Neuroprotective effects of lixisenatide and liraglutide in the MPTP mouse model of Parkinson's disease

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Abstract

Glucagon-like peptide 1 (GLP-1) is a growth factor. GLP-1 mimetics are on the market as treatments for type 2 diabetes and are well tolerated. These drugs 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 clinical trial in PD patients showed promising first results. Liraglutide and lixisenatide are two newer GLP-1 mimetics which have a longer biological half-life than exendin-4. We previously showed that these drugs have neuroprotective properties in an animal model of Alzheimer's disease. Here we demonstrate the neuroprotective effects in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. MPTP was injected once-daily (20mg/kg i.p.) for 7 days, and drugs were injected once-daily for 14 days i.p.. When comparing exendin-4 (10nmol/kg), liraglutide (25nmol/kg) and lixisenatide (10nmol/kg), it was found that exendin-4 showed no protective effects at the dose chosen. Both liraglutide and lixisenatide showed effects in preventing the MPTP-induced motor impairment (Rotarod, open field locomotion, catalepsy test), reduction in Tyrosine Hydroxylase (TH) levels (dopamine synthesis) in the substantia nigra and basal ganglia, a reduction of the pro-apoptotic signaling molecule BAX and an increase in the anti-apoptotic signaling molecule Bcl-2. The results demonstrate that in this study, both liraglutide and lixisenatide are superior to exendin-4, and both drugs show promise as a novel treatment of PD.

Keywords: neurodegeneration, growth factor, apoptosis, insulin, incretin, basal ganglia

1. Introduction

Parkinson's disease (PD) is a chronic neurodegenerative disorder of motor control, characterised by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the degeneration of projection axons and dopaminergic synapses to the striatum, which leads to tremors, muscular rigidity, bradykinesia, and postural and gait abnormalities (Langston, 2002). There is currently no treatment for this condition. Recently, the neuroprotective and restorative effect of growth factors such as glia-derived neurotrophic factor (GDNF) in cell culture and animal models of Parkinson's disease has drawn attention to the potential of using growth factors as a treatment (Mickiewicz and Kordower, 2011). One major stumbling block for this strategy is the fact GDNF and other growth factors do not cross the blood-brain barrier (BBB) (Holscher, 2014b). The growth factor glucagon-like protein 1 (GLP-1) and its analogues have shown neuroprotective effects in several disease models of neurodegeneration (Perry and Greig, 2004, Holscher, 2013). Several GLP-1 receptor agonists have been developed as treatments for type 2 diabetes, and some of these can cross the BBB (Kastin et al., 2002, Kastin and Akerstrom, 2003, McClean et al., 2011, Hunter and Holscher, 2012). Previous investigations found that the GLP-1 receptor agonist exendin-4 showed good neuroprotective effects in animal models of PD (Bertilsson et al., 2008, Harkavyi et al., 2008, Kim et al., 2009, Li et al., 2009). Exendin-4 also had been tested in a pilot clinical trial in people with PD and showed encouraging effects (Aviles-Olmos et al., 2013, Aviles-Olmos et al., 2014). Exendin-4 is superior to endogenous GLP-1 as it is resistant to cleavage by the protease DPP-IV and has a much enhanced biological half-life in the blood (Baggio and Drucker, 2007). Newer GLP-1 mimetics have been developed since, and they have longer survival times in the blood stream (Tan and Bloom, 2013). Liraglutide is an acetylated form of the GLP-1 peptide that has a much enhanced half-life and that is on the market as a drug treatment for diabetes (Victoza) (Raun et al., 2007). Lixisenatide also is a novel GLP-1 mimetic that recently has been approved in Europe as a treatment for diabetes (Lyxumia) (Elkinson and Keating, 2013). Both drugs have shown neuroprotective effects in animal models of Alzheimer's disease (McClean et al., 2011, McClean and Holscher, 2014b). We therefore tested the effects of exendin-4, liraglutide and lixisenatide in the MPTP mouse model of PD at doses that showed effect in previous in vivo studies for comparison. MPTP (1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine is a neurotoxin precursor to MPP+, which induces classic symptoms of Parkinson's disease by impairing or destroying dopaminergic neurons in the substantia nigra (Nakamura and Vincent, 1986, Gerlach et al., 1991). MPTP is a widely used chemical to induce a Parkinson-like state in animals (Nakamura and Vincent, 1986, Kopin and Markey, 1988, Kim et al., 2009, Li et al., 2009).

2. Materials and methods

2.1 Animals

C57Bl6 male mice, 8 weeks old, 25-30g in weight were randomly divided into 8 groups: (1) control group, (2) MPTP/vehicle group (3) exendin-4 group (4) MPTP/exendin-4 group, (5) liraglutide group, (6) MPTP/liraglutide group, (7) lixisenatide group, (8) MPTP/Lixisenatide group. N=12 animals per group). Mice received 20 mg/kg/day MPTP in normal saline intraperitoneally for 7 consecutive days. Drugs dissolved in saline were administered after each MPTP injection. After the 7 days, mice received a further 7 days of drug or saline injection once-daily (Fig. 1). Drug concentrations were exendin-4: 10nmol/kg i.p., lixisenatide: 10nmol/kg i.p., liraglutide: 25nmol/kg i.p.. Animals were handled for one week and exposed to the experimental room and the apparatus 2-3 times before commencement of experiments. All experiments were licenced by the UK Home Office (PPL 70 8236).

2.2 Peptides and chemicals

The peptides were synthetised by ChinaPeptides Co., Ltd (Shanghai, China) to 95% purity. 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. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraformaldehyde (PFA) was purchased from Sigma-Aldrich (St Louis, MO, USA). Rabbit anti- tyrosine hydroxylase (TH), Bcl-2, BAX, Goat Anti-rabbit IgG H&L (HRP) secondary antibody were obtained from Abcam (Cambridge, UK). The Bicinchoninic acid (BCA) protein assay kit was purchased from Applygen Technologies Inc. (Beijing, China). Sodium chloride, ethylene glycol, and 3,3-

diaminobenzidine (DAB) were purchased from ZSGB-BIO Co. (Beijing, China). The transfer polyvinylidene difluoride membranes and Amersham ECL Prime western blotting detection reagent were purchased from GE Life Sciences (USA).

2.3 Open field motor activity test

The open field is a square box (45cm×45cm) with 40cm high walls that prevent escape. The floor is marked with a grid of lines (separated by 9cm), The mice were individually placed in one corner of the open field. The number of crossed lines during 10min of testing session was recorded as the total distance. Animals were tracked by a computerised video system that measured path length (Biosignals, USA).

2.4 Rotarod

Mice were placed on a Rotarod system (II-755, IITC Life Science, USA) that accelerated from 5 to 20 rpm over a period of 50s. The length of time that each animal was able to stay on the rod was recorded as the latency to fall, registered automatically by a trip switch under the floor of each rotating drum. A maximum trial length of 180s was given.

2.5 Catalepsy Bar test

Catalepsy was evaluated in the bar test immediately after MPTP drug treatment and on every second day after the drug treatment. The frame was made of wood (25 cm long; 5 cm wide, 10 cm high) with a horizontal bar (0.8 cm diameter, 20 cm long) suspended 30 cm above the floor. Catalepsy was evaluated by measuring the mean time taken for a mouse to climb over the bar after being laid across it with its hind limbs on the floor.

2.6 Histology

After the experiments, animals were perfused transcardially with PBS buffer followed by ice-cold 4% paraformaldehyde in PBS. Brains were removed and fixed in 4% paraformaldehyde for at least 24h before being transferred to 30% sucrose solution overnight. Brains were then snap frozen and coronal sections of 30-micron thickness were cut at a Leica cryostat. Sections were taken for the basal ganglia from Bregma 1.54mm to Bregma -0.34mm and for Substantia nigra from -2.46mm to Bregma -3.88mm Sections were chosen according to stereological rules (Bondolfi et al., 2002) with the

first section taken at random and every 3th section afterwards. Between 3 and 6 sections were analysed per brain. The sections were analysed on an Axio Scope 1 (Zeiss, Germany) and photographed with a digital camera (AxioCam). A dissector was applied to the images in random orientation to avoid sampling bias (Bondolfi et al., 2002). In the SN, TH positive cells were counted per dissector. In the other immunohistological assessments, the greyscale (optical density) of stained area per dissector was analysed using the image analysis programme Image J 1.41o with the Multi threshold plug (NIH, USA) (http://rsb.info.nih.gov/ij) (McClean et al., 2011). The scale of staining correlates with the stained antigen.

2.7 Western blot

Brains were snap frozen at -20 degrees. Striatum tissue containing 5 µg of protein was separated on 4–12% gradient Bis-Tris gel with molecular marker and electrophoresed in running buffer at 200 mV for 40 min followed by transfer to the transfer membrane. Following protein transfer, the membrane was washed in 1X TBST (tris-buffered saline with 0.05% Tween-20, pH 8) and blocked in 5% skimmed milk for 1 h. The membrane was then incubated with anti-Bax (1 : 200), anti-Bcl-2 (1 : 200), and anti-ß-actin (1:400) antibodies for 1 h and after three washes in PBS further incubated with 1 : 400 horseradish peroxidase-conjugated antirabbit IgG. The protein bands were visualized by Amersham ECL Prime western blotting detection reagent according to the manufacturer's recommendation. Bands were analysed using a. image analysis system (BioRad, USA). Bands were normalised for loading control. Each run was repeated three times.

2.8 Statistics

Data were analysed using the program Prism (Graphpad software Inc., USA), results are expressed as means ± SEM. Data were analyzed by 1-way or 2-way ANOVA, followed by post hoc tests, or student t-tests.

3. Results

3.1 Rotarod motorsensory performance

The injection of MPTP impaired the ability of mice to stay on the rotating rod for 3 min. In a two-way ANOVA, an overall difference was found between groups ($p<0.0001$ drug, interaction ns., time ns.). In subsequent two-way ANOVAs, a difference was found between groups injected with saline or drugs and groups injected with MPTP ($p<0.0001$ drug, interaction ns., time ns.). Furthermore, a difference was found between MPTP + Lira / MPTP + Lixi and the groups MPTP + saline / MPTP + exendin-4 ($p<0.0001$ drug, interaction ns., time ns.). The drugs liraglutide and lixisenatide were able to reverse some of the impairments of MPTP while exendin-4 was not. N=12 per group, fig. 2.

3.2 Open field distance traveled

The injection of MPTP reduced the spontaneous locomotion of mice and the overall time traveled in 10 min. In a two-way ANOVA, an overall difference was found between groups ($p<0.0001$ drug, interaction ns., time ns). In subsequent two-way ANOVAs, a difference was found between groups injected with saline or drugs and groups injected with MPTP ($p<0.0001$ drug, interaction ns., time ns.). Furthermore, a difference was found between MPTP + Lira / MPTP + Lixi and the groups MPTP + saline / MPTP + exendin-4 ($p<0.0001$ drug, interaction ns., time $p<0.001$). The drugs liraglutide and lixisenatide were able to reverse some of the impairments of MPTP while exendin-4 was not. The improvement / reversal of the symptoms induced by liraglutide and lixisenatide was dependent on time, fig. 3.

3.4 Catalepsy test

The injection of MPTP induced catalepsy in mice. In a two-way ANOVA, an overall difference was found between groups ($p<0.0001$ drug, interaction ns., time ns.). In subsequent two-way ANOVAs, a difference was found between groups injected with saline or drugs and groups injected with MPTP ($p<0.0001$ drug, interaction ns., time ns.). Furthermore, a difference was found between MPTP + Lira / MPTP + Lixi and the groups MPTP + saline / MPTP + exendin-4 ($p<0.0001$ drug, interaction ns., time ns.). The drugs liraglutide and lixisenatide were able to reverse some of the impairments of MPTP while exendin-4 was not. N=12 per group, fig. 4.

3.5 TH positive neurons in the substantia nigra, pc.

In the histological analysis of the dopamine biomarker tyrosine hydroxilase in the SN, MPTP reduced the numbers of TH positive cells significantly. In a one-way ANOVA with Bonferroni post-hoc test, MPTP + saline and MPTP+exendin-4 groups showed fewer TH- positive neurons in the SN than drug treated mice ($p<0.001$; $n=6$ per group). MPTP treated mice injected with Lixisenatide or liraglutide showed no difference to non-lesioned mice, fig. 5.

3.6 Tyrosine hydroxylase relative expression levels in the striatum.

In the histological analysis of relative TH expression levels in the striatum, MPTP reduced the expression levels of TH significantly. In a one-way ANOVA with Bonferroni post-hoc test, MPTP + saline and MPTP+exendin-4 groups showed fewer TH- positive neurons in the SN than drug treated mice ($p<0.001$). Mice treated with MPTP and injected with liraglutide or lixisenatide showed reduced TH levels compared to non-lesioned mice ($p<0.01$). Mice treated with MPTP and injected with liraglutide or lixisenatide showed higher TH levels compared to MPTP + saline or MPTP + exendin-4 ($p<0.05$); $n=6$ per group, fig. 6.

3.7 Expression levels of BAX and Bcl-2 in the striatum

MPTP reduced expression levels in the striatum of the anti-apoptotic signaling peptide B-cell lymphoma -2 (Bcl-2) in western blot quantifications (one-way ANOVA with Bonferroni post hoc test, $p<0.001$). Injection of liraglutide or lixisenatide enhanced the expression after MPTP treatment compared to controls ($p<0.05$). MPTP + Exendin-4 showed no enhancement compared to MPTP only. Injection of liraglutide or lixisenatide enhanced the expression after MPTP treatment compared to MPTP + Exendin-4 ($p<0.05$). MPTP enhanced expression levels in the striatum of the apoptotic signaling peptide BAX (one-way ANOVA with Bonferroni post hoc test, $p<0.001$). Injection of exendin-4, liraglutide or lixisenatide reduced the expression after MPTP treatment compared to controls ($p<0.05$). $N=4$, fig. 7.

4. Discussion

The results demonstrate for the first time that that the novel GLP-1 mimetics liraglutide and lixisenatide have protective effects in a PD mouse model. Both drugs are superior to the older drug exendin-4 in protecting mice from the toxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is a neurotoxin precursor to MPP+, which causes symptoms of Parkinson's disease by damaging or destroying dopaminergic neurons in the substantia nigra (Nakamura and Vincent, 1986, Gerlach et al., 1991). MPTP is lipophilic and can cross the blood–brain barrier. There, MPTP is metabolised into the toxic cation 1-methyl-4-phenylpyridinium (MPP+) by the monoamine oxidase B (Glover et al., 1986). MPP+ kills primarily dopamine-producing neurons in the substantia nigra, pars compacta (Nakamura and Vincent, 1986). MPP+ interferes with complex 1 of the mitochondrial electron transport chain, which leads to the production of free radicals and eventually to neuronal death (Kinemuchi et al., 1987, Smith and Bennett, 1997). MPTP is a widely used chemical to induce a Parkinson-like state in animals (Nakamura and Vincent, 1986, Kopin and Markey, 1988).
In the present study, MPTP induced Parkinson-like motor impairments, reduced the expression of the dopamine- synthetising enzyme tyrosine hydroxilase (TH) in the substantia nigra pars compacta and also in the striatum. Furthermore, expression of the anti-apoptotic protein Bcl-2 were much reduced by MPTP, and levels of the pro-apoptotic signaling molecule BAX were enhanced. While the MPTP model of PD does not recapitulate all aspects and symptoms of PD, it is an acceptable chemically induced model for drug testing (Kinemuchi et al., 1987, Kopin and Markey, 1988).
In our study, the older GLP-1 mimetic exendin-4 did not protect mice from the MPTP induced effects. This result is surprising, as two previous studies showed good protection of MPTP treated mice using exendin-4 (Kim et al., 2009, Li et al., 2009). However, the exact conditions of the experiments are different, suggesting that under the conditions chosen in our study, exendin-4 could not protect neurons. In the study by Li et al. (2009), exendin-4 was injected directly into the brain via an Alzet pump, and the C57B/l6 mice received only 4 MPTP doses (20mg/kg i.p.). In the study of Kim et al. (2009) using C57B/l6 mice, only 4 MPTP injections were made with exendin-4 injections 30 min previously (10mg/kg i.p.). Our study treated the mice 7

times over 7 days with MPTP (20mg/kg i.p.). Therefor, it is feasible that exendin-4 is not potent enough to protect neurons using this dose of MPTP.

Exendin-4 has shown 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 study in patients with PD demonstrated encouraging protective effects (Aviles-Olmos et al., 2013, Aviles-Olmos et al., 2014). A phase II clinical study testing Bydureon, the once-weekly formulation of exendin-4 is currently ongoing. Therefore, we can assume that exendin-4 does have some neuroprotective effects in PD, even if this was not visible in our study.

Interestingly, the two newer GLP-1 receptor agonists liraglutide and lixisenatide showed good protective effects in the present study. Both drugs have never been tested in animal models of PD before, so this study presents the novel findings that they are superior to exendin-4 and are neuroprotective from the effects of MPTP in the conditions chosen in our study. As all three drugs are currently on the market as treatments for type 2 diabetes (Campbell and Drucker, 2013, Elkinson and Keating, 2013), it is possible to fast-track these into clinical trials in PD. Both liraglutide and lixisenatide are newer drug developments and are suprior to exendin-4 in terms of biological half-life in the body and efficacy in treating diabetes (Lovshin and Drucker, 2009, Campbell and Drucker, 2013). Both drugs also show good neuroprotective effects in animal models of Alzheimer disease (McClean and Holscher, 2014a, b). Two clinical trial testing exendin-4 or liraglutide in Alzheimer patients are currently ongoing (for a summary, see (Holscher, 2014a). Liraglutide also showed good neuroprotective effects in an animal model of stroke (Sato et al., 2013, Briyal et al., 2014) and head trauma (DellaValle et al., 2014, Hakon et al., 2015).

The basis for the neuroprotective effects of GLP-1 receptor activation are most likely based on the growth-factor like activity of GLP-1. GLP-1 activates the GLP-1 receptor that is coupled to a growth factor related second messenger signaling cascade such as MAPk2 and PKA and reduces apoptosis signaling (Sharma et al., 2013) and also enhances the release of other growth factors such as BDNF and NGF in the brain (DellaValle et al., 2014, Ohtake et al., 2014).

Since exendin-4 showed first promising neuroprotective effects in a pilot study in PD patients, newer GLP-1 mimetics such as liraglutide and lixisenatide may be more effective in treating PD patients. The results of the present study support the proposal

of testing of liraglutide and lixisenatide in clinical trials in PD as they promise better neuroprotection in this disease. Further tests of these drugs in different animal models of PD will be conducted to collect further data on their neuroprotective properties.

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Figure captions

Fig. 1

Study design and timelines.

Fig. 2: Rotarod sensorymotor performance

Latencies of mice to stay on the rotating rod for 3 min. N=12 per group. One-way ANOVA with subsequent post hoc tests. *=$p<0.05$; ***=$p<0.001$.

Fig. 3: Open field locomotion test

The injection of MPTP reduce the spontaneous locomotion of mice and the overall time traveled in 10 min. A difference was found between groups injected with saline or drugs and groups injected with MPTP ($p<0.0001$; two-way ANOVA). Furthermore, a difference was found between MPTP + Lira / MPTP + Lixi and the groups MPTP + saline / MPTP + exendin-4 ($p<0.0001$).

Fig. 4: Catalepsy test

The injection of MPTP induced catalepsy in mice. A difference was found between groups injected with saline or drugs and groups injected with MPTP ($p<0.0001$). Furthermore, a difference was found between MPTP + Lira / MPTP + Lixi and the groups MPTP + saline / MPTP + exendin-4 ($p<0.0001$). The drugs liraglutide and lixisenatide were able to reverse some of the impairments of MPTP while exendin-4 was not. N=12 per group.

Fig. 5: TH positive neurons in the substantia nigra, pc.

In a one-way ANOVA with Bonferroni post-hoc test, numbers of TH positive cells were compared (***=$p<0.001$; n=6 per group). Scale bar 100µm.

Fig. 6: Relative expression of TH in the striatum.

One-way ANOVA with post hoc test, (*=$p<0.05$; **=$p<0.01$; ***=$p<0.001$, n=6 per group). Scale bar 1mm.

Fig. 7: Expression levels of BAX and Bcl-2 in the striatum

MPTP reduced expression levels in the striatum of the anti-apoptotic signaling peptide B-cell lymphoma -2 (Bcl-2) in western blot quantifications (one-way ANOVA with Bonferroni post hoc test, *=$p<0.05$; ***=$p<0.001$). N=4 per group.

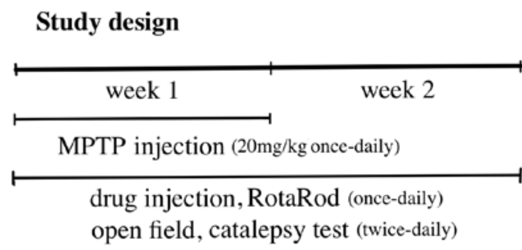
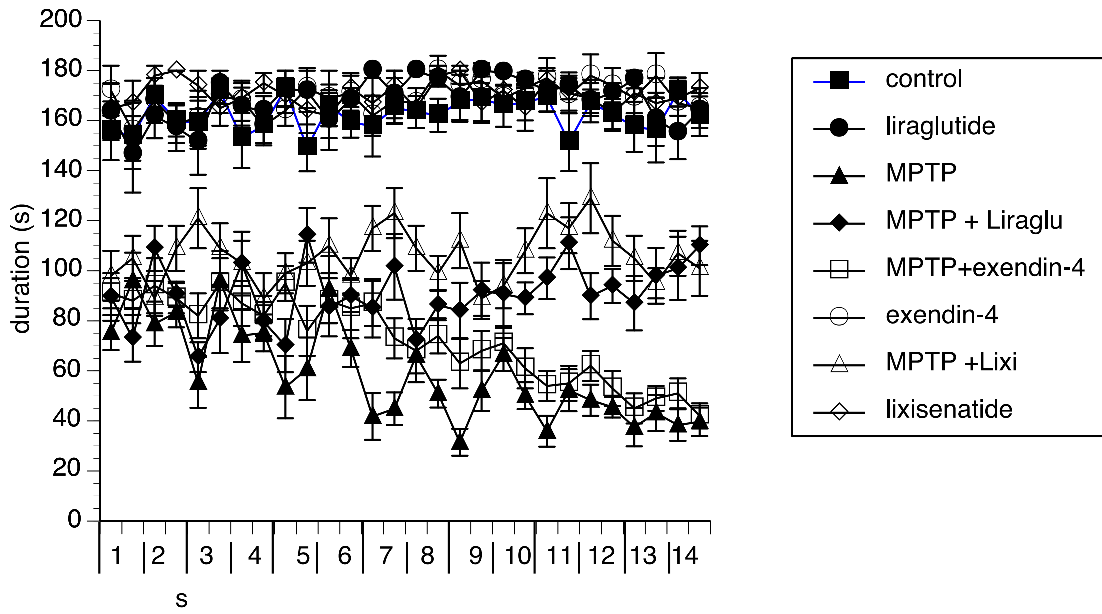


Fig. 1



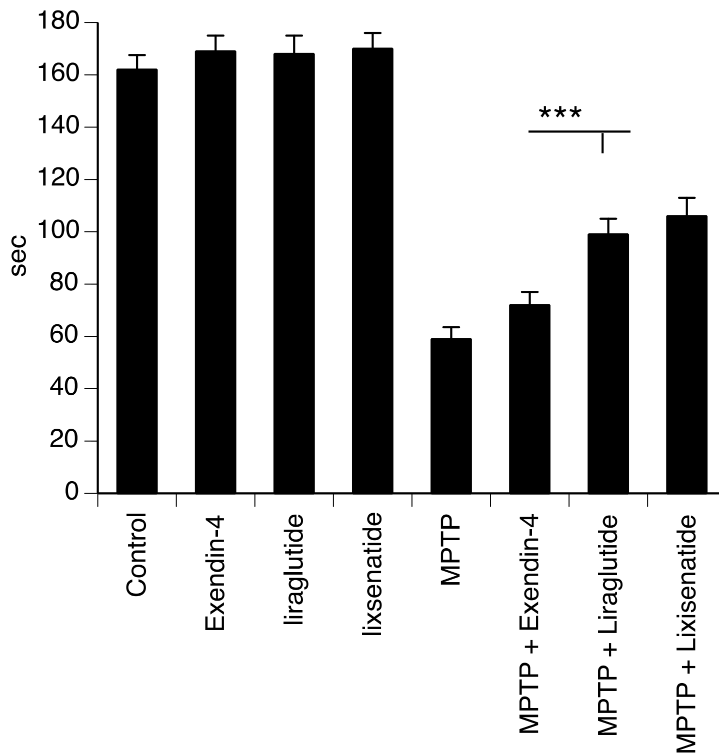


fig. 2

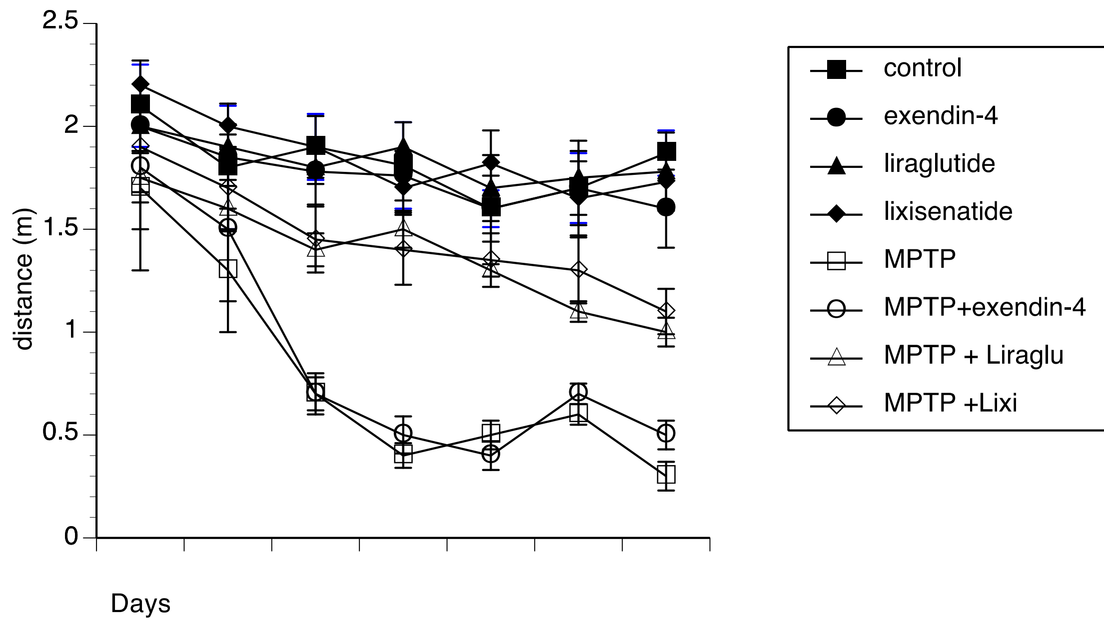


Fig. 3

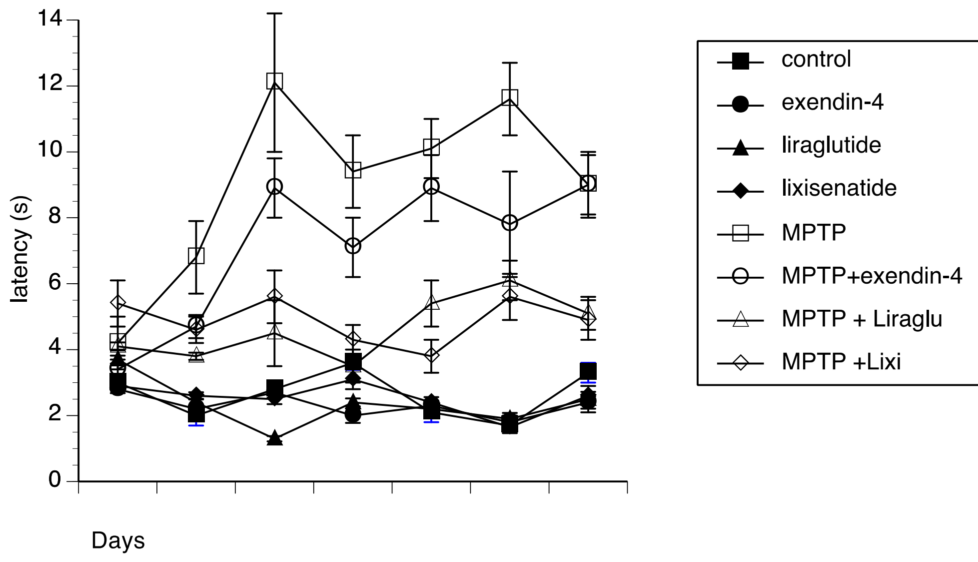


Fig. 4

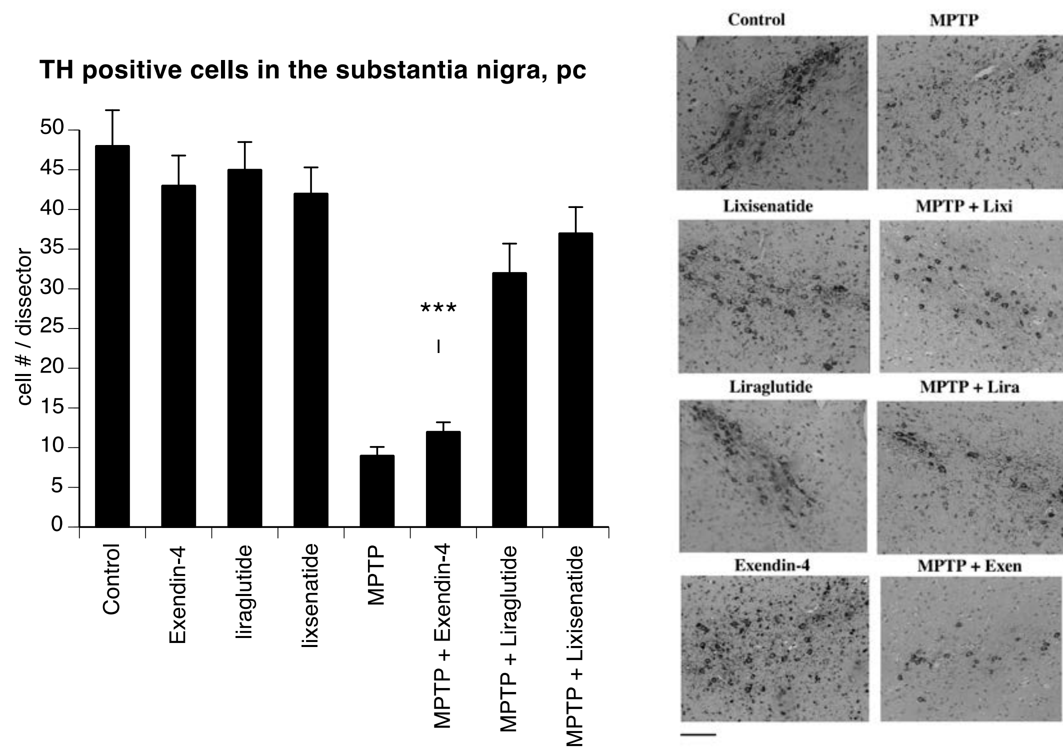


Fig. 5

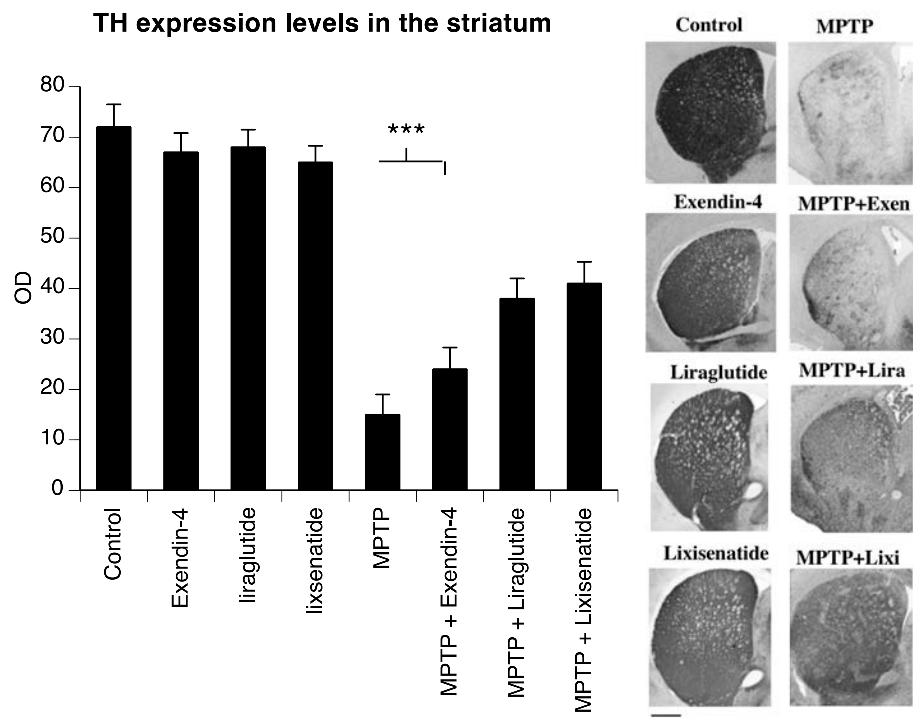


Fig. 6

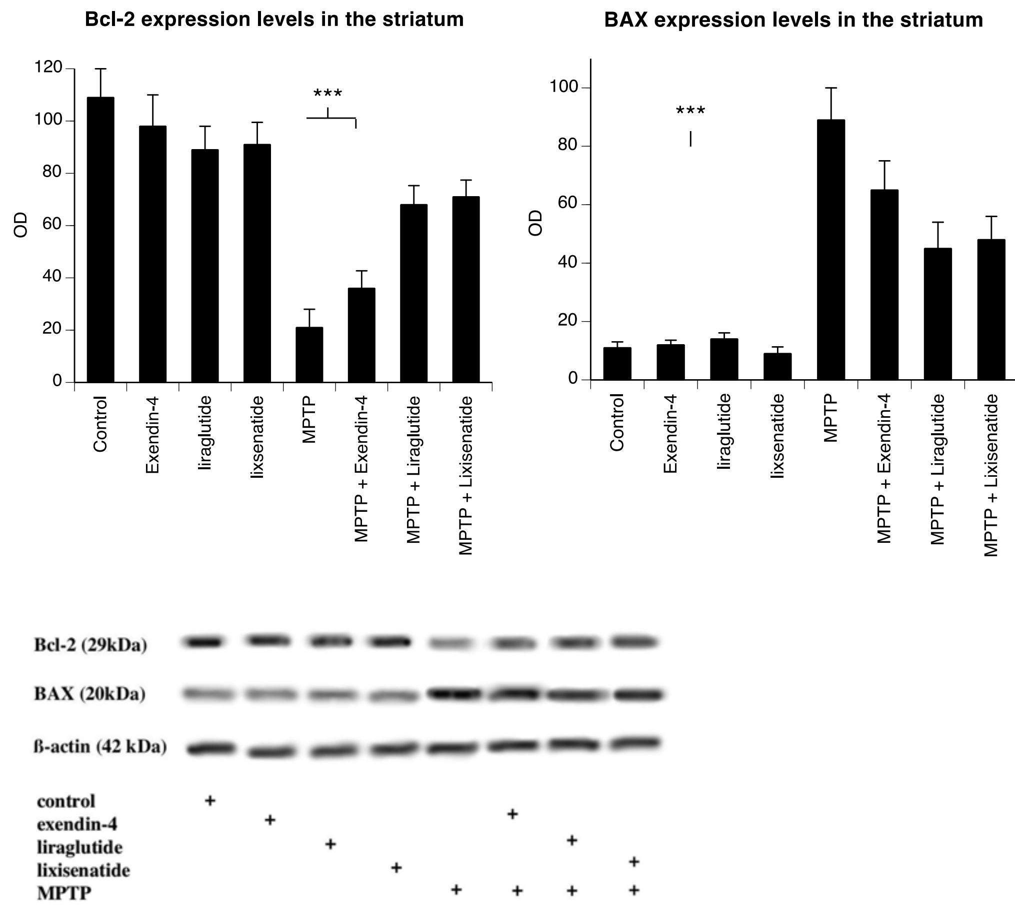


Fig. 7