Amino Acids (2012) 43:1359–1367
DOI 10.1007/s00726-011-1210-x

ORIGINAL ARTICLE

Molecular mechanism underlying the cerebral effect of Gly-Pro-Glu tripeptide bound to L-dopa in a Parkinson's animal model

Alba Minelli · C. Conte · I. Cacciatore · C. Cornacchia · F. Pinnen

Received: 8 September 2011 / Accepted: 22 December 2011 / Published online: 5 January 2012

Abstract Oxidative stress is a critical contributing factor to neurodegenerative disorders. Therefore, the inhibition of ROS formation, responsible for chronic detrimental neuroinflammation, is an important strategy for preventing the neurodegenerative disease and for neuroprotective therapy. Gly-Pro-Glu (GPE) is the N-terminal tripeptide of insulin-like growth factor-I, which is naturally cleaved in the plasma and brain tissues. GPE has neuroprotective effects since it crosses the blood–CSF and the functional CSF–brain barriers and binds to glial cells. It has been shown that GPE improves motor behaviour in rats after 6-OHDA lesion, although it does not rescue dopaminergic neurons. Thus, we hypothesized that the GPE therapeutic efficacy in a Parkinson model might be improved by combining GPE to L-dopa. Here, we used an animal model that represents a progressive chronic Parkinson's disease (PD) model, characterized by high levels of oxidative stress and inflammation. We showed that the co-drug, in which L-dopa is covalently linked to the GPE tripeptide, by down-regulating the expression of inflammatory genes, decreases the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced inflammatory response and, by up-regulating tyrosine hydroxylase, reduces MPTP-induced neurotoxicity. Furthermore, by determining the nuclear translocation/activation of Nrf2 and NF-κB, we showed that systemic administration of the co-drug activates Nrf2-induced antioxidant response while suppressing NF-κB inflammatory pathway. Data suggest that the binding of L-dopa to GPE tripeptide might represent a promising strategy to supply L-dopa to parkinsonian patients.

Keywords MPTP · PD animal model · LD-GPE · Nrf2 · NF-κB

Introduction

Gly-Pro-Glu (GPE) is the N-terminal tripeptide of insulin-like growth factor-I, which is naturally cleaved in the plasma and brain tissues (Sara et al. 1989; Batchelor et al. 2003; Baker et al. 2005; Sizonenko et al. 2001). It has long been known that GPE has neuroprotective effects and, that, following peripheral administration, crosses the blood–CSF and the functional CSF–brain barriers and binds to glial cells (Saura et al. 1999; Alexi et al. 1999; Guan et al. 1999, 2000, 2004; Sizonenko et al. 2001; Aguado-Llera et al. 2004; Baker et al. 2005; Zhao et al. 2005; Shapira et al. 2009). The effectiveness of central uptake of GPE was confirmed by the improvement of functional deficits after 6-OHDA lesion in rats (Krishnamurthi et al. 2004), by the neuroprotective effects in the CA1-2 sub-regions of the hippocampus of hypoxic–ischaemic injured rats (Shapira et al. 2009) and in the somatotropin release-inhibiting factor (SRIF) system of the temporal cortex of

Electronic supplementary material The online version of this article (doi:10.1007/s00726-011-1210-x) contains supplementary material, which is available to authorized users.

A. Minelli (✉) · C. Conte
Dipartimento Medicina Sperimentale Scienze Biochimiche, Sezione Biochimica Cellulare, Università di Perugia, Via del Giochetto, 06124 Perugia, Italy
e-mail: aminelli@unipg.it

I. Cacciatore · C. Cornacchia · F. Pinnen
Dipartimento di Scienze del Farmaco, Università "G. D'Annunzio", Via dei Vestini 31, 66100 Chieti, Italy

Aβ25-35-treated rats (Burgos-Ramos et al. 2009). Parkinson's disease (PD), a common neurodegenerative disorder with a complex aetiology, is characterized by a massive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) that is responsible for neurological sequelae (Shapira et al. 2009). Although the cause of selective dopaminergic neuronal degeneration in PD has still not been resolved, initiation and progression of the disease seem to be linked to oxidative stress, closely related to decreased mitochondrial function and ubiquitin proteasome system dysfunction (Branco et al. 2010). To date, 3,4-dihydroxyphenyl-L-alanine (L-dopa, LD) is the most effective medication for controlling PD symptoms, particularly those related to bradykinesia (Nagatsua and Sawadab 2009). Although still a controversial issue (Weiner 2006), LD itself may exacerbate PD via oxidative stress. Its auto-oxidative potential from a catechol to a quinone can generate reactive oxygen species (ROS) leading to neuronal damage and/or apoptotic or non-apoptotic cell death (Du et al. 2009). Therefore, the inhibition of oxidation of dopamine (DA) and the inhibition of ROS formation, both leading to chronic detrimental neuroinflammation, are important strategies for preventing the neurodegenerative disease and for neuroprotective therapy. The multitarget-directed drug design strategy has already been used to obtain promising multifunctional drugs for the treatment of PD and Alzheimer's disease (AD). Several dual acting drugs in which LD/DA are linked covalently to an antioxidant molecule provided sustained release in rat striatum and seemed to protect against the oxidative stress deriving from autoxidation of DA (Pinnen et al. 2007, 2009). We showed that *N*-acetyl-L-methionyl-L-dopa-methyl ester (Ac-Met-LD-OMe), a dual acting drug in which LD is linked to methionine, successfully spares LD-induced neuronal loss in a Parkinson animal model (Minelli et al. 2010). Given the fact that systemic GPE, although improving the long-term Parkinsonian motor deficit in 6-OHDA injured rats did not prevent the loss of dopamine neurons in the SNpc and striatum (Krishnamurthi et al. 2004), we hypothesized that a co-drug in which LD is covalently linked to GPE may represent a valuable therapeutic improvement in the delivery of LD to Parkinsonian patients. To address this issue, we examined the effect of LD-GPE on an in vivo progressive chronic PD animal model generated by chronic administration of low doses of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) that represents a preferential counterpart of idiopathic PD (Bezard et al. 1997a, b). By immunohistochemical analyses and studies of transcription factors activity, we showed that systemic administration of LD-GPE reduces the oxidative stress and the inflammatory response thus attenuating the factors underlying neurodegenerative disorders.

Materials and methods

Materials

All the reagents, unless otherwise stated, were from Sigma-Aldrich (St. Louis, MO, USA). All the antibodies, unless otherwise stated, were from Santa Cruz Biotech (Santa Cruz, CA, USA).

Synthesis of Ac-LD(Ac)2-Gly-Pro-Glu(OMe)-OMe (LD-GPE)

Triethanolamine (TEA) (1.14 ml, 8.17 mmol) and isobutyl chloroformate (IBCF) (1.08 ml, 8.17 mmol) were added under stirring to an ice-cold solution of Ac-LD$(Ac)_2$-OH (2.64 g, 8.17 mmol) in dry dimethylformamide (DMF). After 15 min at 15°C, H-Gly-Pro-Glu(OMe)-OMe (3.62 g, 8.17 mmol) in dry DMF was added to the mixture at 15°C with stirring. After 3 h at 0°C and 16 h at 5°C, the reaction mixture was filtered and the resulting solution was evaporated under vacuum. The residue was taken up in AcOEt and the organic layers were washed with 1 N $KHSO_4$, saturated aqueous $NaHCO_3$, and brine. The residue obtained after drying and evaporation was chromatographed on silica gel with $CHCl_3$/MeOH (97:3) as eluant to give 1.74 g of LD-GPE (1, MW 634.25). Structure and spectra of LD-GPE were determined by full analysis of the ^{1}H- and ^{13}C-NMR and mass spectral data; homogeneity was confirmed by TLC on silica gel Merck 60 F_{254}. Solutions were routinely dried over anhydrous sodium sulphate prior to evaporation. Chromatographic purifications were performed by Merck 60 70-230 mesh ASTM silica gel column. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian VXR 300-MHz spectrometer. Chemical shifts are reported in parts per million (δ) downfield from the internal standard tetramethylsilane (Me_4Si). The LC–MS/MS system consisted of an LCQ (Thermo Finnigan) ion trap mass spectrometer (San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. The capillary temperature was set at 300°C and the spray voltage at 4.25 kV. The fluid was nebulized using nitrogen (N_2) as both the sheath gas and the auxiliary gas.

Analytical data of LD-GPE: Synthesis yield 33%; $R_f = 0.22$, CHCl3/MeOH (97:3); 1H NMR (CDCl3) δ 1.90-2.12 (4H, m, Pro β-CH2 and Glu β-CH2), 2.01 (3H, s, Ac), 2.10–2.17 (2H, m, Glu γ-CH2), 2.25 and 2.28 (6H, 2× s, Ac), 2.29–2.40 (2H, m, Pro γ-CH2), 3.05–3.12 (2H, m, LD β-CH2), 3.31–3.50 (2H, m, Pro δ-CHA), 3.59–3.65 (2H, m, Pro δ-CHB), 3.61 (3H, s, OMe), 3.73 (3H, s, OMe), 3.80–4.10 (2H, m, Gly α-CH2), 4.49 (2H, m, Pro α-CH and Glu α-CH), 4.80 (1H, m, LD α-CH), 6.45 (1/2 H, t, LD NH), 6.61 (1/2 H, t, LD NH), 6.90–7.12 (4H, m, ArH and Gly NH), 7.30 (1/2 H, d, Glu NH), 7.35 (1/2 H, d, Glu

NH). 13C NMR (CDCl3) δ 20.83 and 20.87 (2× Ac), 23.14 (Ac), 24.93 (Pro β), 27.12 (Glu β), 28.26 (Pro γ), 30.31 (Glu γ), 37.77 (LD β), 42.25 (Gly α), 46.76 (Pro δ), 52.02 (OCH3), 52.27 (LD α), 52.78 (OCH3), 54.06 (Glu α), 60.37 (Pro α), 123.61–142.05 (Ar), 168.06, 168.64, 169.03, 170.80, 171.12, 171.28, 172.27, 173.66 (8× CO). MS (ESI) m/z 634 (M–H).

Animals

Two month-old male C57BL/6 mice (22–25 g) were housed at the Laboratory Animal Research Centre of Perugia University. The animals were fed ad libitum and maintained at a constant temperature of 24°C and 12-h light/dark cycle.

Ethics statement

All experimental procedures were carried out in accordance with European Directives, approved by the Institutional Animal Care and Use Committee of Perugia University (N33/2009). Efforts were made to minimise animal number, stress/discomfort.

Animal treatments

Progressive chronic PD animal model was generated with low doses of MPTP for 20 days (Bezard et al. 1997a, b; Schmidt and Ferger 2001). Thirty animals were divided in four groups as follows: (1) one group (6 animals) was treated with saline for 20 days (control), (2) one group (8 animals) was treated with daily (0900 hours) injections of MPTP hydrochloride (4 mg/kg, i.p., Toronto Research chemicals Inc., Canada) in saline for 20 days, (3) one group (8 animals) was treated with daily (0900 hours) injections of MPTP hydrochloride (4 mg/kg, i.p.) and LD (100 mg/kg, i.p), (4) one group (8 animals) was treated with daily (0900 hours) injections of MPTP hydrochloride (4 mg/kg, i.p.) and LD-GPE (254 mg/kg, i.p). Neither body weight changes nor mortality were observed during treatments. All the animals were killed at the end of the treatments. One hemisphere was processed for immunohistochemistry and the isolated striatum was used for other determinations.

Glutathione determination

Fresh striatum samples, washed in ice-cold isotonic saline solution, were homogenized with 0.6% sulfosalicylic acid/0.01 Triton X-100 on ice to remove proteins, and centrifuged at 10,000×g for 10 min. The supernatant was immediately used for glutathione (GSH) determination with dithionitrobenzoic acid (DTNB) at 412 nm (molar extinction coefficient 13.6 mM^{-1} cm^{-1}) as described (Rahman et al. 2006).

Real time PCR

Striatum RNA was isolated with TRIZOL Reagent (Invitrogen Ltd., Paisley, UK) according to the manufacturer's instructions and cDNA was synthesised using iScript cDNA synthesis kit (Bio-Rad Lab, Hercules, CA, USA) Real time PCR was performed using the iCycler iQ detection system (Bio-Rad) and SYBR Green chemistry. Mouse primer sequences, obtained from Invitrogen (Invitrogen Ltd., Paisley, UK) are listed in Table S1.

SYBR Green RT-PCR amplifications were carried out in a 96-well plate in a 25 µl reaction volume that contained 12.5 µl of 2× iQ™ SYBR® Green SuperMix (Bio-Rad), 400 nM forward and reverse primers, and 5–40 ng of cDNA. In each assay, no-template controls were included and each sample was run in triplicates. The thermal profile consisted of incubation at 95°C for 3 min, followed by 40 cycles of denaturation for 10 s at 95°C and an annealing/extension step of 30 s at 62°C. Mean of Ct values of the stimulated sample was compared to the untreated control sample. ΔCt is the difference in Ct values derived from the target gene (in each assayed sample) and GAPDH, while ΔΔCt represents the difference between the paired samples. The n-fold differential ratio was expressed as $2^{-\Delta\Delta Ct}$.

Tyrosine hydroxylase and glial fibrillar acidic protein immunohistochemistry

Immediately after killing, brains were removed and one hemisphere was immersion-fixed in Carnoy solution and embedded in paraffin. Coronal sections (10 µm) were obtained from the frontal to the caudal pole. Cerebral sections at defined levels were processed to assess immunoreactivity for TH and GFAP (Minelli et al. 2010). Briefly, sections were sequentially incubated with: (1) 20% normal horse serum in phosphate buffered saline (PBS) for 20 min; (2) anti-TH and anti-GFAP (DakoCytomation, Denmark) antibodies (1:100) in PBS containing 20% normal serum and 0.03% Triton X-100 overnight at 4°C; (3) biotinylated secondary antibody (1:40, Vector Laboratories, Burlington, Ontario, CA, USA) for 60 min; (4) avidin–biotin peroxidase complex (Vectastain ABC Kit, Vector Labs, Ontario, CA, USA) for 45 min. The reaction product was visualized by incubation with H_2O_2 and 3-3′-diaminobenzidine (DAB) and slices analysed by Olympus B×50 system microscope. Immunohistochemical reactivity was semi-quantified by analysing photomicrographs with ImageJ software (National Institute of Health).

Subcellular fractionation

Striatum samples were washed three times with PBS on ice, homogenised in PBS, and collected by centrifugation for 5 min at 500×*g*. Pellets were then processed with NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA) according to manufacturer's instruction. Protein concentrations of nuclear and cytoplasmic fractions were determined by Bio-Rad protein assay kit (Bio-Rad).

Western blotting analyses

Striatum nuclear/cytosolic extracts (60 μg) were loaded on 12% SDS-PAGE and protein levels determined by Western blotting using appropriate antibodies Nrf2 (C-20), NF-κB p65(A), inducible nitric oxide synthase (iNOS,C-11), 1:200; heme oxygenase 1 (HO-1, C-18), 1:50). Lamin B(H-90) and β-actin (I-19), antibodies (1:400) were used as marker proteins for nuclear/cytosolic extracts. After secondary incubation in horseradish peroxidase-conjugated anti IgG antibody (1:5,000) (Amersham Bioscience, Little Chalfont, UK), the immunocomplexes were visualized with an enhanced chemiluminescence kit (ECL, Pierce Biotechnology, Rockford, IL, USA). Bands were analysed with the Bandscan software.

Striatal dopamine and 1-methyl-4-phenylpyridinium levels

Striatal DA levels were individually determined by HPLC-EC assay, as described (Pinnen et al. 2007). 1-methyl-4-phenylpyridinium (MPP^+) levels were individually determined by HPLC–UV, as described (Andrews et al. 2005). Concentration of DA/MPP^+ were expressed as nanogram/milligram of striatum.

Statistical analysis

Each assay was performed at least in triplicate unless otherwise indicated. Data are expressed as mean values ± SD, and significance was assessed by a one-way ANOVA followed by Tukey's test. A P value <0.05 was considered significant in all cases.

Results

Chemistry

Ac-LD$(Ac)_2$-Gly-Pro-Glu(OMe)-OMe (LD-GPE, 1, MW 634.25) was synthesized by solution phase procedures through the coupling of Ac-LD$(Ac)_2$-OH (2) with H-Gly-Pro-Glu(OMe)-OMe (3) (Brimble et al. 2005). Compound 2 was obtained after N-acetylation of H-LD$(Ac)_2$-OH (Bodor et al. 1977; Dahmer and Whitesides 1989) (Fig. 1).

LD-GPE reduces MPTP neurotoxicity and gliosis

Systemic administration of MPTP to C57BL/6 mice and the subsequent degeneration of dopaminergic neurons in the substantia nigra can produce striking similarities to PD diagnosed in humans (Schmidt and Ferger 2001). The schedule of MPTP administration used in the study provides a progressive chronic PD model, successfully used for studies on neuroprotection and compensatory mechanisms (Schmidt and Ferger 2001). We analysed MPTP-induced neurotoxicity using TH immunoreactivity as a phenotypic marker for dopaminergic neurons (Fig. 2a). Immunostaining for TH-containing dopaminergic neurons in the midbrain of chronically treated animals showed a decline in TH-immunoreactivity. Semi-quantitative analyses showed that the significant reduction (55 ± 7%) in TH-density by the end of the chronic MPTP administration was attenuated by treatment with LD and LD-GPE (78 ± 9 vs. 89 ± 8%). Real time PCR analyses of the expression of TH gene showed that TH gene expression was restored to control value only by LD-GPE treatment (Fig. 2b). Given that MPTP administration causes a reactive gliosis (Innamorato et al. 2010), we verified whether LD-GPE was also capable of attenuating MPTP-inflammatory effects by GFAP immunoreactivity (Fig. 2a). Treatment with LD-GPE nearly restored the resting morphology. Real time PCR analyses of each striatum were used to quantify mRNA levels of astrocyte GFAP (glial fibrillar acidic protein), microglia Iba-1 (ionized calcium binding adaptor molecule 1), and brain macrophages CD11b (integrin α_M chain), as markers of inflammation (Rojo et al. 2010). LD-GPE treatment resulted in a marked down-regulation of the expression of GFAP and CD11b, showing a reduced

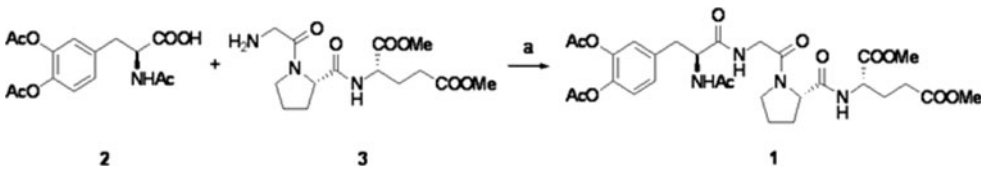


Fig. 1 Synthesis of LD-GPE. Reagents and conditions: Isobutyl chloroformate (IBCF), triethanolamine (TEA), and dimethylformamide (DMF), 3 h at 0°C, and then 15 h at 4°C

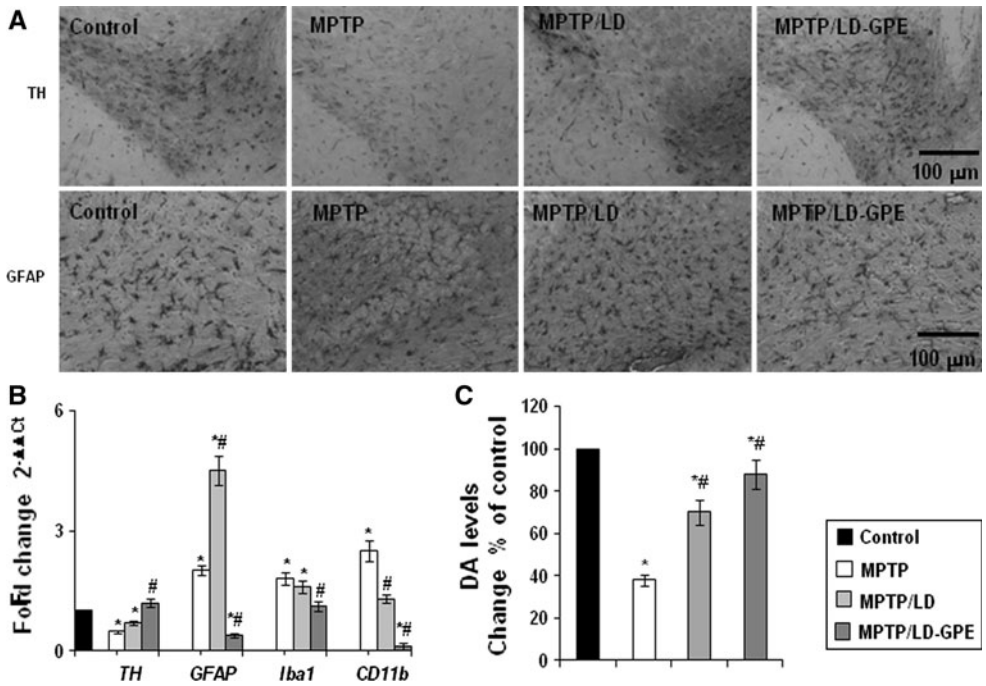


Fig. 2 LD-GPE protects from dopaminergic neuronal death in mice Parkinson's model. MPTP hydrochloride (4 mg/kg, dissolved in saline) was injected daily i.p. for 20 days to male mice (C57BL/6 mice, 8 weeks old). Animals were divided in four groups (6–8 mice) and treated with MPTP (4 mg/kg), MPTP plus LD (100 mg/kg), and MPTP plus LD-GPE (254 mg/kg). Control mice were treated with saline for 20 days and killed after the last injection. After killing, brains were sagittally divided and hemispheres used for immunohistochemical analyses. **a** TH- and GFAP-stained sections of brain. Strong decline in TH immunoreactivity was detected after MPTP treatment. Mice treated with LD-GPE showed an increased TH-density and glial resting morphology. One representative experiment is shown; **b** real time PCR analyses showing expression of TH and inflammatory genes in the isolated striatum from each animal. Changes in gene expression were normalized to GAPDH expression and presented as $2^{-\Delta\Delta Ct}$. Relative mRNA abundance of each gene in striatum of untreated animals was assumed to be 1.0 (control); **c** striatal DA levels determination. Each striatum was used for determinations by HPLC-EC. Dopamine levels are given as percentage of control (100% control: 15 ± 4 ng/mg of striatum). Data represent mean values ± SD of six/eight experiments. $^{*}P < 0.05$ versus control striata, $^{\#}P < 0.05$ versus MPTP-treated striata

inflammatory response to MPTP treatment. LD treatment did not significantly decrease GFAP and Iba-1 mRNA levels (Fig. 2b). Striatal dopamine confirmed that peripheral LD-GPE administration has central effects (Fig. 2c). MPP^{+} levels (2.24 ± 0.7 ng/mg of striatum) of MPTP-treated animals were not significantly modified by LD and LD-GPE treatment (2.10 ± 0.5 vs. 2.18 ± 0.6), showing that LD-GPE does not interfere with MPTP access.

LD-GPE increases Nrf2 nuclear translocation

GPE administration to Aβ25-35-treated rats is coincident with activation of protective pathways via cAMP response element-binding (CREB) activation (Burgos-Ramos et al. 2009). CREB is a transcription factor closely related to Nrf2 and NF-κB (Katoh et al. 2001; Zou and Crews 2006). To investigate whether and how LD-GPE interferes with the activation/suppression of Nrf2 and NF-κB, we analysed by Western blotting the nuclear translocation of these transcription factors, responsible for the integrated transcriptional responses to cellular stressors (Brigelius-Flohe

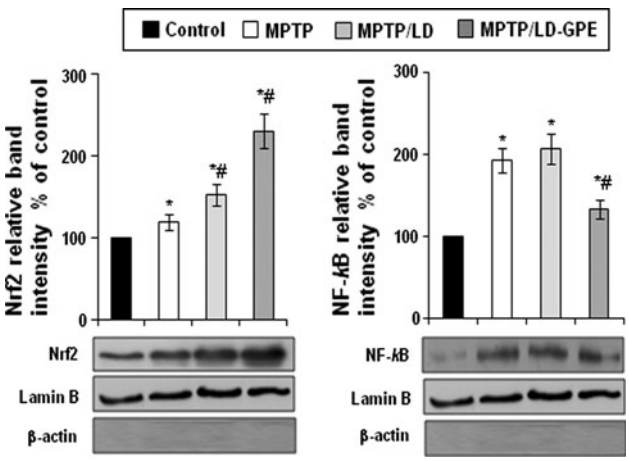


Fig. 3 LD-GPE increases Nrf2 nuclear translocation while reducing NF-κB. Nuclear extracts were obtained from striatum of each animal and used for Western blotting analysis. 60 µg of proteins were immunodetected with the indicated antibodies. Anti-lamin B and anti-β-actin antibodies were used as markers for nuclear and cytosolic extracts. One representative experiment is shown. Data represent mean values ± SD of six/eight experiments. $^{*}P < 0.05$ versus control striata, $^{\#}P < 0.05$ versus MPTP-treated striata

and Flohè 2011).We found that MPTP treatment resulted in a significantly increased NF-κB nuclear translocation accompanied by a modest Nrf2 translocation whereas LD-GPE treatment caused a marked translocation of Nrf2 while suppressing NF-κB. LD treatment did not significantly modify NF-κB nuclear translocation compared to MPTP treatment, although increasing the nuclear translocation of Nrf2, confirming that LD interferes with cellular redox homeostasis (Fig. 3). Analyses of gene expression by real time PCR confirmed the suppression of the NF-κB signalling and the activation of the Nrf2-antioxidant response by LD-GPE treatment (Fig. 4a). We found a significant up-regulation of the expression of Nrf2-driven genes, responsible for endogenous cellular defence and a down-regulation of NF-κB-driven genes, responsible for inflammatory responses. These findings indicate that the beneficial effects of LD-GPE depend on the simultaneous regulation of Nrf2 and NF-κB within striatum. We next decided to assess the efficiency of the transcriptional regulation of these two transcription factors, which, in addition to the nuclear translocation of protein components, is of paramount importance for the efficiency of the NF-κB and Nrf2 systems (Salminen et al. 2008). Determination of protein expression levels of heme oxygenase 1 (HO-1) and inducible nitric oxide synthase (iNOS) confirmed the efficiency of the transcriptional regulation of these transcription factors (Fig. 4b). Striatal glutathione (GSH) level determinations were compatible with the efficacy of the co-drug treatment that restored control GSH levels (Fig. 4c).

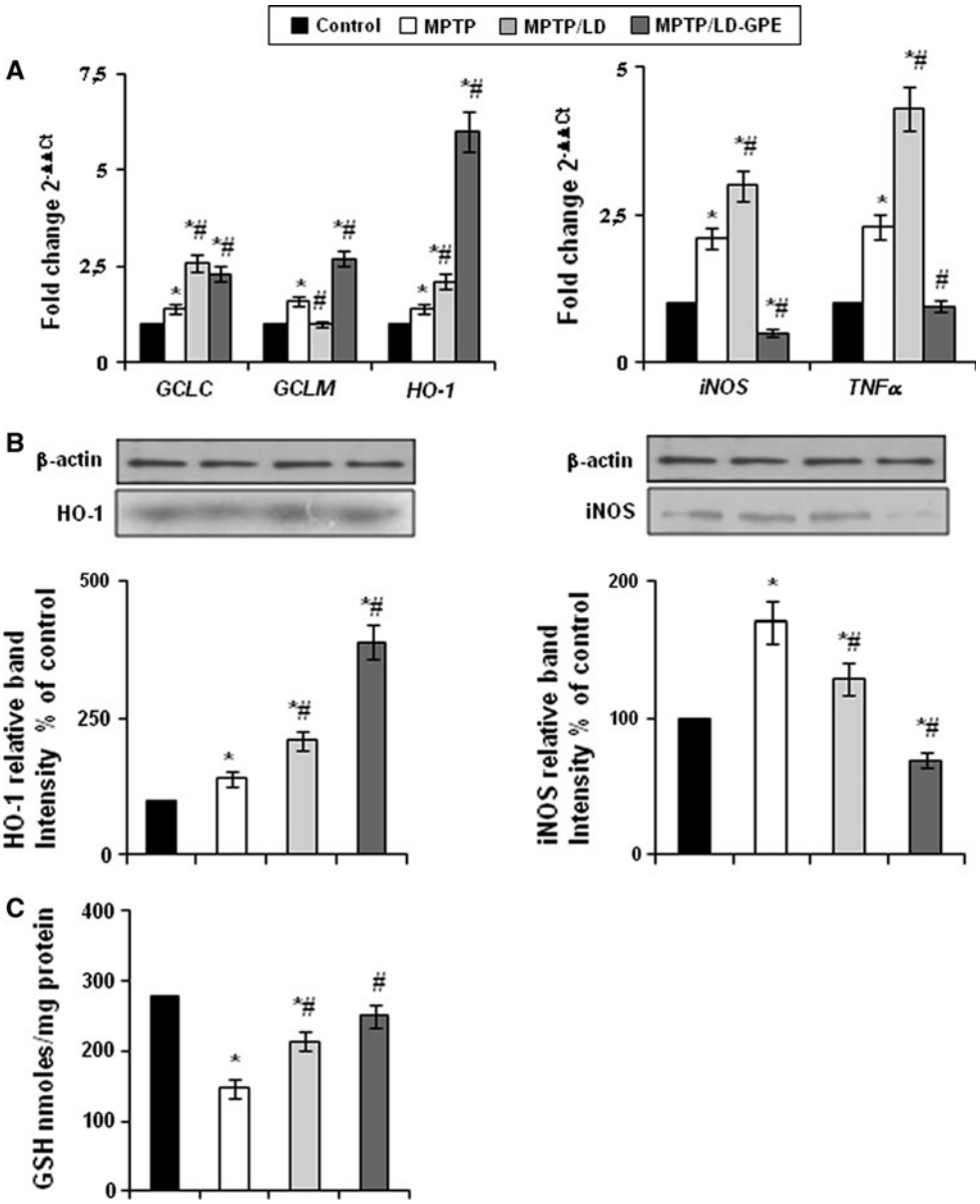
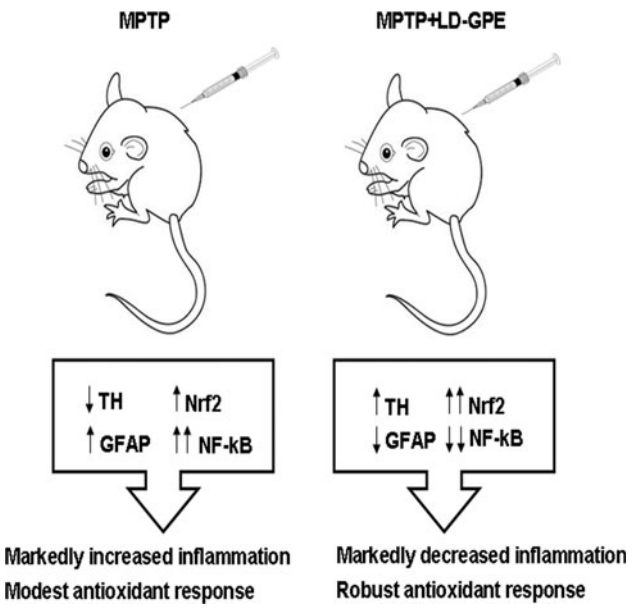


Fig. 4 LD-GPE up-regulates Nrf2-antioxidant while suppressing NF-κB-pro-inflammatory genes. **a** Real time PCR analyses of mRNA level of Nrf2- and NF-κB-driven genes. Changes in gene expression were normalized to GAPDH expression and presented as $2^{-\Delta\Delta Ct}$. Relative mRNA abundance of each gene in untreated striata was assumed to be 1.0 (control); **b** Western blotting analyses of HO-1 and iNOS. 60 μg of cytosolic extract were immunodetected with the indicated antibodies and β-actin used as loading control. One representative experiment is shown; **c** striatum GSH content (100% control GSH: 278 ± 17 nmol/mg protein). Data represent mean values ± SD from six/eight animal per group from two separate experiments. $^{*}P < 0.05$ versus control striata; $^{\#}P < 0.05$ versus MPTP-treated striata

Discussion

Two major observations are reported in this study. First, we showed that the new dual acting drug LD-GPE has potential application in PD therapy, as confirmed by immunohistochemical and real time PCR analyses; second, by analysing the mechanism underlying the beneficial effects of LD-GPE, we showed the simultaneous regulation of Nrf2 and NF-κB, and, more importantly, the cellular consequences of such co-regulation.

Parkinson's disease is the most common neurodegenerative movement disorder whose symptoms, in the beginning, can be successfully treated with LD (Schapira 2009; Nagatsua and Sawadab 2009). In PD, loss of striatal dopaminergic terminals and degeneration of DA neurons in the substantia nigra (SN) are associated with glial reactions that, in turn, cause an inflammation-derived oxidative stress. LD and its metabolite dopamine (DA) can enhance oxidative stress and accelerate the degenerative process of residual cells in patients with PD (Weiner 2006; Hattoria et al. 2009). LD itself may contribute to the progression of PD via oxidative stress: its auto-oxidative potential from a catechol to a quinone can generate reactive oxygen species (ROS) leading to neuronal damage and/or apoptotic or non-apoptotic cell death (Du et al. 2009). Therefore, activation of antioxidant responses and inhibition of inflammatory/apoptotic pathways are important strategies for preventing the neurodegenerative disease and for neuroprotective therapy. Here, LD was bound to GPE, the N-terminal tripeptide of insulin-like growth factor-I. Data from the literature (Sizonenko et al. 2001; Saura et al. 1999; Alexi et al. 1999; Guan et al. 1999, 2000, 2004; Aguado-Llera et al. 2004; Zhao et al. 2005; Shapira et al. 2009; Krishnamurthi et al. 2004; Burgos-Ramos et al. 2009) indicate that peripheral GPE administration can have central neuroprotective effect, even though it does not prevent the loss of tyrosine hydroxylase in the SNpc and the striatum (Krishnamurthi et al. 2004). Thus, we investigated the therapeutic efficacy of LD-GPE in a progressive chronic PD animal model. We found that the toxicity of MPTP treatment was significantly reduced by LD-GPE by immunohistochemical and real time PCR analyses. mRNA levels of TH and inflammation marker genes were restored to control values by the co-drug. The marked anti-inflammatory effect of LD-GPE was not unexpected since GPE, by binding to glial cells, leads to a reduced release of neurotoxic products (Baker et al. 2005; Sizonenko et al. 2001; Guan et al. 2004). GPE administration to Aβ25-35-treated rats activates protective pathways via CREB activation (Burgos-Ramos et al. 2009), which, in turn, is closely related to Nrf2 and NF-κB modulation (Katoh et al. 2001; Zou and Crews 2006). The interplay between Nrf2 and NF-κB is responsible for cellular responses to chemical and inflammatory stressors (Brigelius-Flohe and Flohè 2011). In this study, by investigating in our animal models the role of these transcription factors and their cellular outcomes, we showed that both transcription factors sense and respond to cell stress and that their simultaneous regulation dictates the cellular response. Striata of animals treated with MPTP showed a marked nuclear translocation/activation of NF-κB and a more moderate increase in Nrf2 nuclear levels, resulting in a significant up-regulation of NF-κB-driven genes and a modest induction of Nrf2-driven genes. This mutual modulation resulted in a marked cell loss in the MPTP treatment (Bezard et al. 1997a, b). Treatment with LD-GPE suppressed NF-κB signalling while strongly activating Nrf2, confirming that the induction of Nrf2-mediated antioxidant enzymes and the reduction of NF-κB-mediated inflammatory mediators/enzymes exert beneficial effects (Jung et al. 2010; Minelli et al. 2009). In LD-GPE-treated animals, glial reactivity was reduced while striatum GSH levels and HO-1 expression/protein levels restored/increased, resulting in a neuroprotective effect. Nrf2 activation and HO-1 activity contribute to the anti-inflammatory activity of cells and tissues (Innamorato et al. 2010; Rojo et al. 2010; Chen et al. 2009; Jadhav et al. 2008; Lee and Chau 2002; Jung et al. 2001), thus an increased expression of antioxidant genes via Nrf2 activation might be a feasible therapeutic target to reduce neuronal death and inflammation in PD patients. Here we provided the first evidence that the dual acting drug LD-GPE induces the expression of *HO-1* via Nrf2 activation in animal striatum while suppressing the expression of NF-κB-driven genes (Scheme 1) thus



Scheme 1 LD-GPE is neuroprotective against MPTP neurotoxicity in mice via Nrf2/NF-κB modulation

holding great potential for the therapeutic strategies to PD treatment. Indeed, although in our study the co-drug was administered with the neurotoxin, a common pitfall of animal models, our results showed a therapeutic superiority of LD-GPE over LD, which, to date, is the most effective medication for controlling PD symptoms. We also showed an inverse correlation between Nrf2 and NF-κB, not found in the LD treated animals, that might explain the observed reduction in glial activation, thus supporting the proposal that increased expression of antioxidant genes via Nrf2 activation might be a feasible therapeutic target to reduce neuronal death and inflammation in PD patients.

Acknowledgments The authors thank Mary Kerrigan (MA, Cantab) for helpful linguistic suggestions. The study was funded by Fondazione Cassa di Risparmio, Perugia, Italy (Grant 2009-010-0437). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of interest The authors declare no conflict of interest.

References

Aguado-Llera D, Martín-Martínez M, García-López MT, Arilla-Ferreiro E, Barrios V (2004) Gly-Pro-Glu protects beta-amyloid-induced somatostatin depletion in the rat cortex. Neuroreport 15:1979–1982

Alexi T, Hughes PE, van Roon-Mom WM, Faull RL, Williams CE, Clark RG, Gluckman PD (1999) The IGF-I amino-terminal tripeptide glycine-proline-glutamate (GPE) is neuroprotective to striatum in the quinolinic acid lesion animal model of Huntington's disease. Exp Neurol 159:84–97

Andrews ZB, Horvath B, Barnstable CJ, Elsworth J, Yang L, Beal MF, Roth RH, Matthews RT, Horvath TL (2005) Uncoupling protein-2 is critical for nigral dopamine cell survival in a mouse model of Parkinson's disease. J Neurosci 25:184–191

Baker AM, Batchelor DC, Thomas GB, Wen JY, Rafiee M, Lin H, Guan J (2005) Central penetration and stability of N-terminal tripeptide of insulin-like growth factor-I, glycine–proline–glutamate in adult rat. Neuropeptides 39:81–87

Batchelor DC, Lin H, Wen JY, Keven C, Van Zijl PL, Breier BH, Gluckman PD, Thomas GB (2003) Pharmacokinetics of glycine–proline–glutamate, the N-terminal tripeptide of insulin-like growth factor-1, in rats. Anal Biochem 323:156–163

Bezard E, Dovero S, Bioulac B, Gross CE (1997a) Effects of different schedules of MPTP administration on dopaminergic neurodegeneration in mice. Exp Neurol 148:288–292

Bezard E, Dovero S, Bioulac B, Gross CE (1997b) Kinetics of nigral degeneration in a chronic model of MPTP-treated mice. Neurosci Lett 234:47–50

Bodor N, Sloan KB, Higuchi T, Sasahara K (1977) Improved delivery through biological membranes. 4 Prodrugs of L-dopa. J Med Chem 20:1435–1445

Branco DM, Arduino DM, Esteves AR, Silva DF, Cardoso SM, Oliveira CR (2010) Cross-talk between mitochondria and proteasome in Parkinson's disease pathogenesis. Front Aging Neurosci 2:17. doi:10.3389/fnagi.2010.00017

Brigelius-Flohe R, Flohé L (2011) Basic principles and emerging concepts in the redox control of transcription factors. Antioxid Redox Signal. doi:10.1089/ars.2010.3534

Brimble MA, Trotter NS, Harris PW, Sieg F (2005) Synthesis and pharmacological evaluation of side chain modified glutamic acid analogues of the neuroprotective agent glycyl-L-prolyl-L-glutamic acid (GPE). Bioorg Med Chem 13:519–532

Burgos-Ramos E, Martos-Moreno GA, López MG, Herranz R, Aguado-Llera D, Egea J, Frechilla D, Cenarruzabeitia E, León R, Arilla-Ferreiro E, Argente J, Barrios V (2009) The N-terminal tripeptide of insulin-like growth factor-I protects against beta-amyloid-induced somatostatin depletion by calcium and glycogen synthase kinase 3 beta modulation. J Neurochem 109:360–370

Chen PC, Vargas MR, Pani AK, Smeyne RJ, Johnson DA, Kan YW, Johnson JA (2009) Nrf2-mediated neuroprotection in the MPTP mouse model of Parkinson's disease: critical role for the astrocyte. Proc Natl Acad Sci USA 106:2933–2938

Dahmer J, Whitesides GM (1989) Kinetic resolution of unnatural and rarely occurring amino acids: enantioselective hydrolysis of *N*-acyl amino acids catalyzed by acylase I. J Am Soc 111:6354–6364

Du F, Qian ZM, Zhu L, Wu XM, Yung WH, Tsim TY, Ke Y (2009) L-dopa neurotoxicity is mediated by up-regulation of DMT1-IRE expression. PLoS One 4:e4593

Guan J, Waldvogel HJ, Faull RL, Gluckman PD, Williams CE (1999) The effects of the N-terminal tripeptide of insulin-like growth factor-1, glycine–proline–glutamate in different regions following hypoxic-ischemic brain injury in adult rats. Neuroscience 89:649–659

Guan J, Krishnamurthi R, Waldvogel HJ, Faull RL, Clark R, Gluckman P (2000) N-terminal tripeptide of IGF-1 (GPE) prevents the loss of TH positive neurons after 6-OHDA induced nigral lesion in rats. Brain Res 859:286–292

Guan J, Thomas GB, Lin H, Mathai S, Bachelor DC, George S, Gluckman PD (2004) Neuroprotective effects of the N-terminal tripeptide of insulin-like growth factor-1, glycine–proline–glutamate (GPE) following intravenous infusion in hypoxic–ischemic adult rats. Neuropharmacology 47:892–903

Hattoria N, Wanga M, Taka H, Fujimura T, Yoritaka A, Kubo S, Mochizuki H (2009) Toxic effects of dopamine metabolism in Parkinson's disease. Parkinsonism Relat Disord 15:S35–S38

Innamorato NG, Jazwa A, Rojo AI, García C, Fernández-Ruiz J, Grochot-Przeczek A, Stachurska A, Jozkowicz A, Dulak J, Cuadrado A (2010) Different susceptibility to the Parkinson's toxin MPTP in mice lacking the redox master regulator Nrf2 or its target gene heme oxygenase-1. PLoS One 5:e11838

Jadhav A, Torlakovic E, Ndisang JF (2008) Interaction among heme oxygenase, nuclear factor-kappaB, and transcription activating factors in cardiac hypertrophy in hypertension. Hypertension 52:910–917

Jung KH, Hong SW, Zheng HM, Lee HS, Lee H, Lee DH, Lee SY, Hong SS (2001) Targeted expression of heme oxygenase-1 prevents the pulmonary inflammatory and vascular responses to hypoxia. Proc Natl Acad Sci USA 98:8798–8803

Jung KH, Hong SW, Zheng HM, Lee HS, Lee H, Lee DH, Lee SY, Hong SS (2010) Melatonin ameliorates cerulein-induced pancreatitis by the modulation of nuclear erythroid 2-related factor 2 and nuclear factor-kappaB in rats. J Pineal Res 48:239–250

Katoh Y, Itoh K, Yoshida E, Miyagishi M, Fukamizu A, Yamamoto M (2001) Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. Genes Cells 6:857–868

Krishnamurthi R, Stott S, Maingay M, Faull RL, McCarthy D, Gluckman P, Guan J (2004) N-terminal tripeptide of IGF-1 improves functional deficits after 6-OHDA lesion in rats. Neuroreport 15:1601–1604

Lee TS, Chau LY (2002) Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. Nat Med 8:240–246

Minelli A, Conte C, Grottelli S, Bellezza I, Emiliani C, Bolaños JP (2009) Cyclo(His-Pro) up-regulates heme oxygenase 1 via activation of Nrf2-ARE signalling. J Neurochem 11:956–966

Minelli A, Conte C, Prudenzi E, Cacciatore I, Cornacchia C, Taha E, Pinnen F (2010) *N*-acetyl-L-methionyl-L-dopa-methyl ester as a dual acting drug that relieves L-dopa-induced oxidative toxicity. Free Radic Biol Med 49:31–39

Nagatsua T, Sawadab M (2009) L-dopa therapy for Parkinson's disease: past, present, and future. Parkinsonism Relat Disord 15(Suppl 1):S3–S8

Pinnen F, Cacciatore I, Cornacchia C, Sozio P, Iannitelli A, Costa M, Pecci L, Nasuti C, Cantalamessa F, Di Stefano A (2007) Synthesis and study of L-dopa-glutathione codrugs as new anti-Parkinson agents with free radical scavenging properties. J Med Chem 50:2506–2510

Pinnen F, Cacciatore I, Cornacchia C, Sozio P, Cerasa LS, Iannitelli A, Nasuti C, Cantalamessa F, Sekar D, Gabbianelli R, Falcioni ML, Di Stefano A (2009) Codrugs linking L-dopa and sulfur-containing antioxidants: new pharmacological tools against Parkinson's disease. J Med Chem 52:559–563

Rahman I, Kode A, Biswas SK (2006) Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. Nat Protoc 1:3159–3165

Rojo AI, Innamorato NG, Martin-Moreno AM, De Ceballos ML, Yamamoto M, Cuadrado A (2010) Nrf2 regulates microglial dynamics and neuroinflammation in experimental Parkinson's disease. Glia 58:588–598

Salminen A, Huuskonen J, Ojala J, Kauppinen A, Kaarniranta K, Suuronen T (2008) Activation of innate immunity system during aging: NF-kB signaling is the molecular culprit of inflamm-aging. Ageing Res Rev 7:83–105

Sara VR, Carlsson-Skwirut C, Bergman T, Jörnvall H, Roberts PJ, Crawford M, Håkansson LN, Civalero I, Nordberg A (1989) Identification of Gly-Pro-Glu (GPE), the aminoterminal tripeptide of insulin-like growth factor 1 which is truncated in brain, as a novel neuroactive peptide. Biochem Biophys Res Commun 165:766–771

Saura J, Curatolo L, Williams CE, Gatti S, Benatti L, Peeters C, Guan J, Dragunow M, Post C, Faull RL, Gluckman PD, Skinner SJ (1999) Neuroprotective effects of Gly-Pro-Glu, the N-terminal tripeptide of IGF-1, in the hippocampus in vitro. Neuroreport 10:161–164

Schapira AH (2009) Etiology and pathogenesis of Parkinson disease. Neurol Clin 27:583–603

Schmidt N, Ferger B (2001) Neurochemical findings in the MPTP model of Parkinson's disease. J Neural Transm 108:1263–1282

Shapira S, Mathai S, Zhang R, Guan J (2009) Delayed peripheral administration of the N-terminal tripeptide of IGF-1 (GPE) reduces brain damage following microsèhere induced embolic damage in young adult and aged rats. Neurosci Lett 454:53–57

Sizonenko SV, Sirimanne ES, Williams CE, Gluckman PD (2001) Neuroprotective effects of the N-terminal tripeptide of IGF-1, glycine-proline-glutamate, in the immature rat brain after hypoxic-ischemic injury. Brain Res 922:42–50

Weiner WJ (2006) Levodopa-toxic or neuroprotective? Nat Clin Pract Neurol 2:518–519

Zhao X, Liu SJ, Zhang J, Strong R, Aronowski J, Grotta JC (2005) Combining insulin-like growth factor derivatives plus caffeinol produces robust neuroprotection after stroke in rats. Stroke 36:129–134

Zou J, Crews F (2006) CREB and NF-kappaB transcription factors regulate sensitivity to excitotoxic and oxidative stress induced neuronal cell death. Cell Mol Neurobiol 26:385–405