

Neuroprotective effects of a GIP analogue in the MPTP Parkinson's disease mouse model

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ABSTRACT

Parkinson's disease (PD) is a chronic neurodegenerative disease, and there is no cure for it at present. Recent research has indicated a link between type 2 diabetes mellitus (T2DM) and PD, which suggested that a treatment to improve insulin resistance for T2DM may be useful for PD patients. Glucose-dependent insulintropic polypeptide (GIP) belongs to the incretin hormone family, which can promote insulin release and improve insulin resistance. Several GIP analogues have been developed as potential treatments for T2DM. In the present study, a novel long-lasting GIP analogue, D-Ala2-GIP-glu-PAL, has been tested in an acute PD mouse model induced by four 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intraperitoneal injections. D-Ala2-GIP-glu-PAL treatment (25 nmol/kg ip.) for 7 days after MPTP treatment improved the locomotor and exploratory activity of mice, and improved bradykinesia and movement balance of mice. D-Ala2-GIP-glu-PAL treatment also restored tyrosine hydroxylase (TH) positive dopaminergic neuron numbers in the substantia nigra and TH levels in the striatum. D-Ala2-GIP-glu-PAL also reduced the chronic inflammation response as seen in astrocyte and microglia activation in the substantia nigra *pars compacta* (SNpc). D-Ala2-GIP-glu-PAL reversed the reduction of synapse numbers (synaptophysin levels), decreased the ratio of growth factor and apoptosis signaling molecules Bax/Bcl-2, and improved the decrease of p-CREB^{S133} growth factor signaling in the substantia nigra. Therefore, D-Ala2-GIP-glu-PAL promotes cell survival of dopaminergic neuron in the SNpc by activating the cAMP/PKA/CREB growth factor second messenger pathway that also inhibits apoptosis. The present results demonstrate that D-Ala2-GIP-glu-PAL shows promise as a novel treatment of PD.

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1. Introduction

Parkinson's disease (PD) is the second most common chronic neurodegenerative disease afflicting about 1% of people over 65 years old and 4–5% of people over 85 years. The pathology of PD is characterized by a progressive loss of dopaminergic neurons in the substantia nigra *pars compacta* (SNpc) and the degeneration of projecting nerve fibers to the striatum, which leads to tremors, muscular rigidity, bradykinesia, and postural and gait abnormalities (Kowal et al., 2013). Current treatment strategies for PD have mostly targeted the dopamine system. Levodopa is the most

effective drug used in the treatment of PD. However, the long-term use of levodopa produces complications such as highly disabling fluctuations of motor activity and dyskinesias (Sharma et al., 2015). Other treatment options include dopamine agonists, anticholinergics, amantadine, monoamine oxidase (MAO) inhibitors, and deep brain stimulation (Giugni and Okun, 2014). However, neuroprotective treatments that delay or cure PD remain an unrealised goal.

Recently, studies have shown a link between diabetes and PD. Both diabetes and PD are age-related chronic diseases and some pathogenic processes may underlie both conditions (Chen et al., 2008; Henchcliffe and Beal, 2008; Pradhan, 2007). Insulin desensitisation may be one mechanism that underlies both conditions. Clinical data evaluation showed that an 8–30 percentage of

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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; Schernhammer et al., 2011). PD and diabetes also share several genetic susceptibilities, such as single nucleotide polymorphisms in the growth factor signaling kinase gene Akt, which increase an individual's risk for developing PD and diabetes (Jain et al., 2012; Xiomerisiou et al., 2008). Common pathways that link PD, diabetes, and inflammation have been identified by genome-wide transcriptome profiling (Moran and Graeber, 2008). In preclinical studies, systemic administration of drugs for T2DM, such as insulin (Holscher, 2014b), rosiglitazone (Schintu et al., 2009), and metformin (Patil et al., 2014), significantly attenuate neuropathology, including the loss of SNpc neurons and the striatal dopaminergic fibers, microglial activation, or the expression of pro-inflammatory cytokines. Also, a drug that is on the market to treat type 2 diabetes, Exenatide (exendin-4), showed a therapeutic effect in preclinical tests (Harkavyi et al., 2008; Kim et al., 2009; Li et al., 2009) and in a pilot clinical trial in PD patients (Aviles-Olmos et al., 2013a, 2014). In light of these recent findings, a hypothesis has emerged that suggests that mitochondrial dysfunction, endoplasmic reticulum stress, inflammation, and alterations in metabolism may lead to insulin resistance and, ultimately, to diabetes and/or neurodegeneration (Lima et al., 2014; Santiago and Potashkin, 2013). Hence, the treatment to improve insulin resistance for T2DM may be useful for PD patients (Aviles-Olmos et al., 2013b; Holscher, 2014b).

Glucose-dependent insulintropic polypeptide (GIP), also named gastric inhibitory polypeptide, is an endogenous 42 amino acid peptide hormone (Baggio and Drucker, 2007). GIP belongs to the incretin family, which can promote insulin release and lower blood glucose, and has growth factor like properties (Holscher, 2014a). As GIP is quickly degraded by the enzyme dipeptidyl peptidase IV (DPP-IV) in the blood stream (Kieffer et al., 1995), several enzyme-resistant GIP analogues have been developed, such as D-Ala2GIP and D-Ala2-GIP-GLU-PAL, as potential treatments for type 2 diabetes (Irwin et al., 2006a).

In the central nervous system, GIP is expressed in neurons and acts as a neurotransmitter, and GIP receptors are also found in multiple brain regions, including the olfactory bulb, hippocampus, cerebellum, cerebral cortex, substantia nigra, thalamus, and brainstem (Nyberg et al., 2005, 2007). Activation of the GIPR leads to proliferation of neuronal progenitor cells and therefore may contribute to neurogenesis (Holscher, 2014b). GIP not only has growth-factor like properties in the brain, but also modulates synaptic activity. GIP prevented the detrimental effects of beta-amyloid on synaptic plasticity (LTP) in the hippocampus (Gault and Holscher, 2008) and on spatial learning and memory (Figueiredo et al., 2010). Furthermore, GIP has been shown to promote axonal regeneration after sciatic nerve injury (Buhren et al., 2009). In our previous study, chronic injection of D-Ala2GIP enhanced memory formation, synaptic neurotransmission (LTP) in the hippocampus, and progenitor cell proliferation in the dentate gyrus of wild type mice and mice on a high-fat diet (Faivre et al., 2012; Porter et al., 2011). Moreover, D-Ala2GIP can improve cognitive function and prevent deficits of learning and memory in APP/PS1 transgenic mice, and reduces the beta amyloid plaque load and neuroinflammation in the brain. Synaptic plasticity and synapse numbers were also normalised by the drug (Duffy and Holscher, 2013; Faivre and Holscher, 2013a,b).

Although GIP analogues showed a potential therapeutic effect for AD in animal experiments, the protective effects in PD have not been investigated. In the present study, a new long lasting GIP analogue, D-Ala2-GIP-GLU-PAL, was tested in an acute PD mouse model induced by 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

2. Materials and methods

2.1. Chemicals and peptides

Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) were purchased from Sigma–Aldrich (St Louis, MO, USA). Other chemicals used were of the highest quality commercially available. D-Ala2-GIP-GLU-PAL was purchased from 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

Male C57BL/6 mice (20–25 g) were purchased from the Academy of Military Medical Sciences (AMMS, China). The animals were maintained on 12-h light/dark cycle at $24 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ humidity with food and water *ad libitum*, and were acclimatized for at least 1 week prior to the MPTP procedure. 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 ($n = 8$ 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 alone; and (iv) a group treated with D-Ala2GIP-GIP-Glu-PAL in combination with MPTP. Mice received four intraperitoneal injections of MPTP (20 mg/kg body weight; Sigma–Aldrich) or saline (0.9%) control at 2-h intervals. D-Ala2-GIP-GLU-PAL was dissolved in saline and drug (25 nmol/kg body weight) ip. injection continued for 7 days after MPTP injection. Control mice received equal volumes of the vehicle solution. At the end of drug treatments, measurements of behavioral changes, neuronal damage, inflammatory markers, and biomarkers were made.

2.3. Behavioral assessment

2.3.1. Open-field test

To assess locomotor and exploratory activity of PD mice, the open-field test was conducted on the 6th day after MPTP treatment as previously described (Rial et al., 2014). 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 rat was placed in the center of the apparatus and was observed. After acclimatizing 10 min, the number of crossing line and rearings made by the animal was recorded within 5 min. The apparatus was then cleaned with 70% alcohol and dried between trials. 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 PD mice, the pole test was conducted on the 5th day after MPTP treatment as previously described (Park et al., 2013). In brief, animals were placed facing upward near the top of a wooden pole with a rough surface (10 mm in diameter and 55 cm in height). The time taken until they turned completely downward (defined as turn time, T-turn) and the time taken to

arrive at 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. The mice were pre-trained before MPTP injections. Every mouse was tested for 3 times and the average of the three trials was calculated for statistical analyses.

2.4. Sample preparation

All animals were sacrificed on the 7th day after MPTP injection. After anesthetization with ethyl carbamate, whole brains of 4 mice per group were removed immediately and substantia nigra was dissected out and stored at -80°C for immunoblot analysis. And another 4 mice per group were transcardial perfused with ice-cold PBS and 4% paraformaldehyde/PBS (pH7.4), and brains were removed and postfixed for 24 h in the same fixative, and the brains were embedded in paraffin.

2.5. Immunohistochemistry

Brain tissue samples were embedded in paraffin, and sections were cut at $4\text{ }\mu\text{m}$ 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; Abcam, Cambridge, UK), GFAP (rabbit anti-GFAP; 1:100; Boster Biotechnology Co., Ltd. Wuhan, China) and IBA1 (goat anti-IBA1; 1:500; Abcam, Cambridge, UK) at 37°C for 1 h. They were rinsed in PBS and incubated with biotinylated goat anti-rabbit IgG or biotinylated rabbit anti-goat IgG at 37°C for 0.5 h and incubated with followed by the avidin-biotin peroxidase complex reagent (Boster Biotechnology Co., Ltd. Wuhan, China) at 37°C for 0.5 h. Samples were colored in 3,3'-diaminobenzidine (DAB) solution in the presence of H_2O_2 (Zhongshan Golden Bridge Biotechnology Co., Ltd. Beijing, China). To verify the immune specificity, control sections were processed with the same protocol, except that omitting the primary antibody. 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). 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 or IBA1 positive cells were 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 (Beyotime Institute of Biotechnology, Shanghai, China) containing phenyl-methylsulfonyl fluoride (PMSF). After 30 min, tissue lysates were obtained by centrifugation at 12,000 rpm for 5 min at 4°C . The protein concentration of the samples then was quantified by BCA protein assay (Beyotime Institute of Biotechnology, Shanghai, China), using bovine serum albumin as standard. After tissue lysate was mixed in loading buffer and boiled for 5 min, equivalent amounts of protein were separated on 12% or 10% SDS-polyacrylamide gel and transferred to

polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5%BSA in Tris-buffered saline (Boster Biotechnology Co., Ltd. Wuhan, China) and incubated overnight at 4°C with rabbit anti-mouse synaptophysin (SYN) (1:5000; Abcam, Cambridge, UK), rabbit anti-mouse Bcl-2 (1:400; Boster Biotechnology Co., Ltd. Wuhan, China), rabbit anti-mouse Bax (1:400; Boster Biotechnology Co., Ltd. Wuhan, China), rabbit anti-mouse p-CREB^{S133} (1:500; Abcam, Cambridge, UK) and rabbit anti-mouse CREB (1:500; Abcam, Cambridge, UK), followed by incubation for 2 h at room temperature with the HRP-labeled goat anti-rabbit immunoglobulin (1:1000; Abcam, Cambridge, UK). The bound antibodies were then visualized by ECL-enhanced chemiluminescence (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. In some cases, the blots were stripped and reprobed with an rabbit anti-mouse β -actin (1:1000; Abcam, Cambridge, UK) 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 were expressed as means \pm S.E.M. All analysis was conducted using GraphPad Prism (Graph-Pad software Inc., San Diego, CA, USA). Statistical significance was performed by one-way ANOVA for multiple comparisons followed by Bonferroni's post-hoc test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. D-Ala2-GIP-GLU-PAL normalised the MPTP-induced impairments in locomotor and exploratory activity of mice

A one-way ANOVA found an overall difference between groups ($P < 0.01$). The results showed that the number of crossings in MPTP group mice exhibited a significant reduction versus the control group mice ($P < 0.01$ in the Bonferroni's post hoc test). However, D-Ala2-GIP-GLU-PAL treatment reversed these effects on crossing ($P < 0.01$). MPTP procedure also decreased the number of rearing ($P < 0.01$), and D-Ala2-GIP-GLU-PAL could improve the rearing number ($P < 0.05$). Each group was $n = 8$, see Fig. 1A.

3.2. D-Ala2-GIP-GLU-PAL improved the bradykinesia and movement imbalance of mice induced by MPTP

A one-way ANOVA found an overall difference between groups ($P < 0.01$). MPTP injection induced the bradykinesia of mice, with more time to turn at the top (T-turn) ($P < 0.001$) compared with the control. And MPTP injection also induced movement imbalance of mice, with more time to climb down (T-LA) ($P < 0.001$) compared with the control. However, treatment with D-Ala2-GIP-GLU-PAL significantly reversed these effects induced by MPTP ($P < 0.01$). $N = 8$ per group, see Fig. 1B.

3.3. D-Ala2-GIP-GLU-PAL protected dopaminergic neuronal damage induced by MPTP

A one-way ANOVA found an overall difference between groups ($P < 0.01$). On the 7th day after MPTP injection, the number of TH-positive cells in the SNpc was decreased to 27.00% ($P < 0.001$), and the average optical density of TH staining in the striatum was decreased to 56.86% ($P < 0.001$). There is no difference between control group and D-Ala2-GIP-GLU-PAL group. However, D-Ala2-GIP-GLU-PAL treatment restored numbers of dopaminergic

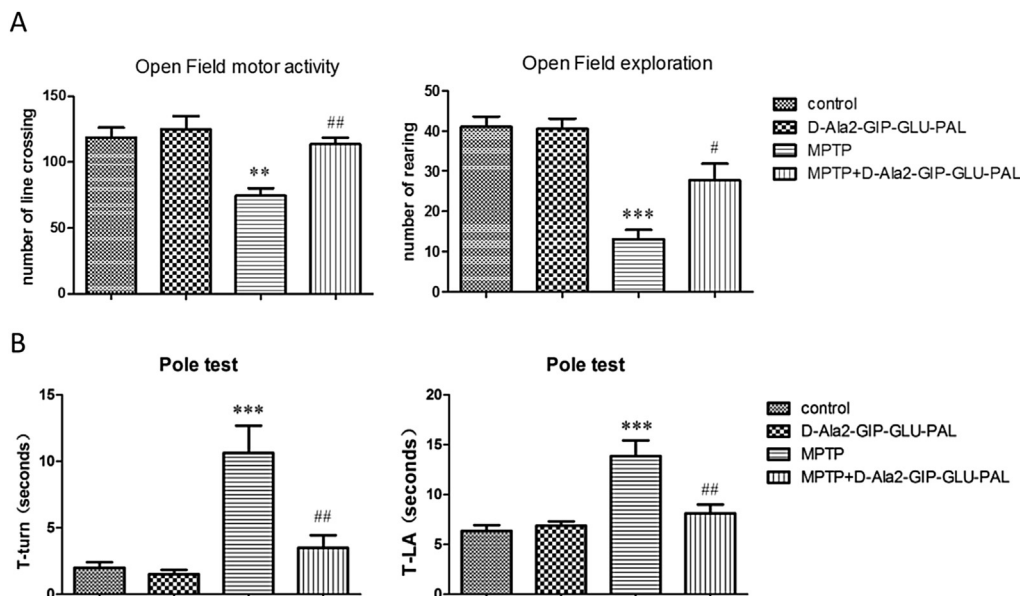


Fig. 1. A: D-Ala2-GIP-GLU-PAL protects from the MPTP-induced impairment in motor activity and exploration of mice. A difference was found between the control group and MPTP group. Furthermore, a difference was found between MPTP + D-Ala2-GIP-GLU-PAL and MPTP group. **B:** D-Ala2-GIP-GLU-PAL improved the bradykinesia and unbalance of mice induced by MPTP. The values represent the means \pm S.E.M.; *** P < 0.001 compared with the control group. # P < 0.05, ## P < 0.01, and ### P < 0.001 compared with the MPTP group. n = 8 per group.

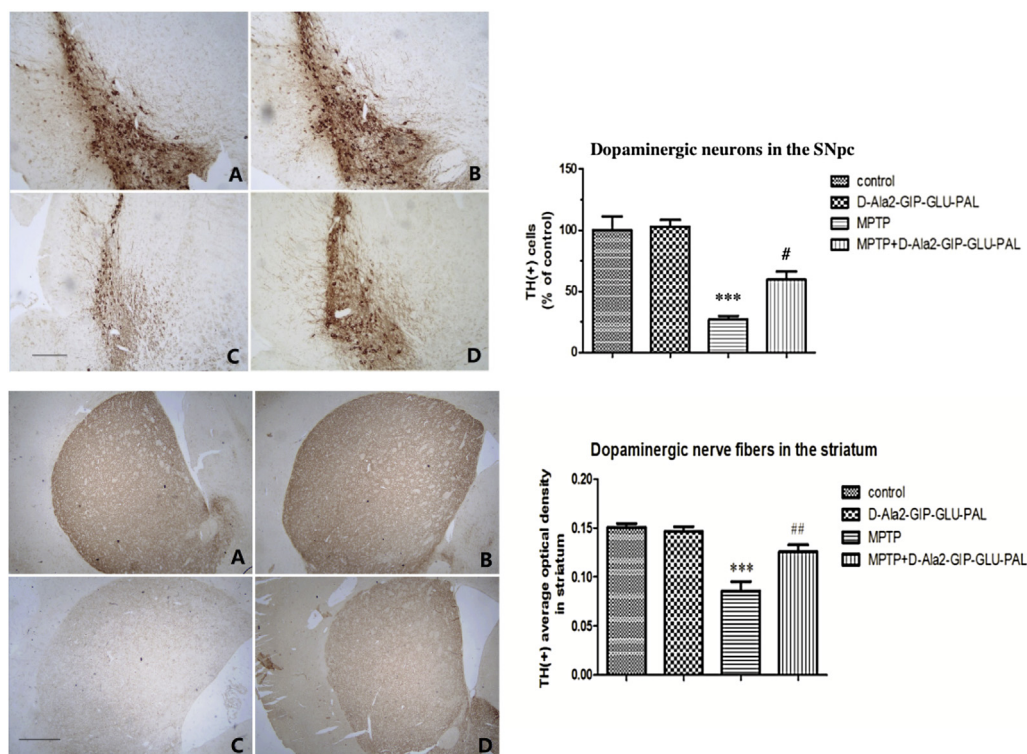


Fig. 2. **Top:** D-Ala2-GIP-GLU-PAL restored tyrosine hydroxylase (TH) positive dopaminergic neuron numbers in the substantia nigra. (A–D) – TH positive neuron numbers were reduced following treatment with MPTP, and increased by D-Ala2-GIP-GLU-PAL. A: Saline; B: D-Ala2-GIP-GLU-PAL; C: MPTP; D: MPTP + D-Ala2-GIP-GLU-PAL. Scale bar in image C: 200 μ m. **Graph:** D-Ala2-GIP-GLU-PAL treatment restored numbers of dopaminergic neurons in MPTP-treated mice. **Bottom:** D-Ala2-GIP-GLU-PAL restored tyrosine hydroxylase (TH) expression in the striatum. (A–D) – TH positive nerve fibers were reduced following treatment with MPTP, and increased by D-Ala2-GIP-GLU-PAL. A: Saline; B: D-Ala2-GIP-GLU-PAL; C: MPTP; D: MPTP + D-Ala2-GIP-GLU-PAL. Scale bar in image C: 500 μ m. **Graph:** D-Ala2-GIP-GLU-PAL treatment restored levels of TH staining in MPTP-treated mice. The values represent the means \pm S.E.M. *** P < 0.001 compared with the control group. # P < 0.05, and ## P < 0.01 compared with the MPTP group. n = 4 per group.

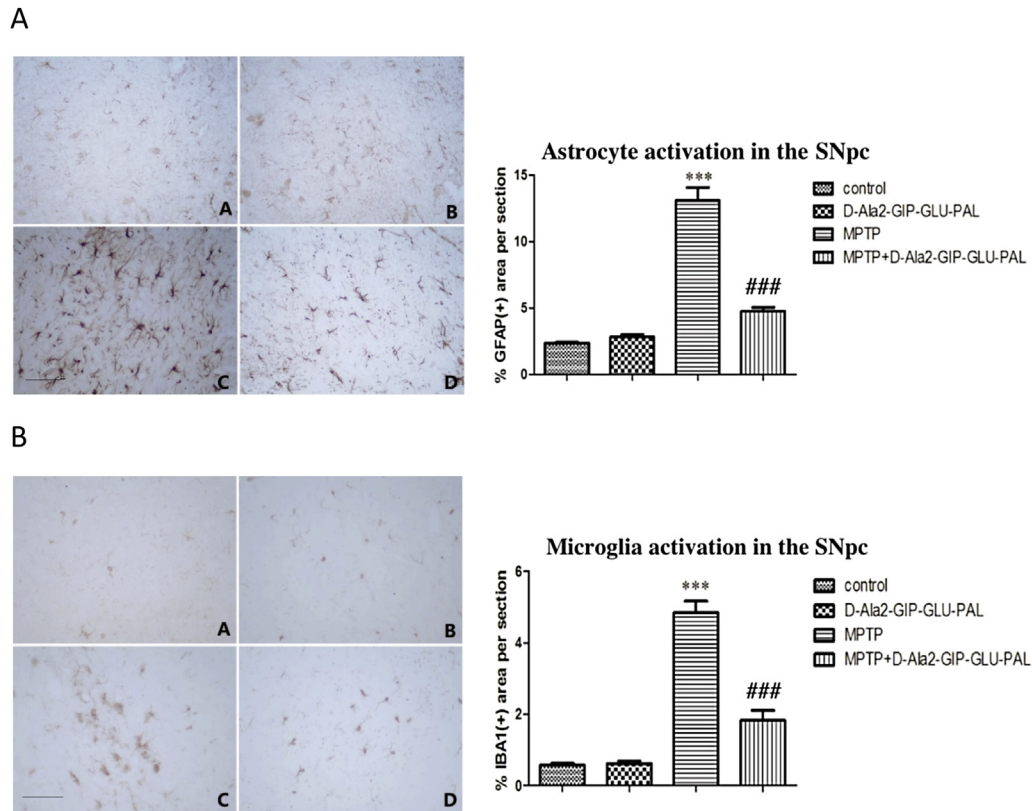


Fig. 3. A: D-Ala2-GIP-GLU-PAL reduced the astrocyte activation in the substantia nigra of mice induced by MPTP. (A–D) – GFAP positive cell numbers were increased following treatment with MPTP, and reduced by D-Ala2-GIP-GLU-PAL. A: Saline; B – D-Ala2-GIP-GLU-PAL; C – MPTP; D – MPTP + D-Ala2-GIP-GLU-PAL. Scale bar in image C: 50 μ m. Graph: Quantification of area of GFAP positive cell in the substantia nigra demonstrates that saline-treated and D-Ala2-GIP-GLU-PAL-treated mouse groups had significantly lower expression of GFAP than the MPTP-treated mouse group. B: D-Ala2-GIP-GLU-PAL reduced the microglia activation in the substantia nigra of mice induced by MPTP. (A–D) – IBA1 expression was increased following treatment with MPTP, and reduced by D-Ala2-GIP-GLU-PAL. A: Saline; B: D-Ala2-GIP-GLU-PAL; C: MPTP; D: MPTP + D-Ala2-GIP-GLU-PAL. Scale bar in image C: 50 μ m. Graph: Quantification of area of IBA1 positive cell in the substantia nigra demonstrates that saline-treated and D-Ala2-GIP-GLU-PAL-treated mouse groups had significantly lower expression of IBA1 than the MPTP-treated mouse group. The values represent the means \pm S.E.M. *** P < 0.001 compared with the control group. ### P < 0.001 compared with the MPTP group. n = 4 per group.

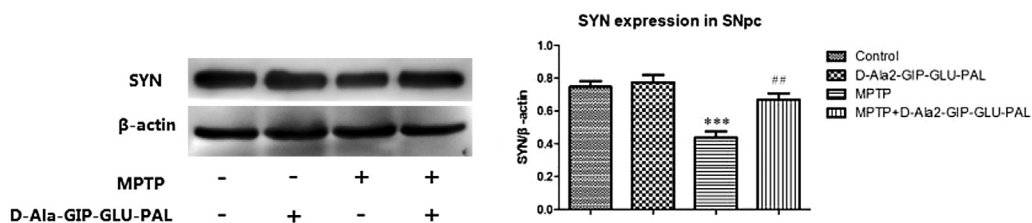


Fig. 4. D-Ala2-GIP-GLU-PAL reversed the reduction of synaptophysin in the substantia nigra of mice induced by MPTP. The values represents the means \pm S.E.M. *** P < 0.001 compared with the control group. ## P < 0.01 compared with the MPTP group. n = 4 per group.

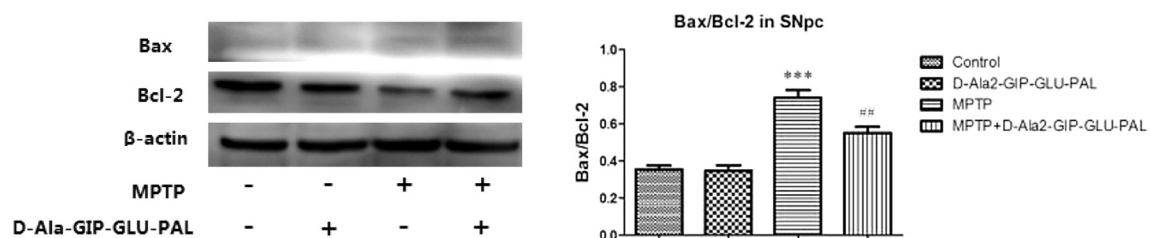


Fig. 5. D-Ala2-GIP-GLU-PAL reversed the increase of ratio of Bax/Bcl-2 in the substantia nigra of mice induced by MPTP. The values represents the means \pm S.E.M. *** P < 0.001 compared with the control group. ## P < 0.01 compared with the MPTP group. n = 4 per group.

neurons to 59.4% in SNpc ($P < 0.05$) and the overall levels of TH to 83.64% in the striatum ($P < 0.01$) in MPTP-treated mice. See Fig. 2.

3.4. D-Ala2-GIP-GLU-PAL reduced the chronic inflammation and astrocyte and microglia activation in the substantia nigra induced by MPTP

A one-way ANOVA found an overall difference between groups ($P < 0.001$). Anti-GFAP was used as an indicator of activated astrocytes. GFAP-positive astrocytes covered an average of 2.38% of each section in the SNpc of control mice, and covered an average of 2.81% in alone D-Ala2-GIP-GLU-PAL injection group. There was no difference between control and alone D-Ala2-GIP-GLU-PAL injection group. GFAP levels increased to 13.10% after MPTP treatment, which was significantly higher than in control mice ($P < 0.001$). While D-Ala2-GIP-GLU-PAL treatment made the areas of GFAP-positive astrocytes decrease to 4.74%, which was significantly less than MPTP group ($P < 0.001$). See Fig. 3A.

Anti-IBA1 was used as a biomarker for microglia activation. IBA1-positive microglia covered an average of 0.57% of each section in the SNpc of control mice, and covered an average of 0.63% in alone D-Ala2-GIP-GLU-PAL injection group. There was no difference between control and alone D-Ala2-GIP-GLU-PAL injection group. This was rise to 4.85% by MPTP procedure, which was significantly higher than control mice ($P < 0.001$). While D-Ala2-GIP-GLU-PAL treatment made the areas of IBA1-positive microglia decrease to 1.83%, which was significantly less than MPTP group ($P < 0.001$). $N = 4$ per group, see Fig. 3B.

3.5. D-Ala2-GIP-GLU-PAL reversed the reduction of synaptophysin levels in the substantia nigra of mice induced by MPTP

A one-way ANOVA found an overall difference between groups ($P < 0.001$). The marker for synapse numbers, synaptophysin (SYN), was much reduced in mice treated with MPTP ($P < 0.001$) compared to control. D-Ala2-GIP-GLU-PAL treatment partially reversed the reduction ($P < 0.01$) compared to the MPTP group. $N = 4$ per group, see Fig. 4.

3.6. D-Ala2-GIP-GLU-PAL reversed the increase of Bax/Bcl-2 levels in the substantia nigra of mice induced by MPTP

A one-way ANOVA found an overall difference between groups ($P < 0.001$). The overall levels of the anti-apoptotic signaling molecule Bcl-2 in SNpc was reduced by MPTP treatment, levels of the pro-apoptotic signaling molecule Bax in SNpc was increase by MPTP treatment, and ratio of Bax/Bcl-2 was increased ($P < 0.01$), compared with control group. While D-Ala2-GIP-GLU-PAL partly decreased the ratio of Bax/Bcl-2 by enhancing Bcl-2 levels ($P < 0.01$). $N = 4$ per group, see Fig. 5.

3.7. D-Ala2-GIP-GLU-PAL moderate reversed the decrease of p-CREB^{S133} in the substantia nigra of mice induced by MPTP

The levels of total CREB did not change in any of the groups. The level of p-CREB^{S133} in alone D-Ala2-GIP-GLU-PAL group was slight higher than control group, but there was no significance ($P > 0.05$). There was a significant decrease in levels of p-CREB^{S133} in MPTP-treated group ($P < 0.001$), compared with control group. However, D-Ala2-GIP-GLU-PAL treatment moderate reversed the decrease ($P < 0.05$). $N = 4$ per group, see Fig. 6.

4. Discussion

In our study we show for the first time that GIP receptor activation has neuroprotective effects in an animal model of PD. We have previously shown that a protease resistant GIP analogue has protective effects in a mouse model of Alzheimer's disease (Duffy and Holscher, 2013; Faivre and Holscher, 2013a,b), but no data has been published previously that demonstrates that GIP analogues may also have protective effects in PD. In the present study, the optimal dose of D-Ala2-GIP-GLU-PAL (25 nmol/kg body weight) for the evaluation of the neuroprotective effects in the PD mouse model was selected based on the previous findings (Faivre et al., 2012; Irwin et al., 2006b; Martin et al., 2013; Tatarkiewicz et al., 2014). We chose the MPTP mouse of PD model for testing this novel GIP analogue. MPTP is a commonly used chemical to induce a Parkinson-like state in rodents (Kopin and Markey, 1988; Nakamura and Vincent, 1986). It was discovered when a contaminated batch of heroin was sold in New York and the users developed PD like symptoms (Langston et al., 1999; Morin et al., 2014). MPTP is lipophilic and can cross the blood–brain barrier. There, MPTP is metabolised into the toxic cation 1-methyl-4-phenylpyridinium (MPP⁺) by the monoamine oxidase B (Glover et al., 1986). MPP⁺ kills primarily dopamine-producing neurons in the substantia nigra, pars compacta (Gerlach et al., 1991; Nakamura and Vincent, 1986). MPP⁺ interferes with complex 1 of the mitochondrial electron transport chain, which leads to the production of free radicals and eventually to neuronal death in the SN (Kinemuchi et al., 1987; Smith and Bennett, 1997). As animals do not naturally develop PD, the MPTP lesion model is one of several models that are in use to investigate the underlying mechanisms of PD and also to test new drug candidates for their neuroprotective properties (Morin et al., 2014).

Locomotor dysfunction including tremors, rigidity and bradykinesia are the classic clinical symptoms of PD. In the present study, open field test analysis suggests that D-Ala2-GIP-GLU-PAL improved the locomotor and exploratory activity of mice induced by MPTP, and the improved bradykinesia, movement coordination and balance of mice also were shown in the pole test. The impairment in locomotor performance was consistent with the reduced levels of TH protein levels. On the 7th day after acute MPTP injection, the number of TH-positive cell bodies in the SNpc was decreased to 27.00%, and the level of TH in the striatum was decreased to 56.86%.

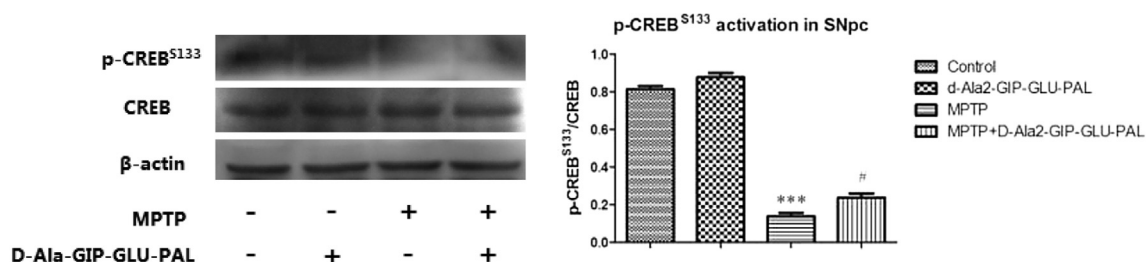


Fig. 6. D-Ala2-GIP-GLU-PAL moderate reversed the decrease of p-CREB^{S133} in the substantia nigra of mice induced by MPTP. The values represents the means \pm S.E.M. *** $P < 0.001$ compared with the control group, # $P < 0.05$ compared with the MPTP group. $n = 4$ per group.

However, D-Ala2-GIP-GLU-PAL treatment restored numbers of TH positive neurons to 59.4% in SNpc and the nerve fibers to 83.64% in the striatum. 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. Furthermore, as shown in the results of synapse numbers biomarker synaptophysin, the synapse numbers are reduced by the MPTP treatment, and D-Ala2-GIP-GLU-PAL was able to partially prevent or reverse this effect. Similar protection of synapse numbers and of synaptic plasticity by GIP analogues was observed in a mouse model of AD (Faivre and Holscher, 2013a,b; Gault and Holscher, 2008). The results demonstrate protective effects of D-Ala2-GIP-glu-PAL on synapses and synaptic function in the MPTP treated mice.

A key element of disease progression in PD is the development of a chronic inflammation response in the brain. The reduction of inflammation in the brain was shown by the reduced levels of GFAP levels in astrocytes and IBA1 in microglia after D-Ala2-GIP-glu-PAL treatment. 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, cerebral spinal fluid, and blood of PD patients (Chen et al., 2008). Chronic inflammation is thought to play a central role in PD pathogenesis because the release of cytokines promotes disease progression (Ferrari and Tarelli, 2011). Initially, an acute inflammatory response may be beneficial to clear out necrotic cells. Once the chronic inflammation is established, however, the inflammation response becomes neurotoxic due to the production of free radicals and pro-inflammatory cytokines. Abnormal production of pro-inflammatory cytokines by activated microglia and astrocyte can lead to synapse dysfunction and ultimately synapse loss (Tansey and Goldberg, 2010). We previously reported that another GIP analogue, D-Ala2-GIP, also decreased neuroinflammation in the cortex of APP/PS1 mice (Duffy and Holscher, 2013). The reduction of the inflammation response is most likely one of the mechanisms by which GIP analogues exert their neuroprotective effects.

Apoptosis also plays a role in neurodegenerative disorders such as PD. GIP acts as an endogenous neurotrophic factor and supports neuronal survival (Holscher, 2014a; Maino et al., 2014; Paratore et al., 2011); and in cultured cerebellar granule neurons, GIP reduced the extent of apoptotic death (Paratore et al., 2011). As neurons in the SN are not replaced, the inhibition of apoptotic signaling will be of benefit (Schulz, 2006). MPTP has been shown to induce apoptosis by inhibiting the mitochondrial multi-subunit enzyme complex I (Bove and Perier, 2012). We analysed the protein levels of the anti-apoptotic signaling molecule Bcl-2 (Maino et al., 2014) and the pro-apoptotic signaling molecule Bax (Kim et al., 2005) in the SNpc in this study. MPTP treatment led to a decline of Bcl-2 levels and an increase of Bax in the SNpc, and D-Ala2-GIP-glu-PAL partly reversed this process by promoting Bcl-2 expression. Previous studies have shown that GIP enhances cell survival and increases Bcl-2 levels in cultured neurons (Maino et al., 2014) and in islet beta cells (Kim et al., 2005; Lupi et al., 2010). Other studies showed that GIP promotes the survival of cells by increasing Bcl-2 promoter activity and upregulating the synthesis of Bcl-2 in vivo or in vitro (Kim et al., 2008). Microarray analysis of GIP treated cells also showed down-regulation of Caspase 8 associated protein 2, a multifunctional protein of the cysteine proteases family which plays a key role in apoptosis. Additionally, GIP treatment up-regulated the expression of cytoglobin, a protein involved in the antioxidant defense system and regulation of apoptosis (Maino et al., 2014). Therefore, D-Ala2-GIP-glu-PAL may promote cell survival of dopaminergic neurons in the SNpc by blocking apoptosis induced by MPTP.

GIP receptors activate an adenyl cyclase and enhance intracellular cAMP levels, which in turn activates Protein Kinase A (PKA),

which phosphorylates the cAMP-response element binding protein (CREB) at Serine 133 and the cAMP-responsive CREB coactivator 2 (TORC2) through a pathway involving phosphatidylinositol 3-kinase (PI3-K), PKB, and AMP-activated protein kinase (AMPK) (Holscher, 2014b; Kim et al., 2010). The cAMP/PKA/CREB cell signaling cascade is a classic growth-factor cell signaling cascade (Hussain et al., 2006; Li et al., 2010), and plays a regulatory role in the nervous system and promotes neuronal survival, precursor cell proliferation and synaptic activity (Holscher, 2014b; Sharma et al., 2013). An impairment in the CREB-induced transcription cascade correlates with neuronal loss and the resulting motor disorders in PD (Kano et al., 1995). GIP stimulates expression of the anti-apoptotic Bcl-2 gene in pancreatic beta cells through a pathway involving AMP-activated protein kinase (AMPK), TORC2, and CREB (Holscher, 2014a; Kim et al., 2008). We therefore analysed the levels of CREB phosphorylation at serine 133 in the SNpc. There was a significant decrease in levels of p-CREB^{S133} in the MPTP-treated group. The result was consistent with previous studies (Sa et al., 2015). However, D-Ala2-GIP-glu-PAL treatment partially reversed the decrease of p-CREB^{S133} levels. These findings demonstrate that D-Ala2-GIP-GLU-PAL can facilitate CREB activation, and ultimately promote cell survival of dopaminergic neuron in SNpc. Further research is required to elucidate the cellular signaling pathways that are involved in neurodegeneration in PD and in neuroprotection by GIP analogues.

The MPTP mouse model is considered an animal model of PD, but it has its limitations and does not recapitulate all pathological processes found in PD. The results presented here show that GIP analogues hold promise as a potential treatment for PD. Further tests will be conducted in other animal models of PD using different chemicals to induce PD-like symptoms such as 6-OHDA or LPS injection into the brain, and transgenic mouse models that express human mutated genes that are known to induce PD.

In conclusion, the results from the in vivo PD mouse models demonstrate the neuroprotective effects of D-Ala2-GIP-glu-PAL and provide insight into its mechanism of action. Our findings demonstrate that treatment with D-Ala2-GIP-glu-PAL significantly inhibits MPTP-induced Parkinsonism-like symptoms in mice. D-Ala2-GIP-glu-PAL also reduces MPTP-induced damage in the dopaminergic neurons in this mouse model. Moreover, the neuroprotective activity of D-Ala2-GIP-glu-PAL may result from increasing CREB-mediated Bcl-2 expression to prevent apoptosis and from the reduction of chronic neuroinflammation. Long-acting GIP analogues hold promise as novel treatments that not only improve the symptoms but prevent the neurodegenerative processes underlying PD.

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.

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