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Neuroprotective role of (Val^8)GLP-1-Glu-PAL in an *in vitro* model of Parkinson's disease

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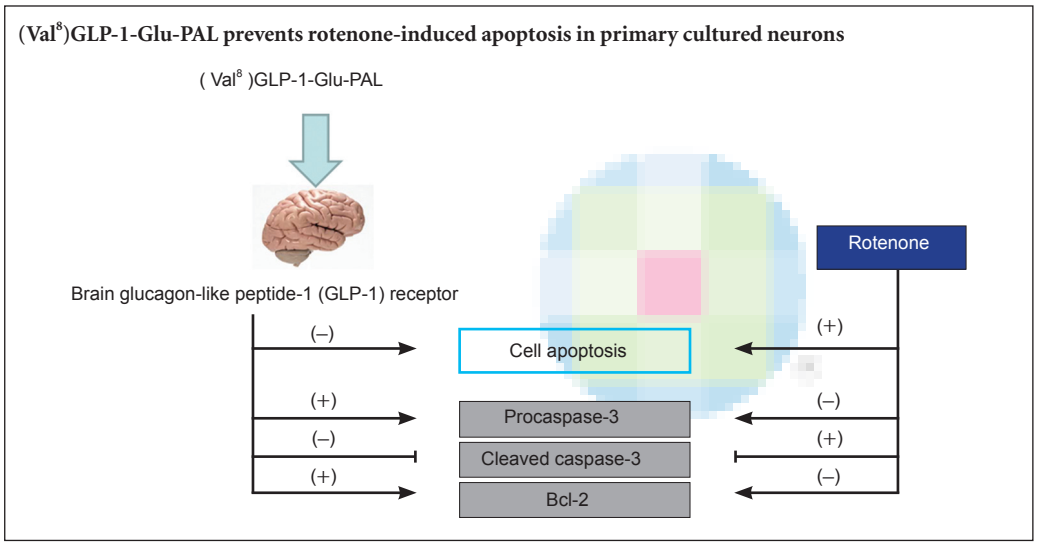
Graphical Abstract



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Abstract

The growth factor glucagon-like peptide-1 (GLP-1) is neuroprotective in several animal models of neurodegeneration. Here, we analyzed the neuroprotective effects of a novel protease-resistant GLP-1 analogue, (Val^8)GLP-1-Glu-PAL, which has advantages over older analogues, such as improvement of hippocampal neurogenesis, glucose homeostasis, and insulin secretion. We established an *in vitro* model of Parkinson's disease using the mitochondrial stressor rotenone in primary cultured mouse neurons pretreated with (Val^8)GLP-1-Glu-PAL. (Val^8)GLP-1-Glu-PAL alone did not affect neuronal viability, but prevented the rotenone-induced reduction in cell viability in a dose-dependent manner. In addition, (Val^8)GLP-1-Glu-PAL pretreatment prevented rotenone-induced proapoptotic changes manifesting as downregulation of procaspase-3 and Bcl-2 and upregulation of cleaved caspase-3. These results demonstrate that the novel agent (Val^8)GLP-1-Glu-PAL shows promise as a drug treatment for Parkinson's disease.

Key Words: *nerve regeneration; Parkinson's disease; GLP-1; neurodegenerative disease; apoptosis; caspase-3; Bcl-2; cellular culture; rotenone; neural regeneration*

Introduction

Parkinson's disease is the second most common adult-onset neurodegenerative disorder. To date, no agent has been found that successfully prevents or reverses neurodegenerative processes in the brain (Bagetta et al., 2010). Mitochondrial toxins, such as the complex 1 inhibitor rotenone, are widely used as pesticides, and have been found to induce Parkinson's disease in farmers who are exposed to the toxin (Chin-Chan et al., 2015). In rodents, administration of rotenone can lead to biochemical and histological changes similar to those observed in Parkinson's disease (Fleming et al., 2004; Zhu et al., 2004).

The growth factor glucagon-like peptide-1 (GLP-1) is a member of the incretin hormone family (Perry and Greig, 2002; Hölscher, 2014a), and the GLP-1 receptor is expressed in neurons in the central nervous system (Hamilton and Hölscher, 2009; Lee et al., 2011; Darsalia et al., 2012). Agonists at the GLP-1 receptor were originally developed as a treatment for type 2 diabetes, and several remain on the

market (Campbell and Drucker, 2013). GLP-1 and long-acting, protease-resistant GLP-1 receptor agonists have shown a range of neuroprotective effects in cell culture (Perry and Greig, 2002; Sharma et al., 2014) and in animal models of Parkinson's (Bertilsson et al., 2008; Li et al., 2009) and Alzheimer's diseases (McClean et al., 2011). In cellular and functional studies in rodents, the GLP-1 analogue exendin-4 protects against Parkinson's disease-like pathologic changes such as 6-hydroxydopamine-induced dopaminergic neuronal loss (Bertilsson et al., 2008). This was confirmed in another model of Parkinson's disease in which 6-hydroxydopamine and lipopolysaccharide were used to lesion the substantia nigra (Harkavyi et al., 2008). Exendin-4 also protects dopaminergic neurons and rescues motor function in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced mouse model of Parkinson's disease (Li et al., 2009). Based on these encouraging preclinical studies, a pilot clinical trial of exendin-4 was conducted in patients with Parkinson's disease (Aviles-Olmos et al., 2013). The study examined the effects of exendin-4 in a randomized single-blind trial in 45 patients. The drug was given for 12 months followed by a 2-month wash-out period. Clinically relevant improvements in motor and cognitive measures were observed. At 12 months, patients who had received exendin-4 showed a mean improvement of 2.7 points on the Movement Disorder Society–Sponsored Revision of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS), compared with a mean decline of 2.2 points in control patients taking conventional Parkinson's disease medication. Most interestingly, patients taking exendin-4 showed a clear improvement in the Mattis DRS-2 cognitive score, suggesting that the drug has beneficial effects on cognition and memory (Aviles-Olmos et al., 2013). A follow-up study showed that the protection of motor skills and cognitive scores remained at 12 months after cessation of exendin-4 treatment (Aviles-Olmos et al., 2014). These results support the hypothesis that GLP-1 receptor agonists may also be effective in the treatment of Alzheimer's disease (Hölscher, 2014b).

Type 2 diabetes is a risk factor for Alzheimer's and Parkinson's diseases. Insulin signaling is impaired in the brains of patients with these diseases, and recent studies have shown that the pharmacologic agents used to treat diabetes also improve symptoms in Alzheimer's and Parkinson's diseases. In particular, three licensed GLP-1 mimetics are very effective in crossing the blood-brain barrier, and show good effects in animal models of Alzheimer's and Parkinson's diseases (Hölscher, 2014c). GLP-1 itself has a relatively short circulating half-life, making it impractical as a therapeutic agent (Kieffer et al., 1995). Therefore, the focus of ongoing research is the development of new GLP-1 analogues with increased enzymatic stability and improved biological efficacy (Vilsbøll and Knop, 2008). (Val^8)GLP-1 is a human GLP-1 analogue developed by substitution of Ala with a Val residue at N-terminal position 8. This renders the peptide resistant to degradation by dipeptidyl peptidase-4 by masking its cleavage site (Green et al., 2006), but it is still subject to rapid renal clearance. Renal filtration can be minimized by the incorporation of a fatty acid moiety into a peptide chain, thereby facilitating binding to serum proteins such as albumin and thus prolonging the duration of action (Kurtzhals et al., 1995). Liraglutide, a GLP-1 analogue, is characterized by a C16 fatty acid moiety conjugated to Lys26 (Madsen et al., 2007). Based on the structural properties of liraglutide, Lennox et al. (2013) developed a novel GLP-1 peptide, (Val^8)GLP-1-Glu-PAL, which contains a C16 fatty acid moiety (designated PAL) conjugated to Lys26 *via* a glutamic acid linker (Glu) in addition to an amino acid substitution at position 8. This Glu-PAL moiety is the same linker-fatty acid conjugate found in GLP-1. They examined the enzymatic stability and *in vitro* insulinotropic activity together with the acute and persistent *in vivo* actions of (Val^8)GLP-1-Glu-PAL on glucose tolerance and insulin response. Furthermore, the longer-term effects of (Val^8)GLP-1-Glu-PAL were assessed by measuring bodyweight, food intake, glycemic and insulinotropic responses, insulin sensitivity, and hippocampal neurogenesis. Their study demonstrated that (Val^8)GLP-1-Glu-PAL is a long-acting GLP-1 peptide that significantly improves hippocampal neurogenesis, glucose homeostasis, and insulin secretion in high-fat-fed mice (Lennox et al., 2013). In the present study, we investigated whether (Val^8)GLP-1-Glu-PAL prevents rotenone-induced apoptosis in primary cultured neurons.

Materials and Methods

Primary culture of mouse neurons

Swiss mouse pups (1–3 days old) were obtained from the Animal Center at Shanxi Medical University, China (license No. SCXK (Jin) 2009-0001). All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications, No. 80-23, revised 1978) and approved by the Animal Ethics Committee in Shanxi Medical University, China. All procedures involving fresh tissue were performed on ice. Neonatal mice were sacrificed by decapitation. Cortical and hippocampal tissues (Paxinos and Watson, 2005) were isolated and washed in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution. The tissue was cut into small pieces and incubated with 0.25% trypsin for 10 minutes at 37°C. The trypsin was inactivated using fetal bovine serum to prevent excessive dissociation. The dissolved tissues were centrifuged (1,000 r/min for 5 minutes) and cells were taken. The cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (Trans, Beijing, China) in a humidified atmosphere at 37°C with 5% CO_2 for 6 hours. Neurobasal medium (Gibco, Carlsbad, CA, USA) containing 2% B27 supplement, 1% L-glutamine and 1% penicillin and streptomycin was used. Neurons were cultured and grew for 7 days until they reached 70–80% confluency.

Cell counting kit-8 (CCK-8) assay

We used the CCK-8 assay (Dojindo Laboratories, Tokyo, Japan) to identify the lowest concentration of rotenone that would inhibit cellular survival. Primary neurons were seeded at a concentration of 1×10^6 cells per well in 96-well cell

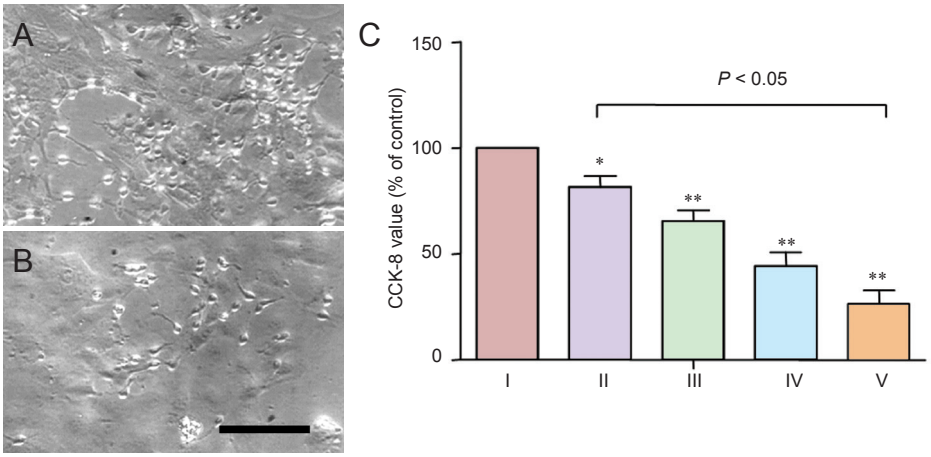

Figure 1 Viability of neurons exposed to rotenone at different concentrations.
(A, B) Hippocampal and cortical neuronal density was markedly lower after exposure to rotenone (5 nM) (B) than in control cells not exposed to rotenone (A). Scale bar: 50 μm. (C) Number of viable neurons after exposure to rotenone. Data are expressed as the mean ± SEM. All experiments were performed independently in triplicate. Intergroup comparison was done by one-way analysis of variance and Dunnett's *t*-test. **P* < 0.05, ***P* < 0.01, *vs.* I. CCK-8: Cell counting kit-8; I: control group; II: 0.5 nM rotenone group; III: 1 nM rotenone group; IV: 3 nM rotenone group; V: 5 nM rotenone group.

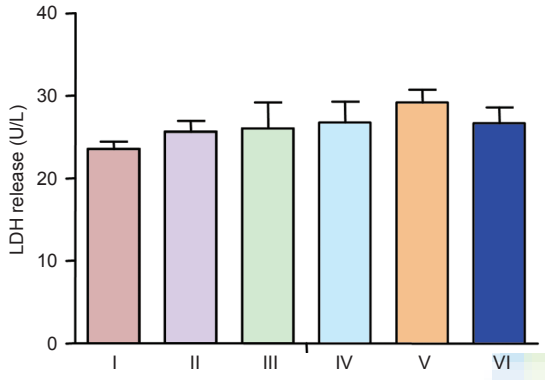

Figure 2 LDH release in cultured neurons treated with different concentrations of (Val^8)GLP-1-Glu-PAL.
Data are expressed as the mean ± SEM. All experiments were performed independently in triplicate. Groups were compared by one-way analysis of variance and Dunnett's *t*-test. There was no significant difference in LDH release among groups. LDH: Lactate dehydrogenase; GLP-1: glucagon-like peptide-1; I: control group; II: Val^8-PAl (10 nM) group; III : Val^8-PAl (50 nm) group; IV: Val^8-PAl (100 nM) group; V: Val^8-PAl (200 nm) group; VI: Val^8-PAl (500 nM) group.

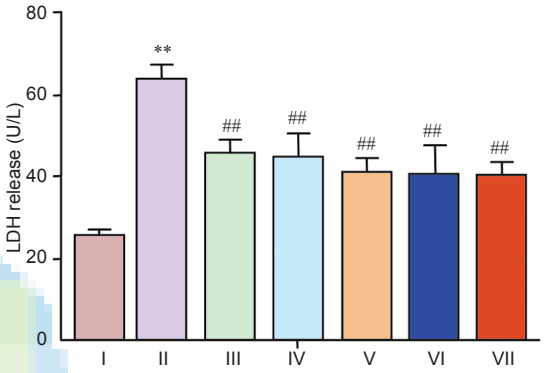

Figure 4 Rotenone-induced elevation of LDH release was inhibited by (Val^8)GLP-1-Glu-PAL.
Data are expressed as the mean ± SEM. All experiments were performed independently in triplicate. Groups were compared by one-way analysis of variance and Dunnett's *t*-test. ***P* < 0.01, *vs.* I; ##*P* < 0.01, *vs.* II. LDH: Lactate dehydrogenase; GLP-1: glucagon-like peptide-1; I: control group; II: rotenone group; III: Val^8-PAL + rotenone (10 nM) group; IV: Val^8-PAL + rotenone (50 nM) group; V: Val^8-PAL + rotenone (100 nM) group; VI: Val^8-PAL + rotenone (200 nM) group; VII: Val^8-PAL + rotenone (500 nM) group.

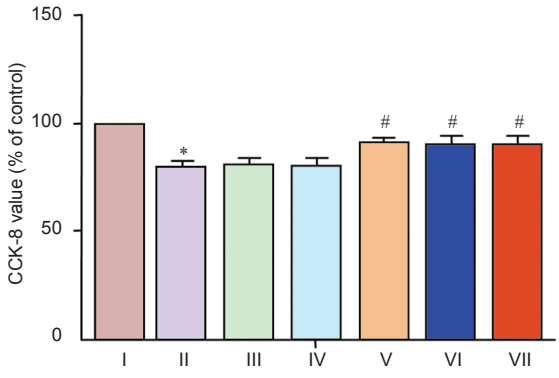

Figure 3 Neuroprotective effects of (Val^8)GLP-1-Glu-PAL on neuron survival after rotenone exposure.
Data are expressed as the mean ± SEM. All experiments were performed independently in triplicate. Groups were compared by one-way analysis of variance and Dunnett's *t*-test. **P* < 0.05, *vs.* I; #*P* < 0.05, *vs.* rotenone. CCK-8: Cell counting kit-8; GLP-1: glucagon-like peptide-1; I: control group; II: rotenone (0.5 nM); III: Val^8-PAL (10 nM) + rotenone (0.5 nM); IV: Val^8-PAL (50 nM) group + rotenone (0.5 nM) group; V: Val^8-PAL (100 nM) + rotenone (0.5 nM) group; VI: Val^8-PAL (200 nM) + rotenone (0.5 nM) group; VII: Val^8-PAL (500 nM) + rotenone (0.5 nM) group.

culture plates at 37°C for 7 days. Rotenone (Solarbio, Beijing, China) was prepared in 1 mM dimethyl sulfoxide and diluted in the culture medium at concentrations of 0.5, 1, 3, and 5 nM. Cell survival was assessed at 48 hours by adding CCK-8 solution (10 μL per well) directly to the cell suspension and incubating for 2 hours at 37°C. Absorbance values of all wells were measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The results are expressed as a percentage of values in control cells not exposed to rotenone.

Lactate dehydrogenase (LDH) assay

The neuroprotective effects of (Val^8)GLP-1-Glu-PAL against rotenone were explored at a range of concentrations using the LDH cell viability assay (de Carvalho et al., 2014). Cultured neurons were incubated for 7 days, then pretreated with 0, 10, 50, 100, 200 and 500 nM (Val^8)GLP-1-Glu-PAL for 2 hours, before being exposed to rotenone (0.5 nM) for 48 hours. Activity of LDH released into the medium after freeze-thaw lysing of the cells was evaluated using an LDH assay kit (Jiancheng Company, Nanjing, Jiangsu Province, China) according to the manufacturer's instructions, and

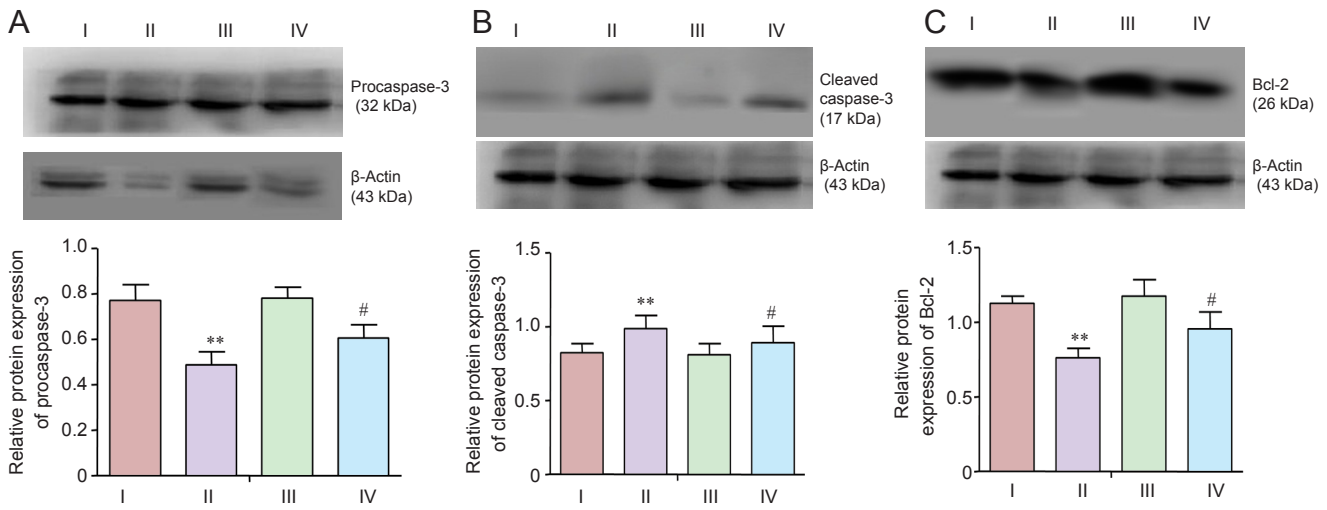

Figure 5 (Val^8)GLP-1-Glu-PAL inhibited apoptosis signaling induced by rotenone.
Optical density ratio of target protein/β-actin representing expression of procaspase 3 (A), cleaved caspase 3 (B), and Bcl-2 (C). Data are expressed as the mean ± SEM. All experiments were performed independently in triplicate. Intergroup comparison was done by one-way analysis of variance and Dunnett's *t*-test. $^{**}P < 0.01$, *vs.* I; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, *vs.* II. GLP-1: Glucagon-lide peptide-1; I: control group; II: rotenone group; III: (Val^8)GLP-1-Glu-PAL group; IV: (Val^8)GLP-1-Glu-PAL + rotenone group.

quantified by measuring the optical density at 440 nm with a 722 visible spectrophotometer (Shanghai Optical Instrument Factory, Shanghai, China). The released LDH activity was expressed as U/L.

Western blot analysis

Western blot analysis was used to determine expression of the apoptotic signaling proteins procaspase-3, cleaved caspase-3 and Bcl-2. Cultured neurons were incubated for 7 days, then treated with (Val^8)GLP-1-Glu-PAL (100 nM) for 2 hours before exposure to rotenone (0.5 nM) for 48 hours. Cells were plated at a concentration of 1×10^6 per dish in cell culture dishes of 100 mm × 20 mm, and grown for 7 days until they reached 70–80% confluence. Cells were collected in ice-cold RIPA lysis buffer (Beyotime, Shanghai, China). Samples were centrifuged (12,000 r/min for 5 minutes at 4°C) before the supernatant was taken. Total protein concentration of the cells was determined using the BCA Protein Assay Kit (Beyotime), and an equal concentration of loading buffer was added to the samples. Proteins (30 μg) were run in a 15% Tris-Tricine gel and electrophoretically transferred onto polyvinylidene fluoride membranes. After blocking with 5% bovine serum albumin in PBS containing 0.05% Tween-20 (PBST) at room temperature for 2 hours, the membranes were incubated overnight at 4°C with rabbit anti-procaspase 3 polyclonal antibody (1:500; Abcam, Cambridge, UK), rabbit anti-cleaved caspase 3 polyclonal antibody (1:1,000; Cell Signaling Technology, Boston, MA, USA), rabbit anti-Bcl 2 polyclonal antibody (1:500; Abcam), or rabbit anti-mouse β-actin monoclonal antibody (1:500; Abcam). After washing with PBST, the membranes were incubated with goat anti-rabbit IgG (1:5,000, Abcam) for 2 hours at room temperature. The bands were detected using an enhanced chemiluminescence system (Transgen, Beijing, China). The blots were scanned and the relative protein content quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Protein content was expressed as the target protein/β-actin optical density ratio.

Statistical analysis

Data was expressed as the mean ± SEM and were compared between groups by one-way analysis of variance (SPSS 16.0 software; SPSS, Chicago, IL, USA). Dunnett's *t*-test was used for pairwise comparison between groups. Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used to present the data. $P < 0.05$ was considered statistically significant.

Results

Neurotoxicity of rotenone in primary cultured neurons

Evident morphological changes such as cell loss, shrinkage, enhanced refraction, string-of-beads appearance, and even floating and cell death were seen in the cultured hippocampal and cortical neurons exposed to rotenone (5 nM) for 48 hours, compared with control. Neuronal density was markedly reduced (**Figure 1**).

The CCK-8 assay showed a dose-dependent decrease in the number of viable cultured neurons after rotenone exposure, with minimal cellular loss observed after low concentrations and a steady decline in number at higher concentrations. Of the concentrations tested, the lowest to reduce cell numbers was 0.5 nM (**Figure 1**).

LDH release in cultured neurons treated with different concentrations of (Val^8)GLP-1-Glu-PAL

Cellular viability was determined by measuring LDH release into the culture medium from dead or dying cells. (Val^8)GLP-1-Glu-PAL alone did not affect total LDH release at any concentration tested ($P > 0.05$, *vs.* control; **Figure 2**).

Protective effect of (Val^8)GLP-1-Glu-PAL on cell survival after rotenone treatment

A 20% decrease in CCK-8 value was shown in cultured neurons exposed to 0.5 nM rotenone compared with control ($P < 0.05$), which was prevented by pretreatment with (Val^8)GLP-1-Glu-PAL. No significant difference was observed between rotenone-damaged neurons after pretreatment with 10 or 50 nM (Val^8)GLP-1-Glu-PA ($P > 0.05$); however, significantly higher CCK-8 values were observed after pretreatment with higher concentrations of (Val^8)GLP-1-Glu-PAL (100, 200 and 500 mN; $P < 0.05$). The lowest concentration of (Val^8)GLP-1-Glu-PAL to prevent rotenone damage was 100 nM (**Figure 3**).

Neuroprotective effect of (Val^8)GLP-1-Glu-PAL against rotenone toxicity

Release of LDH in cultured neurons exposed to 0.5 nM rotenone was significantly greater than in control cells ($P < 0.05$). This increase was prevented by pretreatment with different concentrations of (Val^8)GLP-1-Glu-PAL ($P < 0.01$, *vs.* rotenone; **Figure 4**).

(Val^8)GLP-1-Glu-PAL inhibited apoptosis signaling induced by rotenone

Western blot analysis showed that 48 hours after exposure to rotenone (0.5 nM), neurons expressed a lower level of procaspase-3, and higher level of cleaved caspase-3, than control neurons ($P < 0.01$). These effects were significantly reduced by pretreatment with 100 nM (Val^8)GLP-1-Glu-PAL ($P < 0.01$ or $P < 0.05$). A rotenone-induced decrease in Bcl-2 expression was also prevented by pretreatment with 100 nM (Val^8)GLP-1-Glu-PAL ($P < 0.05$; **Figure 5**).

Discussion

The mechanisms underlying the initiation of neurodegenerative processes in Parkinson's disease are incompletely understood. Chemicals known to induce disease-like symptoms and depletion of dopaminergic metabolism and transmission in neurons, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Herrero et al., 1995), 6-hydroxydopamine (Jolicoeur et al., 1991), and rotenone, are used to study the disease and test potential treatments. The mode of action appears to be that these chemicals are preferentially taken up or metabolized by dopaminergic neurons, increasing oxidative stress and blocking mitochondrial activity. We therefore used the rotenone-induced cellular model of Parkinson's disease to investigate the neuroprotective properties of (Val^8)GLP-1-Glu-PAL *in vitro*.

Exendin-4 was the first GLP-1 receptor agonist developed, and was originally used to treat type 2 diabetes. It has a biological half-life of about 4 hours, and has to be injected subcutaneously twice daily. Several GLP-1 receptor agonists have since been developed, with longer half-lives and superior effects than exendin-4 (Campbell and Drucker, 2013). For example, liraglutide (GLP-1-Glu-PAL) only needs to be injected once daily (Vilsbøll et al., 2008). (Val^8)GLP-1 is a protease-resistant GLP-1 analogue that is also neuroprotective (Wang et al., 2010; Gengler et al., 2012; Li et al., 2012). The combination of these two analogues creates a powerful new GLP-1 analogue, (Val^8)GLP-1-Glu-PAL (Lennox et al., 2013).

In the present study, we have shown that (Val^8)GLP-1-Glu-PAL protects primary cultured neurons from the toxic effects of rotenone. It preserved neuronal cell viability, suggesting that it may prevent dopaminergic degenerative processes induced by rotenone or similar oxidants. Importantly, (Val^8)GLP-1-Glu-PAL antagonized rotenone-activated apoptotic signaling pathways. Caspase-3 is a key protease activated by mitochondrial damage (Kashyap et al., 2010). Rotenone-induced oxidative mitochondrial stress activated this apoptotic pathway, upregulating total procaspase and cleaved activated caspase-3 expression. This was inhibited by (Val^8)GLP-1-Glu-PAL, suggesting that (Val^8)GLP-1-Glu-PAL prevents apoptosis and neuronal death. The effect is most likely due to the activation of growth factor signaling *via* the GLP-1 receptor, which inhibits apoptotic signaling (Li et al., 2010; Sharma et al., 2014). In addition, the GLP-1 receptor induces upregulation of the anti-apoptotic protein Bcl-2 (Li et al., 2010; Sharma et al., 2014). Bcl-2 acts to preserve mitochondrial integrity by preventing the loss of mitochondrial membrane potential and/or release of pro-apoptotic proteins such as cytochrome C into the cytosol (Harada and Grant, 2003). In the present study, (Val^8)GLP-1-Glu-PAL was also able to restore normal levels of Bcl-2 that had been reduced by rotenone.

In conclusion, (Val^8)GLP-1-Glu-PAL shows good effects in this neuronal culture assay. Preclinical tests in rodent models of Parkinson's disease will determine its effects on motor activity, dopamine transmission and neuronal survival. Direct comparisons with exendin-4, liraglutide and other drugs for Parkinson's disease will have to be conducted before a conclusion on the efficacy of this GLP-1 analogue can be drawn. The primary neuronal cell culture results presented here are the first encouraging demonstrations of neuroprotection that pave the way to further studies of this novel analogue.

Author contributions: *LL and YZL wrote the paper and were responsible for the study protocol, design, and grant support. CH directed the study and was responsible for chemical supply. GLL performed western blot analysis. KL and JZ performed all remaining experimental procedures. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

Plagiarism check: *This paper was screened twice using Cross-Check to verify originality before publication.*

Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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