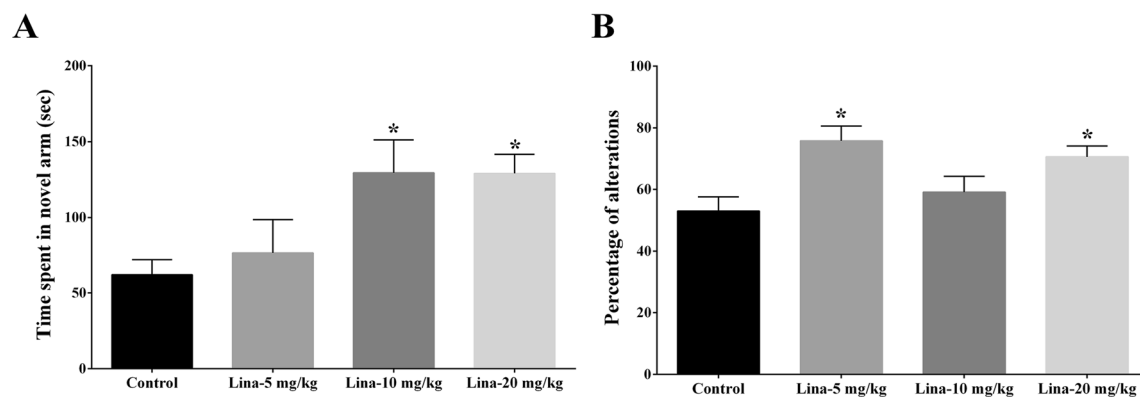


Fig. 3 Linagliptin improved cognitive function in the Y-maze. **a** Time spent in the novel arm and **b** percentage of alterations. Significance was analyzed by one-way ANOVA ($n = 8$, mean \pm SEM) followed by Bonferroni post hoc test using Graphpad Prism. * $p < 0.05$, compared with control mice

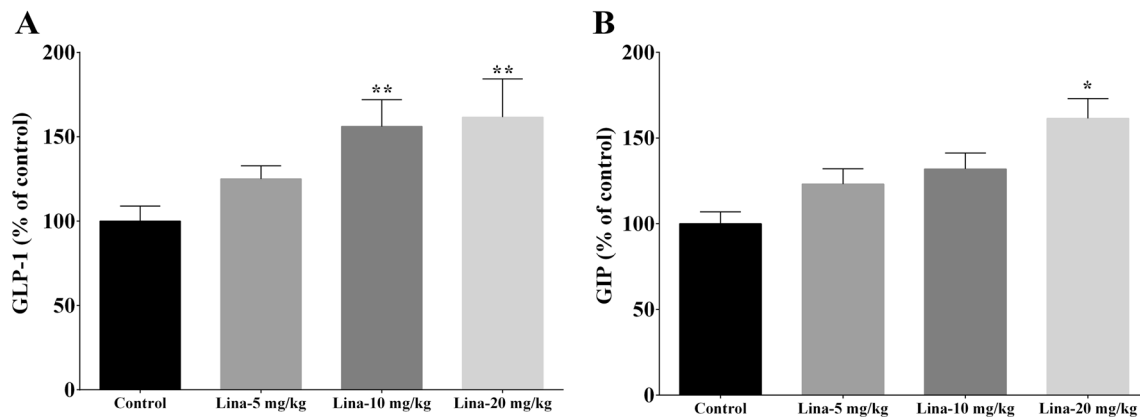


Fig. 4 Linagliptin increases glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) levels in the brain following 8 weeks of treatment. Significance was analyzed by one-way

ANOVA ($n = 6$, mean \pm SEM) following Bonferroni post hoc test using Graphpad Prism. * $p < 0.05$ and ** $p < 0.01$, compared with control mice. Levels of GLP-1 (a) and GIP (b) in the brain

Linagliptin Reduces Phosphorylated Tau and Neuroinflammation

Tau phosphorylation is also a major feature in the progression of AD and also worsens with age in 3xTg-AD mice [32]. To access the outcome of linagliptin treatment on tau phosphorylation, we performed Western blotting using AT8 primary antibody that detects phosphorylated tau at Ser202/Thr205. A significant ($p < 0.01$) reduction in p-tau at 20 mg/kg of linagliptin as compared to control mice was observed. Chronic neuroinflammation is an additional pathological feature in the AD brain. Interestingly, linagliptin at 20 mg/kg also reduced ($p < 0.05$) GFAP immunoreactivity, a marker for inflammation. We also examined levels of the amyloid precursor protein (APP) (Fig. 7) and total tau (data not shown) following linagliptin treatment but failed to observe any changes in these proteins.

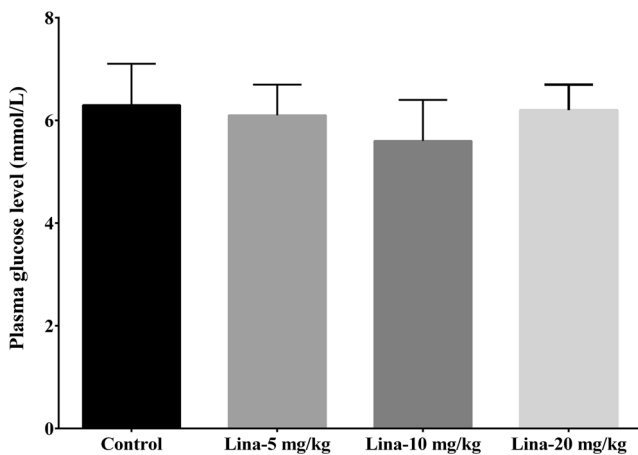


Fig. 5 Linagliptin does not alter the plasma glucose level following 8 weeks of treatment. Significance was analyzed by one-way ANOVA ($n = 6$, mean \pm SEM) following Bonferroni post hoc test using Graphpad Prism

Histology of Brain Sections Following Linagliptin Treatment

The efficacy of treatment on plaque deposition was examined by immunohistochemistry using the mouse monoclonal anti-A β 42 antibody 12F4 and by immunofluorescence using thioflavin S staining. Figures 8 and 9 represent images from brain sections of control and treated mice after immunohistochemistry and thioflavin S staining. Plaque load in the control mice was taken as reference (100 %) to determine the treatment effects of linagliptin. Immunohistochemistry of different groups proved that A β deposits in the linagliptin-treated (20 mg/kg) animals were reduced significantly ($p < 0.01$) as compared with control mice (Fig. 8). Plaque load was also confirmed by thioflavin S staining and quantification of plaques. Thioflavin S positive plaques were also significantly reduced ($p < 0.05$ and $p < 0.01$) by linagliptin treatment at 10 and 20 mg/kg, respectively (Fig. 9). Taken together, linagliptin treatment for 8 weeks effectively ameliorated plaque deposition in the 3xTg-AD mouse model of AD.

Discussion

Aging is a major risk factor contributing to AD. A wide range of diseases are linked with aging including diabetes and cardiovascular disorders [29]. Type-2-diabetes (T2D) is a recently identified risk factor and shares many features with AD [33, 34] that has led to the suggestion that treatments used for T2D might be beneficial in treating the pathologies associated with AD. Metformin and pioglitazone, two drugs used for T2D, have demonstrated promising results in preclinical models of AD [35, 36]. DPP-4 inhibitors are a novel class of T2D medications that have also shown beneficial effects in AD animal models [19–21]. We previously demonstrated that

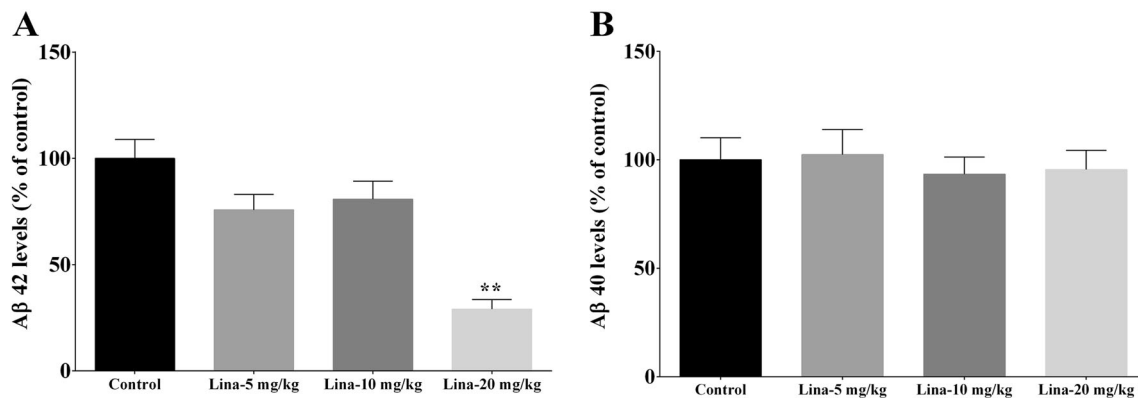


Fig. 6 Linagliptin decreases brain amyloid burden following 8 weeks of treatment by decreasing amyloid beta 1–42 levels. Significance was analyzed by one-way ANOVA ($n = 6$, mean \pm SEM) following

Bonferroni post hoc test using Graphpad Prism. $**p < 0.01$, compared with control mice. **a** Levels of A β 42 and **b** A β 40 in the brain

administration of vildagliptin [20] and saxagliptin [21] to streptozotocin-induced AD rats afforded neuroprotective effects. In the present study, 9–10-month-old female 3xTg-AD mice were treated daily with a DPP-4 inhibitor, linagliptin for a period of 8 weeks. This treatment period was chosen based on a previous study by Ma and colleagues [25] who demonstrated that treatment with linagliptin for 8 weeks resulted in the amelioration of

cognitive impairment and brain atrophy induced by transient cerebral ischemia in type 2 diabetic mice. Our data reveal that, under controlled environmental conditions, linagliptin improves learning and memory and alleviates clinical manifestations observed in 3xTg-AD mice. Taken together, the present data enhance the existing knowledge and contribute to the basic understanding of the role of DPP-4 inhibitors in AD.

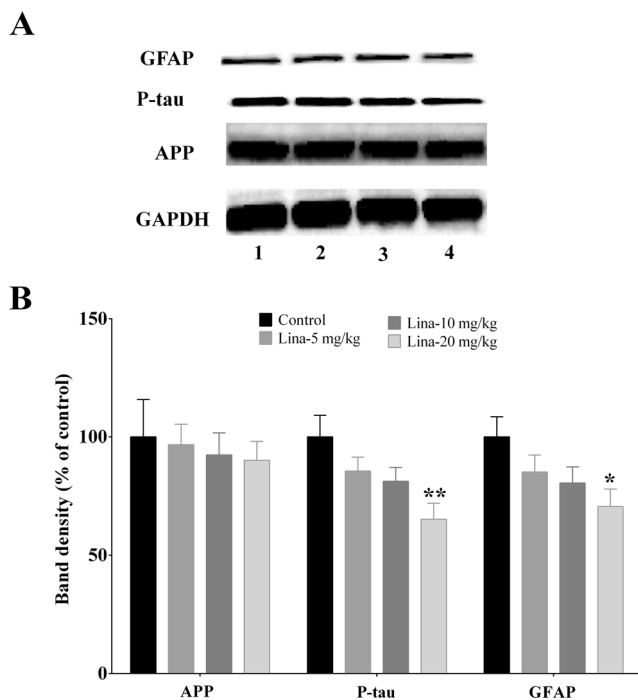
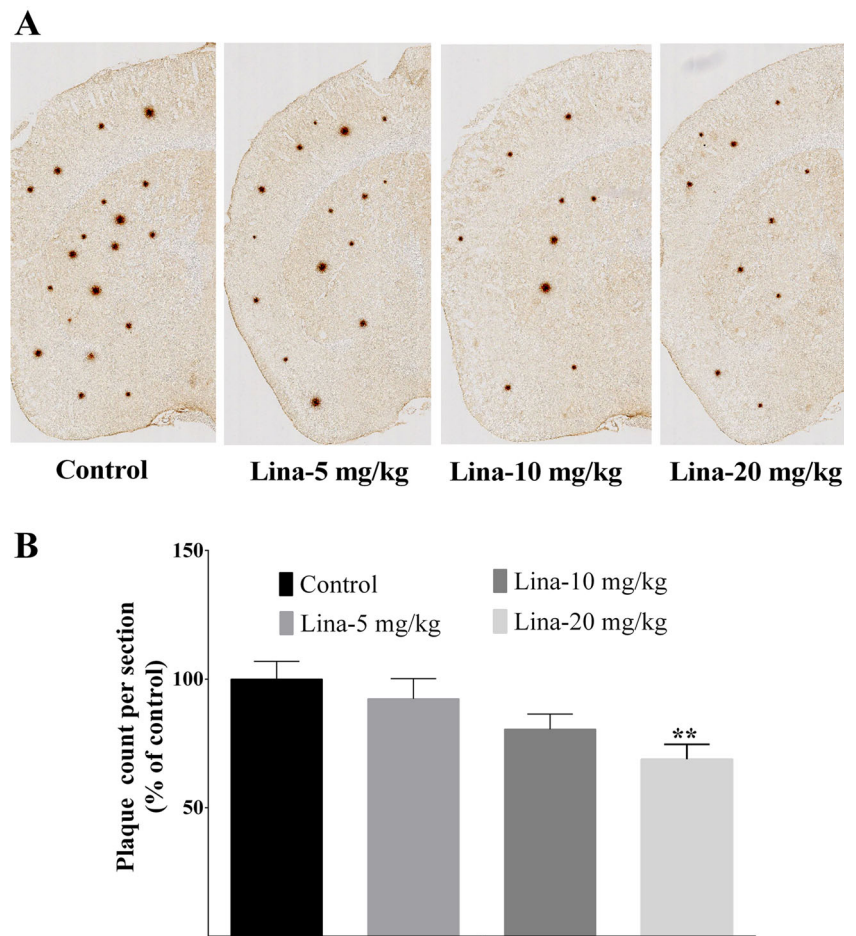


Fig. 7 Linagliptin reduces tau phosphorylation and neuroinflammation following 8 weeks of treatment. Significance was analyzed by one-way ANOVA ($n = 5$, mean \pm SEM) following Bonferroni post hoc test using Graphpad Prism. $*p < 0.05$ and $**p < 0.01$, compared with control mice. **a** Representative immunoreactive species of GFAP, P-tau, APP, and GAPDH in control and treated mice and **b** their corresponding densities. Lane 1: control, Lane 2: linagliptin 5 mg/kg, Lane 3: linagliptin 10 mg/kg, and Lane 4: linagliptin 20 mg/kg

The triple transgenic mouse model of AD (3xTg-AD) is a phenotypically well-characterized model and is ideally suited to screen therapeutic molecules against AD. 3xTg-AD mice have been reported to show cognitive deficits by 6 months, which worsens with age [37]. In the current study, the MWM and Y-maze tasks were used to test memory deficits in 3xTg-AD female mice at the age of 11–12 months. The MWM was carried out to observe spatial learning and memory whereas the Y-maze was used to monitor working memory; both tasks are highly sensitive tests and ideally suited to determine the effects of therapeutics in 3xTg-AD mice [38, 39]. Learning and memory enhancement was observed following linagliptin treatment and this is consistent with previous reports employing other DPP-4 inhibitors [19–21, 40, 41]. Sitagliptin and vildagliptin were also shown to improve cognitive function in insulin-resistant animal models [42, 43]. In the current study, linagliptin-treated mice demonstrated improved memory in the MWM compared to control mice. The treated mice also demonstrated enhanced spatial awareness by spending increased periods of time in the platform quadrant during the probe trial. The role of GLP-1 and GIP in cognitive function has been proved in receptor-knockout mice [44, 45]. We now extend this work by demonstrating that blocking the peripheral action of DPP-4 and thereby increasing levels of GLP-1 and GIP crossing the blood-brain barrier leads to cognitive enhancement in a disease-relevant animal model. Additional work will be necessary to decipher the

Fig. 8 Immunohistochemical analysis of amyloid plaques shows that linagliptin reduces amyloid burden following 8 weeks of treatment. Significance was analyzed by one-way ANOVA ($n = 5$, mean \pm SEM) following Bonferroni post hoc test using Graphpad Prism. $**p < 0.01$ compared with control mice. **a** Representative images of immunohistochemistry at $2\times$, and **b** the plaque count per section



mechanisms underlying the cognitive improvement observed in the present study.

Endogenous GLP-1 and GIP have a very short half-life and in order to prevent degradation from DPP-4, incretins that are DPP-4 resistant and possess longer half-life have been developed. Endogenous incretins and their analogs can cross the BBB, and a few studies suggest that DPP-4 inhibition may also increase GLP-1 levels in the brain [13, 22]. GLP-1 and GIP as well as their agonists promote cell differentiation, proliferation, and growth in the brain [46, 47]. In the current study, linagliptin administration for 8 weeks enhanced incretin levels in the brain. This suggests that there is a direct effect of incretins in mitigating the pathological markers of AD, including amyloid and tau in 3xTg-AD mice. Our study demonstrates that linagliptin reduced amyloid burden and tau phosphorylation following 8 weeks of treatment in 3xTg-AD mice. Reduced plaque load might be due to reduced production of A β since GLP-1 reduces APP processing by binding to its C-terminus. Similarly, GLP-1 protects SH-SY5Y neuroblastoma cells against A β -induced apoptosis [48, 49]. Prior reports have also shown that exendin-4, a GLP-1 receptor analog, downregulates glycogen synthase kinase 3 β

(GSK3 β) and decreases the phosphorylation of tau [50]. It is plausible that the reduced tau hyperphosphorylation observed in our study might be due to downregulation of GSK3 β . However, the relationship between tau phosphorylation and amyloid burden is highly complex. GLP-1 and its analogs have been proposed to prevent neurodegeneration by improving long-term potentiation and modifying synaptic plasticity [51, 52]. The neuroprotection afforded by incretins is well reported in animal models [4, 6–11, 15, 53, 54]. Clinical trials on exendin-4 and liraglutide in patients with AD are ongoing. Furthermore, previous studies on DPP-4 inhibitors against AD have shown consistent results on reducing both amyloid burden and tau phosphorylation in animal models [19–21]. In addition, our immunohistochemistry and thioflavin S staining results also reveal the reduction of overall plaque load in 11–12-month-old 3xTg-AD mice following linagliptin treatment.

Neuroinflammation is another well-known feature observed in the brain of Alzheimer's patients. The decreased cognitive capacity in AD might be attributed to increased production of cytokines including interleukins and tumor necrosis factor. A β production and tau phosphorylation are upregulated by activated glia which appears to be an

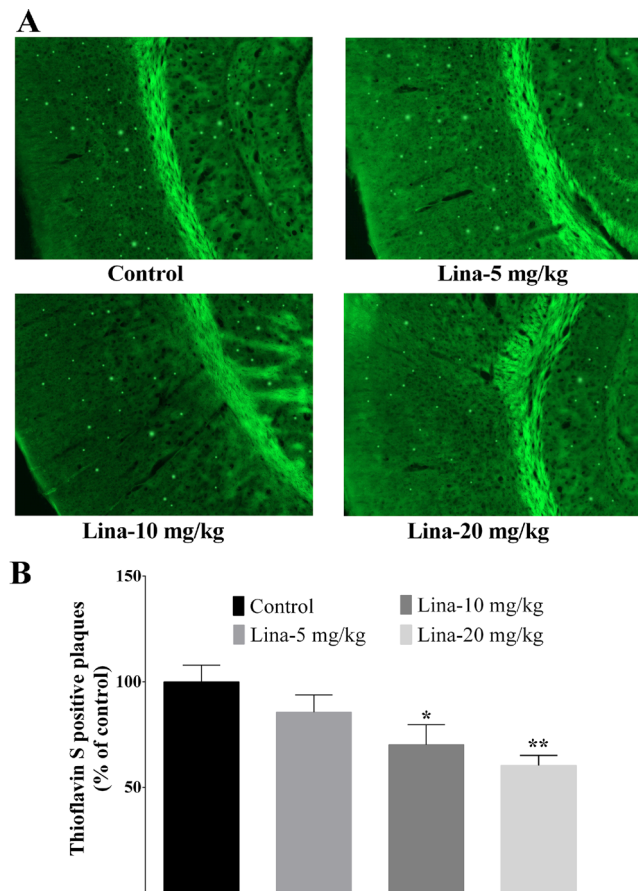


Fig. 9 Thioflavin S staining of amyloid plaques shows that linagliptin reduces amyloid plaques following 8 weeks of treatment. Significance was analyzed by one-way ANOVA ($n = 5$, mean \pm SEM) following Bonferroni post hoc test using Graphpad Prism. * $p < 0.05$ and ** $p < 0.01$, compared with control mice. **a** Representative images of thioflavin S staining at 5 \times , and **b** the plaque count per section

initial cause in AD pathophysiology [55]. In the present study, linagliptin mitigates neuroinflammation by significantly reducing GFAP, which is consistent with the previous reports on DPP-4 inhibitors [19–21]. Recent studies on DPP-4 inhibitors have demonstrated the reduction of inflammatory cytokine levels in chronic inflammatory conditions including atherosclerosis and myocarditis [56, 57]. In addition, GLP-1 and GIP act as anti-inflammatory mediators and counteract neuroinflammation in AD [34, 58]. GLP-1, GIP, and their analogs are also well known for their neuroprotective activity by reducing neuroinflammation [9, 59, 60]. This demonstrates that DPP-4 inhibition and incretins have independent roles in mitigating neuroinflammation.

Collectively, our investigation shows that the DPP-4-targeting molecule linagliptin mitigates plaque load and reduces tau phosphorylation, two cardinal pathologies observed in Alzheimer's disease. 3xTg-AD mice administered 20 mg/kg linagliptin demonstrate reduced cognitive deficits and improved performance on MWM and Y-

maze. Linagliptin dose-dependently improves brain levels of both GLP-1 and GIP incretins and reduces neuroinflammation, which may be attributed to the reduced amyloid burden and increased incretin levels. In conclusion, we have shown that DPP-4 inhibitors represent a promising therapeutic avenue in the management of AD.

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Authors' Contribution JK executed most of the study including dosing, behavioral studies, immunoblotting, histology, data interpretation, and manuscript writing. RMDH supported in analyzing the results and manuscript editing. LG involved in data interpretation. KYT supervised and guided all phases of the project, including the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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