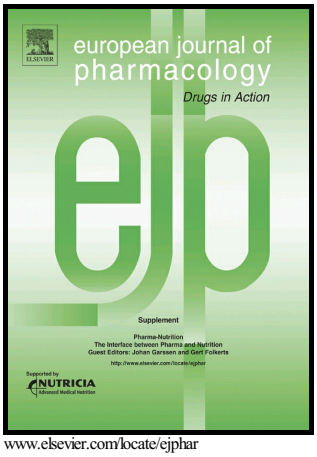

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PII: S0014-2999(16)30762-2
DOI: http://dx.doi.org/10.1016/j.ejphar.2016.11.050
Reference: EJP70955

To appear in: *European Journal of Pharmacology*

Received date: 22 September 2016
Revised date: 18 November 2016
Accepted date: 28 November 2016

Cite this article as: Yanwei Li, WeiZhen Liu, Lin Li and Christian Hölscher, D-Ala2-GIP-glu-PAL is neuroprotective in a chronic Parkinson's disease mouse model and increases BNDF expression while reducing neuroinflammation and lipid peroxidation, *European Journal of Pharmacology* http://dx.doi.org/10.1016/j.ejphar.2016.11.050

D-Ala2-GIP-glu-PAL is neuroprotective in a chronic Parkinson's disease mouse model and increases BNDF expression while reducing neuroinflammation and lipid peroxidation

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Abstract

Type 2 diabetes mellitus (T2DM) is a risk factor for Parkinson's disease (PD). Therefore, treatment to improve insulin resistance in T2DM may be useful for PD patients. Glucose dependent insulinotropic polypeptide (GIP) is a member of the incretin hormone family that can promote insulin release and improve insulin resistance. Several GIP analogues have been developed as potential treatments for T2DM. We had shown previously that D-Ala2-GIP-glu-PAL, a novel long-acting GIP analogue, can play a neuroprotective role in the PD mouse model induced by acute MPTP injection. The drug reduced damage to the dopaminergic neurons and increased CREB-mediated Bcl-2 expression to prevent apoptosis and reduced chronic inflammation in the brain. In the present study, we further tested the effects of chronic treatment by D-Ala2-GIP-glu-PAL in a chronic PD mouse model induced by MPTP (25 mg/kg ip.) combination with probenecid (250 mg/kg ip.) injection for 5 weeks. The results demonstrated that chronic treatment with D-Ala2-GIP-glu-PAL inhibits MPTP -induced Parkinsonism-like motor disorders in mice, and that the drug prevents dopaminergic neuronal loss in the substantia nigra pars compacta (SNpc). Moreover, D-Ala2-GIP-glu-PAL also inhibited the increased levels of expression of α-synuclein in the SNpc and striatum induced by MPTP. Furthermore, drug treatment reduced chronic neuroinflammation, oxidative stress and lipid peroxidation, and increased the expression of BDNF. These findings show that GIP signaling is neuroprotective and holds promise as a novel treatment of PD.

Keywords: neurodegeneration; insulin; brain; incretin; growth factor; dopamine

1. Introduction

Parkinson's disease (PD) is a degenerative neurological disorder affecting over 1% of the population. A key aspect of the disease is the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), and in some cases, the aggregation of α-synuclein in neurons (Kowal et al., 2013). Disease- modifying treatments for PD are still lacking. Similarities between PD and Type 2 diabetes mellitus (T2DM) have been discovered recently. A clinical data meta-analysis found that 8-30 % of PD patients were diabetic, a significantly higher percentage compared to age matched controls (Cereda et al., 2011; Hu et al., 2007; Miyake et al., 2010). A histological study found a loss of insulin receptor immunoreactivity in the SNpc neurons in PD, and a dysfunction of the insulin signaling system may precede death of the dopaminergic neurons (Moroo et al., 1994). Also, exendin-4, an agonist for the glucagon-like peptide-1 receptor (GLP-1R) that is on the market to treat T2DM, showed therapeutic effects in preclinical tests (Kim et al., 2009; Li et al., 2009). Furthermore, a pilot clinical trial in PD patients showed good effects with this drug (Aviles-Olmos et al., 2013; Foltynie and Aviles-Olmos, 2014; Simuni and Brundin, 2014). Hence, a treatment to improve insulin resistance may be useful for PD patients (Athauda and Foltynie, 2016).

Glucose-dependent insulinotropic polypeptide (GIP) is a member of the incretin hormone family (Baggio and Drucker, 2007). Several protease-resistant GIP analogues have been developed as potential treatments for type 2 diabetes (Irwin et al., 2006). In the central nervous system, GIP and the GIPR is found in the hippocampus, cerebellum, cerebral cortex, substantia nigra, thalamus, the hypothalamus, and the brainstem (Nyberg et al., 2005; Nyberg et al., 2007). GIP modulates synaptic activity – D-Ala2GIP prevented the detrimental effects of Aβ on synaptic plasticity in the hippocampus (Gault and Holscher, 2008) and on spatial learning and memory (Figueiredo et al., 2011). In our previous studies, chronic injection of D-Ala2GIP enhanced memory formation, long-term potentiation (LTP) in the hippocampus, and progenitor cell proliferation in the dentate gyrus of mice (Faivre et al., 2012; Porter et al., 2011). Moreover, D-Ala2GIP improved cognitive function and prevented memory deficits in the APP/PS1 mouse model of Alzheimer's disease, and reduced the Aβ plaque load and chronic inflammation in the brain (Duffy and Holscher, 2013; Faivre and Holscher, 2013a, b).

Based on this information, we previously have tested the effects of a novel GIP analogue, D-Ala2-GIP-glu-PAL in a PD mouse model induced by acute MPTP injection without added probenecid. The results demonstrated that treatment with D-Ala2-GIP-glu-PAL significantly inhibited MPTP-induced Parkinsonism-like motor impairments in mice, and reduced damage to dopaminergic neurons. Moreover, the drug increased CREB-mediated Bcl-2 expression to prevent apoptosis and reduced chronic neuroinflammation (Li et al., 2016). To further analyse the underlying molecular mechanisms, we studied the effects of systemic administration of D-Ala2-GIP-glu-PAL in a chronic mouse model of PD induced by MPTP in combination with probenecid treatment which enhances the toxic MPTP effect (Korecka et al., 2013; Meredith et al., 2008). Chronic lesion with MPTP is a more realistic model of the progressive neurodegenerative development in PD.

2. Material and methods

2.1 Reagents and peptides

Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and probenecid were purchased from Sigma-Aldrich (St Louis, MO, USA). RIPA lysis buffer, BCA protein assay kit and ECL-enhanced chemoluminescence were purchased from Beyotime Institute of Biotechnology (Shanghai, China). The primary antibodies recognising TH, IBA1, 4-Hydroxynonenal (4-HNE), BDNF and β-actin were obtained from Abcam (Cambridge, UK). The primary antibody against α-synuclein was obtained from Cell Signaling Biotechnology (Beverly, MA, USA) and the primary antibody against GFAP was obtained from Boster Biotechnology (Wuhan, China). HRP-labeled goat anti-rabbit immunoglobulin was obtained from Abcam (Cambridge, UK). The biotinylated goat anti-rabbit IgG, biotinylated rabbit anti-goat IgG, avidin-biotin peroxidase complex reagent was purchased from Boster Biotechnology (Wuhan, China). The 3, 3-diaminobenzidine (DAB) solution was obtained from Zhongshan Golden Bridge Biotechnology (Beijing, China). All other chemicals used were of analytical grade and of the highest quality commercially available.

D-Ala2-GIP-glu-PAL was synthesized by GL Biochem Ltd (Shanghai). The purity of the peptide

was analysed by reversed-phase high performance liquid chromatography (HPLC) and characterised using matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry. D-Ala2-GIP-glu-PAL sequence:

Tyr-**(D-Ala)**-Glu-Gly-Thr-Phe-Ile-Ser-Asp-Tyr-Ser-Ile-Ala-Met-Asp-Lys-Ile-His-Gln-Gln-Asp-Phe-Val-Asn-Trp-Leu-Leu-Ala-Gln-Lys-Gly-Lys-Lys-Asn-Asp-Trp-Lys**(glu-PAL)**-His-Asn-Ile-Thr-Gln

(PAL = C16 fatty acid)

2.2 Animals and drug treatments

Adult male C57BL/6 mice (25–30 g) were purchased from the Academy of Military Medical Sciences (AMMS, China). The animals were maintained on 12-h light/dark cycle at 24 ℃ ± 1℃ and 55 ± 5% humidity with food and water *ad libitum*, and were acclimatized for at least 1 week prior to the medication. All experimental procedures involving animals in the present study were approved by the Institutional Animal Care Committee of Shanxi Medical University and conform to the guidelines of National Institute of Health (NIH) guideline (NIH publication NO. 85-23. Revised 1985).

Mice were divided into four different groups (n=12 per group) consisting of (i) a control group treated with saline alone; (ii) a group treated with D-Ala2-GIP-glu-PAL; (iii) a group treated with MPTP and probenecid; and (iv) a group treated with D-Ala2-GIP-glu-PAL in combination with MPTP and probenecid. Mice were intraperitoneally injected with 10 doses of MPTP (25 mg/kg in saline) and probenecid (250 mg/kg in dimethyl sulfoxide) for 5 weeks with an interval of 3.5 days between consecutive doses. D-Ala2-GIP-glu-PAL was dissolved in saline and drug (25nmol/kg body weight,) ip. was treated one time per day during MPTP and probenecid injection and continued for 1 week after MPTP and probenecid injection. Control mice received equal volumes of saline solution. At the end of drug treatments, measurements of behavioral changes, neuronal damage, and pathologic biomarkers were conducted.

2.3 Behavioral assessment

2.3.1 Open-field test

To assess locomotor and exploratory activity of mice, the open-field test was conducted on the 6th weeks as previously described (Li et al., 2016). The open-field apparatus consisted of a square arena (50 cm*50 *cm *40 cm) with a floor divided into 25 equal-sized squares with grids. Each mouse was placed in the center of the square arena and allowed to move freely for 5 min. Two parameters of mouse were measured in this experiment: the number of crossing line (crossing the squares boundaries with both forepaws) and the number of rearing (standing on its hind legs and sometimes leaning on the wall with forelegs, sniffing and looking around). The surface of the arena was then cleaned with 70% alcohol and dried between trials. The evaluation was conducted by an investigator who was unaware of the information of grouping and drug administration. The experiment was repeated 2 times for each animal, and the average of 2 trials was calculated for statistical analyses.

2.3.2 Pole test

To determine the degree of bradykinesia and ability to movement balance of mice, the pole test was conducted on the 6th weeks as previously described (Li et al., 2016). In brief, animals were positioned head upward near the top of a rough-surfaced wooden pole (10 mm in diameter and 55 cm in height). The time taken until animals turned around completely and face the floor downward (defined as turn time, T-turn) and the time taken to climb down the pole and place all four feet on the floor (locomotor activity time, T-LA) were recorded. If the mouse did not descend in 30s, it was guided, and T-turn was recorded as 30s. Before drug injections, all mice were pre-trained for pole test procedure for three times a day for five consecutive days. Every mouse was tested for 3 times and the average of the three trials was calculated for statistical analyses. The investigator was blind to the treatments given to mice.

2.3.3 Rotarod test

To determine the degree of hypokinesia and motor coordination of mice, the rotarod test was conducted on the 6th weeks as previously described (Liu et al., 2015). The rotarod equipment (YLS-4C, Academy of medical sciences in Shandong, China) consisted of a rotating spindle (3 cm in diameter) and five individual compartments able to simultaneously test five mice. Before initiation of the experiment, all mice were trained on rotarod for three times a day for five

consecutive days. On the 6th weeks of experiment, mice were placed on a rod that accelerated smoothly from 5 to 20 rpm over a period of 180s. The time that each mouse was able to stay on the rod was recorded as the latency period to fall. The experiment was repeated 3 times for each mouse at 10 min rest intervals to prevent stress and fatigue. The average of the three trials was calculated for statistical analyses. The investigator was blind to the treatments given to mice.

2.4 Sample preparation

All animals were killed on the end of the 6^{th} weeks. After anesthetization with ethyl carbamate, whole brains of half of mice per group were removed immediately and ventral midbrain was dissected out and stored at −80 °C for western blot analysis. And another half of mice per group were transcardial perfused with ice-cold PBS and 4% paraformaldehyde/PBS (pH7.4), and brains were removed and post-fixed for 24 h in the same fixative, and the brains were embedded in paraffin.

2.5 Immunohistochemistry

The sections of brain tissue samples were cut at 4 μm with a microtome. The sections encompassing the SNpc and the striatum were placed on coated slides. Paraffin was removed from the tissue sections with xylene, and the sections were rehydrated in graded ethanol solutions. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 min. Antigen retrieval was performed by heating in 10 mmol/l citrate buffer (pH 6.0) for 10 min. After blocked with 5% BSA, sections were incubated with the primary antibody for TH (rabbit anti-TH; 1:500), GFAP (rabbit anti-GFAP; 1:100), IBA1 (goat anti-IBA1; 1:500), α-synuclein (rabbit anti-α-synuclein; 1:200) and 4-HNE (rabbit anti- 4-HNE; 1:200) at 37℃ for 1h. After rinsed in PBS, the sections were incubated with biotinylated goat anti-rabbit IgG or biotinylated rabbit anti-goat IgG at 37℃ for 0.5h and incubated with followed by the avidin-biotin peroxidase complex reagent at 37℃ for 0.5h. Samples were colored in 3,3-diaminobenzidine (DAB) solution in the presence of H_2O_2. To verify the immune specificity, control sections were processed with the same protocol, except that omitting the primary antibody. And some sections were retained used with hematoxylin. All stained sections were viewed and photographed under a Zeiss light microscope, and images were captured by digital camera (Motic BA210).

Quantitative analysis of DA neurons in SNpc was carried out by serial section analysis of the total number of TH positive (TH+) neurons throughout the rostro-caudal axis. Only the region corresponding to the SNpc was carefully delineated, according to the mouse brain atlas of Paxinos (Paxinos et al., 1999). Using the method of unbiased 2D stereology, a dissector was overlaid onto the micrographs of the brain section. The ratio percent of the total number of TH+ neurons of every mouse and average TH+ neurons of control group was calculated for statistical analyses. The optical density of dopaminergic neuron termini in the striatum were determined from scanned TH+ sections by digital densitometry using Image-pro plus6.0 software. The areas of GFAP and IBA1 positive cells, the average optical density of α-synuclein and 4-HNE positive staining in the SNpc and striatum were also determined using Image-pro plus6.0 software.

2.6 Western blot

Western blot assay was performed using standard protocols. Midbrain tissues containing substantia nigra were homogenized on ice in RIPA lysis buffer containing phenyl-methylsulfonyl fluoride (PMSF). After 30 min, tissue lysates were obtained by centrifugation at 12000 rpm (28000 G) for 5 min at 4°C. The protein concentration of the samples then was quantified by BCA protein assay. After tissue lysate was mixed in loading buffer and boiled for 5 min, equivalent amounts of protein were separated on 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% milk in Tris-buffered saline and incubated overnight at 4°C with rabbit anti-mouse α-synuclein(1:1000,) and anti-mouse BDNF(1:5000), followed by incubation for 2h at room temperature with the HRP-labeled goat anti-rabbit immunoglobulin (1:10000). The bound antibodies were then visulalized by ECL-enhanced chemoluminescence according to the manufacturer's instructions. In some cases, the blots were stripped and reprobed with a rabbit anti-mouse β-actin (1:5000) to ensure equal sample loading. Western blot images were captured with a chemiluminescent imaging system (Sagecreation, Beijing, China). All bands were quantified using the image system of Quantity one 4.31(Bio-Rad, Hercules, CA, USA).

2.7 Statistical analysis

All data are presented as the mean ±standard error of mean (SEM). Statistical assessment was

analyzed by one way analysis of variance (ANOVA) followed with post-hoc Bonferroni tests between four different groups. A value of $P < 0.05$ was considered statistically significant. All analyses were performed with the Graph Pad Prism version 5.0 Software (GraphPad Software Inc, La Jolla, CA, USA).

3. Results

3.1 D-Ala2-GIP-glu-PAL alleviated motor impairments induced by MPTP and probenecid

In the open field test, D-Ala2-GIP-glu-PAL alleviated the impairments of locomotor and exploratory activity of mice induced by MPTP and probenecid. A one-way ANOVA found an overall difference on the numbers of crossings ($F=9.83$, $df=11$; $P<0.001$) and the numbers of rearing ($F=17.87$, $df=11$; $P<0.001$) of mice between groups. In post-hoc Bonferroni tests, the results showed that the numbers of crossings in MPTP and probenecid group mice exhibited a significant reduction versus the control group mice ($P<0.01$). However, D-Ala2-GIP-glu-PAL treatment reversed these effects on crossing ($P<0.05$). Furthermore, MPTP and probenecid procedure also decreased the numbers of rearing ($P<0.001$), and D-Ala2-GIP-glu-PAL could improve the numbers of rearing ($P<0.05$). Although numbers of crossings and rearing in MPTP and probenecid + D-Ala2-GIP-glu-PAL group mice were still less than control group, there was no significant($P>0.05$). N =12 mice in each group, see Fig. 1A.

In the pole test, D-Ala2-GIP-glu-PAL improved the bradykinesia and movement imbalance of mice induced by MPTP and probenecid. A one-way ANOVA found an overall difference on T-turn ($F= 19.78$, $df=11$; $P<0.001$) and T-LA ($F=10.66$, $df=11$; $P<0.001$) of mice between groups. In post-hoc Bonferroni tests, the results showed that MPTP and probenecid injection induced the bradykinesia of mice, with more time to turn at the top (T-turn) ($P<0.001$) compared with the control. The MPTP and probenecid injection also induced movement imbalance of mice, with more time required by mice to climb down (T-LA) ($P<0.001$) compared to controls. However, treatment with D-Ala2-GIP-glu-PAL significantly reversed these effects induced by MPTP and probenecid ($P<0.05$). But T-turn in MPTP and probenecid + D-Ala2-GIP-glu-PAL group mice were still more than control group($P<0.01$). Although T-LA in MPTP and probenecid + D-Ala2-GIP-glu-PAL group mice were still more than control group, there was no

significant(P>0.05).N =12 mice in each group, see Fig. 1B.

In the rotarod test, D-Ala2-GIP-glu-PAL improved muscular strength and hypokinesia of mice induced by MPTP and probenecid. A one-way ANOVA found an overall difference between groups (F=16.97, df=11; P<0.001). In post-hoc Bonferroni tests, the results showed that MPTP and probenecid injection induced the impairments in muscular strength and movement balance of mice, with less time staying on the rotating rod (drop latency) (P<0.001) compared with the control. However, treatment with D-Ala2-GIP-glu-PAL significantly reversed these effects induced by MPTP and probenecid (P<0.05). But drop latency in MPTP and probenecid + D-Ala2-GIP-glu-PAL group mice were still less than control group(P<0.05). N =12 mice in each group, see Fig. 1C.

3.2 D-Ala2-GIP-glu-PAL reduced dopaminergic neuronal loss induced by MPTP and probenecid

The expression of tyrosine hydroxylase (TH), a key enzyme for the synthesis of dopamine, was analyzed by immunohistochemical staining which identifies dopaminergic neurons. A one-way ANOVA found an overall difference on the numbers of TH positive cells in the SNpc (F= 36.70, df=5; P<0.001) and the striatum (F= 30.54, df=5; P<0.001) of mice between groups. There is no difference between control group and D-Ala2-GIP-glu-PAL group. The numbers of TH positive cells in the SNpc was decreased to 33.99% of control group (P<0.001), and the average optical density of TH staining in the striatum was decreased to 56.75% of control group (P<0.001) by MPTP and probenecid injection for 5 weeks. However, D-Ala2-GIP-glu-PAL treatment restored numbers of dopaminergic neurons to 59.21% in SNpc (P<0.05) and the overall levels of TH to 73.33% in the striatum (P<0.05) in MPTP and probenecid -treated mice. But the numbers of TH positive cells in the SNpc and the average optical density of TH staining in the striatum in MPTP and probenecid + D-Ala2-GIP-glu-PAL group mice were still less than control group(P<0.001). N =6 mice in each group, see Fig. 2.

3.3 D-Ala2-GIP-glu-PAL inhibited the increase of α-synuclein expression in the SNpc and striatum induced by MPTP and probenecid

The level of α-synuclein, a primary structural component of Lewy body, was analyzed by Western

blot and immunohistochemical staining.

In the Western blot, a one-way ANOVA found an overall difference between groups (F=11.49, df=5; P<0.001). The expression of α-synuclein in SNpc was increased in mice treated with MPTP and probenecid (P<0.001), compared to control. While D-Ala2-GIP-glu-PAL treatment partially reversed the increase (P <0.05) compared to the MPTP and probenecid group. Although the expression of α-synuclein in SNpc in MPTP and probenecid + D-Ala2-GIP-glu-PAL group mice were still higher than control group, there was no significant(P>0.05).N =6 mice in each group, see Fig. 3.

In the immunohistochemical staining, a one-way ANOVA found an overall difference on the α-synuclein expression in the SNpc (F=37.32, P<0.001) and the striatum(F=18.34, df=5; P<0.001) of mice between groups. The expression of α-synuclein in the SNpc and striatum of control mice was moderate. There is no difference between control group and D-Ala2-GIP-glu-PAL group. The average optical density of α-synuclein positive staining in the SNpc and striatum was significantly increased by MPTP and probenecid injection for 5 weeks (P<0.001) compared with control mice. However, D-Ala2-GIP-glu-PAL treatment decreased the effects induced by MPTP and probenecid (P<0.05). But the average optical density of α-synuclein positive staining in the SNpc(P<0.001) and striatum(P<0.05) in MPTP and probenecid + D-Ala2-GIP-glu-PAL group mice were still higher than control group. N =6 mice in each group, see Fig. 3.

3.4 D-Ala2-GIP-glu-PAL reduced astrocyte and microglia activation in the SNpc and striatum induced by MPTP and probenecid

In the immunohistochemical analysis, an anti-GFAP antibody was used as an indicator of activated astrocytes. GFAP is expressed at a much higher rate in activated astrocytes and indicates an inflammation response. A one-way ANOVA found an overall difference on the GFAP expression in the SNpc (F= 121.7, df=5; P<0.001) and the striatum (F= 94.13, df=5; P<0.001) of mice between groups. GFAP-positive stain covered an average of 2.22% of each section in the SNpc of control mice, and covered an average of 0.85% of each section in the striatum. There was no difference between the control and the D-Ala2-GIP-glu-PAL injection group. GFAP levels in the SNpc increased to 14.83% after MPTP and probenecid treatment for 5 weeks, and in the striatum increased to 10.92%, which was significantly higher than in control mice (P<0.001).

While D-Ala2-GIP-glu-PAL treatment reduced the area of GFAP-positive stain in the SNpc to 8.52%, in the striatum to 4.82%, which was significantly less than in the MPTP and probenecid procedure group ($P<0.001$). But the areas of GFAP-positive astrocytes in the SNpc and striatum in MPTP and probenecid + D-Ala2-GIP-glu-PAL group mice were still higher than control group($P<0.001$). N =6 mice in each group, see Fig. 4.

In the immunohistochemical staining of IBA1, which is expressed at a much higher rate in activated microglia, a one-way ANOVA found an overall difference on the IBA1 expression in the SNpc ($F= 45.92$, $df=5$; $P<0.001$) and the striatum ($F= 43.24$, $df=5$; $P<0.001$) of mice between groups. IBA1-positive stain covered an average of 0.21% of each section in the SNpc of control mice, and covered an average of 0.40% of each section in the striatum. There was no difference between control and the D-Ala2-GIP-glu-PAL injection group. IBA1 levels in the SNpc increased to 1.27% after MPTP and probenecid treatment for 5 weeks, and in the striatum increased to 1.11%, which were significantly higher than in control mice ($P<0.001$). While D-Ala2-GIP-glu-PAL treatment decreased the areas of IBA1-positive microglia in SNpc to 0.58% and in the striatum to 0.79%, which was significantly less than in the MPTP and probenecid procedure group ($P<0.01$). But the areas of IBA1-positive microglia in the SNpc ($P<0.01$) and striatum ($P<0.001$) in MPTP and probenecid + D-Ala2-GIP-glu-PAL group mice were still higher than control group. N =6 mice in each group, see Fig. 5.

3.5 D-Ala2-GIP-glu-PAL reduced lipid peroxidation in the SNpc and striatum induced by MPTP and probenecid

In the immunohistochemical analysis, 4-HNE was measured as an indicator of lipid peroxidation. A one-way ANOVA found an overall difference on 4-HNE expression in the SNpc ($F=13.76$, $df=5$; $P<0.001$) and the striatum ($F=10.25$, $df=5$; $P<0.001$) of mice between groups. There is no difference between control group and the D-Ala2-GIP-glu-PAL group. The average optical density of 4-HNE staining in the SNpc and striatum was significantly increased by MPTP and probenecid injection for 5 weeks ($P<0.001$), compared with the control group. However, D-Ala2-GIP-glu-PAL treatment decreased the levels induced by MPTP and probenecid ($P<0.001$). Although the expression of 4-HNE in SNpc and striatum in MPTP and probenecid +

D-Ala2-GIP-glu-PAL group mice were still higher than control group, there was no significant(P>0.05).N =6 mice in each group, see Fig. 6.

3.6 D-Ala2-GIP-glu-PAL inhibited the decrease of BDNF expression in the SNpc induced by MPTP and probenecid

The expression of BDNF, an important neurotrophic factor in the brain, was analyzed by Western blot. A one-way ANOVA found an overall difference between groups (F=15.39, df=5; P<001). The expression of BDNF in SNpc was much decreased in mice treated with MPTP and probenecid (P<0.001), compared to control. While D-Ala2-GIP-glu-PAL treatment partially reversed the decrease (P <0.05) compared to the MPTP and probenecid group. Although the expression of BDNF in SNpc in MPTP and probenecid + D-Ala2-GIP-glu-PAL group mice were still less than control group, there was no significant(P>0.05).N =6 mice in each group, see Fig. 7.

4. Discussion

In the present study, we showed antiparkinsonian effects of chronic systemic administration of D-Ala2-GIP-glu-PAL in a chronic mouse model of PD induced by MPTP in combination with probenecid. The neurotoxin MPTP has been shown to induce Parkinsonism in humans (Przedborski and Jackson-Lewis, 1998), and is widely used for modeling Parkinsonism in rodents and non-human primates (Fox and Brotchie, 2010). The chemical MPTP can cross the blood–brain barrier and is converted in the cell to 1-methyl-4-phenylpyridium ion (MPP+) by the monoamine oxidase B in glia and dopaminergic neurons (Glover et al., 1986). MPP+ is selectively transported into neurons by the dopamine transporter. In the cell it inhibits the mitochondria complex I which induces oxidative stress and exerts cytotoxic effects on dopaminergic neurons (Gerlach et al., 1991). Probenecid is known to promote MPTP/MPP+ build -up in the brain and potentiates its neurotoxic effect by impeding the renal excretion and neuronal clearance of MPTP and its toxic metabolites. As animals do not naturally develop PD, the MPTP and probenecid chronic lesion mouse model is used to test new drug candidates for their Neuroprotective properties (Meredith et al., 2008).

In the present study, the behavioral assessment demonstrated that D-Ala2-GIP-glu-PAL

significantly alleviates motor disorders induced by MPTP and probenecid. This finding was consistent with the results of our previous study (Li et al., 2016). As TH is a key enzyme in the dopamine synthesis, the changes in TH levels indicate a reduction in the synthesis of dopamine in SNpc neurons. Following lesion with MPTP and probenecid for 5 weeks, TH positive neuron numbers in SNpc were reduced to 33.99% of controls, and TH levels in the striatum was decreased to 56.75%. However, D-Ala2-GIP-glu-PAL treatment for 6 weeks restored numbers of dopaminergic neurons to 59.21% in SNpc and the overall levels of TH to 73.33% in the striatum. A similar protection of neurons by GIP analogues was observed in a mouse model of AD (Faivre and Holscher, 2013a, b; Gault and Holscher, 2008). GIP treatment also showed neuroprotective effects in a rat model of traumatic brain injury by reversing cognitive impairments and reducing the chronic inflammation response in the brain (Yu et al., 2016).

Besides progressive loss of dopaminergic neurons in the SNpc, the formation of Lewy Bodies (LB), which are cytoplasmic inclusions mainly containing α-synuclein, are among the pathological hallmarks of PD (Yasuda et al., 2013). In the present study, the expression of α-synuclein was analyzed by Western blot and immunohistochemistry. The results showed that α-synuclein expression in SNpc and striatum was increased following treatment with MPTP and probenecid, which confirms previous studies in which α-synuclein was up-regulated in MPTP-treated animals (Kuhn et al., 2003) and α-synuclein-positive neurons that were also TH-positive (Vila et al., 2000). Transgenic α-synuclein overexpressing mice develop deficits in motor function at 12 weeks when the loss of DA neurons exceeds a threshold of around 50%. The overexpression of α-synuclein induced progressive nigrostriatal degeneration and increased the susceptibility of DA neurons to MPTP (Song et al., 2015). The results of immunohistochemistry showed that in normal mice, detectable levels of α-synuclein protein were seen in all brain regions studied and especially in the ventral midbrain. In the latter, there was a dense α-synuclein-positive nerve fiber network in the SNpc, and only few scattered α-synuclein-positive neurons. After MPTP and probenecid treatment, numbers of α-synuclein-positive neurons were increased in the SNpc and there was an increase of α-synuclein levels in some neurons. However, D-Ala2-GIP-glu-PAL significantly inhibited the increase of α-synuclein expression, by which D-Ala2-GIP-glu-PAL may reduce the α-synuclein-related pathological mechanism of PD.

Chronic inflammation is thought to play a central role in PD pathogenesis (Ferrari and Tarelli,

2011). In clinical studies, high concentrations of pro-inflammatory cytokines such as interleukin (IL)-1b, IL-6, and tumor necrosis factor (TNF)-α have been found in the brain, cerebrospinal fluid, and blood of PD patients (Chen et al., 2008). Abnormal production of pro-inflammatory cytokines by activated microglia and astrocytes can lead to synapse dysfunction and ultimately synapse loss (Herrero et al., 2015). Co-culture of activated astrocytes with primary dopaminergic cultures leads to increased susceptibility of dopaminergic neurons to MPP+ and 6-OHDA -mediated cell death (McNaught and Jenner, 1999). Hence, anti-inflammatory medication is an attractive therapeutic strategy for PD (Hirohata et al., 2008). In the present study, a chronic inflammation response in the SNpc and striatum of mice was indicated by the increased levels of GFAP in astrocytes and IBA1 in microglia after MPTP and probenecid treatment. Importantly, the chronic inflammation response was reduced by D-Ala2-GIP-glu-PAL treatment. We previously have reported that another GIP analogue, D-Ala2-GIP, also decreased neuroinflammation in the cortex of APP/PS1 mice (Duffy and Holscher, 2013). In an acute PD model, we previously demonstrated that D-Ala2-GIP-glu-PAL also inhibited neuroinflammation in the SNpc induced by MPTP short-term injection (Li et al., 2016). Growth factors in general have anti-inflammatory properties which is an important part of their cytoprotective and regenerative effects (Cotman et al., 2007). Therefore, the reduction of the inflammation response may be one of the mechanisms by which GIP analogues inhibit the pathogenesis of neurodegenerative diseases.

There is growing evidence that oxidative stress and following lipid peroxidation contribute the pathogenesis of PD (Andersen, 2004). 4-HNE is one of the markers of membrane lipid peroxidation induced by cytotoxic radicals such as •OH. 4-HNE has cytotoxic, mutagenic and genotoxic properties and 4-HNE is reported to stimulate apoptosis via caspase 3 activation and mitochondrial damage as shown in cytochrome c release (Ji et al., 2001). In PD, lipid peroxidation and the level of 4-HNE in SNpc are increased (Andersen, 2004; Dexter et al., 1989). Moreover, conjugates of 4-HNE and α-synuclein have been reported to increase protein misfolding and impair degradation, resulting in its buildup in the neuron (Friguet and Szweda, 1997; Okada et al., 1999). In our present study, the levels of 4-HNE in the SNpc and striatum was significantly increased by MPTP and probenecid injection for 5 weeks, which was consistent with previous studies in which protein level of 4-HNE in midbrain was increased when MPTP was administered (Aras et al., 2014; Liang et al., 2007; Selley, 1998). However, D-Ala2-GIP-glu-PAL

treatment decreased the levels of 4-HNE of the regions studied. Hence, D-Ala2-GIP-glu-PAL reduces oxidant stress, which may promote cell survival of dopaminergic neuron in the SNpc when treated with MPTP and probenecid. However, whether these effects of GIP analogue arose from elevation of endogenous antioxidant substances cannot be resolved by the present data. As GIP itself is a trophic factor, it is possible to promote the production of endogenous antioxidant substance by activating the GIPR signal transduction pathway (Maino et al., 2014).

BDNF is a member of the nerve growth factor (NGF) family in the brain. Clinical data have demonstrated that DA neurons which survive in the substantia nigra of PD brains express low levels of BDNF mRNA with significantly low levels of BDNF protein (Chauhan et al., 2001; Howells et al., 2000). Inhibiting BDNF expression by antisense oligonucleotide infusion causes loss of nigral dopaminergic neurons (Porritt et al., 2005). In contrast, in a MPTP-induced parkinsonian primate model, intrathecal injection of BDNF has shown neuroprotective effects on DA neurons (Tsukahara et al., 1995). Treatment with BDNF increased the number of surviving dopaminergic neurons and promoted sprouting of dopaminergic axons in mesencephalic cultures (Hyman et al., 1991). Hence, reduced BDNF expression in the substantia nigra may contribute to the death of nigral dopaminergic neurons and the development of PD. In the present study, the expression of BDNF in SNpc was much decreased in mice treated with MPTP and probenecid compared to control. However, D-Ala2-GIP-glu-PAL treatment partially reversed the decrease of BDNF induced by MPTP. The findings are consistent with the results of our previous study, in which a novel dual GLP-1 and GIP receptor agonist increased the expression of BNDF in the brain (Ji et al., 2016).

In the central nervous system, the expression of BNDF can be enhanced by CREB signaling activation (Zhen et al., 2016), and the neuroprotective effect of CREB/BDNF signaling is correlated with a decrease in oxidative load (Lee et al., 2009). GIP receptors activate an adenylyl-cyclase and enhance intracellular cAMP levels, which in turn activates Protein Kinase A (PKA), which phosphorylates the cAMP-response element binding protein (CREB) (Holscher, 2014; Kim et al., 2008). The cAMP/PKA/CREB cell signaling cascade is most likely the pathway via which GIP enhances BDNF expression. It is a classic growth-factor cell signaling cascade and plays a regulatory role in the nervous system and promotes neuronal survival, precursor cell proliferation and synaptic activity (Hussain et al., 2006). In our previous study,

D-Ala2-GIP-glu-PAL treatment partially reversed the decrease of p-CREB^{S133} levels induced by the MPTP treatment. These findings demonstrated that D-Ala2-GIP-glu-PAL can facilitate CREB activation, enhance the expression of BDNF, and ultimately promote cell survival of dopaminergic neurons in the SNpc.

In conclusion, long-acting GIP analogues show promise as novel treatments that not only improve the symptoms but prevent the neurodegenerative processes underlying PD. The combination of GIP and GLP-1 receptor agonists may be a promising strategy to treat PD.

Acknowledgements

This work was supported by grants from the Cure Parkinson's Trust UK and a Shanxi province government grant to C Holscher under the '100 Foreign talent' grant scheme.

Conflict of interest: Dr. Holscher is a named inventor on a patent held by Ulster University on the use of GIP analogues in diseases of loss of synaptic transmission. The other authors do not declare a conflict of interest.

References

Andersen, J.K., 2004. Oxidative stress in neurodegeneration: cause or consequence? Nature medicine 10 Suppl, S18-25.

Aras, S., Tanriover, G., Aslan, M., Yargicoglu, P., Agar, A., 2014. The role of nitric oxide on visual-evoked potentials in MPTP-induced Parkinsonism in mice. Neurochemistry international 72, 48-57.

Athauda, D., Foltynie, T., 2016. The glucagon-like peptide 1 (GLP) receptor as a therapeutic target in Parkinson's disease: mechanisms of action. Drug discovery today 21, 802-818.

Aviles-Olmos, I., Dickson, J., Kefalopoulou, Z., Djamshidian, A., Ell, P., Soderlund, T., Whitton, P., Wyse, R., Isaacs, T., Lees, A., Limousin, P., Foltynie, T., 2013. Exenatide and the treatment of patients with Parkinson's disease. The Journal of clinical investigation 123, 2730-2736.

Baggio, L.L., Drucker, D.J., 2007. Biology of incretins: GLP-1 and GIP. Gastroenterology 132, 2131-2157.

Cereda, E., Barichella, M., Pedrolli, C., Klersy, C., Cassani, E., Caccialanza, R., Pezzoli, G., 2011. Diabetes and risk of Parkinson's disease: a systematic review and meta-analysis. Diabetes care 34, 2614-2623.

Chauhan, N.B., Siegel, G.J., Lee, J.M., 2001. Depletion of glial cell line-derived neurotrophic factor in substantia nigra neurons of Parkinson's disease brain. Journal of chemical neuroanatomy 21, 277-288.

Chen, H., O'Reilly, E.J., Schwarzschild, M.A., Ascherio, A., 2008. Peripheral inflammatory biomarkers and risk of Parkinson's disease. American journal of epidemiology 167, 90-95.

Cotman, C.W., Berchtold, N.C., Christie, L.A., 2007. Exercise builds brain health: key roles of growth factor cascades

and inflammation. Trends Neurosci 30, 464-472.

Dexter, D.T., Carter, C.J., Wells, F.R., Javoy-Agid, F., Agid, Y., Lees, A., Jenner, P., Marsden, C.D., 1989. Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. Journal of neurochemistry 52, 381-389.

Duffy, A.M., Holscher, C., 2013. The incretin analogue D-Ala2GIP reduces plaque load, astrogliosis and oxidative stress in an APP/PS1 mouse model of Alzheimer's disease. Neuroscience 228, 294-300.

Faivre, E., Hamilton, A., Holscher, C., 2012. Effects of acute and chronic administration of GIP analogues on cognition, synaptic plasticity and neurogenesis in mice. European journal of pharmacology 674, 294-306.

Faivre, E., Holscher, C., 2013a. D-Ala2GIP facilitated synaptic plasticity and reduces plaque load in aged wild type mice and in an Alzheimer's disease mouse model. Journal of Alzheimer's disease 35, 267-283.

Faivre, E., Holscher, C., 2013b. Neuroprotective effects of D-Ala2GIP on Alzheimer's disease biomarkers in an APP/PS1 mouse model. Alzheimer's research & therapy 5, 20-28.

Ferrari, C.C., Tarelli, R., 2011. Parkinson's disease and systemic inflammation. Parkinson's disease 2011, 436813.

Figueiredo, C.P., Antunes, V.L., Moreira, E.L., de Mello, N., Medeiros, R., Di Giunta, G., Lobao-Soares, B., Linhares, M., Lin, K., Mazzuco, T.L., Prediger, R.D., Walz, R., 2011. Glucose-dependent insulinotropic peptide receptor expression in the hippocampus and neocortex of mesial temporal lobe epilepsy patients and rats undergoing pilocarpine induced status epilepticus. Peptides 32, 781-789.

Foltynie, T., Aviles-Olmos, I., 2014. Exenatide as a potential treatment for patients with Parkinson's disease: first steps into the clinic. Alzheimer's & dementia : the journal of the Alzheimer's Association 10, S38-46.

Fox, S.H., Brotchie, J.M., 2010. The MPTP-lesioned non-human primate models of Parkinson's disease. Past, present, and future. Progress in brain research 184, 133-157.

Friguet, B., Szweda, L.I., 1997. Inhibition of the multicatalytic proteinase (proteasome) by 4-hydroxy-2-nonenal cross-linked protein. FEBS letters 405, 21-25.

Gault, V.A., Holscher, C., 2008. Protease-resistant glucose-dependent insulinotropic polypeptide agonists facilitate hippocampal LTP and reverse the impairment of LTP induced by beta-amyloid. J Neurophysiol 99, 1590-1595.

Gerlach, M., Riederer, P., Przuntek, H., Youdim, M.B., 1991. MPTP mechanisms of neurotoxicity and their implications for Parkinson's disease. European journal of pharmacology 208, 273-286.

Glover, V., Gibb, C., Sandler, M., 1986. The role of MAO in MPTP toxicity--a review. Journal of neural transmission. Supplementum 20, 65-76.

Herrero, M.T., Estrada, C., Maatouk, L., Vyas, S., 2015. Inflammation in Parkinson's disease: role of glucocorticoids. Frontiers in neuroanatomy 9, 32.

Hirohata, M., Ono, K., Morinaga, A., Yamada, M., 2008. Non-steroidal anti-inflammatory drugs have potent anti-fibrillogenic and fibril-destabilizing effects for alpha-synuclein fibrils in vitro. Neuropharmacology 54, 620-627.

Holscher, C., 2014. Insulin, incretins and other growth factors as potential novel treatments for Alzheimer's and Parkinson's diseases. Biochemical Society transactions 42, 593-599.

Howells, D.W., Porritt, M.J., Wong, J.Y., Batchelor, P.E., Kalnins, R., Hughes, A.J., Donnan, G.A., 2000. Reduced BDNF mRNA expression in the Parkinson's disease substantia nigra. Experimental neurology 166, 127-135.

Hu, G., Jousilahti, P., Bidel, S., Antikainen, R., Tuomilehto, J., 2007. Type 2 diabetes and the risk of Parkinson's disease. Diabetes care 30, 842-847.

Hussain, M.A., Porras, D.L., Rowe, M.H., West, J.R., Song, W.J., Schreiber, W.E., Wondisford, F.E., 2006. Increased pancreatic beta-cell proliferation mediated by CREB binding protein gene activation. Molecular and cellular biology 26, 7747-7759.

Hyman, C., Hofer, M., Barde, Y.A., Juhasz, M., Yancopoulos, G.D., Squinto, S.P., Lindsay, R.M., 1991. BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature 350, 230-232.

Irwin, N., Clarke, G.C., Green, B.D., Greer, B., Harriott, P., Gault, V.A., O'Harte, F.P., Flatt, P.R., 2006. Evaluation of the

antidiabetic activity of DPP IV resistant N-terminally modified versus mid-chain acylated analogues of glucose-dependent insulinotropic polypeptide. Biochemical pharmacology 72, 719-728.

Ji, C., Amarnath, V., Pietenpol, J.A., Marnett, L.J., 2001. 4-hydroxynonenal induces apoptosis via caspase-3 activation and cytochrome c release. Chemical research in toxicology 14, 1090-1096.

Ji, C., Xue, G.F., Lijun, C., Feng, P., Li, D., Li, L., Li, G., Holscher, C., 2016. A novel dual GLP-1 and GIP receptor agonist is neuroprotective in the MPTP mouse model of Parkinson's disease by increasing expression of BNDF. Brain research 1634, 1-11.

Kay, D.M., Factor, S.A., Samii, A., Higgins, D.S., Griffith, A., Roberts, J.W., Leis, B.C., Nutt, J.G., Montimurro, J.S., Keefe, R.G., Atkins, A.J., Yearout, D., Zabetian, C.P., Payami, H., 2008. Genetic association between alpha-synuclein and idiopathic Parkinson's disease. American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics 147B, 1222-1230.

Kim, S., Moon, M., Park, S., 2009. Exendin-4 protects dopaminergic neurons by inhibition of microglial activation and matrix metalloproteinase-3 expression in an animal model of Parkinson's disease. The Journal of endocrinology 202, 431-439.

Kim, S.J., Nian, C., Widenmaier, S., McIntosh, C.H., 2008. Glucose-dependent insulinotropic polypeptide-mediated up-regulation of beta-cell antiapoptotic Bcl-2 gene expression is coordinated by cyclic AMP (cAMP) response element binding protein (CREB) and cAMP-responsive CREB coactivator 2. Molecular and cellular biology 28, 1644-1656.

Korecka, J.A., Eggers, R., Swaab, D.F., Bossers, K., Verhaagen, J., 2013. Modeling early Parkinson's disease pathology with chronic low dose MPTP treatment. Restorative neurology and neuroscience 31, 155-167.

Kowal, S.L., Dall, T.M., Chakrabarti, R., Storm, M.V., Jain, A., 2013. The current and projected economic burden of Parkinson's disease in the United States. Movement disorders : official journal of the Movement Disorder Society 28, 311-318.

Kuhn, K., Wellen, J., Link, N., Maskri, L., Lubbert, H., Stichel, C.C., 2003. The mouse MPTP model: gene expression changes in dopaminergic neurons. The European journal of neuroscience 17, 1-12.

Lee, B., Cao, R., Choi, Y.S., Cho, H.Y., Rhee, A.D., Hah, C.K., Hoyt, K.R., Obrietan, K., 2009. The CREB/CRE transcriptional pathway: protection against oxidative stress-mediated neuronal cell death. Journal of neurochemistry 108, 1251-1265.

Li, Y., Liu, W., Li, L., Holscher, C., 2016. Neuroprotective effects of a GIP analogue in the MPTP Parkinson's disease mouse model. Neuropharmacology 101, 255-263.

Li, Y., Perry, T., Kindy, M.S., Harvey, B.K., Tweedie, D., Holloway, H.W., Powers, K., Shen, H., Egan, J.M., Sambamurti, K., Brossi, A., Lahiri, D.K., Mattson, M.P., Hoffer, B.J., Wang, Y., Greig, N.H., 2009. GLP-1 receptor stimulation preserves primary cortical and dopaminergic neurons in cellular and rodent models of stroke and Parkinsonism. Proceedings of the National Academy of Sciences of the United States of America 106, 1285-1290.

Liang, L.P., Huang, J., Fulton, R., Day, B.J., Patel, M., 2007. An orally active catalytic metalloporphyrin protects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity in vivo. The Journal of neuroscience 27, 4326-4333.

Lin, M.K., Farrer, M.J., 2014. Genetics and genomics of Parkinson's disease. Genome medicine 6, 48.

Liu, W., Li, Y., Jalewa, J., Saunders-Wood, T., Li, L., Holscher, C., 2015. Neuroprotective effects of an oxyntomodulin analogue in the MPTP mouse model of Parkinson's disease. European journal of pharmacology 765, 284-290.

Maino, B., Ciotti, M.T., Calissano, P., Cavallaro, S., 2014. Transcriptional analysis of apoptotic cerebellar granule neurons following rescue by gastric inhibitory polypeptide. Int J Mol Sci 15, 5596-5622.

McNaught, K.S., Jenner, P., 1999. Altered glial function causes neuronal death and increases neuronal susceptibility to 1-methyl-4-phenylpyridinium- and 6-hydroxydopamine-induced toxicity in astrocytic/ventral mesencephalic co-cultures. Journal of neurochemistry 73, 2469-2476.

Meredith, G.E., Totterdell, S., Potashkin, J.A., Surmeier, D.J., 2008. Modeling PD pathogenesis in mice: advantages of a chronic MPTP protocol. Parkinsonism & related disorders 14 Suppl 2, S112-115.

Miyake, Y., Tanaka, K., Fukushima, W., Sasaki, S., Kiyohara, C., Tsuboi, Y., Yamada, T., Oeda, T., Miki, T., Kawamura, N., Sakae, N., Fukuyama, H., Hirota, Y., Nagai, M., Fukuoka Kinki Parkinson's Disease Study, G., 2010. Case-control study of risk of Parkinson's disease in relation to hypertension, hypercholesterolemia, and diabetes in Japan. Journal of the neurological sciences 293, 82-86.

Moroo, I., Yamada, T., Makino, H., Tooyama, I., McGeer, P.L., McGeer, E.G., Hirayama, K., 1994. Loss of insulin receptor immunoreactivity from the substantia nigra pars compacta neurons in Parkinson's disease. Acta neuropathologica 87, 343-348.

Nyberg, J., Anderson, M.F., Meister, B., Alborn, A.M., Strom, A.K., Brederlau, A., Illerskog, A.C., Nilsson, O., Kieffer, T.J., Hietala, M.A., Ricksten, A., Eriksson, P.S., 2005. Glucose-dependent insulinotropic polypeptide is expressed in adult hippocampus and induces progenitor cell proliferation. The Journal of neuroscience 25, 1816-1825.

Nyberg, J., Jacobsson, C., Anderson, M.F., Eriksson, P.S., 2007. Immunohistochemical distribution of glucose-dependent insulinotropic polypeptide in the adult rat brain. Journal of neuroscience research 85, 2099-2119.

Okada, K., Wangpoengtrakul, C., Osawa, T., Toyokuni, S., Tanaka, K., Uchida, K., 1999. 4-Hydroxy-2-nonenal-mediated impairment of intracellular proteolysis during oxidative stress. Identification of proteasomes as target molecules. The Journal of biological chemistry 274, 23787-23793.

Paxinos, G., Huang, X., Toga, A., 1999. The rat Brain in Stereotaxic Coordinates. Academic Press, San Diego.

Porritt, M.J., Batchelor, P.E., Howells, D.W., 2005. Inhibiting BDNF expression by antisense oligonucleotide infusion causes loss of nigral dopaminergic neurons. Experimental neurology 192, 226-234.

Porter, D.W., Irwin, N., Flatt, P.R., Holscher, C., Gault, V.A., 2011. Prolonged GIP receptor activation improves cognitive function, hippocampal synaptic plasticity and glucose homeostasis in high-fat fed mice. European journal of pharmacology 650, 688-693.

Przedborski, S., Jackson-Lewis, V., 1998. Mechanisms of MPTP toxicity. Movement disorders : official journal of the Movement Disorder Society 13 Suppl 1, 35-38.

Selley, M.L., 1998. (E)-4-hydroxy-2-nonenal may be involved in the pathogenesis of Parkinson's disease. Free radical biology & medicine 25, 169-174.

Simuni, T., Brundin, P., 2014. Is exenatide the next big thing in Parkinson's disease? Journal of Parkinson's disease 4, 345-347.

Song, L.K., Ma, K.L., Yuan, Y.H., Mu, Z., Song, X.Y., Niu, F., Han, N., Chen, N.H., 2015. Targeted Overexpression of alpha-Synuclein by rAAV2/1 Vectors Induces Progressive Nigrostriatal Degeneration and Increases Vulnerability to MPTP in Mouse. PloS one 10, e0131281.

Tsukahara, T., Takeda, M., Shimohama, S., Ohara, O., Hashimoto, N., 1995. Effects of brain-derived neurotrophic factor on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonism in monkeys. Neurosurgery 37, 733-739; discussion 739-741.

Vila, M., Vukosavic, S., Jackson-Lewis, V., Neystat, M., Jakowec, M., Przedborski, S., 2000. Alpha-synuclein up-regulation in substantia nigra dopaminergic neurons following administration of the parkinsonian toxin MPTP. Journal of neurochemistry 74, 721-729.

Yasuda, T., Nakata, Y., Mochizuki, H., 2013. alpha-Synuclein and neuronal cell death. Molecular neurobiology 47, 466-483.

Zhen, J.L., Chang, Y.N., Qu, Z.Z., Fu, T., Liu, J.Q., Wang, W.P., 2016. Luteolin rescues pentylenetetrazole-induced cognitive impairment in epileptic rats by reducing oxidative stress and activating PKA/CREB/BDNF signaling. Epilepsy & behavior : E&B 57, 177-184.

Figure captions

Fig. 1. D-Ala2-GIP-glu-PAL alleviated motor symptoms of PD mice induced by MPTP and probenecid. **A**: The results of the open field test showed that D-Ala2-GIP-glu-PAL protects from the MPTP-induced impairment in motor activity and exploration of mice. **B**: The results of the pole test showed that D-Ala2-GIP-glu-PAL improves the bradykinesia and imbalance induced by MPTP and probenecid. **C**: The results of the rotarod test showed that D-Ala2-GIP-glu-PAL improves muscular strength and hypokinesia induced by MPTP and probenecid. The values represent the means±S.E.M.; **P<0.01, ***P<0.001 compared with the control group, $^{\#}$P<0.05 compared with the MPTP and probenecid group, n=12 per group.

Fig. 2. **Top:** D-Ala2-GIP-glu-PAL restored tyrosine hydroxylase (TH) positive dopaminergic neuron numbers in the SNpc. (A-D)-TH positive neuron numbers were reduced following treatment with MPTP and probenecid, and increased by D-Ala2-GIP-glu-PAL. A: Saline; B: D-Ala2-GIP-glu-PAL; C: MPTP + probenecid; D: MPTP + probenecid + D-Ala2-GIP-glu-PAL. Scale bar in image C: 200μm. Graph: D-Ala2-GIP-glu-PAL treatment restored numbers of dopaminergic neurons in the SNpc of mice treated with MPTP and probenecid. **Bottom:** D-Ala2-GIP-GLU-PAL restored TH expression in the striatum. (A-D)-TH positive nerve fibers were reduced following treatment with MPTP and probenecid, and increased by D-Ala2-GIP-glu-PAL. A: Saline; B: D-Ala2-GIP-glu-PAL; C: MPTP + probenecid; D: MPTP + probenecid + D-Ala2-GIP-glu-PAL. Scale bar in image C: 500μm. Graph: D-Ala2-GIP-glu-PAL treatment restored levels of TH staining in the striatum of mice treated with MPTP and probenecid. The values represent the means±S.E.M. ***P<0.001 compared with the control group. $^{\#}$P<0.05 compared with the MPTP and probenecid group, n=6 per group.

Fig. 3. **Top:** Western blot showed that D-Ala2-GIP-glu-PAL inhibited the increase of α-synuclein expression in the SNpc induced by MPTP and probenecid. **Middle:** D-Ala2-GIP-glu-PAL inhibited the increase of α-synuclein expression in the SNpc induced by MPTP and probenecid. (A-D)-α-synuclein expression in SNpc were increased following treatment with MPTP and probenecid, and reduced by D-Ala2-GIP-glu-PAL. A: Saline; B: D-Ala2-GIP-glu-PAL; C: MPTP + probenecid; D: MPTP + probenecid + D-Ala2-GIP-glu-PAL. Scale bar in image C: 50μm. Graph: Quantification of average optical density of α-synuclein positive staining in the SNpc demonstrated that MPTP and probenecid -treated mouse group showed significantly higher expression of α-synuclein than saline-treated mouse group while D-Ala2-GIP-glu-PAL reversed the effects induced by MPTP and probenecid. **Bottom:** D-Ala2-GIP-glu-PAL inhibited the increase of α-synuclein expression in the striatum induced by MPTP and probenecid. (A-D)-α-synuclein expression in the striatum were increased following treatment with MPTP and probenecid, and reduced by D-Ala2-GIP-glu-PAL. A: Saline; B: D-Ala2-GIP-glu-PAL; C: MPTP + probenecid; D: MPTP + probenecid + D-Ala2-GIP-glu-PAL. Scale bar in image C: 50μm. Graph: Quantification of average optical density of α-synuclein positive staining in the striatum demonstrated that MPTP and probenecid -treated mice had a higher expression of α-synuclein than saline-treated mice while D-Ala2-GIP-glu-PAL reversed the effects induced by MPTP and

probenecid. The values represent the means±S.E.M. ***P<0.001 compared with the control group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 compared with the MPTP and probenecid group, n=6 per group.

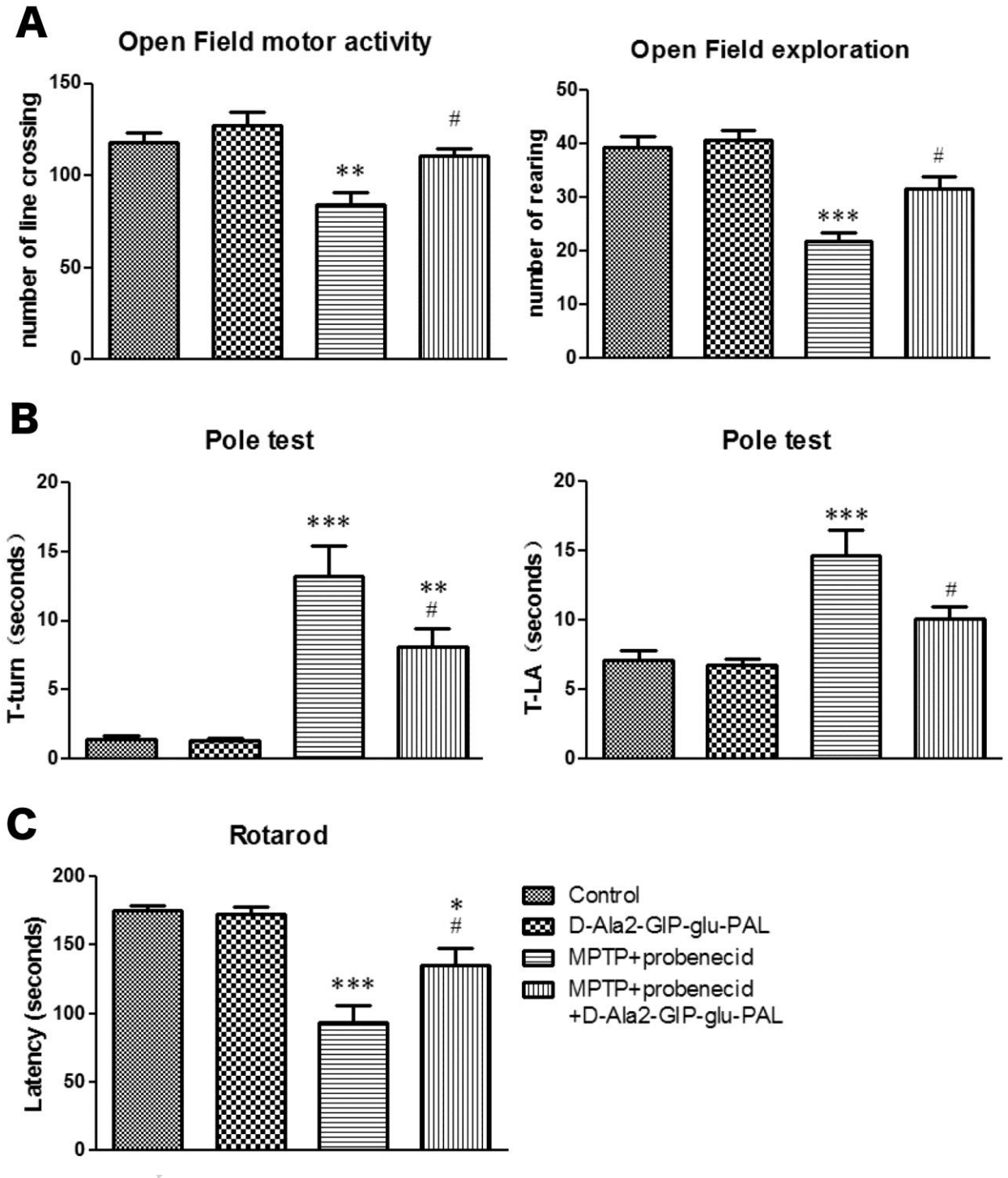
Fig.4. **Top:** D-Ala2-GIP-glu-PAL reduced astrocyte activation in the SNpc induced by MPTP and probenecid. (A-D)-GFAP positive cell numbers were increased following treatment with MPTP and probenecid, and reduced by D-Ala2-GIP-glu-PAL. A: Saline; B: D-Ala2-GIP-glu-PAL; C: MPTP + probenecid; D: MPTP + probenecid + D-Ala2-GIP-glu-PAL. Scale bar in image C: 200μm. A close-up of the micrograph is shown as an insert to demonstrate that the staining is specific for astrocytes with little background staining. Graph: Quantification of the area of GFAP positive stain in the SNpc demonstrated that the MPTP and probenecid -treated mouse groups had significantly higher expression of GFAP than saline-treated mice while D-Ala2-GIP-glu-PAL reversed the effects induced by MPTP and probenecid. **Bottom:** D-Ala2-GIP-glu-PAL reduced the astrocyte activation in the striatum of mice induced by MPTP and probenecid. (A-D)-GFAP positive cell numbers were increased following treatment with MPTP and probenecid, and reduced by D-Ala2-GIP-glu-PAL. A: Saline; B: D-Ala2-GIP-glu-PAL; C: MPTP + probenecid; D: MPTP + probenecid + D-Ala2-GIP-glu-PAL. Scale bar in image C: 200μm. Graph: Quantification of area of GFAP positive cell in the striatum demonstrated that MPTP and probenecid -treated mouse groups had significantly higher expression of GFAP than saline-treated mouse group. While D-Ala2-GIP-glu-PAL reversed the effects induced by MPTP and probenecid. The values represent the means±S.E.M. ***P<0.001 compared with the control group. $^{\#\#\#}$P<0.001 compared with the MPTP and probenecid group. n=6 per group.

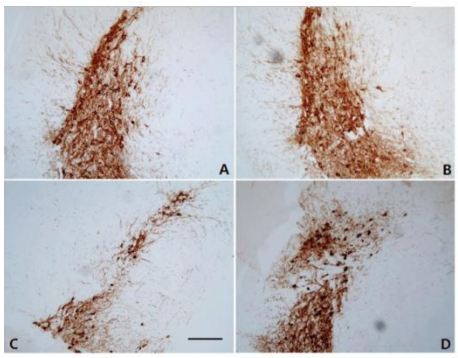
Fig.5. **Top:** D-Ala2-GIP-glu-PAL reduced microglia activation in the SNpc induced by MPTP and probenecid. (A-D)-IBA1 positive cell numbers were increased following treatment with MPTP and probenecid, and reduced by D-Ala2-GIP-glu-PAL. A: Saline; B: D-Ala2-GIP-glu-PAL; C: MPTP + probenecid; D: MPTP + probenecid + D-Ala2-GIP-glu-PAL. Scale bar in image C: 200μm. A close-up of the micrograph is shown as an insert to demonstrate that the staining is specific for microglia. Graph: Quantification of area of IBA1 positive cell in the SNpc demonstrated that MPTP and probenecid -treated mouse groups had significantly higher expression of IBA1 than saline-treated mouse group. While D-Ala2-GIP-glu-PAL reversed the effects on microglia activation. **Bottom:** D-Ala2-GIP-glu-PAL reduced microglia activation in the striatum induced by MPTP and probenecid. (A-D) IBA1 positive cell numbers were increased following treatment with MPTP and probenecid, and reduced by D-Ala2-GIP-glu-PAL. A: Saline; B: D-Ala2-GIP-glu-PAL; C: MPTP + probenecid; D: MPTP + probenecid + D-Ala2-GIP-glu-PAL. Scale bar in image C: 200μm. Graph: Quantification of area of IBA1 positive cell in the striatum demonstrated that MPTP and probenecid -treated mice had significantly higher expression of IBA1 than saline-treated mice while D-Ala2-GIP-glu-PAL reversed the effects on microglia activation. The values represent the means±S.E.M. ***P<0.001 compared with the control group. $^{\#\#}$P<0.01 compared with the MPTP and probenecid group. n=6 per group.

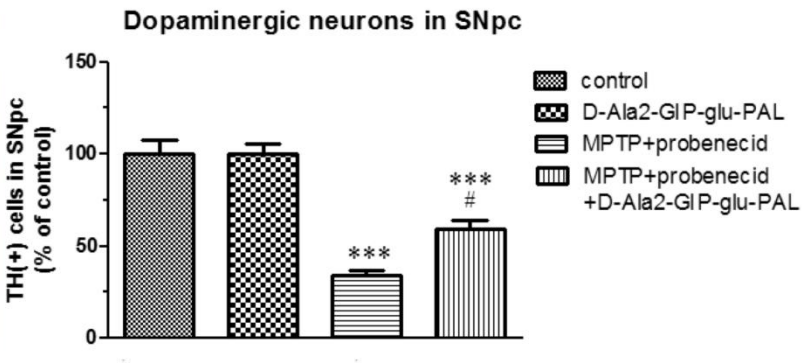
Fig.6. **Top:** D-Ala2-GIP-glu-PAL reduced lipid peroxidation in SNpc of mice induced by MPTP and probenecid. (A-D)-The expression of 4-HNE was increased following treatment with MPTP and probenecid, and reduced by D-Ala2-GIP-glu-PAL. A: Saline; B: D-Ala2-GIP-glu-PAL; C: MPTP + probenecid; D: MPTP + probenecid + D-Ala2-GIP-glu-PAL. Scale bar in image C:

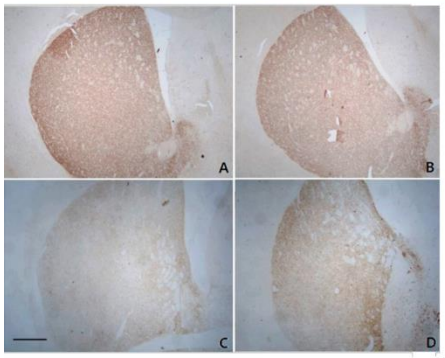
50μm. Graph: Quantification of average optical density of 4-HNE positive staining in the SNpc demonstrated that MPTP and probenecid -treated mouse groups had significantly higher expression of 4-HNE than saline-treated mouse group. While D-Ala2-GIP-glu-PAL reversed the effects induced by MPTP and probenecid. **Bottom:** D-Ala2-GIP-glu-PAL inhibited the lipid peroxidation in the striatum induced by MPTP and probenecid. (A-D) The expression of 4-HNE in the striatum was increased following treatment with MPTP and probenecid, and reduced by D-Ala2-GIP-glu-PAL. A: Saline; B: D-Ala2-GIP-glu-PAL; C: MPTP + probenecid; D: MPTP + probenecid + D-Ala2-GIP-glu-PAL. Scale bar in image C: 50μm. Graph: Quantification of the average optical density of 4-HNE positive staining in the striatum demonstrated that MPTP and probenecid -treated mice had significantly higher expression of 4-HNE than saline-treated mice while D-Ala2-GIP-glu-PAL reversed the effects induced by MPTP and probenecid. The values represent the means±S.E.M. ***$P<0.001$ compared with the control group. $^{\#}P<0.05$ compared with the MPTP and probenecid group. n=6 per group.

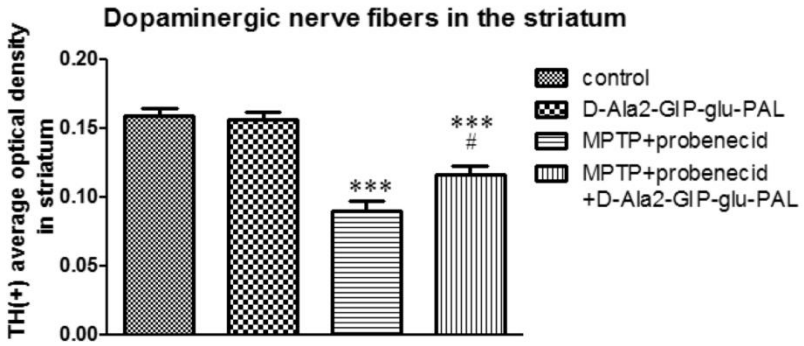
Fig.7. D-Ala2-GIP-glu-PAL inhibited the decrease of BDNF expression in the SNpc induced by MPTP and probenecid treatment. The values represents the means±S.E.M. ***$P<0.001$ compared with the control group, $^{\#}P<0.05$ compared with the MPTP and probenecid group. n=6 per group.

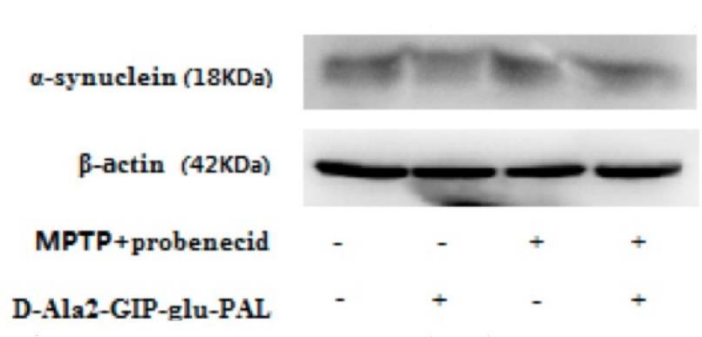

A
Open Field motor activity
number of line crossing
150
100
50
0
**
#
Open Field exploration
number of rearing
50
40
30
20
10
0
***
#
B
Pole test
T-turn（seconds）
20
15
10
5
0
***
**
#
Pole test
T-LA（seconds）
20
15
10
5
0
***
#
C
Rotarod
Latency (seconds)
200
150
100
50
0
***
*
#
Control
D-Ala2-GIP-glu-PAL
MPTP+probenecid
MPTP+probenecid
+D-Ala2-GIP-glu-PAL

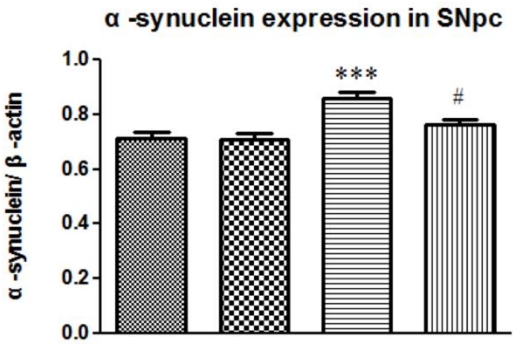


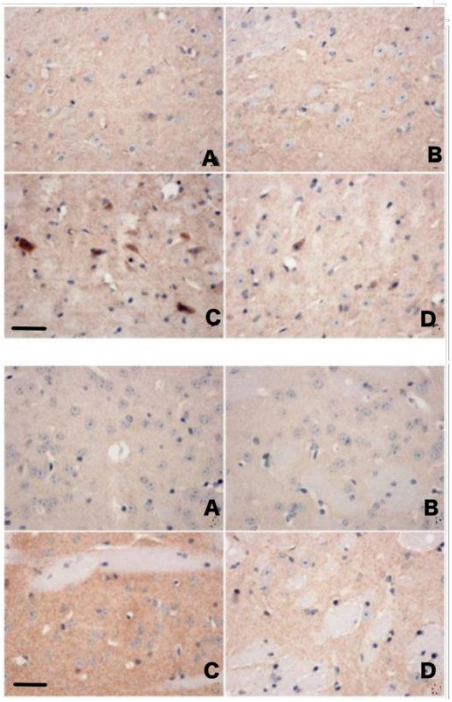


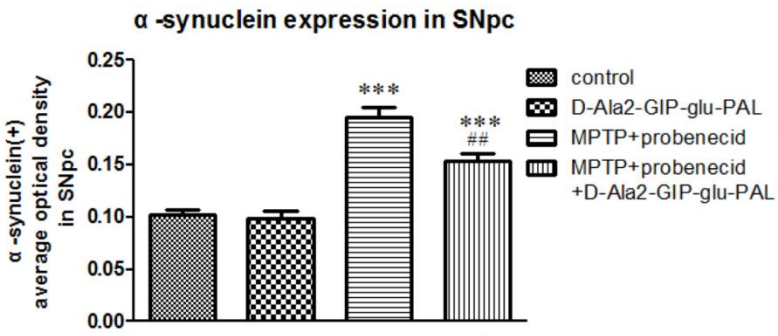





α-synuclein (18KDa)
β-actin (42KDa)
MPTP+probenecid - - + +
D-Ala2-GIP-glu-PAL - + - +


α -synuclein expression in SNpc
α -synuclein/ β -actin
1.0
0.8
0.6
0.4
0.2
0.0
***
#


A
B
C
D
A
B
C
D


α -synuclein expression in SNpc
α -synuclein(+) average optical density in SNpc
0.25
0.20
0.15
0.10
0.05
0.00
***
***
##
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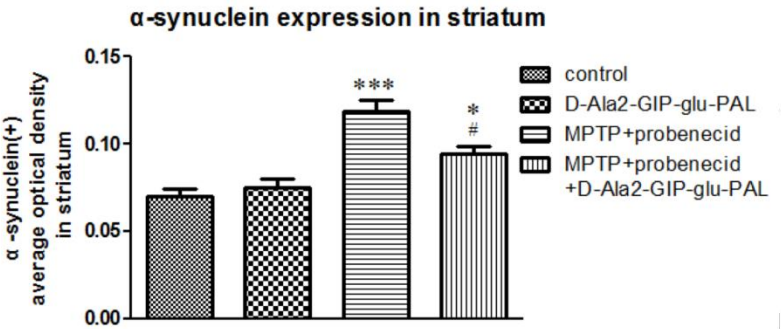
D-Ala2-GIP-glu-PAL
MPTP+probenecid
MPTP+probenecid +D-Ala2-GIP-glu-PAL


α-synuclein expression in striatum
α -synuclein(+) average optical density in striatum
0.15
0.10
0.05
0.00
***
*
#
control
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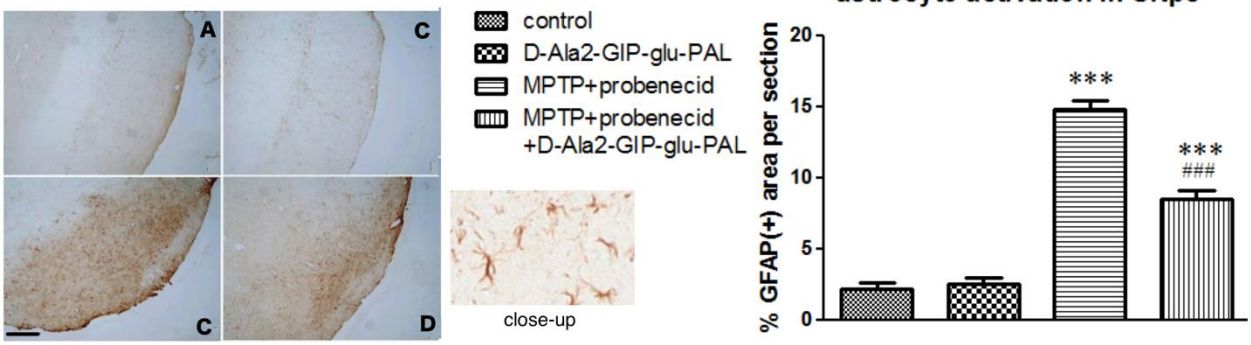
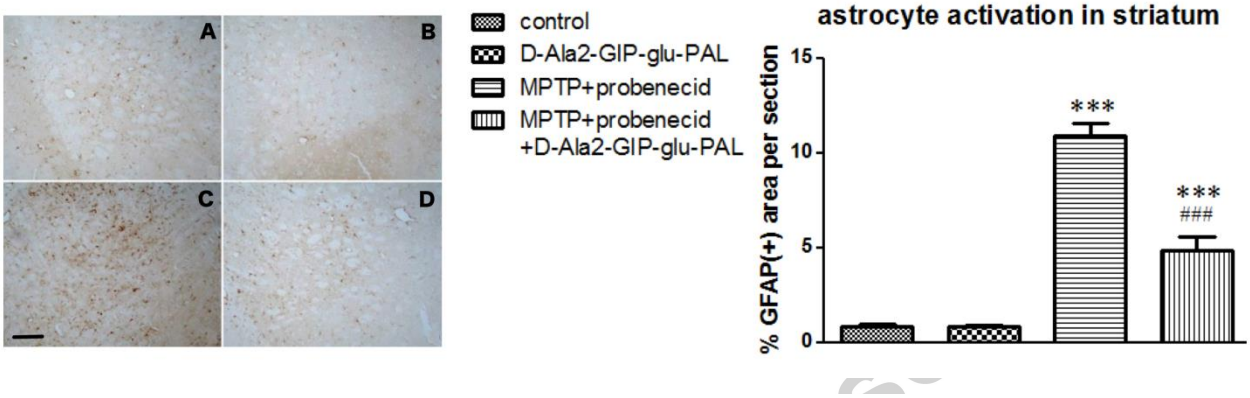
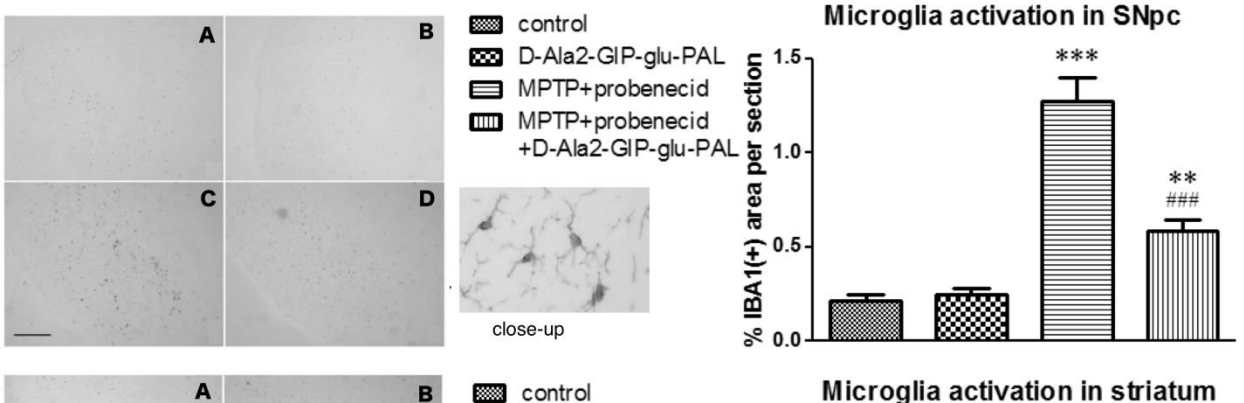
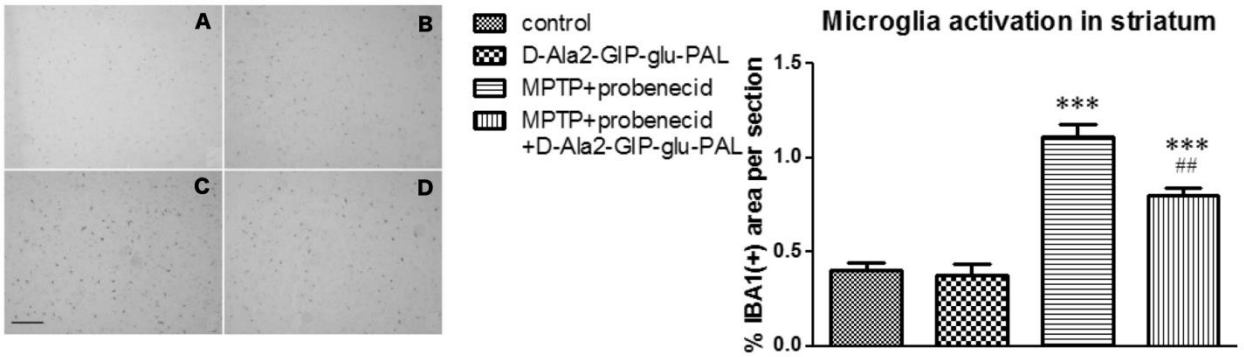
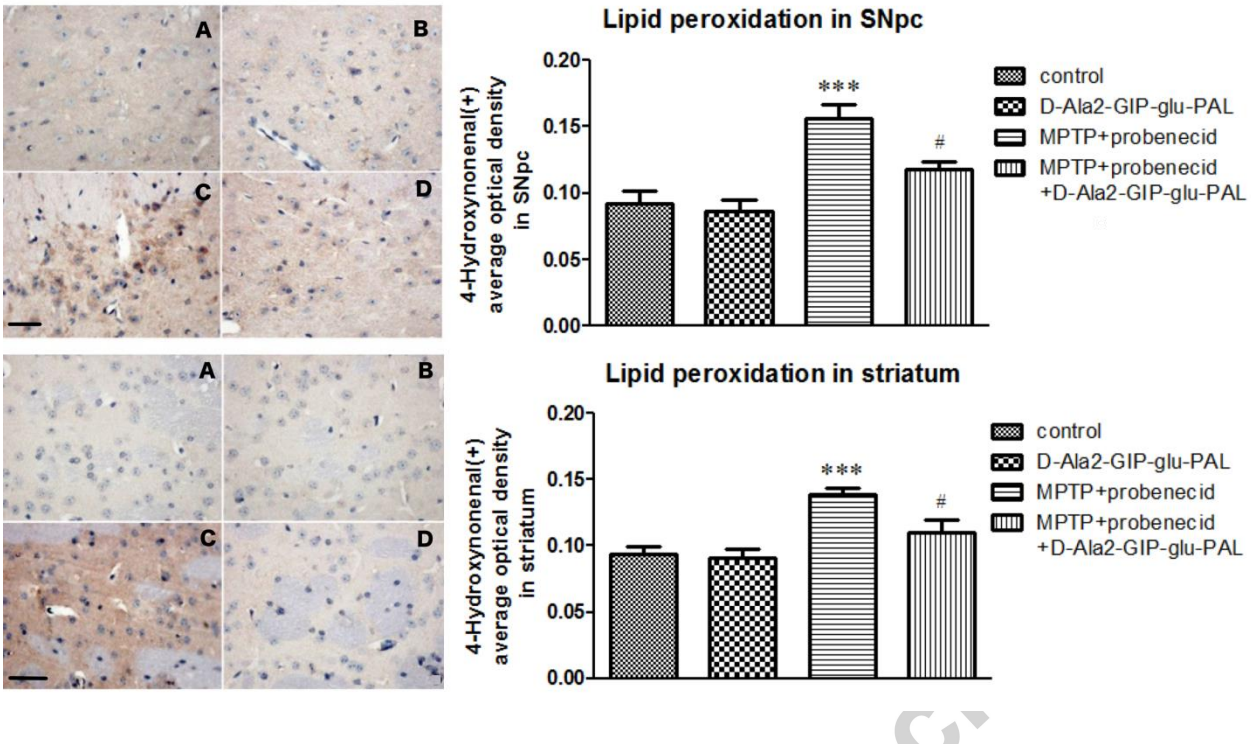
MPTP+probenecid
MPTP+probenecid +D-Ala2-GIP-glu-PAL

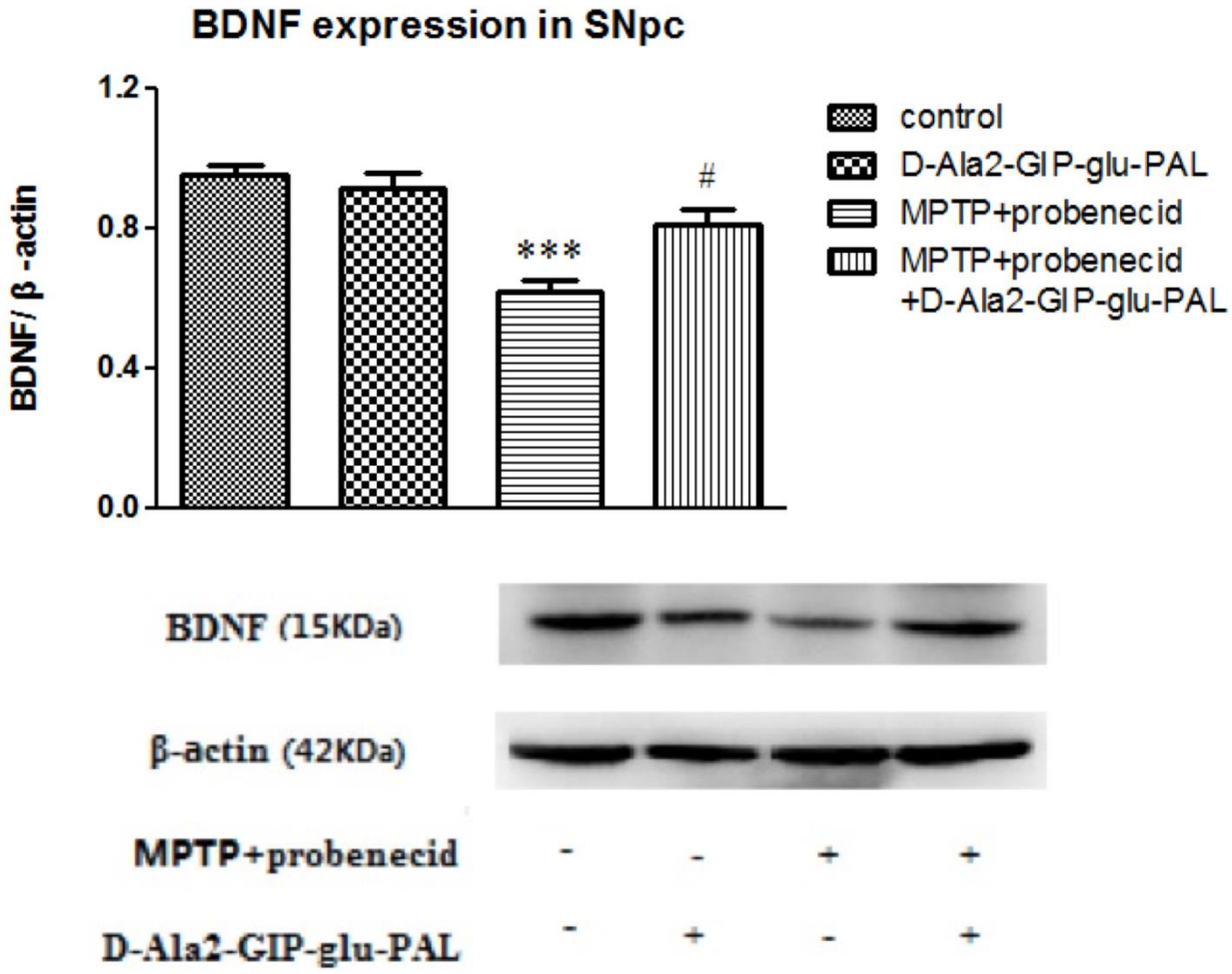

A
C
C
D
control
D-Ala2-GIP-glu-PAL
MPTP+probenecid
MPTP+probenecid
+D-Ala2-GIP-glu-PAL
close-up
astrocyte activation in SNpc
% GFAP(+) area per section
0
5
10
15
20
***
***
###


A
B
C
D
control
D-Ala2-GIP-glu-PAL
MPTP+probenecid
MPTP+probenecid
+D-Ala2-GIP-glu-PAL
astrocyte activation in striatum
% GFAP(+) area per section
0
5
10
15
***
***
###


A
B
C
D
control
D-Ala2-GIP-glu-PAL
MPTP+probenecid
MPTP+probenecid
+D-Ala2-GIP-glu-PAL
close-up
Microglia activation in SNpc
% IBA1(+) area per section
0.0
0.5
1.0
1.5
***
**
###


A
B
C
D
control
D-Ala2-GIP-glu-PAL
MPTP+probenecid
MPTP+probenecid
+D-Ala2-GIP-glu-PAL
Microglia activation in striatum
% IBA1(+) area per section
0.0
0.5
1.0
1.5
***
***
##


A
B
C
D
Lipid peroxidation in SNpc
4-Hydroxynonenal(+) average optical density in SNpc
0.20
0.15
0.10
0.05
0.00
***
#
control
D-Ala2-GIP-glu-PAL
MPTP+probenecid
MPTP+probenecid +D-Ala2-GIP-glu-PAL
Lipid peroxidation in striatum
4-Hydroxynonenal(+) average optical density in striatum


BDNF expression in SNpc
BDNF/ β -actin
1.2
0.8
0.4
0.0
***
#
control
D-Ala2-GIP-glu-PAL
MPTP+probenecid
MPTP+probenecid +D-Ala2-GIP-glu-PAL
BDNF (15KDa)
β-actin (42KDa)
MPTP+probenecid - - + +
D-Ala2-GIP-glu-PAL - + - +