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A novel GLP-1/GIP dual agonist is more effective than liraglutide in reducing inflammation and enhancing GDNF release in the MPTP mouse model of Parkinson's disease

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Abstract

Type 2 diabetes mellitus (T2DM) is one of the risk factors for Parkinson's disease (PD). Insulin desensitisation has been observed in the brains of patients, which may promote neurodegeneration. Incretins are a family of growth factors that can re-sensitise insulin signalling. We have previously shown that mimetics of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) have neuroprotective effects in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropypridine (MPTP) mouse model of PD. Recently, dual GLP-1/GIP receptor agonists have been developed. We therefore tested the novel dual agonist DA3-CH in comparison with the best GLP-1 analogue currently on the market, liraglutide (both drugs 25nmol/kg ip once-daily for 7 days) in the MPTP mouse model of PD

(25mg/kg ip once-daily for 7 days). In the Rotarod and grip strength assessment, DA3-CH was superior to liraglutide 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 DA3-CH reversed this while liragutide only partially reversed this. The chronic inflammation response as shown in increased levels of activated microglia and astrocytes was reduced by both drugs. Importantly, expression levels of the neuroprotective growth factor Glial Derived Neurotrophic Factor (GDNF) was much enhanced by both DA3-CH and liraglutide. The results demonstrate that the combination of GLP-1 and GIP receptor activation is superior to single GLP-1 receptor activation alone. Therefore, new dual agonists may be a promising treatment for PD. The GLP-1 receptor agonist exendin-4 has already shown disease modifying effects in clinical trials in PD patients.

Keywords: insulin; growth factor; incretins; dopamine; inflammation; brain; GLP-1; GIP

1. Introduction

Parkinson disease (PD) is a neurodegenerative chronic disorder. Because of the increase in life expectancy in the industrialised nations, patient numbers are on the increase (Schapira, 2013). PD is diagnosed by characteristic motor impairments (Langston, 2002), caused by a reduction in striatal dopamine levels, which is due to the loss of dopaminergic neurons in the substantia nigra (Moore et al., 2005; Wakamatsu et al., 2008). Importantly, type 2 diabetes (T2DM) 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 an important role in neuronal metabolism, repair, and synaptic efficacy (Freiherr et al., 2013; Ghasemi et al., 2013; van der Heide et al., 2006). Insulin desensitisation has been observed in

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the brains of patients with PD (Aviles-Olmos et al., 2013b; Moroo et al., 1994; Morris et al., 2011). Protease-resistant analogues of incretin hormones have been developed to treat T2DM (Campbell and Drucker, 2013; Holst, 2004). The incretin hormones are glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (Baggio and Drucker, 2007; Campbell and Drucker, 2013; Doyle and Egan, 2003). Mimetics 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 were found to re-sensitise insulin signaling in the brain (Long-Smith et al., 2013). 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 protective effects in a pilot clinical study in PD patients (Aviles-Olmos et al., 2013a; Aviles-Olmos et al., 2014). We previously tested GLP-1 or GIP analogues in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropypridine (MPTP) mouse model of PD, demonstrating that both incretin signaling pathways protect the brain from the MPTP induced pathology such as motor impairment, increase of α-synuclein levels, chronic inflammation in the brain, loss of dopaminergic neurons, oxidative stress and growth factor expression (Ji et al., 2016a; Li et al., 2016; Liu et al., 2015a; Liu et al., 2015b; Zhang et al., 2015).

Recently, dual GLP-1/GIP receptor agonist peptides have been developed as a treatment of T2DM. These dual receptor agonists show superior effects to single GLP-1 analogues such as liraglutide (Finan et al., 2013). We tested a novel dual agonist (DA-JC1) in rotenone stressed SH-SY5Y cells and demonstrated that it was cytoprotective at much lower doses than single GLP-1 or GIP receptor agonists (Jalewa et al., 2016). We furthermore tested DA-JC1 in the MPTP mouse model of PD, which showed some protective effect but which was not superior to liraglutide (Cao et al., 2016; Ji et al., 2016b). On the basis of this, we tested the novel

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GLP-1/GIP dual agonist DA3-CH and compared it to the currently most effective GLP-1 analogue liraglutide on the market (Lovshin and Drucker, 2009) in the MPTP mouse model of PD to investigate if this dual receptor agonist is more effective in protecting the brain than a single GLP-1 receptor agonist.

2. Materials and methods

2.1 Animals and drug treatment

Eight weeks 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 $22 \pm 3°C$ and 50-55% humidity with food and water ad libitum. All animal procedures were licensed by the Shanxi Medical University ethics committee and performed in accordance to National Institute of Health (NIH) guideline (NIH publication NO. 85-23. Revised 1985). Mice were randomly divided into four groups: 1) control (saline treated) group, 2) MPTP only treated group (once daily 25mg/kg ip. for 7 days), 3) MPTP (once daily 25mg/kg ip. for 7 days) followed immediately by liraglutide treated group (25nmol/kg ip. once daily), and 4) the MPTP (once daily 25mg/kg ip. for 7 days) followed immediately by DA3-CH (25nmol/kg ip. once daily) treated group. N=10 per group. The experiment lasted 7 days. A timeline is shown in fig. 1.

2.2 Peptides

The dual agonist DA3-CH and liraglutide (Peptide Purity: 95.77%) were obtained from Chinapeptides Ltd (Shanghai, China). The purity of the peptide was confirmed by reversed-

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phase HPLC and characterised using matrix assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry.

Peptide sequence for GLP-1 (human)

HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH2

Peptide sequence for GIP (human)

YAEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ-NH2

Peptide sequence of the GLP-1/GIP dual agonist DA3-CH (Finan et al., 2013):

Y**X**EGTFTSDYSIYLDKQAA**X**EFVNWLLAGGPSSGAPPPSK-NH2

X = aminoisobutyric acid

2.3 Glucose measurements

Blood plasma glucose levels were tested two h after injection on the first and last day. The Sannuo rapid detection blood glucose meter, product number 1304110 (Sinocare Inc. in Changsha, China) was used to test blood sampled from the tail vein. Levels are reported as mmol/l.

2.4 Motor activity tests

RotaRod test

Motor coordination was evaluated on a RotaRod (YLS-4C, Academy of medical sciences in Shandong, China) consisted of a five-lane rotating rod (diameter 7.5 cm) designed for mice.

Animals were tested with a few runs to familiarize the animals to the procedure. Animals were positioned on a rotating rod accelerating from 5 rpm-20 rpm (14,000 g) over a 180 s time period. Mice that completed the task received a final latency time of 180 s. The length of time the mouse was able to stay on the rotating rod was recorded.

Grip strength test

Muscle grip strength was assessed 4 h after MPTP injection by a grip test as previously published (Luo et al., 2011). The mouse was placed onto a horizontal wire, which the mouse gripped by its front paws. The mice were scored 3 for gripping the wire with both paws, 2 for gripping the wire with one paw, and 1 for not gripping the wire with either paw. The test was repeated three times for each mouse on day 1 and on day 7 of the experiment.

2.5 Immunohistochemistry

Anesthetized mice were transcardially perfused with cold paraformaldehyde 4 %. The brain was isolated and fixed in 4%PFA. Then the brains were embedded in paraffin. Coronal sections were cut at a 5-μm thickness with a microtome (Leica, Germany). Sections were incubated with primary Ab for 2h at 37°C: anti-TH 1:200 (Abcam, Cambridge, UK), GFAP 1:500 (Abcam, Cambridge, UK), Iba1 1:4000 (Abcam, Cambridge, UK), anti-GDNF 1:100 (Abcam, Cambridge, UK), Sections were then incubated with secondary Ab biotin-conjugated goat anti- mouse (KPL) for 30min at 37°. Staining was visualized using a solution of 0.05 % 3,3'-diaminobenzidine (DAB), 50 mM Tris–HCl pH 7.2, 0.02 % H_2O_2. Images were captured with an Olympus BX51 microscope. Microglial and astroglia cells were analysed with ImageJ program in a double- blind design. The images were analysed for staining intensity which represents the level of target protein present. Four sections were randomly chosen through the midbrain, with a total of 12 sections per individual animal using

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unbiased stereological rules. The striatum and the substantia nigra was analysed as shown in the sample images. All analysis was done blind to treatment.

2.6 Western blots

At the end of a week, animals were anesthetised with pentobarbital (40 mg/kg body weight), and cardiac perfusion was carried out using 0.01 M phosphate-buffered saline (PBS) at pH 7.4 to wash out the blood. The brains were snap frozen and stored at -8 0 °C for further use. The midbrain and striatum regions were dissected out from one hemisphere. Protein extracts were prepared from the ventral midbrain regions and homogenized in an ice cold RIPA buffer (containing1% deoxycholate,1% Triton X-100,0.1%SDS) and phenyl-methylsulfonyl fluoride (PMSF). Homogenates were then centrifuged at 12,000rpm, at 4 °C for 10 min, and the supernatant was collected for analysis. Protein concentration was determined by BCA Protein Assay Kit. Samples were then added with loading buffer to the same concentration, boiled, cooled and centrifuged. Upon use, SDS (12%) was added to the homogenate. Approximately 10-30 μg of protein was run on a Tris–HCl gel (Bio-Rad, Richmond, USA) which was run at 80V for 30 min, and 120V for 1h, and transferred to a PVDF membranes (Bio-Rad, Richmond, USA) at 100-120mA for 40 min. Membranes were blocked in 5% milk for 2 h at room temperature. Primary antibodies, which were incubated overnight at 4 °C, were: anti-Iba1 1:1000 (Abcam, UK), anti-GFAP (glial fibrillary acidic protein) 1:10000 (Abcam, Cambridge, UK), anti-GDNF 1:500 (Abcam, Cambridge, UK), β-actin 1:3000. Secondary antibodies used anti-rabbit IgG peroxidase conjugated (Boster, China) at 1:3000 to 1:5000 concentrations respectively, and incubated at room temperature for 1 h. Before this, PVDF membranes were washed three times in TBST for 10 min. Blots were visualized with Molecular Imager ChemiDoc™ XRS+ (Bio-Rad, Richmond, USA). Optical density measurements were taken by Image Lab™ Software version 2.0.

2.7 Statistical analysis

All data were displayed as means standard, error bars represent S.E.M.. Data were analysed using the statistical package Prism (Graphpad software, USA). Repeated measures analysis of variance using a one-way or two-way ANOVA were used, followed by Tukey's multiple comparison test for post-hoc comparison. Probability value less than 0.05 was considered to be statistically significant.

3. Results

3.1 Body weight and blood plasma glucose levels are not affected by the drugs

When measuring blood plasma glucose levels and body weight of the duration of the experiment, a repeated measure one-way ANOVA did not find an overall difference between groups in glucose levels (p>0.05, Fig. 2A) and a repeated measure two-way ANOVA did not find a difference in body weight between groups (p>0.05, Fig. 2B). N=10 per group.

3.2 Motor performance in the Rotarod test is rescued by the drugs

The ability of remaining on the Rotarod was severely impaired by MPTP and improved by liraglutide and by DA3-CH. A repeated measure one-way ANOVA found an overall difference between groups (P<0.001), and Tukey's multiple comparisons post-hoc tests found that the MPTP group and the Liraglutide +MPTP group showed impaired motor control than from the control group (P<0.001). However, the MPTP group was more impaired when compared to the Liraglutide +MPTP group (P<0.01) or to the DA3 +MPTP group (P<0.001). Importantly,

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the Liraglutide +MPTP group was more impaired compared to the DA3 +MPTP group (P<0.001) (Fig. 3A). When plotting the results for each day, a repeated measure two-way ANOVA found a difference between groups (P<0.001) and over time (P<0.05), demonstrating that animals improved over time (Fig. 3B). DA3-CH was the more potent drug. N=10 per group.

3.3 Grip strength was impaired by MPTP and was improved by drug treatment

When testing grip strength on day 1 and 7 (4h after MPTP injection), a repeated measures two-way ANOVA found a difference between groups (P<0.001) but not over time (p>0.05). A Tukey post-hoc test found differences between groups. On day 1 and 7, the control group performed best compared to the other groups (P<0.001). The Liraglutide +MPTP group performed worse compared to the DA3+MPTP group (P<001). On day 1, the MPTP group performed worse when compared to the Liraglutide +MPTP group (P<0.001) or to the DA3 +MPTP group (P<0.001). On day 7, however, the MPTP group impairment differed from the Liraglutide +MPTP group impairment only moderately (P<0.05), while the difference to the DA3 +MPTP group performance was much higher at (P<0.001). This demonstrates that DA3-CH was the more potent. See fig. 4. N=10 per group.

3.4 Dopamine synthesis is impaired by MPTP and rescued by the two drugs

When assessing the levels of tyrosine hydroxylase (TH) in neurons in the substantia nigra and in axonal fibres in the striatum, a one-way ANOVA found an overall difference between groups (P<0.001). In Tukey's multiple comparison's tests, MPTP was shown to clearly reduce the expression levels, and the two drugs were able to reverse this to some extent. DA3-CH was the more potent drug. In the substantia nigra, TH levels in the control group were

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highest in all groups (P<0.001). Levels in the DA3+MPTP group were highest compared to the Liraglutide+MPTP group (P<0.001) and the MPTP group (P<0.001). See Fig. 5A. In the striatum, TH levels in the control group were highest in all groups (P<0.001) except the DA3+MPTP group, where the difference was only (P<0.05), indicating that DA3-CH had improved the levels. The Liraglutide +MPTP group showed lower levels than the DA3+MPTP group (P<0.01), but higher than the MPTP group (P<0.001). The DA3+MPTP group also showed higher levels than the MPTP group (P<0.001). N=10 per group. See Fig. 5B.

3.5 The chronic inflammation response in the brain was improved

When analysing GFAP levels (astrogliosis) and IBA-1 levels (microgliosis), a one-way ANOVA found an overall difference between groups (P<0.001). GFAP levels in the control group were found to be far lower compared to the other groups as shown in a Tukey's post hoc test (P<0.001), except for the DA3+MPTP group, where the difference to controls was only (P<0.05), indicating an improvement and a reduction of the MPTP-induced inflammation by DA3-CH. This was verified in the statistical analysis comparing the DA3+MPTP group with the MPTP and the Liraglutide+MPTP group, showing reduced inflammation (P<0.001). The Liraglutide+MPTP group showed a clear reduction of inflammation compared to the MPTP group (P<0.001). See Fig. 6A. When assessing IBA-1 levels in the control group, they were found to be lower than in the other groups as shown in a Tukey's post hoc test (P<0.001) but identical to the DA3+MPTP group (n.s.). This shows that the DA3-CH drug reduced microgliosis. The DA3+MPTP group differed from the MPTP and the Liraglutide+MPTP group, demonstrating that DA3-CH reduced microgliosis (P<0.001). The Liraglutide+MPTP group showed reduced microgliosis compared to the MPTP group (P<0.001). DA3-CH was the more potent drug. N=10 per group. See Fig. 6B.

3.6 Levels of the growth factor GDNF were improved

When analysing GDNF levels, a one-way ANOVA found an overall difference between groups (P<0.001). GDNF levels in the DA3+MPTP group were found to be higher compared to the other groups in a Tukey's post hoc test (P<0.001). The DA3+MPTP group showed higher levels compared to the MPTP and the Liraglutide+MPTP group (P<0.001). The Liraglutide+MPTP group showed higher levels compared to the MPTP group (P<0.001). DA3-CH was the more potent drug. N=10 per group. See Fig. 7.

3.7 Western blot analysis of GDNF, GFAP and IBA-1 levels

When analysing GDNF levels using the western blot technique, a one-way ANOVA found an overall difference between groups (P<0.001). The control group had the highest levels compared to the MPTP group (P<0.001) and the Liraglutide+MPTP group (P<0.01) but not to the DA3+MPTP group. The impairment caused by MPTP was reversed by DA3-CH. The MPTP group showed lower levels compared to the Liraglutide+MPTP group (P<0.01) and the DA3+MPTP group (P<0.001). The Liraglutide+MPTP group had lower levels compared to the DA3+MPTP group (P<0.01), so DA3-CH was the more potent drug. See Fig. 8A.

When analysing GFAP levels, a one-way ANOVA found an overall difference between groups (P<0.001). The control group showed lower levels than the MPTP group (P<0.001) and the Liraglutide+MPTP group (P<0.01) but not the DA3+MPTP group. Again, this shows that DA3-CH prevented the inflammation induced by MPTP. The MPTP group had higher levels than the Liraglutide+MPTP group (P<0.01) and the DA3+MPTP group (P<0.001). The Liraglutide+MPTP group had higher levels than the DA3+MPTP group (P<0.01). DA3-CH was the more potent drug. See Fig. 8B.

When analysing IBA-1 levels, a one-way ANOVA found an overall difference between groups (P<0.001). The control group had lower levels than the MPTP group (P<0.001) and the Liraglutide+MPTP group (P<0.05) but did not differ from the DA3+MPTP group. The MPTP group had higher levels than the DA3+MPTP group (P<0.001). The Liraglutide+MPTP group had higher levels from the DA3+MPTP group (P<0.01). DA3-CH was the more potent drug in reducing microgliosis. See Fig. 8C. N=4.

4. Discussion

The data presented here demonstrate that both the GLP-1 analogue liraglutide and the novel GLP-1/GIP dual receptor agonist DA3-CH have neuroprotective properties in the MPTP mouse model of PD. MPTP is lipophilic and can cross the blood–brain barrier (BBB). In neurons, MPTP is metabolised into the toxic cation 1-methyl-4-phenylpyridinium (MPP+) by monoamine oxidase B (Glover et al., 1986). MPP+ has been shown to kill primarily dopamine-producing neurons in the substantia nigra (Nakamura and Vincent, 1986). MPP+ blocks complex 1 in the mitochondrial electron transport chain, which leads to the production of free radicals and to cell death (Kinemuchi et al., 1987; Smith and Bennett, 1997). Liraglutide and DA3-CH have been developed as treatments for type 2 diabetes (Finan et al., 2013; Lovshin and Drucker, 2009). Importantly, as shown previously, GLP-1 analogues do not greatly affect blood plasma glucose levels or body weight in non-diabetic or non-obese animals or humans (Faivre et al., 2012; Gallwitz, 2006; George et al., 2014). We were able to show that neither liraglutide nor DA3-CH had prominent effects on body weight or blood glucose levels. In the motor tasks, both drugs were able to counteract the detrimental effects of MPTP on motor coordination and grip strength. DA3-CH was significantly more effective than liraglutide. MPTP causes a decrease of protein synthesis of key enzymes such as

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tyrosine hydroxylase (TH), an enzyme that is required for dopamine synthesis. Both drugs were able to protect dopaminergic neurons to some degree, with DA3-CH again being more effective than liraglutide. It is of interest to note that grip strength was very much impaired on day 1 after MPTP treatment, in contrast to rotarod performance. This result is in line with previous results using these techniques (Cao et al., 2016). This difference demonstrates that the toxin affects muscle strength to a much higher degree than sensorimotor coordination as measured in the rotarod. Chronic inflammation is a key aspect of PD, and the activation of microglia and astrocytes lead to release of pro-inflammatory cytokines, enhanced oxidative stress, synaptic failure, reduced energy utilisation and mitochondrial damage (Moran and Graeber, 2008; Tansey and Goldberg, 2010). Both drugs tested reduced the activation of microglia and astrocytes by MPTP, which may be an important part in the neuroprotective properties of these incretin analogues. Glial-derive neurotropic factor (GDNF) is a key growth factor that is important 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 are currently under investigation as a potential treatment of PD (see eg. NCT01621581), but as GDNF cannot cross the blood-brain barrier (BBB) (Allen et al., 2013), no effective treatment has been developed yet. GLP-1 and GIP are members of the growth factor family (Holscher, 2014). We have previously shown that GLP-1 or GIP analogues enhance the expression of Brain-Derived Neurotrophic Factor (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 et al., 2002) and enhance the expression of key growth factors such as BDNF or GDNF. Therefore, the increase of the expression of these neuroprotective growth factors by incretin analogues may explain some of the neuroprotective effects that we and others have shown. In our study, the

dual receptor agonist was more potent than the single GLP-1 receptor agonist in upregulating GDNF expression. The slight discrepancy between the results obtained using the histology and the western blot method can be explained by the different sensitivity in detecting the target. The western blot analysis is the more accurate technique in quantifying protein levels. Previously we tested GLP-1 or GIP receptor agonists in the MPTP mouse model of PD. We found that the second generation GLP-1 mimetics liraglutide and lixisenatide were more effective than the first generation drug exendin-4. Motor coordination and activity was improved by the newer GLP-1 mimetics, and the expression of TH was also rescued by both drugs. Importantly, levels of the pro-apoptotic signal BAX were reduced, the cell survival signal Bcl-2 was increased by both drugs (Liu et al., 2015a). Similar protective effects were observed with the GLP-1 analogue (Val8)GLP-1-Glu-PAL (Zhang et al., 2015). We also tested a protease-resistant analogue of GIP, D-Ala2-GIP-glu-PAL, in the acute MPTP mouse model of PD. This GIP analogue also reduced the effects of MPTP on motor activity and coordination, and TH levels in the substantia nigra and striatum were enhanced. MPTP treatment reduced synapse numbers in the striatum, and the GIP analogue reversed this to some degree. Importantly, the cAMP/PKA/CREB growth factor second messenger signalling pathway was activated by this drug (Li et al., 2016). In a chronic MPTP study, we observed further neuroprotective effects induced by this GIP analogue. Moreover, MPTP treatment increased the levels of alpha-synuclein in the brain, and D-Ala2-GIP-glu-PAL reduced these levels to almost normal values. D-Ala2-GIP-glu-PAL also reduced the chronic inflammation response in the brain, reduced oxidative stress and lipid peroxidation, and increased the expression of Brain Derived Neurotrophic Factor (BDNF) (Li et al., 2017).

Based on our findings, novel dual GLP-1/GIP receptor agonists are a promising new strategy that may show improved neuroprotective effects than single GLP-1 or GIP analogues. Such novel dual agonists have already shown superior effects in preclinical and clinical trials in

diabetes. compared to lirglutide (Finan et al., 2013). One of these dual agonists that we named 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 (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 DA3-CH dual agonist in direct comparison with liraglutide and show here for the first time that this dual agonist not only shows good neuroprotective effects in the MPTP mouse model of PD, but that it is superior to liraglutide. DA-JC1 differs from the DA3-CH dual agonist mainly in the addition of a C16 fatty acid that enhances the survival time in the blood stream but reduces BBB permeability.

Based on the extensive preclinical data, clinical trials have started that investigate the neuroprotective effects of exendin-4 (Byetta, Bydureon®) and liraglutide (Victoza®), drugs that are already on the market as T2DM treatments, in PD patients. A pilot trial of exendin-4 in PD patients has already shown good effects (NCT01174810). This pilot study tested the effects of exendin-4 in 45 patients. There were clear improvements in the motor coordination as assessed in the MDS-UPDRS tests, and cognition was improved as measured by the Mattis DRS-2 test battery that had been designed to evaluate cognitive impairments in PD patients (Aviles-Olmos et al., 2013a). Patients were re-tested 12 months later, and the differences in motor performance and cognitive scores was still visible (Aviles-Olmos et al., 2014). This test was important as the pilot study was an open label trial. The fact that the drug improvement was still observed 12 months after the trial had stopped suggests that the

difference in performance is not due to a placebo effect. A phase II double-blind, placebo

controlled clinical trial that tested the once-weekly formulation of exendin-4, Bydureon®,

has been completed (NCT01971242), but the results have not been published at the time of

writing. A phase II trial testing liraglutide in PD patients has started in Sept. 2016, testing

patients in a double blind, placebo controlled trial for one year (NCT02953665). A further

clinical trial testing the GLP-1 receptor agonist Lixisenatide in PD patients is in planning.

As clinical trials of novel dual GLP-1/GIP analogues in diabetes patients are ongoing, it is

highly likely that several of these will be brought to the market. Once these drugs are

licenced for use in patients, they can be fast-tracked into clinical trials in PD patients

(Hölscher, 2016).

Conclusion: The data that we present here demonstrate that novel dual GLP-1/GIP receptor

agonists such as DA3-CH can show superior neuroprotective effects compared to single

GLP-1 receptor agonists, and hold promise to be developed as a drug treatment that is

superior in treating PD.

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Fig. 1: Timeline of the experimental design. Drugs or saline or MPTP or combinations of this were injected once-daily for 7 days.

Fig. 2A: analysis of blood plasma glucose levels for each group. A repeated measures two-way ANOVA found no difference between groups (ns.) but over time ($P<0.05$). 2B: animal weight for 4 days. A repeated measures two-way ANOVA found no difference between groups (ns.) or over time (ns.). N=10 per group.

Fig. 3A: Motor performance in the Rotarod test. A one-way repeated measures ANOVA found an overall difference over all groups, and the post-hoc Tukey's multiple comparisons test found differences Between groups: ***=$P<0.001$ compared to controls; $\$\$$ =$P<0.1$; $\$\$\$$ =$P<0.001$ compared to MPTP group; £££ =$P<0.001$ DA3+MPTP group compared to Liraglutide +MPTP group. 2B: A two-way repeated measures ANOVA found a difference between groups ($P<0.001$) and over time ($P<0.05$).
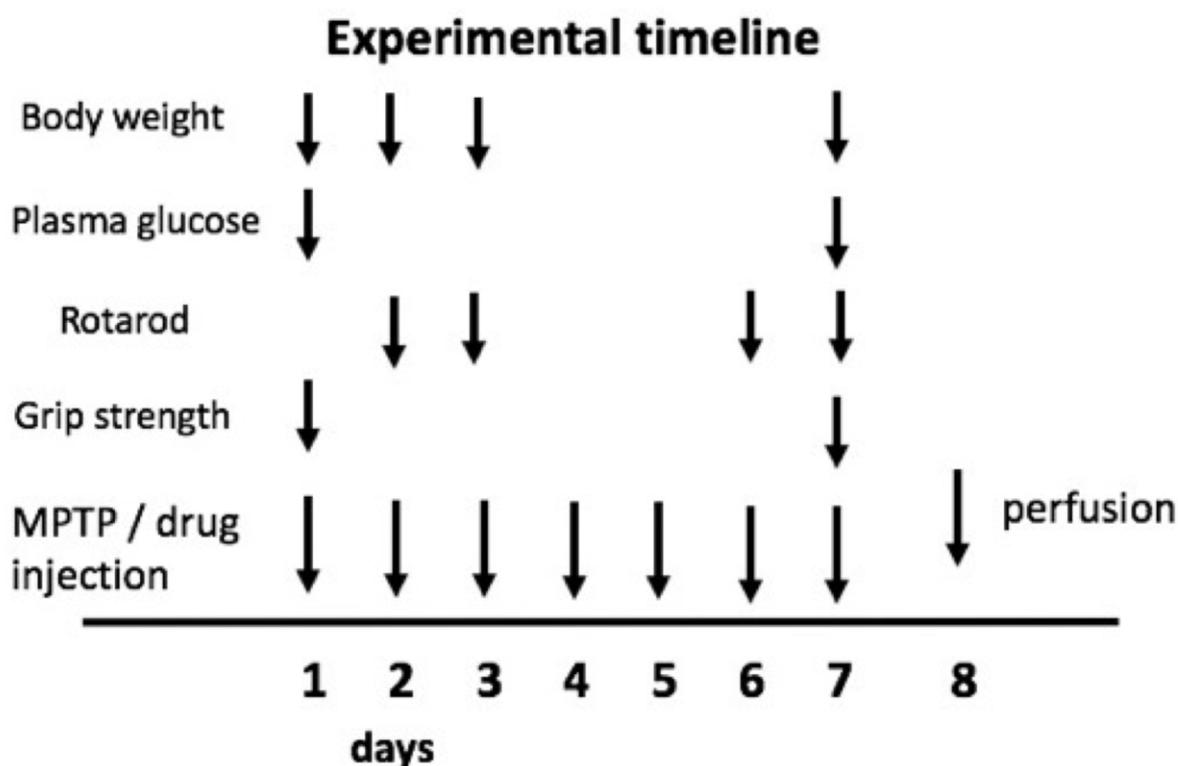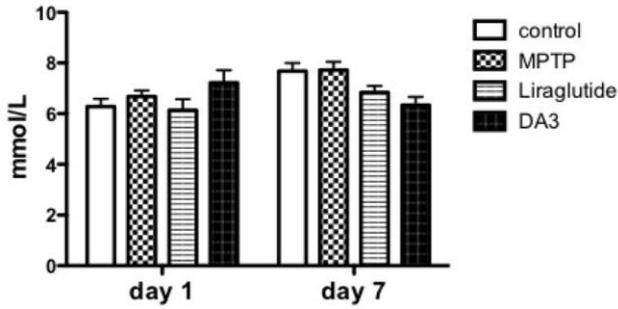
Fig. 4: grip strength test on day 1 and 7 of treatment. A repeated measures two-way ANOVA found a difference between groups (P<0.001) but not over time (p>0.05). A Tukey post-hoc test found differences between groups: ***=P<0.001 compared to controls; $ =P<0.05; $$$ =P<0.001 compared to MPTP group; £££ =P<0.001 DA3+MPTP group compared to Liraglutide +MPTP group. N=10 per group.
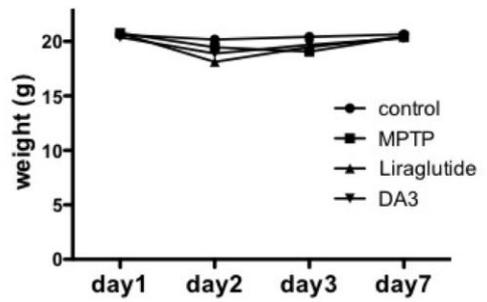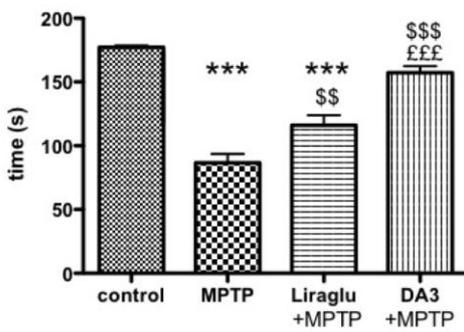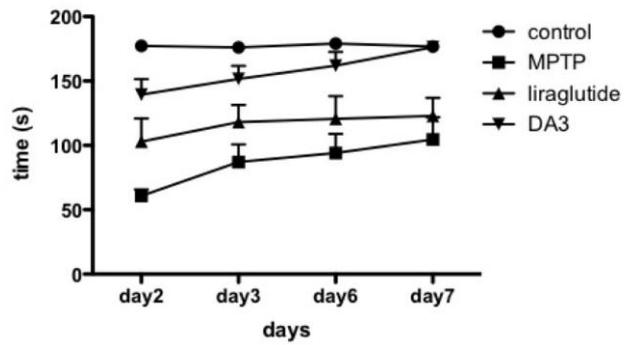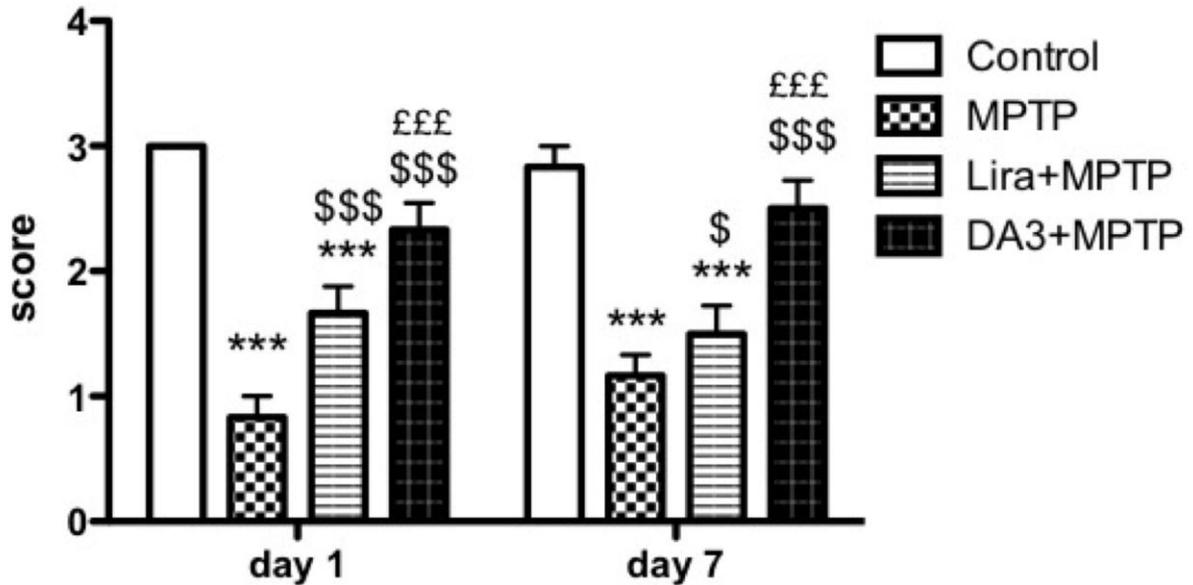
Fig. 5: Expression of tyrosine hydroxylase in. 5A: levels of TH in the substantia nigra. A one-way ANOVA found a difference between groups. A Tukey post-hoc test found differences between groups: ***=P<0.001 compared to controls; $$$ =P<0.001 compared to MPTP group; £££ =P<0.001 DA3+MPTP group compared to Liraglutide +MPTP group. Sample images are shown. 5B: levels of TH in the striatum. A one-way ANOVA found a difference between groups. A Tukey post-hoc test found differences between groups: ***=P<0.001; *=P<0.05 compared to controls; $$ =P<0.01; $$$ =P<0.001 compared to MPTP group; £££ =P<0.001 DA3+MPTP group compared to Liraglutide +MPTP group. Shown are sample images. A = control; B = Liraglutide +MPTP; C = DA3+MPTP; D = MPTP only. Scale bar = 100µm.
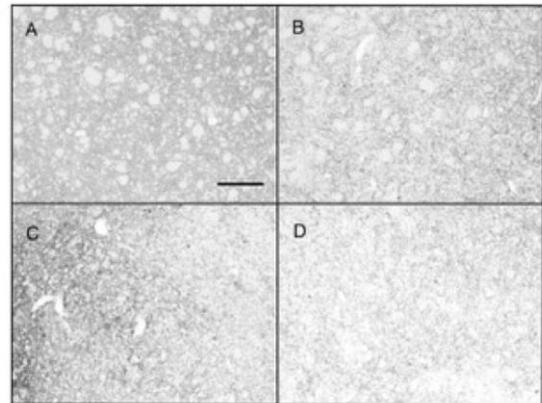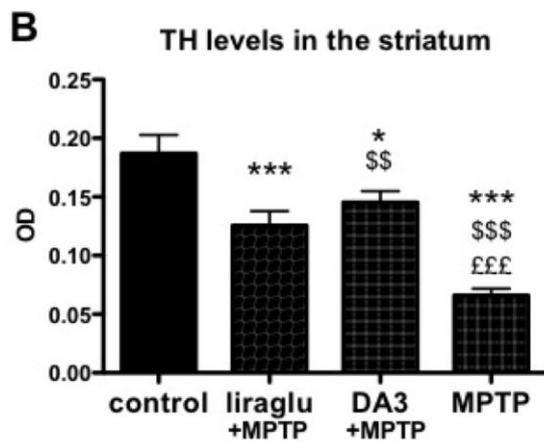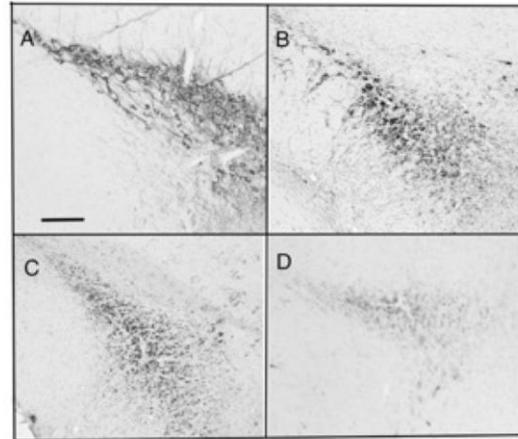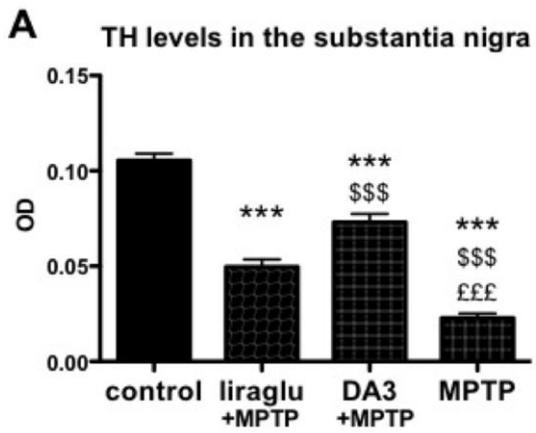
Fig. 6: Chronic inflammation in the brain. 6A: Expression of GFAP in the cortex in activated astroglia. 6B: Activated microglia, expression of IBA-1 in the cortex. A one-way ANOVA found an overall difference, a post-hoc Tukey test found differences between groups: *=P<0.05; ***=P<0.001 compared to controls; $$$ =P<0.001 compared to DA3+MPTP group; £££ =P<0.001 MPTP compared to Liraglutide +MPTP group. Shown are sample micrographs. A = control; B = Liraglutide +MPTP; C = DA3+MPTP; D = MPTP only. Scale bar = 20µm.

Fig. 7: Expression of Glia-Derived Neurotrophic Factor (GDNF) in the cortex. A one-way ANOVA found an overall difference, a post-hoc Tukey test found differences Between groups: ***=P<0.001 compared to controls; $$$ =P<0.001 compared to DA3+MPTP group; £££ =P<0.001 compared to Liraglutide +MPTP group. Shown are sample micrographs. A = control; B = Liraglutide +MPTP; C = DA3+MPTP; D = MPTP only. Scale bar = 20µm.

Fig. 8: Western blot analysis of protein levels of GDNF (A) and the inflammation markers GFAP (B) and IBA-1 (C). A one-way ANOVA found an overall difference and post-hoc Tukey tests showed group differences: *=P<0.05, **=P<0.01, ***=P<0.01 compared to controls. $$=P<0.01, $$$=P<0.01 compared to the MPTP group. ££=P<0.01 comparing the liraglutide +MPTP group with the DA3+MPTP group. (D) Shown are sample western blots.

Experimental timeline

	1	2	3	4	5	6	7	8
Body weight	↓	↓	↓				↓	
Plasma glucose	↓						↓	
Rotarod		↓	↓			↓	↓	
Grip strength	↓						↓	
MPTP / drug injection	↓	↓	↓	↓	↓	↓	↓	↓ perfusion

days

A Plasma glucose levels

B Body Weight

A Rotarod: summary of all days

B Rotarod results for each day

Grip strength test

A

TH levels in the substantia nigra

B

TH levels in the striatum

Chronic inflammation in the brain

A GFAP expression

B IBA-1 expression

GDNF expression

A **GDNF levels**

B **GFAP levels**

C **IBA-1 levels**

D