

Neuroprotective effects of (Val8)GLP-1-Glu-PAL in the MPTP Parkinson's disease mouse model

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short title: *(Val8)GLP-1-Glu-PAL is protective in the MPTP mouse model*

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Abstract

Glucagon-like peptide 1 (GLP-1) is a hormone and a growth factor. GLP-1 mimetics are currently on the market as treatments for type 2 diabetes. They also have shown neuroprotective properties in animal models of neurodegenerative disorders. In addition, the GLP-1 mimetic exendin-4 has shown protective effects in animal models of Parkinson's disease (PD), and a first clinical trial in PD patients showed promising results.

(Val8)GLP-1-glu-PAL is a new GLP-1 analogue which has a longer biological half-life than exendin-4. We previously showed that (Val8)GLP-1-glu-PAL has neuroprotective properties. Here we tested the drug in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. MPTP was injected (30mg/kg i.p.) along with (Val8)GLP-1-glu-PAL (25nmol/kg i.p.) once-daily for 8 days. (Val8)GLP-1-glu-PAL showed good effects in preventing the MPTP- induced motor impairment (Rotarod, open field locomotion, swim test), reduction in Tyrosine Hydroxylase levels (dopamine synthesis) in the substantia nigra, a reduction of activated caspase 3 levels, of TUNEL positive cell numbers, of the pro-apoptotic signaling molecule BAX and an increase in the growth signaling molecule Bcl-2. The results demonstrate that (Val8)GLP-1-glu-PAL shows promise as a novel treatment of PD.

Keywords: Growth factors; incretin; neuroinflammation; apoptosis; neuroprotection; insulin

1. Introduction

Parkinson's disease (PD) is one of the common progressive neurological diseases in the elderly population. Although the pathogenesis and detailed mechanisms of this disease has not been fully elucidated, the main pathological characteristic is the progressive loss of dopaminergic neurons, mainly in the substantia nigra pars compacta (SNpc). It causes the substantia nigra-striatum pathways to degenerate, and induces a decline in the release of the dopaminergic neurotransmitter, and thereby impairs the function of motor activity and coordination. It causes many clinical characteristics such as resting tremors, bradykinesia, rigidity and postural instability, along with non- motor symptoms (Kowal, Dall, Chakrabarti, Storm, & Jain, 2013). Recently, studies have shown a link between diabetes and PD. Both diabetes and PD are age-related chronic diseases and some pathogenic processes may underlie both conditions (Chen, O'Reilly, Schwarzschild, & Ascherio, 2008; Henchcliffe & Beal, 2008; Pradhan, 2007). Insulin de-sensitisation may be one mechanism that underlies both conditions. The analysis of clinical data showed that an 8–30 % of PD patients were diabetic or glucose intolerant, a significantly higher percentage compared to age matched controls (Cereda et al., 2011; Hu, Jousilahti, Bidel, Antikainen, & Tuomilehto, 2007; Lu et al., 2014; Miyake et al., 2010; Schernhammer, Hansen, Rugbjerg, Wermuth, & Ritz, 2011). In preclinical studies, systemic administration of drugs for T2DM, such as insulin (Freiherr et al., 2013), rosiglitazone (Schintu et al., 2009), and metformin (Patil, Jain, Ghumatkar, Tambe, & Sathaye, 2014), significantly attenuate neuropathology, including the loss of SNpc neurons and the striatal dopaminergic fibers, microglial activation, or the expression of pro-inflammatory cytokines, in PD animal models. Also, a drug that is on the market to treat type 2 diabetes, Exenatide (exendin-4), showed a therapeutic effect in preclinical tests (Harkavyi et al., 2008; Kim, Moon, & Park, 2009; Li et al., 2009) and in a pilot clinical trial in PD patients (Aviles-Olmos et al., 2013; Aviles-Olmos et al., 2014). Exendin-4 is a receptor agonist for the incretin hormone Glucagon-like peptide-1 (GLP-1) (Baggio & Drucker, 2007). GLP-1 is a growth factor with neuroprotective properties in a range of disease models of chronic neurodegenerative

disorders (Holscher, 2013). Newer GLP-1 mimetics have been developed, and they have longer survival times in the blood stream than exendin-4 (Sadry & Drucker, 2013; Tan & Bloom, 2013). We have previously reported good neuroprotective effects of GLP-1 mimetics in animal models of Alzheimer's disease (Gengler, McClean, McCurtin, Gault, & Holscher, 2012; P. McClean, Parthasarathy, Faivre, & Hölscher, 2011; P. L. McClean & Holscher, 2014). However, no data have been published that demonstrate neuroprotective effects of these newer and more potent drugs in animal models of PD. We therefore tested the effects of a novel protease-resistant GLP-1 analogue, (Val8)GLP-1-glu-PAL, in a mouse model of PD. (Val8)GLP-1-glu-PAL has shown good neuroprotective effects in an animal model of diabetes (Lennox, Porter, Flatt, & Gault, 2013). We induced PD in mice using the chemical MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), which is a precursor of the neurotoxin MPP+ that induces classic symptoms of Parkinson's disease by impairing or destroying dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Gerlach, Riederer, Przuntek, & Youdim, 1991; Nakamura & Vincent, 1986). MPTP is a widely used chemical to induce a Parkinson-like state in animals for drug discovery (Kim et al., 2009; Kopin & Markey, 1988; Li et al., 2009; Nakamura & Vincent, 1986). Motor activity and key biomarkers for PD were evaluated to analyse the neuroprotective efficacy of (Val8)GLP-1-glu-PAL. The previously established effective dose of 25nmol/kg ip. was used (Lennox et al., 2013).

2. Methods

2.1 Experimental Animals

Adult Male C57BL/6 mice (23 ± 2 g) 10 ± 2 weeks old were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), were maintained in a constant temperature ($25 \pm 2^\circ\text{C}$) and humidity (relative 60%-70%) under a 12h light/dark cycle in animal house, Shanxi Medical University. Animals were allowed free access to food and water. All animals procedures were licenced by the ethics

committee of Shanxi Medical University and performed in accordance to National Institute of Health (NIH) guideline (NIH publication NO. 85-23. Revised 1985). During experimental procedures, all efforts were made to minimize animal suffering and to reduce the number of animal used.

2.2 Chemicals

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 4% paraformaldehyde (PFA) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St Louis, MO, USA). Bicinchoninic acid (BCA) protein assay kit was purchased from Beyotime Biotechnology Inc. (Zhejiang, China). Sodium chloride, ethylene glycol, and 3,3-diaminobenzidine (DAB) were purchased from ZSGB-BIO Co. (Beijing, China). All other reagents used in the research were obtained from ZSGB-BIO Co. (Beijing, China).

2.3 Antibodies

Rabbit anti-tyrosine hydroxylase (TH), Bax, Bcl-2, Anti-Pro-caspase3, Goat Anti-rabbit IgG H&L (HRP) were obtained from Abcam (Cambridge,UK), Anti-Cleaved-caspase3 was purchased from Cell Signaling Technology (Boston, USA).

2.4 (Val8)GLP-1-glu-PAL peptide

The (Val8)GLP-1-glu-PAL used in this study was purchased from ChinaPeptides Co., Ltd (Shanghai, China) to 95% purity. The identity and 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. The peptide was stored in dry form and dissolved in double distilled 0.9%NaCl before experiments.

2.5 Experimental design

In our experimental design, 56 mice were randomly allocated to four groups (n=14). Group1 mice were injected intraperitoneally with saline (0.2ml per mouse) and served as control group. Group2 mice received MPTP (30mg/kg wt. ip.)(Zhao et al) once daily for 8

consecutive days. Group3 mice were injected intraperitoneally with (Val8)GLP-1-glu-PAL (25nmol/kg ip.). Group4 was the MPTP+(Val8)GLP-1-glu-PAL group (intraperitoneally MPTP injected plus Val8 treated group). (Val8)GLP-1-glu-PAL was dissolved in saline (1mg dissolved in 27 ml 0.9%NaCl) was administered after each MPTP injection. After all treatment animals were subjected to behavioral, including swimming, rotarod, open-field test and perfused afterwards for histological and western blot measurements.

2.6 Behavioral Tests

Swim test

In the swim test, each mouse was separately placed in the water tubs (40 cm length \times 25cm width \times 16 cm height), filled up to 12cm with tap water at a temperature of $25 \pm 2^\circ\text{C}$ and let swim for 3min as previously described (Haobam, Sindhu, Chandra, & Mohanakumar, 2005). The animals were wiped dry immediately after the experiment using a dry towel and were returned to cages. Swim score scales were: 0, hind part sinks with head floating (swimming time $<$ 30s); 1, occasional swimming using hind limbs while floating on one side (30s $<$ swimming time $<$ 90s); 2, occasional floating/swimming only (90s $<$ swimming time $<$ 150s); 3, continuous swimming(swimming time $>$ 150s) (Haobam et al., 2005; Kavitha, Nataraj, Essa, Memon, & Manivasagam, 2013). The swimming behavior of each animal was recorded and analyzed by an observer blind to the experimental groups. The experiment was repeated three times for each animal.

Rotarod test

In the Rotarod test, rotorod apparatus diameter of 3cm, rotational speed of 20rpm, the constant temperature of $25 \pm 2^\circ\text{C}$.

The rotarod test is a useful method for measuring hypokinesia 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. Animals were allowed to walk on rotarod before each MPTP injection to assess sensorimotor coordination.

The rotarod test in which animal balances on a rotating drum, is widely used to assess motor deficit in neurodegenerative disease models in rodents (Rial et al., 2014). Mice were placed on a rod that accelerated smoothly from 5 to 20 rpm over a period of 120s. For both protocols, the length of time that each mouse was able to stay on the rod was recorded as the latency period to fall, registered automatically by a trip switch under the floor of each rotating drum at 5 min rest intervals to prevent stress and fatigue and a maximum trial length of 120s pretrial. The experiment was repeated three times for each mouse.

Open field test

Open field exploratory behavior and spontaneous motor activity were analyzed using Open field test (OFT). The environment of detection is quiet and dark. The open field is an enclosure, generally square (45cm length×45cm width×45cm height) in shape with surrounding walls that prevent escape. Distance moved and standing times are among many measures that can be tabulated and reported. Commonly, the field is marked with a grid (area 45cm×45cm; size 9cm×9cm) and square crossings. The mice were individually placed in one corner of the open field. The crossing numbers (defined as at least three paws in a square) were monitored for 5 min by treatment-blinded experimenters seated quietly approximately 0.5m distant. The total distance (crossing numbers) and rearing of each animal in the field during 5mins of testing session was recorded. After 5min, the mouse was removed and the open field was cleaned. The experiment was repeated three times for each mouse.

2. 7 Immunohistochemistry

On the 8th day after the MPTP treatment, mice were deeply anesthetized with urethane (ip) and were intracardially perfused saline(20ml), followed by 4% PFA(20ml). Brains were immediately removed and post-fixed in 4% PFA overnight (>12h). Brains were embedded in paraffin and coronal sections (3µm thick) of the brains were cut on a Leica microtome for immunohistochemical study. The sections were pretreated with 3% hydrogen peroxide(H₂O₂) for 10 min at room temperature to remove endogenous peroxidase activity. Then, sections were incubated with primary antibodies (rabbit

anti-mice monoclonal TH, 1:300, Abcam); diluted in PBS in 4°C for overnight. After this, sections were incubated with their corresponding secondary antibodies (goat anti-rabbit IgG conjugated to horse radish peroxidase, 1:500, Abcam) diluted in PBS for 60min at 37°C. In each treatment, the slides were washed at least 3 times with PBS each for 5 min. The immunoreactivity was visualized with 3,3'-Diaminobenzidine (DAB) (ZSGB-BIO Co., Beijing China) color reaction in SNpc.

Sections were chosen in accordance with stereological rules: the first section was taken at random and every fifth section afterward. Stereology removes bias and possible sources of systematic errors (Bondolfi et al., 2002).

The DAB staining was observed with a ScanScope CS system (APERIO, USA). N=3 sections per brain were analysed, n=6 per group.

2.8 Western Blotting

Fresh sections of the midbrain s. nigra area were homogenized (20s homogenization and 10s pause × 3 times) in an ice cold RIPA buffer (containing 1% Triton X-100, 0.1% SDS, 1% deoxycholate) and phenyl-methylsulfonyl fluoride (PMSF). The homogenate was centrifuged 10,000 rpm for 10 min at 4°C, and the supernatant was collected. Protein concentration was measured by the method of Bradford (cat.500-0006, Bio-Rad, Hercules, CA). Samples were then added with loading buffer to the same concentration, boiled and centrifuged. Protein were separated on 10% SDS-polyacrylamide gel and electrophoretically transferred to PVDF membrane. The membranes were incubated with the blocking buffer containing 5% bovine serum albumin (BSA) for 2h to reduce non-specific binding sites and then probed overnight with Rabbit anti-TH (1:500) and Rabbit anti-BCI-2 (1:100) and Rabbit anti-Bax (1:200) and Rabbit anti-Pro-caspase3 (1:600) (Abcam) and Rabbit anti- Cleaved-caspase3 (1:750)(CST) in 4°C for overnight, then labeled with appropriate secondary antibodies (goat anti-rabbit IgG conjugated to horse radish peroxidase, 1:5000, Abcam) was added in room temperature for 2h. To confirm equal protein loading, blots were reprobed with α -actin antibody (Abcam) at 0.5 μ g/ml. Protein bands were visualized with enhanced chemiluminescence (ECL) (ZSGB-BIO Co., Beijing China) and quantified using the image system of Quantity one 4.31 (Bio-Rad,

Hercules, CA, USA).

2.9 Statistical analysis

All values were presented as the mean \pm standard errors (SEM). The statistical software SPSS 19.0 was used for statistical analysis. One-way ANOVA tests were used to analyse the data with Bonferroni post-hoc tests. A probability value of less than 0.05 was considered to be statistically significant.

3. Results

3.1 (Val8)GLP-1-glu-PAL improved the MPTP-induced motor impairments

In the swimming test, MPTP impaired motor activity compared to saline controls ($p < 0.001$). An overall one-way ANOVA found a difference between all groups ($p < 0.0001$). In post-hoc Bonferroni tests, (Val8)GLP-1-glu-PAL on its own did not affect swimming ability compared to saline control. (Val8)GLP-1-glu-PAL prevented or reversed some of the motor impairment induced by MPTP. A difference between the MPTP treated groups and the (Val8)GLP-1-glu-PAL and MPTP co-administrated group was found ($p < 0.001$). Data are represented as mean \pm SEM, $n=14$ mice in each group. See fig. 1A.

In the rotarod test, (Val8)GLP-1-glu-PAL partially reversed the impairment of staying on the rotating rod for 120 seconds that was induced by MPTP ($p < 0.01$). (Val8)GLP-1-glu-PAL on its own did not affect performance compared to saline controls. (Val8)GLP-1-glu-PAL prevented or reversed some of the motor impairment induced by MPTP. A difference between the MPTP treated groups and the (Val8)GLP-1-glu-PAL and MPTP co-administrated group was found ($p < 0.01$). Data are represented as mean \pm SEM, $n=14$ mice in each group. See fig. 1B.

In the Open field assessment (5 min), MPTP reduced the number of rearings ($p < 0.001$) compared to controls. (Val8)GLP-1-glu-PAL prevented this effect, and a difference between the MPTP treated groups and the (Val8)GLP-1-glu-PAL and MPTP co-administrated group was found ($p < 0.001$). Overall locomotion was severely impaired by MPTP compared to controls ($p < 0.001$). The distance traveled was increased in the (Val8)GLP-1-glu-PAL and MPTP co-administrated group compared to MPTP alone ($p < 0.001$). See fig. 1C.

3.2 Inhibitory Effects of (Val8)GLP-1-glu-PAL on dopaminergic neuronal loss in MPTP treated mice

We determined whether (Val8)GLP-1-glu-PAL protects the dopaminergic neurons against MPTP toxicity. The expression of tyrosine hydroxylase (TH), a key enzyme for the synthesis of dopamine, was analysed. MPTP treatment led to a reduction of TH synthesis and of TH-positive neurons in the SN. In the MPTP-only treated mice, the number of TH-positive cells in the SNpc was decreased to 35% of the saline control group ($p < 0.001$). However, co-administration with (Val8)GLP-1-glu-PAL ameliorated MPTP-induced decrease of TH-positive neuron numbers. In comparison with the saline control group, the number of TH-positive neurons in Val8+MPTP group was about 60% ($p < 0.01$). See fig. 2. For western blot results see fig. 4.

3.3 (Val8)GLP-1-glu-PAL reduces apoptosis induced by MPTP in the SN

To investigate the preventive effect of (Val8)GLP-1-glu-PAL on the rate of apoptotic DNA fragmentation in PD mice brains, the TUNEL assay was performed. Representative TUNEL assay results are shown in Fig. 3. The nuclei of the apoptotic cells were stained brown/yellow, while those of non-apoptotic cells and the negative control were stained blue. Brain sections from the MPTP group showed a significantly higher percentage of cells with DNA damage as indicated when compared with brain sections from control group

($p < 0.001$). Although the apoptosis index of the MPTP+Val8 group was significantly higher compared with that of the control group, (Val8)GLP-1-glu-PAL prevented this effect to some degree, and when compared with the MPTP group, the number of positive cells in the MPTP+(Val8)GLP-1-glu-PAL group decreased significantly ($P < 0.01$). $N=6$ per group, data shown are means \pm SEM. See fig. 3.

3.4 (Val8)GLP-1-glu-PAL normalised TH expression levels, cell growth signaling and reduced apoptotic cell signaling induced by MPTP in the SN

To investigate the effect of (Val8)GLP-1-glu-PAL on key biomarkers, western blot analyses were made on SN brain tissue. The level of TH in the SN was decreased by MPTP ($p < 0.05$) but partially rescued by (Val8)GLP-1-glu-PAL ($p < 0.05$ compared to MPTP group, fig. 4B). Levels of cell growth signaling Bcl-2 molecule were reduced by MPTP ($p < 0.01$) but almost normalised by (Val8)GLP-1-glu-PAL $p < 0.05$ compared to MPTP group, fig. 4C). Levels of the apoptosis cell signaling molecule BAX were increased by MPTP ($p < 0.01$) and normalised by (Val8)GLP-1-glu-PAL ($p < 0.05$ compared to MPTP group, fig. 4D). The levels of the procaspase3 protein expression were slightly reduced overall by MPTP ($p < 0.05$) and normalised by (Val8)GLP-1-glu-PAL ($p < 0.05$ compared to MPTP group, fig. 4E). Levels of the activated cleaved caspase 3 enzyme were much increased by MPTP ($p < 0.01$) and slightly reduced by (Val8)GLP-1-glu-PAL ($p < 0.05$ compared to MPTP group, fig. 4F). $N=6$ per group, data shown are means \pm SEM.

4. Discussion

The results demonstrate that the GLP-1 analogue (Val8)GLP-1-glu-PAL has neuroprotective effects in the MPTP mouse model of PD. The motor impairments induced by MPTP were clearly visible in the rotarod test of sensorimotor coordination, in the swim test and in the open field observation of spontaneous locomotion and exploration. Furthermore, the levels of the cell growth second messenger molecule Bcl-2 was reduced, while the

pro-apoptotic signaling molecule Bax was increased. Activation levels of caspase 3 were enhanced by MPTP treatment as well, and the increase in TUNEL positive cells showed that apoptosis was much increased. MPTP is a neurotoxin precursor to MPP+, which causes symptoms of Parkinson's disease by damaging or destroying dopaminergic neurons in the substantia nigra (Gerlach et al., 1991; Nakamura & Vincent, 1986). MPTP is a widely used chemical to induce a Parkinson-like state in animals (Kopin & Markey, 1988; Nakamura & Vincent, 1986).

(Val8)GLP-1-glu-PAL was able to reverse or prevent some of these symptoms. Motor activity and somatosensory coordination was improved by the drug, and the biomarkers for cell growth and apoptosis were improved. In addition, the expression of the dopamine synthesising enzyme TH was much enhanced, suggesting that dopaminergic neurons in the SN were functioning again. GLP-1 analogues have shown neuroprotective effects in various diseases, such as Alzheimer's disease, Parkinson's disease, head trauma or stroke (Darsalia et al., 2012; Holscher, 2013; P. L. McClean & Holscher, 2014; Perry & Greig, 2004; Sato et al., 2013; Tweedie et al., 2013). Furthermore, two previous studies showed good protection of MPTP treated mice using the GLP-1 agonist exendin-4 (Kim et al., 2009; Li et al., 2009). Exendin-4 also showed good neuroprotective effects in other animal models of PD that use different chemicals to induce PD like symptoms, 6-OHDA or LPS injection into the brain (Bertilsson et al., 2008; Harkavyi et al., 2008). More importantly, a pilot clinical trial in PD patients showed first positive effects (Aviles-Olmos et al., 2013; Aviles-Olmos et al., 2014), and a phase II trial is currently ongoing. Exendin-4 was the first GLP-1 receptor agonist to be marketed as a drug treatment for type II diabetes (Baggio & Drucker, 2007). It has a short half-life of around 4 hours in the blood stream and needs to be injected twice-daily. Therefore, newer GLP-1 analogues have been developed with a much enhanced half-life (Campbell & Drucker, 2013; Holscher, 2010) One of these is liraglutide, which is GLP-1-gluPAL, human GLP-1 that has been acetylated to enhance the half-life in the blood stream. It does not have the amino acid substitution at position 8 that Val(8)GLP-1(glu-PAL) has. This substitution prevents cleavage by the DPP-IV protease (Green, Gault, O'Harte, & Flatt, 2005) and enhances the biological half life in the blood stream. We have shown in a previous study that Val(8)GLP-1(glu-PAL) is neuroprotective

and protects memory and synaptic plasticity in diabetic animals (Lennox et al., 2013). We also have previously shown that liraglutide and the newer diabetes drug lixisenatide are protective in the MPTP mouse model of PD (Liu et al., 2015). Here, we demonstrate for the first time that Val(8)GLP-1(glu-PAL) is also neuroprotective in this mouse model of PD. However, Val(8)GLP-1(glu-PAL) will have to be tested in other animal models of PD that use different chemicals to induce PD- like symptoms such as 6-OHDA or LPS injection into the brain (Bertilsson et al., 2008; Harkavyi et al., 2008), and in transgenic mouse models that express human mutated genes that are known to induce Parkinson's disease (Bobela, Zheng, & Schneider, 2014; Giraldez-Perez, Antolin-Vallespin, Munoz, & Sanchez-Capelo, 2014). Another important test to be conducted is to treat animals *after* inducing the PD lesion in order to assess the regenerative effect of the novel drugs. As GLP-1 mimetics are already on the market as treatments for type II diabetes and are well received, it would be straightforward to test these in patients with PD (Holscher, 2014).

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Figure captions

Fig. 1: (Val8)GLP-1-glu-PAL improved the MPTP-induced motor impairments

1A: In the swimming test, MPTP impaired motor activity compared to saline controls (two-way ANOVA, $p < 0.001$). (Val8)GLP-1-glu-PAL prevented some of the motor impairment induced by MPTP. A difference between the MPTP treated groups and the (Val8)GLP-1-glu-PAL and MPTP co-administrated group was found ($p < 0.001$).

1B: In the rotarod test, (Val8)GLP-1-glu-PAL partially reversed the impairment of staying on the rotating rod for 120 seconds that was induced by MPTP ($p < 0.001$). A difference between the MPTP treated groups and the (Val8)GLP-1-glu-PAL and MPTP co-administrated group was found ($p < 0.001$).

1C: In the Open field assessment (5 min), MPTP reduced the number of rearings ($p < 0.001$) compared to controls. A difference between the MPTP treated groups and the (Val8)GLP-1-glu-PAL and MPTP co-administrated group was found ($p < 0.001$). Overall locomotion was severely impaired by MPTP compared to controls ($p < 0.001$). The distance traveled was increased in the (Val8)GLP-1-glu-PAL and MPTP co-administrated group compared to MPTP along ($p < 0.001$).

Post-hoc analysis of day-to-day differences are shown between the MPTP group and the MPTP+(Val8)GLP-1-glu-PAL group to indicate the protective effect of the drug. $* = p < 0.05$; $** = p < 0.01$; $*** = p < 0.001$. Data are represented as mean \pm SEM, $n = 14$ mice in each group.

Fig 2: Protective effects of (Val8)GLP-1-glu-PAL on dopaminergic neurons in the SN. The expression of tyrosine hydroxylase (TH), a key enzyme for the synthesis of dopamine, was reduced by MPTP in the SN to 35% of the saline control group ($p < 0.001$).

(Val8)GLP-1-glu-PAL ameliorated MPTP-induced decrease of TH-positive neuron numbers. In comparison with the saline control group, the number of TH-positive neurons in Val8+MPTP group was increased ($p < 0.01$). Length of image = 1mm.

Fig. 3: TUNEL assay in the SN. The number of TUNEL positive cells was much increased in the MPTP group. (Val8)GLP-1-glu-PAL prevented this effect to some degree. Data shown

as means±SEM, n=3 per group. Sample images are shown, A=control; B=MPTP; C=(Val8)GLP-1-glu-PAL; D=MPTP+(Val8)GLP-1-glu-PAL. Calibration bar = 100µm. **= P<0.01, ***=P<0.001.

Fig. 4: To investigate the effect of (Val8)GLP-1-glu-PAL on key biomarkers, western blot analyses were made on SN brain tissue. **4A:** sample gel lanes with β-actin loading control. **4B:** The level of TH in the SN was decreased by MPTP but partially rescued by (Val8)GLP-1-glu-PAL. **4C:** Levels of cell growth signaling Bcl-2 molecule were reduced by MPTP but almost normalised by (Val8)GLP-1-glu-PAL. **4D:** Levels of the BAX apoptosis cell signaling molecule were increased by MPTP and normalised by (Val8)GLP-1-glu-PAL. **4E:** levels of the pro-caspase3 protein expression were slightly reduced overall by MPTP and normalised by (Val8)GLP-1-glu-PAL. **4F:** Levels of the activated cleaved caspase 3 enzyme were much increased by MPTP and slightly reduced by (Val8)GLP-1-glu-PAL. N=3 per group, data shown are means±SEM. *=p<0.05, **=p<0.01 compared to control, #=p<0.05 compared to MPTP group.

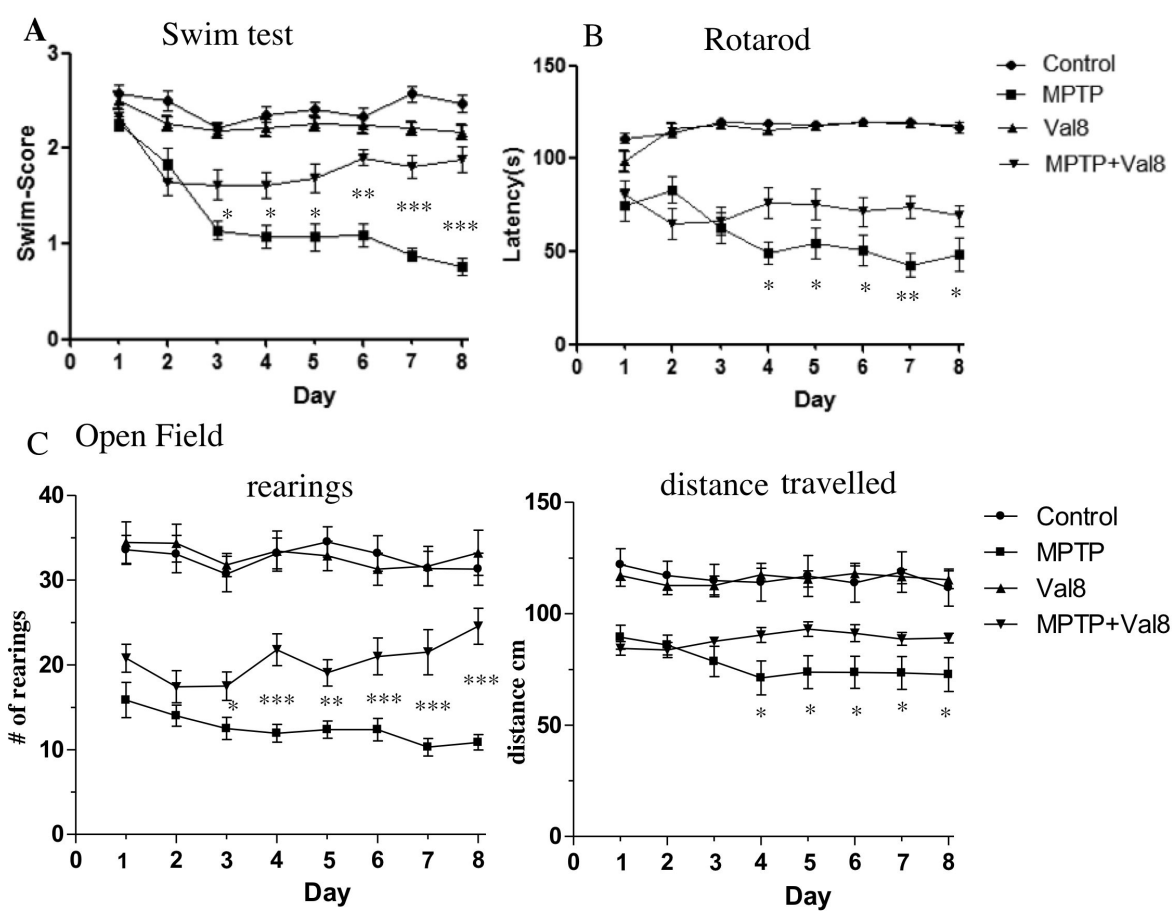


Fig. 1

Number of TH positive neurons in the SN

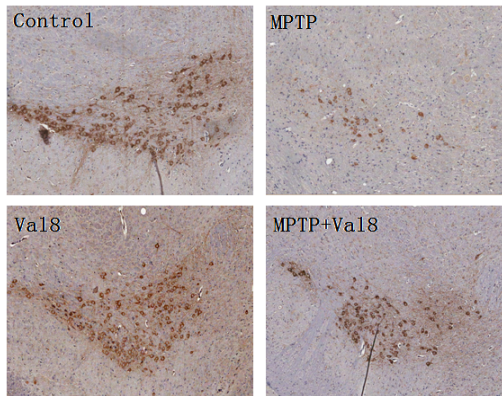
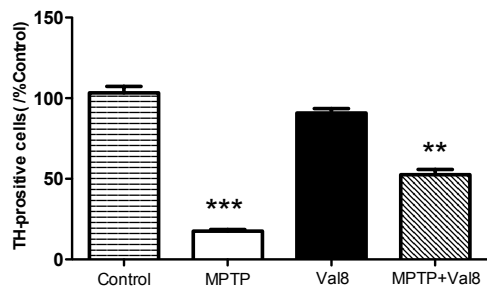


Fig. 2

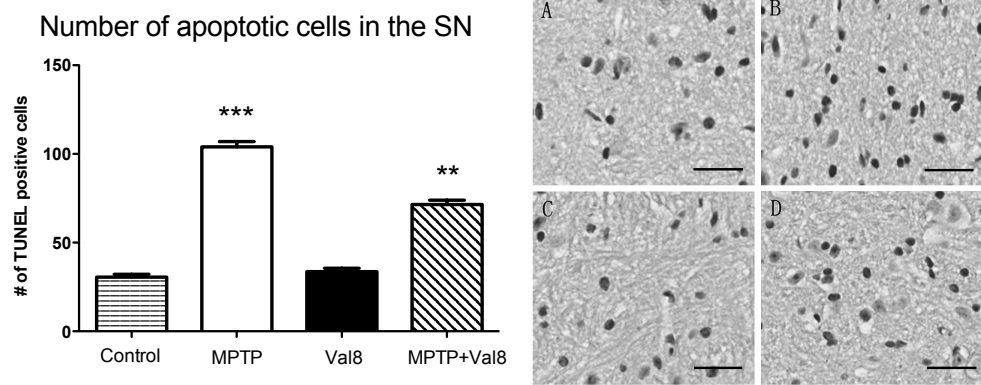


Fig. 3

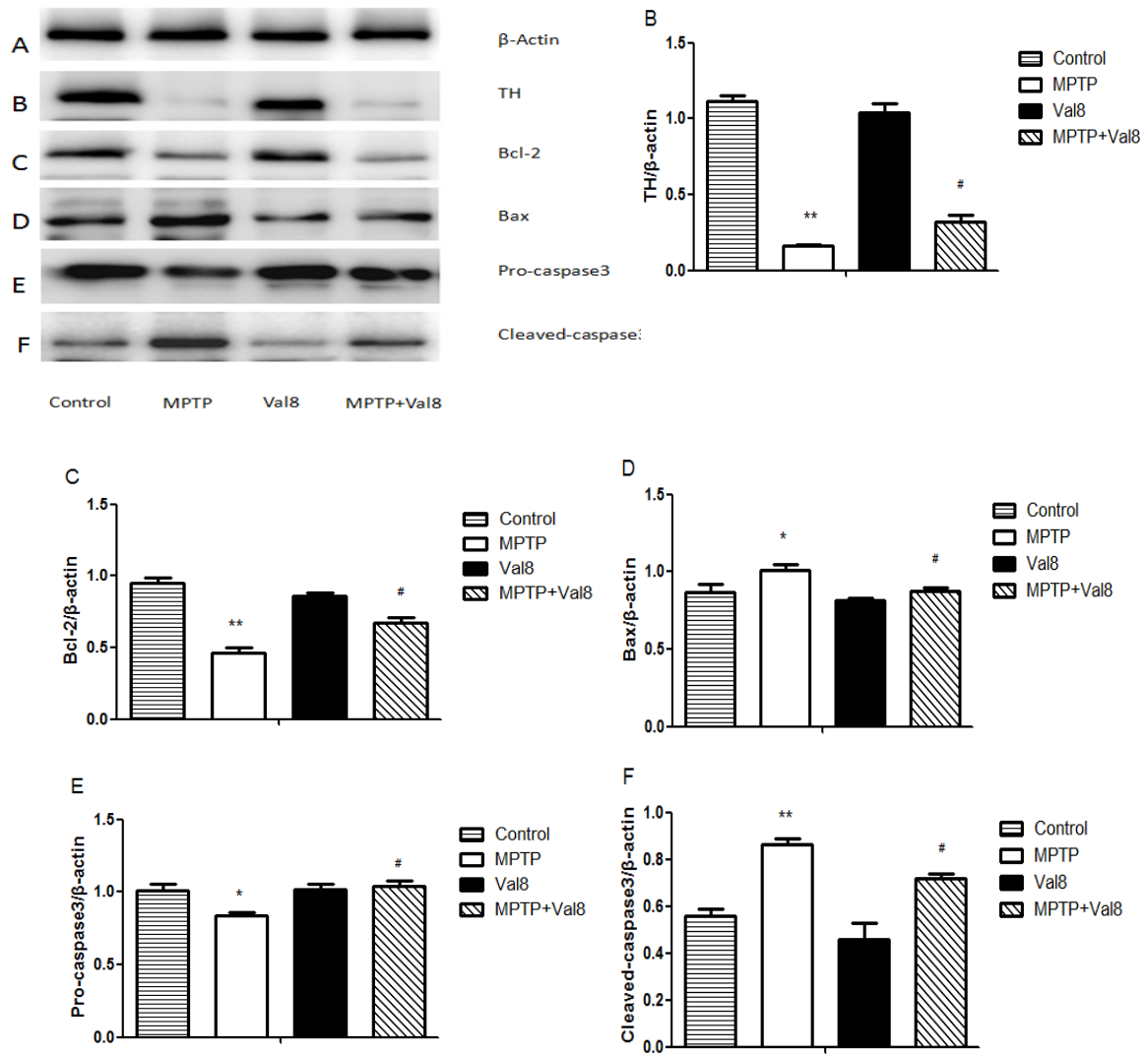


Fig. 4