

Two novel dual GLP-1/GIP receptor agonists are neuroprotective in the MPTP mouse model of Parkinson's disease

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Abstract

Type 2 diabetes mellitus (T2DM) is a risk factor for developing Parkinson's disease (PD). Insulin desensitization is observed in the brains of PD patients, which may be an underlying mechanism that promotes neurodegeneration. Incretin hormones are growth factors that can re-sensitize insulin signalling. We have previously shown that analogues of the incretins GLP-1 or GIP have neuroprotective effects in the MPTP mouse model of PD. Novel dual GLP-1/GIP receptor agonists have been developed as treatments for T2DM. We have tested 3 novel dual receptor agonists DA-JC1, DA-JC4 and DA-CH5 in comparison with the GLP-1 analogue liraglutide (all drugs at 25nmol/kg ip once-daily for 6 days) in the MPTP mouse model of PD (4x 25mg/kg ip). In the Rotarod and grip strength assessment, DA-CH5 performed best in reversing the MPTP-induced motor impairment. Dopamine synthesis as indicated by levels of tyrosine hydroxylase was much reduced by MPTP in the substantia nigra and striatum, and DA-CH5 was the best drug to reverse this. Pro-inflammatory cytokines were best reduced by DA-CH5, while expression levels of the neuroprotective growth factor Glial Derived Neurotrophic Factor (GDNF) was most increased by DA-JC4. Synapses were protected best by DA-JC4 and DA-CH5. Both DA-JC1 and liraglutide showed inferior effects. These results show that a combination of GLP-1 and GIP receptor activation is more efficient compared to single GLP-1 receptor activation. We conclude that dual agonists are a promising novel treatment for PD. The GLP-1 mimetic exendin-4 has previously shown disease modifying effects in two clinical trials in Parkinson patients.

Keywords: brain; insulin; growth factor; incretins; dopamine; inflammation

Introduction

Parkinson disease (PD) is a progressive neurodegenerative disorder, and due to the increase of life expectancy in the industrialized countries, patient numbers are on the increase (Schapira, 2013). PD presents with symptoms such as resting tremor, bradykinesia, rigidity and others (Langston, 2002). The main cause of this appears to be a reduction in striatal dopamine levels, which is caused by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Moore et al., 2005; Wakamatsu et al., 2008). Importantly, type II diabetes has been identified as a risk factor for PD (Hu et al., 2007; Schernhammer et al., 2011; Sun et al., 2012; Wahlqvist et al., 2012). Insulin signaling in the brain plays a key role in neuronal metabolism, repair and synaptic efficacy (Freiherr et al., 2013; Ghasemi et al., 2013; van der Heide et al., 2006). It has been shown that insulin signaling is desensitized in the brains of patients with PD (Aviles-Olmos et al., 2013b; Moroo et al., 1994; Morris et al., 2011). Analogues of incretin hormones have been developed to treat type II diabetes (Campbell and Drucker, 2013; Holst, 2004). The incretin hormones are glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP) (Baggio and Drucker, 2007; Campbell and Drucker, 2013; Doyle and Egan, 2003). Protease-resistant analogues of GLP-1 and GIP have shown neuroprotective effects in animal models of Alzheimer's disease (Bomfim et al., 2012; Duffy and Holscher, 2013; Faivre and Holscher, 2013a, b; Li et al., 2010; McClean et al., 2011) and re-sensitize insulin signaling in the brain (Long-Smith et al., 2013; Shi et al., 2017). Furthermore, previous studies found that GLP-1 receptor agonists also have neuroprotective effects in animal models of PD (Bertilsson et al., 2008; Harkavyi et al., 2008; Li et al., 2009; Liu et al., 2015a; Zhang et al., 2015). The GLP-1 mimetic exendin-4 showed impressive effects in a pilot clinical study and in a phase II double blind, placebo controlled study in PD patients (Athauda et al., 2017; Aviles-Olmos et al., 2013a; Aviles-Olmos et al., 2014). We have shown that GLP-1 and also GIP analogues are neuroprotective in the 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, demonstrating that both incretin signaling pathways protect the brain from the MPTP induced pathology such as motor impairment, an increase of α synuclein levels in the brain, development of chronic inflammation in the brain, loss of dopamine synthesis, and reduced growth factor expression (Ji et al., 2016a; Li et al., 2016, 2017; Liu et al., 2015a; Liu et al., 2015b; Zhang et al., 2015). Recently, several dual GLP-1/GIP receptor agonists have been developed as a treatment of type II

diabetes. These peptides can activate both the GLP-1 and the GIP receptor. Such dual receptor agonists are superior compared to single GLP-1 analogues such as liraglutide (Finan et al., 2013). Previously, we tested the dual agonist (DA-JC1) in cell culture SH-SY5Y cells that were stressed with rotenone and demonstrated that DA-JC1 was neuroprotective at much lower doses than GLP-1 receptor agonists (Jalewa et al., 2016). We tested DA-JC1 in the MPTP mouse model of Parkinson as well. The drug reduced the MPTP induced impairment of motor control, increased the number of TH positive neurons in the substantia nigra, and reduced the chronic inflammation response and apoptotic signalling while increasing the levels of the growth factor Brain Derived Neurotrophic Factor (BDNF) (Cao et al., 2016; Ji et al., 2016b). We also tested DA-JC1 in the 6-OHDA rat model of PD where it displayed good neuroprotective effects and increased dopamine levels in the striatum (Jalewa et al., 2017). However, the efficacy of DA-JC1 is not superior to liraglutide at the same drug dose. We therefore tested the novel GLP-1/GIP dual agonists DA-JC4 and DA-CH5 and compared it to the currently most effective GLP-1 analogue liraglutide that is on the market as a T2DM drug (Lovshin and Drucker, 2009) and to DA-JC1 in the MPTP mouse model of PD.

Materials and methods

Animals and drug treatment

Eight week-old adult male C57BL/6 mice (15-20g) were obtained from the Academy of Military Medical Sciences (AMMS China). The animals were maintained under 12h light/dark cycles, at $22 \pm 3^\circ\text{C}$ and 50-55% humidity with food and water ad libitum. 60 mice were divided into 6 groups, each group contained 10 mice, six of them were used for immunohistochemical analysis, and four mice used in western blot assays. Blood was taken for glucose measurements from 5 mice per group, and the other 5 mice per group were used for the weight test on day 1 and day 7 and 5 for the glucose test on days 1, 2, 3 and 7. The mice were randomly assigned to the following groups. (A) control group: treated with saline; (B) MPTP group: 4 intraperitoneal MPTP injections on day 1 (25mg/kg/2h, i.p.) + saline (0.1ml i.p) for 6 days starting the day after day 1; (C) liraglutide group: 4 intraperitoneal MPTP injections on day 1 (25mg/kg/2h, i.p.)+ liraglutide (25nmol /kg/day i.p.) for 6 days after day 1; (D) DA-JC1 group: 4 intraperitoneal MPTP injections on day 1(25mg/kg/2h, i.p.)+DA-JC1

(25nmol/kg/day i.p.) for 6 days after day 1; (E) DA-JC4 group: 4 intraperitoneal MPTP injections for 1 day (25mg/kg/2h,i.p.) + DA-JC4 (25nmol/kg/day i.p.) for 6 days after day 1; (F) DA-CH5 group: 4 intraperitoneal MPTP injections for 1 day (25mg/kg/2h, i.p.)+ DA-CH5 injection (25nmol/kg/day i.p.) for 6 days after day 1. The work was approved by the ethics committee of Shanxi province. All efforts were made to minimise animal suffering and to reduce the number of animal used during experimental procedures. See Fig.1 for the study design.

Peptides

The dual agonists and liraglutide (Peptide Purity: 95%) were obtained from Chinapeptides Ltd (Shanghai, China). The purity of the peptide was confirmed by reversed-phase HPLC and characterised using matrix assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry.

Peptide sequence of the GLP-1/GIP dual agonist **DA-JC1** (Finan et al., 2013):

YXEGTFTSDYSIYLDKQAAXEFVNWLLAGGPSSGAPPPSK-NH2

X = aminoisobutyric acid; **K** = Lys-C₁₆ acyl

DA-JC4

YXEGTFTSDYSIYLDKQAAXEFVNWLLAGGPSSGAPPPSKKKKKK-NH2

DA-CH5

YXEGTFTSDYSIYLDKQAAXEFVNWLLAGGPSSGAPPPSKRRQRRKKRGY-NH2

X = aminoisobutyric acid

Reagents

MPTP was obtained from Sigma-Aldrich (St Louis, MO, USA). Rabbit anti- Nf- κ B antibody was bought from Cell Signal Technology (Boston, USA), the Tyrosine Hydroxylase (TH), IBA-1, synaptophysin, IL-1 β , Glial cell line Derived Neurotrophic Factor (GDNF), TNF- α monoclonal primary antibodies raised in rabbit were purchased from Abcam (Cambridge, UK). Horseradish

peroxidase (HRP)-conjugated anti-rabbit antibodies were purchased from the Boster Institute of Biotechnology (Wuhan, China). β -actin was obtained from Bioworld (St. Louis, MN, USA). Other materials for Immunohistochemistry and western blotting were obtained from Biotechnology (Wuhan).

Determination of glucose levels

Blood samples were taken from the cut tip of the tail vein of mice. Blood glucose was measured instantly by an automated glucose oxidase procedure using Blood Glucose Meter (Sinocare Inc. in Changsha, China).

Motor activity tests

RotaRod test

The rotarod test is a useful method for measuring motor coordination in a mouse model of PD. The rotarod equipment (YLS-4C, Academy of medical sciences in Shandong, China) consisted of a rotating spindle and five individual compartments able to simultaneously test five mice. Mice were placed on a rod that accelerated smoothly from 5 to 20 rpm over a period of 180s. Mice that completed the task received a final latency time of 180 seconds. The length of time the mouse was able to stay on the rotating rod was recorded. On days 2-7, 5 mice per group were used in this test which started 1 hour after drug injection. The experiment was repeated two times for each mouse with an interval of half an hour.

Wire hanging grip test

Muscle strength was assessed by a traction test as previously published (Liu et al., 2015a). Mice were lifted onto a horizontal wire, which the mouse gripped by its Front paws. The mouse was scored as 3 for gripping the wire with both hind paws, 2 for gripping the wire with one hind paw, and 1 for not gripping the wire with either hind paw. On days 2-7, 5 mice per group were used in this test which started 1 hour after drug injection.

Immunohistochemistry

On the 8th day after the MPTP and drugs treatment, mice were injected with chloral hydrate, then

were perfused by saline, followed by 4% PFA. Brains were immediately removed and post-fixed in 4% PFA for overnight. Then they were paraffin embedded, cut, and coronal sections (5 μ m thick) were cut from -2.5 to -4 mm Bregma and mounted onto slides for immune histochemical study. The sections were pretreated with 3% H₂O₂ for 10 min at room temperature to remove endogenous peroxidase activity. Then, sections were incubated with primary antibodies: rabbit anti-mice monoclonal TH (1:200, Abcam), rabbit anti- IBA-1 (1:200, Abcam), rabbit anti-mice GDNF (1:100); all of the antibodies were diluted in PBS in 37°C for 2h. After this, sections were incubated with their corresponding secondary antibodies for 60min at 37°C. The immunoreactivity was visualized with DAB colour reaction in SNpc. Images were captured with a Zeiss Axioscope 1 (Göttingen, Germany) microscope and a digital Zeiss camera. All morphometric parameters were quantified using the image analysis computer program Image-Pro Plus Version 6.0 (Media Cybernetics, USA). N=6 sections per brain were analysed, with n=6 per group.

Western Blotting

For western blotting, brains were removed from the skull and the midbrain dissected out and snap frozen in liquid nitrogen. Proteins were isolated from the ventral midbrain which have been homogenised in an ice cold RIPA buffer (the Boster Institute of Biotechnology, China) and phenyl-methylsulfonyl fluoride (PMSF). Protein concentration was measured by a BCA Protein Assay Kit, Then, equal amounts of proteins from each sample was subjected to SDS-polyacrylamide gel (PAGE) (12 %) for electrophoresis. The gel was run at 80 V for 30min, and 120 V for 1 h, and proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by using a semidry transfer apparatus (Bio-Rad, Hercules, US). which was subsequently blocked for 2h with 5% milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) at room temperature to block remaining protein binding sites, and then incubated with the rabbit anti-Nf- κ B (1:2,000), the rabbit antibody against IL-1 β (1:2,000), rabbit anti-TNF- α (1:1000), the rabbit antibody against GDNF (1:1000), rabbit anti-SYN (1:1000), and rabbit anti-actin (1:1000) followed by washing six times in TBST for 5 min, the horseradish peroxidase (HRP) conjugated secondary antibody. The immunoreactive bands were visualized with an ECL Western Blot kit (Boster Biotechnology, China) according to the manufacturer's instructions. Densitometric analysis of protein bands in the Western blots and zymograms were done using Image Lab™ Software version 3.0. N=4 per group.

Statistical analysis

All values were displayed as means standard error of the mean \pm (SEM). Repeated measures or standard one-way analysis of variance (ANOVA) with Bonferroni post-hoc tests were used for the analysis of the experimental data. Weight and glucose levels were analysed by repeated measure two-way ANOVA using the program Prism (Graphpad software, USA). A probability value of less than 0.05 was considered to be statistically significant.

Results

Plasma glucose levels and weight changes

When analysing body weights at day 1, 7, a two-way repeated measures ANOVA found no difference between groups (time DF=3, F=15, $p>0.05$; groups DF=5, F=1.3, $p>0.05$; interaction DF=15, F=5.9, $p<0.05$) (Fig. 2A). When analysing plasma glucose levels on day 1, 7, no difference between groups were found (time DF=1, F=3.4, $p>0.05$; groups DF=5, F=1.19, $p>0.05$; interaction DF=5, F=4.9, $p<0.05$) (Fig. 2B).

Rotarod and grip strength test

Rotarod test

A two-way repeated measures ANOVA found an overall difference of treatment (DF=5, F=19.2, $p<0.001$), time (DF=9, F=3.4, $p<0.001$) and interaction (DF=45, F=1.2, $p<0.001$). A Bonferroni post-hoc test found differences between groups. The MPTP group and the Liraglutide +MPTP group was impaired compared to controls ($p<0.001$). The MPTP+Liraglutide, DA-JC1+MPTP, DA-JC4+MPTP and DA-CH5+MPTP groups were performing at a better level compared to the MPTP group ($p<0.001$). The DA-JC4+MPTP group ($p<0.01$) and the DA5+MPTP group was performing better compared to the Liraglutide+MPTP group ($p<0.001$), The DA-JC4+MPTP group was performing better compared to the DA-JC1+MPTP group ($p<0.05$), The DA-CH5+MPTP group was performing better compared to the DA-JC1+MPTP group ($p<0.01$) (Fig. 3A). A two-way ANOVA found a difference between groups ($p<0.001$) and over time ($p<0.05$) when comparing performances for each

day (Fig. 3B). N=5 per group.

Grip strength test.

A two-way repeated measures ANOVA found an overall difference of treatment (DF=4, F=2.5, $p<0.05$), time (DF=5, F=48, $p<0.001$) and interaction (DF=20, F=0.36, $p>0.05$). A post-hoc test found differences between groups. The MPTP group, the Liraglutide +MPTP group and the DA-JC4+MPTP group were all impaired compared to controls ($p<0.001$). The performance in the DA-CH5+MPTP group was improved compared to the DA-JC4+MPTP group ($p<0.05$) (Fig. 3C). N=5 per group. A two-way ANOVA found a difference between groups ($p<0.001$) and over time ($p<0.05$) when comparing performances for each day (Fig. 3D).

Expression of Tyrosine Hydroxylase in the s. nigra

Levels of TH expression in the substantia nigra were different over all groups in a repeated measures one way ANOVA (F=132.571; $P<0.001$). Bonferroni post-hoc tests found differences between groups. The Liraglutide+MPTP, DA-JC1+MPTP, DA-JC4+MPTP showed reduced TH levels compared to controls ($p<0.001$), the DA-CH5+MPTP also showed reduced TH levels compared to controls ($p<0.5$). The Liraglutide+MPTP, DA-JC1+MPTP, DA-JC4+MPTP and DA-CH5+MPTP groups showed higher levels compared to the MPTP group ($P<0.001$). The DA-JC1+MPTP, DA-JC4+MPTP and DA5+MPTP group had higher levels compared to the Liraglutide+MPTP group ($p<0.001$), the DA-JC4+MPTP and DA-CH5+MPTP group showed higher levels compared to the DA1+MPTP group ($p<0.001$). The DA-CH5+MPTP group had higher levels compared to the DA4+MPTP group ($p<0.01$). See Fig. 4.

Levels of glial-derived neurotrophic factor (GDNF)

Levels of GDNF expression in the substantia nigra were different over all groups in a repeated measures one way ANOVA (F=71.369; $P<0.001$), followed by post-hoc tests. All the groups were significantly different from the control group ($p<0.001$). The liraglutide +MPTP($P<0.01$) and DA-JC1+MPTP($P<0.01$), DA4+MPTP($P<0.001$) and DA5+MPTP groups ($P<0.001$) showed higher levels compared to the MPTP group. The DA4+MPTP ($P<0.01$) and DA5+MPTP groups ($P<0.05$) showed higher levels compared to the liraglutide+MPTP group. The DA4+MPTP and DA-

CH5+MPTP groups had higher levels compared to the DA-JC1+MPTP group ($P<0.05$). The DA4+MPTP had higher levels compared to the DA-CH5+MPTP group ($P<0.05$) Fig. 5.

Levels of IBA-1 expression in the s. nigra

A One-way ANOVA showed an overall difference between groups ($F=72.437$; $p<0.001$) followed by post-hoc tests. The MPTP group, Liraglutide +MPTP and DA-JC1+MPTP showed lower levels compared to the control group ($p<0.001$), but the other groups did not. The Liraglutide +MPTP, DA-JC1+MPTP, DA-JC4+MPTP and DA-CH5+MPTP groups showed lower levels compared to the MPTP group ($p<0.001$). The DA-JC1+MPTP, DA-JC4 and DA-CH5 +MPTP groups showed lower levels compared to the liraglutide +MPTP group ($p<0.001$). The DA-JC4 and DA-CH5 +MPTP groups showed lower levels compared to the DA-JC1+MPTP group ($p<0.001$). The DA-CH5 +MPTP groups showed lower levels compared to the DA-JC4+MPTP group ($p<0.05$). See Fig.6

Levels of pro-inflammatory cytokines, synaptophysin and GDNF in western blot analysis

Western blot quantification of protein levels of GDNF, IL-1 β , TNF- α , Nf- κ B. A One-way ANOVA showed a difference between groups ($F=11.664$, $F=140.208$, $F=23.816$, $F=10.458$, $F=21.932$, $P<0.001$) followed by post-hoc tests. Data are averages of 4 repetitions of blotting. IL-1 β levels were higher in the MPTP group than in the control group ($p<0.01$). The Liraglutide+MPTP, DA-JC1+MPTP, DA-JC4, DA-CH5 groups showed lower levels compared to the MPTP group ($P<0.05$), and the DA5+MPTP groups showed lower levels compared to the DA-JC1+MPTP group and DA-JC4 ($P<0.05$), see fig. 7A. Levels of Nf- κ B were lower in the Liraglutide +MPTP, DA-JC1+MPTP, DA-JC4+MPTP and DA-CH5+MPTP group compared to the MPTP group ($P<0.001$). The DA-JC4+MPTP ($P<0.05$), DA-CH5+MPTP group ($P<0.001$) had lower levels compared to the Liraglutide+MPTP group, and the DA-CH5+MPTP group had lower levels compared to the DA4+MPTP group ($P<0.05$), see Fig.7B. TNF- α levels were higher in the MPTP group than in the control group ($P<0.001$). Liraglutide +MPTP, DA-JC1+MPTP, DA-JC4+MPTP and DA-CH5+MPTP group showed lower levels compared to the MPTP group ($P<0.001$). The DA-JC1+MPTP and DA-JC4+MPTP groups had lower levels compared to the Liraglutide+MPTP group ($P<0.05$). The DA-CH5+MPTP group had lower levels compared to the DA4+MPTP group ($P<0.01$), see Fig. 7C. As a measure for the numbers and size of synapses, the levels of synaptophysin were evaluated. The

MPTP ($P < 0.001$), liraglutide +MPTP ($P < 0.01$), DA-JC1+MPTP groups showed reduced levels of synaptophysin compared to controls, the DA-JC4+MPTP and DA-CH5+MPTP groups showed higher levels compared to the MPTP group ($P < 0.01$, $P < 0.001$). The DA-CH5+MPTP group had higher levels compared to the Liraglutide+MPTP group ($P < 0.05$), the DA-JC4+MPTP and DA-CH5+MPTP group showed higher levels compared to the DA1+MPTP group ($P < 0.01$), see Fig.7D. The MPTP, Liraglutide+MPTP, DA-JC1+MPTP, DA-JC4+MPTP groups showed reduced GDNF levels compared to controls ($P < 0.001$). The DA-CH5+MPTP also showed reduced GDNF levels compared to controls ($P < 0.01$). Liraglutide +MPTP ($P < 0.01$), DA-JC1+MPTP ($p < 0.05$) and DA-CH5+MPTP groups ($p < 0.01$) showed higher levels compared to the MPTP group. The DA-JC4+MPTP group had higher levels compared to the Liraglutide+MPTP group ($P < 0.01$), the DA-JC4+MPTP ($P < 0.001$) and DA-CH5+MPTP group ($P < 0.01$) showed higher protection compared to the DA1+MPTP group. The DA-CH4+MPTP group had higher levels compared to the DA4+MPTP group ($P < 0.05$), see Fig.7E.

Discussion

The results presented here show that both the GLP-1 analogue liraglutide and the novel GLP-1/GIP dual receptor agonists have neuroprotective properties in the MPTP mouse model of PD. These drugs have been originally developed as treatments for type II diabetes (Finan et al., 2013; Lovshin and Drucker, 2009). GLP-1 and GIP are incretin hormones that play important physiological roles in the control of metabolism. They are expressed in the brain, as are their receptors (Cork et al., 2015; Farr et al., 2016; Heppner et al., 2015; Merchenthaler et al., 1999; Nyberg et al., 2005; Nyberg et al., 2007). The activation of the receptors enhance cAMP levels and activate key secondary cell signalling cascades that increase gene expression for insulin, the insulin receptor, IRS1, Akt, and growth factors such as IGF-1, GDNF and BDNF (Doyle and Egan, 2007; Hölscher, 2016; Perry and Greig, 2002). They also have anti-inflammatory properties (Duffy and Holscher, 2013; Parthasarathy and Holscher, 2013b). Importantly, GLP-1 and GLP-1/GIP analogues do not affect glycaemia levels or body weight in non-diabetic or non-obese animals or humans (Faivre et al., 2012; Gallwitz, 2006; George et al., 2014). Here we show that neither liraglutide nor the dual agonists tested had prominent

effects on body weight or blood glucose levels. In the motor tasks, all drugs were able to counteract the detrimental effects of MPTP on motor coordination and muscle strength. DA-CH5 appeared to be the most potent and was significantly more effective than liraglutide. MPTP causes a decrease of protein synthesis of key enzymes such as tyrosine hydroxylase, an enzyme that is required for dopamine synthesis. All drugs were able to protect dopaminergic neurons to some degree, with DA-CH5 being the most effective drug in most assessments. Glial-derived neurotrophic factor (GDNF) is a key growth factor that is vital for the differentiation and function of dopaminergic neurons. Its role in neuronal metabolism and its neuroprotective profile has been well established (Airaksinen and Saarma, 2002; Broome et al., 1999) (Drinkut et al., 2012; Kordower and Bjorklund, 2013; Moran and Graeber, 2008). Long-lasting analogues of GDNF and gene therapy strategies are currently under investigation as a potential treatment of PD (see eg. clinical trial NCT01621581), but as GDNF cannot cross the blood-brain barrier (BBB) (Allen et al., 2013), no effective treatment has been developed yet. In our study, levels of GDNF were enhanced by all drugs in the striatum, with DA-JC4 showing the most pronounced effects. GLP-1 and GIP have growth-factor like properties (Hölscher, 2018). We have previously shown that GLP-1 or GIP analogues enhance the expression of BDNF (Ji et al., 2016b; Li et al., 2017), a growth factor that protects synaptic activity from stressors (Nagahara et al., 2009). GLP-1 and GIP can cross the BBB (Dogrukol-Ak et al., 2004; Hunter and Holscher, 2012; Kastin and Akerstrom, 2003; Kastin et al., 2002) and enhance the expression of key growth factors such as BDNF or GDNF. Therefore, the use of GLP-1 and GIP analogues appears to be an effective way of enhancing levels of protective growth factors such as GDNF and BDNF in the brain.

Importantly, the levels of the pro-inflammatory cytokines TNF- α and IL-1 β and Nf- κ B, a key transcription factor in chronic inflammatory responses that is activated by pro-inflammatory cytokines, were also reduced by the drugs, confirming our previous results that demonstrated anti-inflammatory effects with GLP-1 and GIP analogues (Cao et al., 2016; Jalewa et al., 2017; Li et al., 2017; Parthasarathy and Holscher, 2013a). Chronic inflammation is one of the central pathological processes in chronic neurodegenerative disorders such as PD. The inflammation response leads to enhanced oxidative stress and the release of pro-inflammatory cytokines, which affect neuronal activity and synaptic function (Ferrari and Tarelli, 2011). Importantly, synaptic integrity as shown by

levels of synaptophysin was protected by the drugs tested in our study.

We have tested GLP-1 or GIP receptor agonists in the MPTP mouse model of PD and showed that the second generation GLP-1 mimetics liraglutide and lixisenatide were more effective than the older drug exendin-4 (Liu et al., 2015a). The GLP-1 analogue (Val8)GLP-1-Glu-PAL also showed neuroprotective effects (Zhang et al., 2015). We furthermore tested an analogue of GIP, D-Ala2 GIP-glu-PAL, in the acute MPTP mouse model of PD. This protease resistant GIP analogue protected the mice from the effects of MPTP on motor activity and coordination, and dopamine levels in the substantia nigra and striatum were enhanced, too. The MPTP treatment reduced synapse numbers in the striatum, and the GIP analogue reversed this. Importantly, the cAMP/PKA/CREB growth factor second messenger signalling pathway was activated by this drug (Li et al., 2016). In a chronic MPTP treatment design study, we found that MPTP treatment increased the levels of alpha-synuclein in the brain, and D-Ala2-GIP-glu-PAL reduced these levels back to near control values (Li et al., 2017). D-Ala2-GIP-glu-PAL furthermore reduced the chronic inflammation response in the brain, reduced lipid peroxidation and apoptotic signalling, and increased the expression of the growth factor BDNF in the brain (Li et al., 2017). We now show in this study that a combined GLP-1/GIP receptor agonist is superior to liraglutide, and thereby demonstrate that novel dual GLP-1/GIP receptor agonists are a promising strategy to treat PD. Such novel dual agonists have already shown superior effects in preclinical and clinical trials in diabetes when compared to liraglutide (Finan et al., 2013). We found in a previous study that DA-JC1 reduced the MPTP-induced impairment of motor control, increased the level of TH positive neurons in the substantia nigra, reduced the activation of microglia and astrocytes, enhanced growth factor cell signalling such as Pi3k activity and Bcl-2 and BDNF levels, while reducing pro- apoptotic BAX signalling (Cao et al., 2016; Ji et al., 2016b). We also tested DA-JC1 in the 6-OHDA rat model of PD where it displayed good neuroprotective effects and enhanced dopamine levels in the striatum. GDNF levels were also enhanced, and the inflammation response reduced (Jalewa et al., 2017). However, the effects were not superior to liraglutide, and higher doses were required to see the effects previously demonstrated with single GLP-1 analogues. We therefore tested the novel DA-JC4 and DA-CH5 GLP-1/GIP dual agonist in direct comparison with DA-JC1 and liraglutide, and we show here for the first time that these novel dual agonists not only show good neuroprotective effects in the MPTP mouse model of PD, but that they are superior to DA-JC1 and

liraglutide when compared at equal concentrations.

Because of the positive preclinical data, first clinical trials have started to investigate the neuroprotective effects of exendin-4 and liraglutide, drugs that are already on the market as type II diabetes treatments, in PD patients. A pilot study of the effects of exendin-4 (Byetta®) in PD patients has already shown good effects. In the MDS-UPDRS motor tests, the progressive degradation was halted by the drug, and cognition had improved in the Mattis scoring system. In comparison, the control group worsened in cognition and motor control assessments (Aviles-Olmos et al., 2013a). Patients were re-tested 12 months later, and the improvement in motor performance and cognitive scores was still visible in the drug group, while the control group deteriorated further (Aviles-Olmos et al., 2014). The encouraging results of this pilot trial were followed by a phase II double-blind, placebo controlled clinical trial that tested the once-weekly formulation of exendin-4, Bydureon®. The trial showed a clear protective effect in the MDS-UPDRS test battery. After 48 weeks, the drug group was 4.3 points superior to the placebo group. After a wash-out period of 12 weeks when no drug was given, the difference between groups was still 3.5 points, demonstrating that the drug treatment has disease-modifying properties (as defined as improvements still present even when the drug is no longer present in the body). CSF analysis confirmed that exendin-4 had crossed the BBB, and that there was no drug present after wash-out (Athauda et al., 2017). A phase II trial testing liraglutide in PD patients has started in 2017, the duration of drug/placebo treatment is one year (NCT02953665). A third clinical trial testing the GLP-1 receptor agonist Lixisenatide in PD patients is in preparation (Hölscher, 2016).

The data that we present here demonstrate that these novel dual agonists can show superior neuroprotective effects in the MPTP mouse model of PD and hold promise to be effective in treating PD.

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Figure captions

Fig. 1: Schematic representation of the study design.

Fig. 2: Analysis of body weight and plasma glucose levels during the experiment. A two-way repeated measure ANOVA did not find differences between groups or time. N=5 per group.

Fig. 3:

Fig 3A: Motor performance in the Rotarod test. A one-way repeated measures ANOVA found an overall difference over all groups, followed by post-hoc tests: ***= $p < 0.001$ compared to controls; ##= $p < 0.01$ compared to the MPTP group; \$\$= $p < 0.01$ compared to Liraglutide+MPTP group. %= $p < 0.05$ compared to DA-JC1+MPTP group, %%= $p < 0.01$ compared to DA-JC1+MPTP group.

Fig. 3B: A two-way ANOVA found a difference between groups ($p < 0.001$) and over time ($p < 0.05$).

Fig. 3C: Grip strength test. A one-way repeated measures ANOVA found an overall difference over all groups, and a post-hoc found differences between groups: ***= $p < 0.001$ compared to controls; ##= $p < 0.01$ compared to MPTP group; \$\$\$= $p < 0.001$ compared to the Liraglutide+MPTP group. %= $p < 0.05$ compared to DA-JC1+MPTP group.

Fig. 3D: A two-way ANOVA found a difference between groups ($p < 0.001$) and over time ($p < 0.05$).

Fig. 4. Quantification of TH-positive neurons in the substantia nigra pars compacta. A One-way ANOVA showed an overall difference between groups ($F=132.571$; $p=0.398$) followed by post-hoc tests; Data are presented as mean \pm SEM, N=6 per group. ***= $p < 0.001$ compared to control group; ###= $p < 0.001$ compared to MPTP group; \$\$= $p < 0.01$ compared to the Liraglutide group; \$\$\$= $p < 0.001$ compared to the Liraglutide group; %%= $p < 0.01$ compared to the DA-JC1 group; %%%= $p < 0.001$ compared to the DA-JC1 group. Shown are representative images, Scale bar = 200 μ m.

Fig. 5: Quantification of GDNF-positive cells in the substantia nigra pars compacta. A One-way ANOVA showed an overall difference between groups ($F=78.736$; $p=0.03$) followed by post-hoc tests. Data are presented as mean \pm SEM, N=6 per group. *= $p < 0.05$ compared to control group; ***= $p < 0.001$ compared to control group; ##= $p < 0.01$ compared to MPTP group. ###= $p < 0.001$ compared to MPTP group; \$= $p < 0.05$ compared to Liraglutide group; \$\$\$= $p < 0.001$ compared to the Liraglutide group; %%%= $p < 0.001$ compared to DA-JC1 group; &&= $p < 0.01$ compared to the DA-JC4group. Shown are representative images, Scale bar = 50 μ m. The area of where this section was taken from is indicated in the small insert.

Fig. 6: Quantification of chronic inflammation (IBA-1-positive glia) in the substantia nigra pars compacta. A One-way ANOVA showed an overall difference between groups ($F=72.313$; $P=0.156$) followed by post-hoc tests. Data are presented as mean \pm SEM, N=6 per group. ***= $p < 0.001$ compared to control group;##= $p < 0.01$ compared to MPTP group;###= $p < 0.001$ compared to MPTP group; \$\$= $p < 0.01$ compared to the Liraglutide group; \$\$\$= $p < 0.001$ compared to the Liraglutide

group; $p < 0.01$ compared to DA-JC1 group. $p < 0.001$ compared to DA-JC1 group. Shown are representative images, Scale bar = $100\mu\text{m}$.

Fig. 7: Western blot quantification of protein levels of IL-1 β , NF-Kb, TNF- α , synaptophysin, and GDNF. A One-way ANOVA showed an overall difference between groups followed by post-hoc tests. Data are presented as mean \pm SEM, N=4 per group. *= $p < 0.05$ compared to control group; **= $p < 0.01$ compared to control group; ***= $p < 0.001$ compared to control group; #= $p < 0.05$ compared to MPTP group; ##= $p < 0.01$ compared to MPTP group; ###= $p < 0.001$ compared to MPTP group; \$= $p < 0.05$ compared to Liraglutide group; \$\$= $p < 0.01$ compared to the Liraglutide group; \$\$\$= $p < 0.001$ compared to Liraglutide group; %%= $p < 0.01$ compared to DA-JC1 group, %%%= $p < 0.001$ compared to the DA-JC1 group, &= $p < 0.05$ compared to the DA-JC4 group; &&= $p < 0.01$ compared to the DA-JC4 group. F shows sample scans of the western blots.

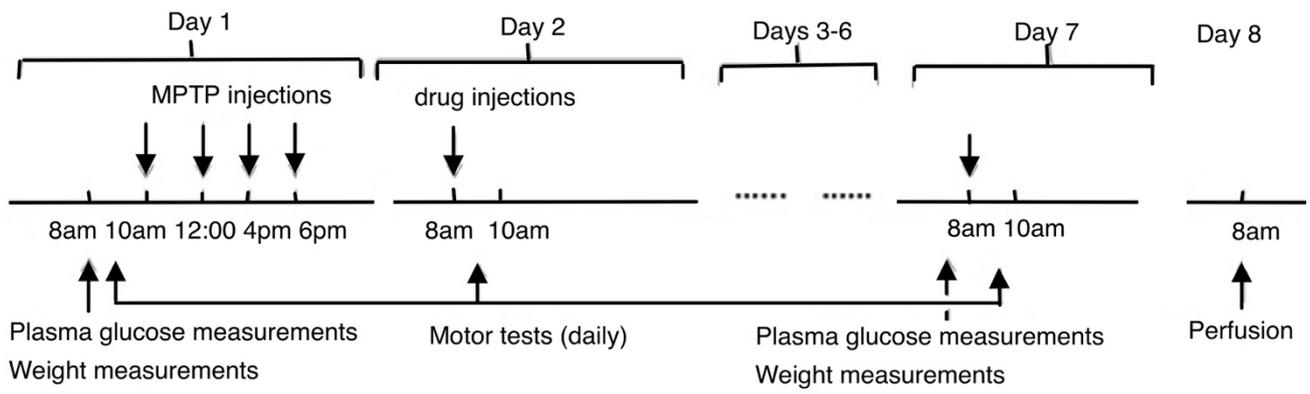


Figure 1

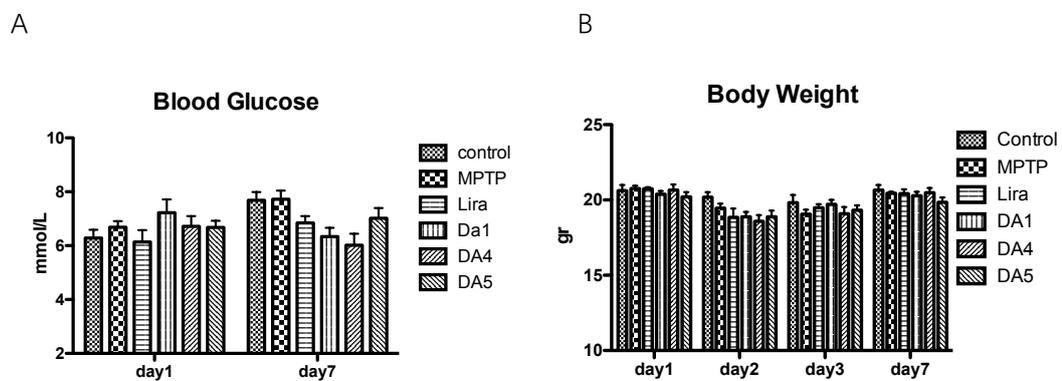


Figure 2

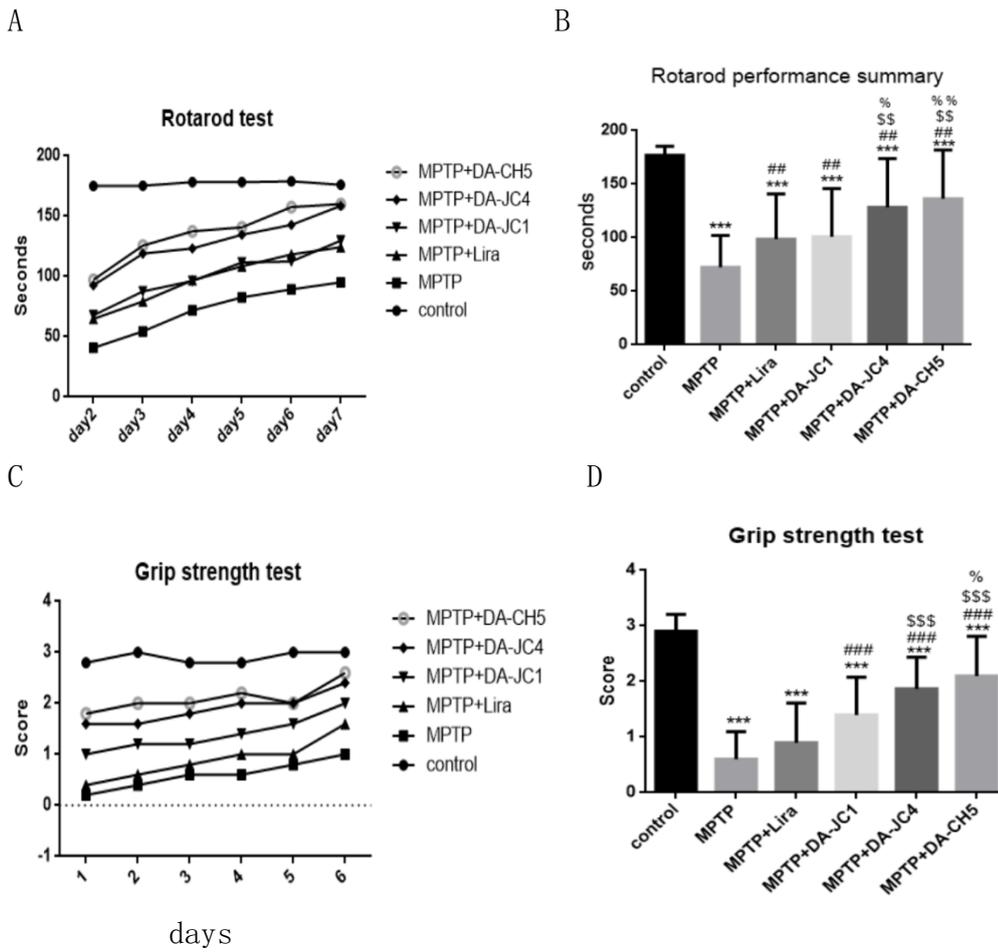


Figure 3

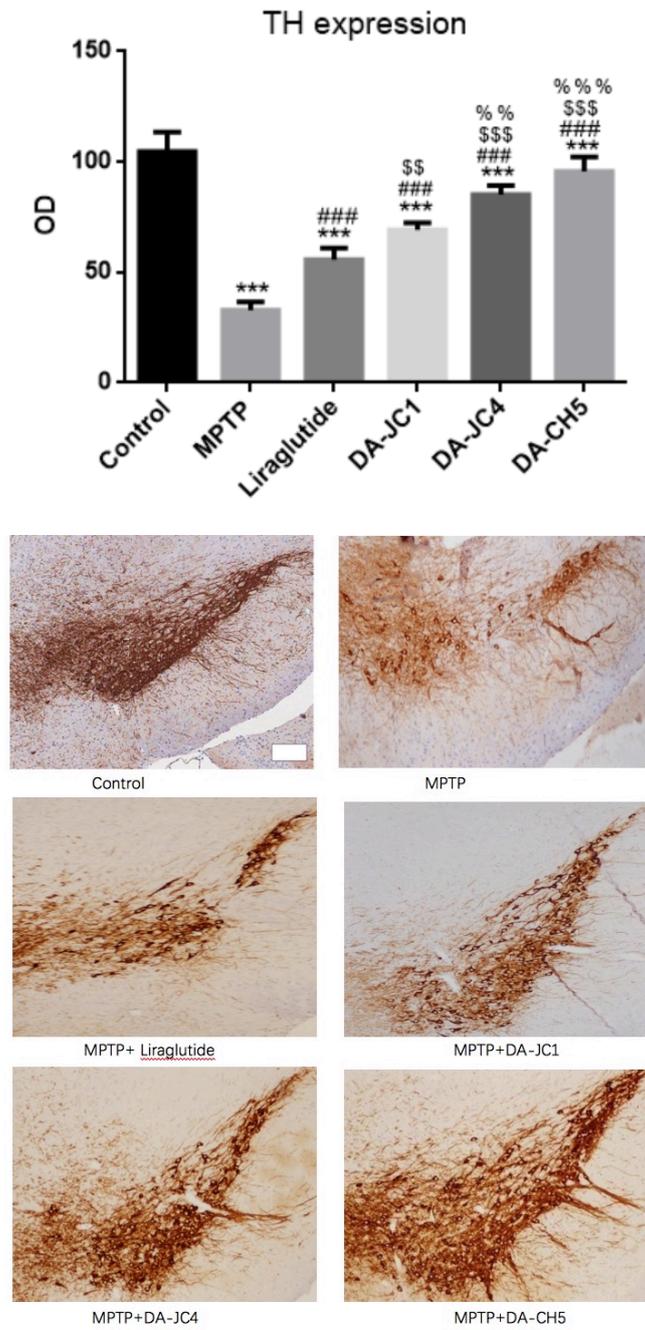


Figure 4

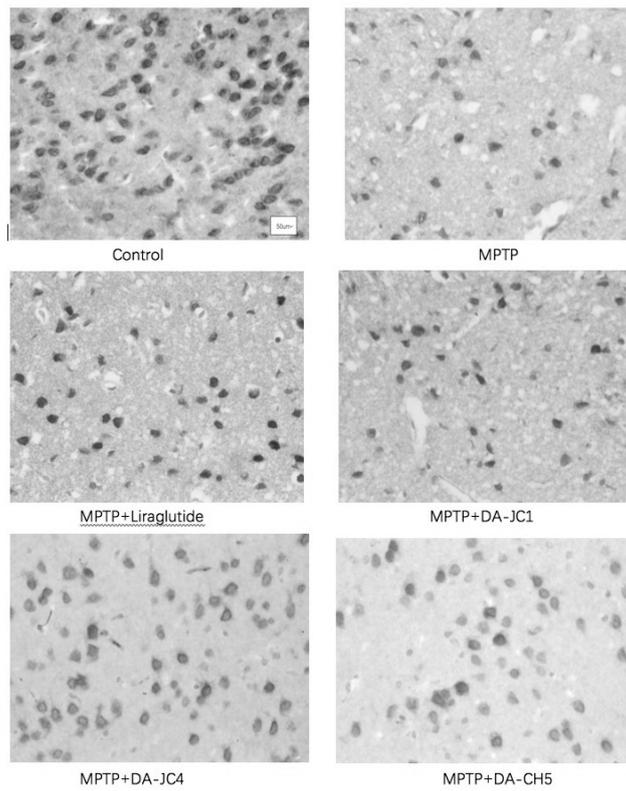
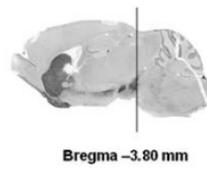
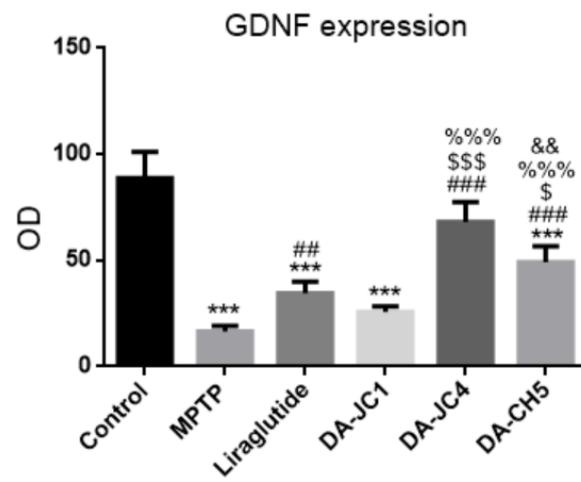


Figure 5

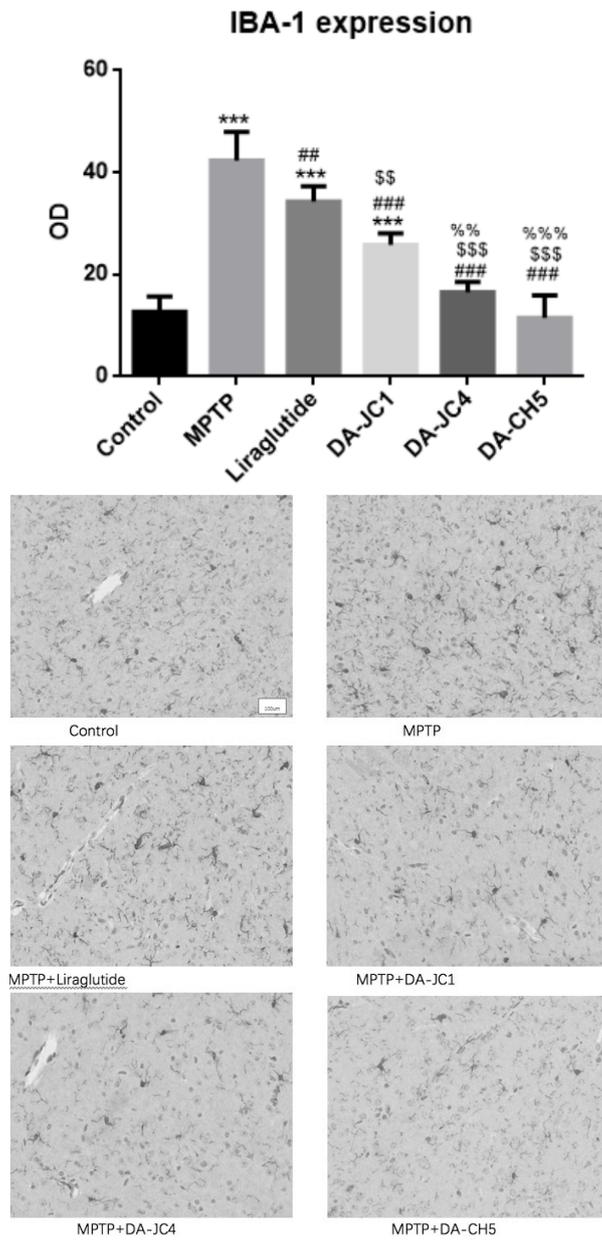


Figure 6

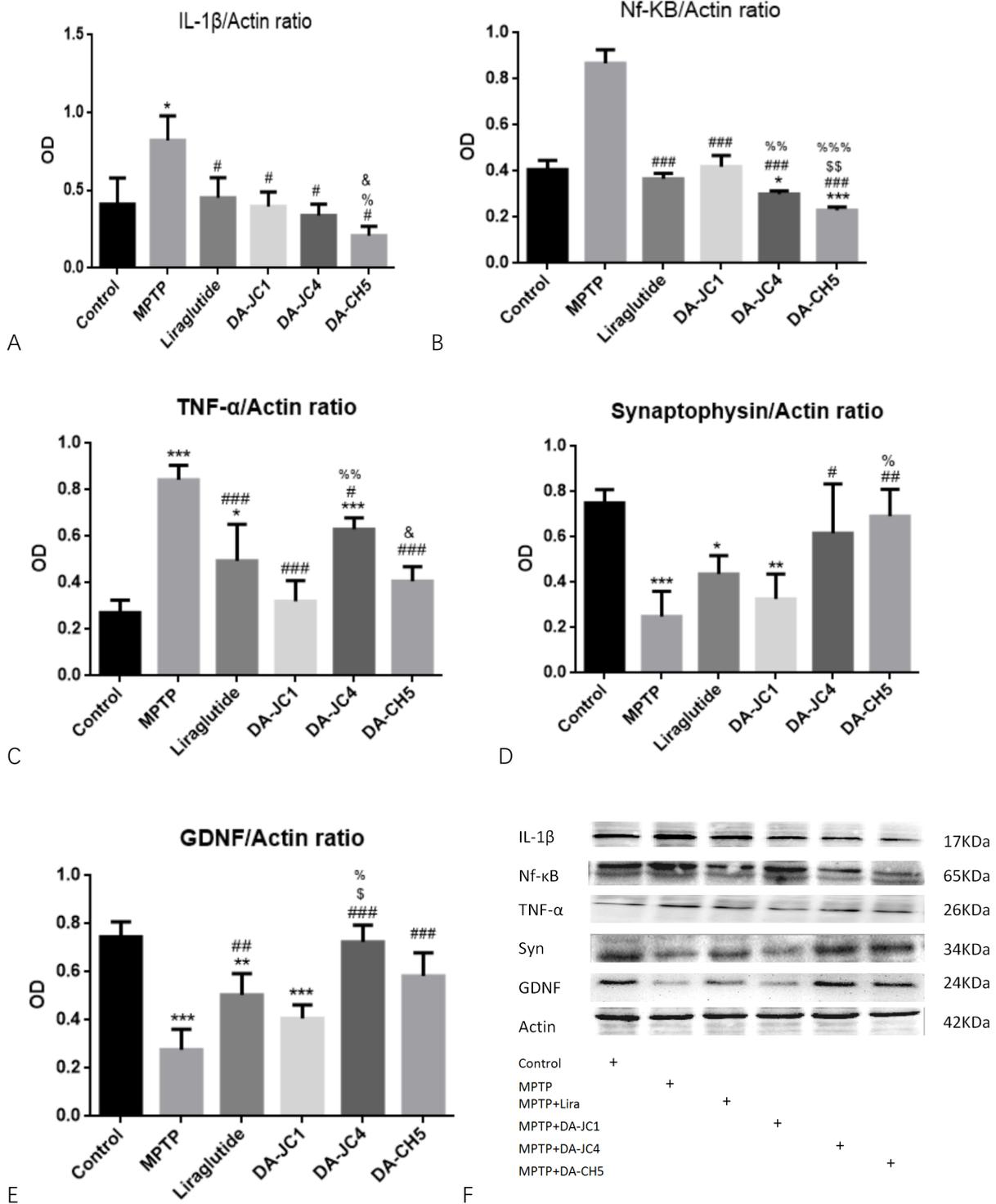


Figure 7