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Tauroursodeoxycholic Acid Prevents MPTP-Induced Dopaminergic Cell Death in a Mouse Model of Parkinson's Disease

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Abstract Mitochondrial dysfunction and oxidative stress are implicated in the neurodegenerative process in Parkinson's disease (PD). Moreover, c-Jun N-terminal kinase (JNK) plays an important role in dopaminergic neuronal death in substantia nigra pars compacta. Tauroursodeoxycholic acid (TUDCA) acts as a mitochondrial stabilizer and anti-apoptotic agent in several models of neurodegenerative diseases. Here, we investigated the role of TUDCA in preventing 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurodegeneration in a mouse model of PD. We evaluated whether TUDCA modulates MPTP-induced degeneration of dopaminergic neurons in the nigrostriatal axis, and if that can be explained by regulation of JNK phosphorylation, reactive oxygen species (ROS) production, glutathione *S*-transferase (GST) catalytic activation, and Akt signaling, using C57BL/6 glutathione *S*-transferase pi (GSTP) null mice. TUDCA efficiently protected against MPTP-induced dopaminergic degeneration. We have previously demonstrated that exacerbated JNK activation in GSTP null mice resulted in increased susceptibility to MPTP neurotoxicity. Interestingly, pre-treatment with TUDCA prevented MPTP-induced JNK phosphorylation in mouse midbrain and striatum. Moreover, the anti-oxidative role of TUDCA was demonstrated in vivo by impairment of ROS production in the presence of MPTP. Finally, results herein suggest that the survival pathway activated by TUDCA involves Akt signaling, including downstream Bad phosphorylation and NF-κB activation. We conclude that TUDCA is neuroprotective in an in vivo model of PD, acting mainly by modulation of JNK activity

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and cellular redox thresholds, together with activation of the Akt pro-survival pathway. These results open new perspectives for the pharmacological use of TUDCA, as a modulator of neurodegeneration in PD.

Keywords Glutathione *S*-transferase pi · Tauroursodeoxycholic acid · 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine · Reactive oxygen species · c-Jun N-terminal kinase · Heat shock protein 27

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by cardinal symptoms (resting tremor, rigidity, postural instability, and bradykinesia) caused by the relentless loss of nigrostriatal dopaminergic neurons [1, 2]. Although PD pathogenesis is still not completely understood, both environmental and genetic factors are thought to play important roles. Several possible mechanisms leading to dopaminergic neurodegeneration in PD have been proposed, including mitochondrial complex I dysfunction, impairment of ATP production, oxidative stress, neuroinflammation, and aberrant proteolytic degradation [3]. In fact, an important trigger of neuronal death in PD is the impairment of mitochondrial function, which may lead to downstream increase in reactive oxygen species (ROS), inflammatory responses, and activation of cell death pathways [4–6].

The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin constitutes the best-characterized toxin paradigm for PD, faithfully replicating most of its clinical and pathological hallmarks in humans and animal models [1]. MPTP and its toxic metabolite, 1-methyl-4-phenylpyridinium (MPP^+), used extensively in in vitro and in vivo models of PD, cause degeneration of nigrostriatal dopaminergic neurons mediated via ROS generation, mitochondrial membrane potential depolarization, and activation of cell death signaling pathways [1, 2, 7, 8].

The c-Jun N-terminal kinase (JNK) pathway constitutes a central stress activated response implicated in the pathogenesis of PD [9]. Indeed, increased levels of phosphorylated JNK and its downstream target c-Jun were found in brains from PD patients [10, 11], and JNK is active in degenerating dopaminergic neurons in the substantia nigra pars compacta (SNpc) of MPTP-treated mice [11–14]. In accordance with this, JNK null mice are resistant to MPTP neurotoxicity, and selective JNK inhibitors were also shown to protect against MPTP-induced neurodegeneration in the nigrostriatal dopaminergic neurons in vivo [11, 15, 16]. Indeed, we have recently demonstrated that mice lacking glutathione *S*-transferase pi (GSTP), an endogenous JNK inhibitor, are more susceptible to MPTP toxicity [14].

Tauroursodeoxycholic acid (TUDCA) is an endogenous bile acid synthesized in the liver conjugation pathway of ursodeoxycholic acid (UDCA). The unconjugated parent molecule is a FDA-approved medical treatment for primary biliary cirrhosis [17]. Several reports have demonstrated that TUDCA is an effective inhibitor of apoptosis through different cellular mechanisms, namely by interfering with the mitochondrial pathway of cell death [18], as well as with targets upstream of mitochondria, including cell cycle related proteins E2F-1 and p53 [19] and endoplasmic reticulum stress [20]. Importantly, TUDCA was also shown to efficiently modulate the activation of the JNK pathway in Aβ-treated PC12 neuronal cells [21]. Indeed, TUDCA has been shown to exhibit antioxidant properties, maintain mitochondrial membrane potential, and prevent cytochrome *c* release, Bax translocation, caspase activation, and apoptosis in hepatocytes exposed to apoptotic stimuli [18, 22, 23]. Growing evidence also point to an important protective role of TUDCA in both in vitro and in vivo models of neurologic disorders, such as Huntington's disease [24, 25], Alzheimer's disease [26–30], and stroke [31, 32]. Additionally, TUDCA was demonstrated to have a beneficial role in survival and function of nigral transplant in a rat model of PD [33] and also to partially rescue mitochondrial dysfunction in genetic PD models in *Caenorhabditis elegans* [34]. Importantly, TUDCA is an endogenous molecule and a central nervous system penetrating agent with no associated toxicity [25, 31].

In this study, we used GSTP null mice to investigate the neuroprotective role of TUDCA against MPTP toxicity. Interestingly, we show that pre-treatment of mice with TUDCA significantly protected against MPTP-induced dopaminergic cell loss in the nigrostriatal axis. The mechanisms through which TUDCA exerts its neuroprotective actions involved mainly the modulation of JNK activity and cellular redox thresholds, together with activation of the Akt pro-survival pathway.

Materials and Methods

Materials

Mouse anti-tyrosine hydroxylase (TH) and mouse anti-β-actin antibodies, MPTP, TUDCA, Hoechst 33258, the GST assay kit, 2′,7′-dichlorofluorescein diacetate (DCF-DA), and amido black were purchased from Sigma Chemical Co (St Louis, MO, USA). Alexa Fluor 488 goat anti-mouse antibody was from Invitrogen/Molecular Probes (Eugene, OR, USA). Rabbit anti-p-Bad (Ser 136) and rabbit anti-IκB-α antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-heat shock protein 27 (Hsp27) was purchased from R&D Systems (Minneapolis,

MN, USA), whereas rabbit anti-JNK and mouse anti-p-JNK (Thr183/Tyr185) antibodies were from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were from Cell Signaling Technology and Promega (Madison, USA), respectively. The DakoCytomation Fluorescent Mounting Medium was from Dako (Copenhagen, Denmark). The bicinchoninic acid/copper (II) sulfate protein assay kit was from Pierce (IL, USA), and the Complete Mini protease inhibitors cocktail was obtained from Roche Diagnostics (Penzberg, Germany). Immobilon P, PVDF membrane was from Millipore (Bedford, MA, USA). ECL and Hyperfilm ECL were purchased from Amersham Biosciences (Piscataway, NJ, USA). Other chemicals and reagents were of the highest analytical grade and were purchased from local commercial sources.

Animals and Treatment

All procedures were carried out in accordance with the National Institutes of Health guidelines for the care and use of animals, and were approved by the local Institutional Animal Care and Use Committee.

C57BL/6 *Gstp1/p2* null mice (Cancer Research UK) were re-derived and maintained at the Gulbenkian Institute of Science Animal House (Oeiras, Portugal). The work we report here his based on this double-knockout line since in the mouse both Gstp genes (*Gstp1* and *Gstp2*) are arranged in tandem on chromosome 1 and were deleted by homologous recombination [35]. Throughout the text and figures, this knockout mice line will be referred to as GSTP null. The animals were housed under standardized conditions on a 12-h light/dark cycle with free access to a standard diet and water ad libitum.

TUDCA was dissolved in phosphate buffer saline (PBS), pH 7.4, and was intra-peritoneally (i.p.) injected. TUDCA daily administration (50 mg/kg body weight) began on day 0, followed by i.p. administration of MPTP at a single dose (40 mg/kg body weight) on day 3, 6 h after the last TUDCA injection [24, 36].

Control mice received saline alone instead of TUDCA and/or MPTP. Another set of controls included mice treated with TUDCA only. Samples from saline-treated and TUDCA-treated mice were collected prior to MPTP injection. The schematic chronogram of TUDCA and MPTP administration is illustrated in Fig. 1.

The time course studies were carried out in three independent experiments (n=3) with groups of three to six mice.

Immunohistochemistry

Mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and transcardially perfused with ice-cold PBS, followed by

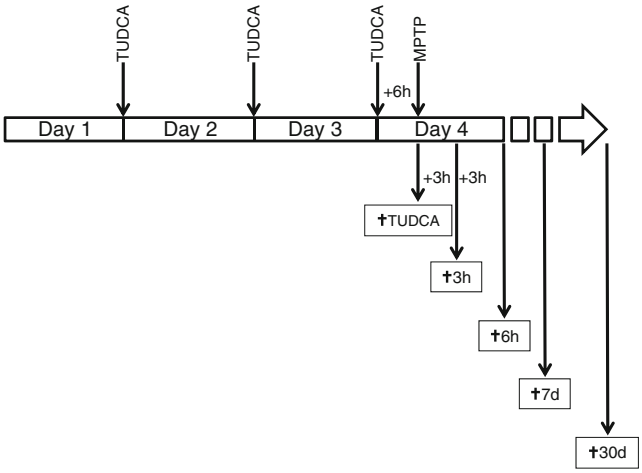

Fig. 1 Schematic representation of the treatment course. C57BL/6 GSTP null mice were i.p. injected with TUDCA (50 mg/kg body weight) for three consecutive days. MPTP (40 mg/kg body weight) single dose i.p. injection was administered 6 h after the last TUDCA injection. Samples for Western blotting were taken 6 h after the last TUDCA injection, or 3 and 6 h after MPTP administration. Mice selected for immunohistochemistry were sacrificed 7 days and 30 days after the last MPTP injection

4% paraformaldehyde. After perfusion, brains were quickly removed and fixed by immersion, at 4°C for 24 h, in a solution containing 85 ml of 2% paraformaldehyde and 15 ml of saturated picric acid per 100 ml of fixative. After rinsing in several changes of PBS containing 15% sucrose and 0.1% sodium azide, brains were processed for cryostat sectioning. Cryostat coronal sections (14 μm thick) were permeabilized with 0.2% Triton X-100 in PBS for 30 min, and then pre-treated with blocking solution [2% bovine serum albumin (BSA), 0.05% Tween-20 in PBS] for 1 h at room temperature. Incubation with anti-TH primary antibody (1:250) was performed overnight at 4°C. After extensive rinsing in PBS, the sections were incubated with Alexa Fluor 488 goat anti-mouse secondary antibody for 1 h at room temperature. Finally, sections were rinsed with PBS, mounted in fluorescent mounting medium containing 5 μg/ml Hoechst 33258, observed under an Axioskop microscope (Carl Zeiss) with an attached Leica DFC490 camera, and photographed using Image Manager 50 software (Leica Microsystems, Inc.). The specificity of the primary antibodies used was previously confirmed by Western blot analysis. Control experiments for non-specific binding were performed in parallel by omission of the primary antibody.

The number of nigral TH-positive cells was counted in four adjacent sections, using three animals per experiment, and averaged.

Western Blot Analysis

After being anesthetized with sodium pentobarbital (50 mg/kg, i.p.), mice were decapitated and brains were quickly removed and placed in ice-cold freshly made PBS. The entire midbrain

region, containing the SNpc, and the whole striatum were dissected as previously described [36]. Dissected mice midbrains (containing the SNpc) and striata were homogenized in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris–HCl, 1% NP40, pH 8) plus Complete Mini protease inhibitors cocktail. After sonication five times for 5 s each, on ice, samples were centrifuged 15,000×*g* for 15 min and the supernatant analyzed for protein concentration using the bicinchoninic acid/copper (II) sulfate protein assay kit. Tissue extracts were added (1:1) to denaturing buffer (0.25 mM Tris–HCl, pH 6.8, 4% SDS, 40% glycerol, 0.2% bromophenol blue, 1% β-mercaptoethanol), boiled for 5 min, resolved on 12% SDS–PAGE, and electrotransferred to PVDF membrane. The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 for 1 h at room temperature and further incubated with the specific primary antibodies (listed in Table 1—Supplementary material), overnight at 4°C, followed by incubation with horseradish peroxidase–conjugated anti-mouse or anti-rabbit secondary antibodies for 1 h at room temperature. The immunocomplexes were detected by the ECL chemiluminescent method and visualized with Hyperfilm ECL. β-Actin expression was analyzed in stripped membranes and used as a loading control. The relative intensities of protein bands were analyzed using the Gel-Pro 32 Analyzer densitometry analysis software (Media Cybernetics, MD, USA).

Measurement of Intracellular ROS Generation

Intracellular ROS generation was measured with the fluorescent probe DCF-DA. Briefly, 50 μg of midbrain and striatum tissue extracts from mice treated with TUDCA or MPTP and with TUDCA plus MPTP were incubated with 10 μM DCF-DA at 37°C for 1 h.

The fluorescence intensity of DCF was measured in a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Each assay was performed in triplicate.

Statistical Analysis

All results are expressed as mean±SEM values. Data were analyzed by one-way ANOVA and Tukey post hoc test (GraphPad, Prism 5.0, San Diego, CA, USA). Means were considered statistically significant at a p value below 0.05.

Results

Neuroprotective Effect of TUDCA Against MPTP-Induced Neurodegeneration

In the present study, we sought to investigate the neuroprotective potential of TUDCA against MPTP toxicity in mice brain. C57BL/6 GSTP null mice were submitted to systemic administration of TUDCA and/or MPTP according to the schematic program depicted in Fig. 1. As a measure of dopaminergic cell death, TH protein expression was evaluated by immunohistochemistry analysis of brain coronal sections at the level of SNpc (Bregma −3.20) and striatum (Bregma 1.00). The number of TH immunoreactive cells in the SNpc was assessed by counting adjacent sections stained for TH (Fig. 2). Confirming our previous results, MPTP administration caused a significant reduction of ~65% in the number of TH-positive cells ($p<0.001$), determined at day 30 after MPTP injection. In the presence of TUDCA, MPTP also induced a significant decrease in the number of nigral dopaminergic neurons ($p<0.001$). However, pre-treatment with TUDCA significantly reduced by ~30% the depletion of DA neurons caused by MPTP ($p<0.001$). Striatal dopaminergic fibers degeneration was evident as early as at day 7 following MPTP administration and became even more marked 30 days after the neurotoxic insult (Fig. 3). Interestingly, TUDCA also reduced dopaminergic fiber loss induced by MPTP since fiber density and TH immunofluorescence intensity were markedly higher in striata from TUDCA-treated animals. Administration of TUDCA in the absence of MPTP treatment did not change the number of dopaminergic neurons, nor dopaminergic fiber density. Taken together, these results indicate that TUDCA exerts a neuroprotective role against MPTP-induced neurodegeneration, delaying and decreasing dopaminergic cell loss triggered by the neurotoxin insult.

Effect of TUDCA on MPTP-Induced JNK Phosphorylation

Recently, we described that the JNK pathway is an important early event regulating MPTP susceptibility in GSTP null mice [14]. To evaluate whether the neuroprotective effect of TUDCA treatment may result from JNK pathway modulation, JNK phosphorylation status was evaluated by Western blot assay using a specific antibody that recognizes dual JNK phosphorylation (Thr183/Tyr85). Levels of phosphorylated JNK (p-JNK) in GSTP null mice treated with TUDCA prior to MPTP administration are similar to those found in controls, both in midbrains and striata (Fig. 4). As expected, administration of a sub-acute dose of MPTP alone induced an important increase in the levels of p-JNK 3 h after the insult. These increments in p-JNK were found to be significantly different from the corresponding controls ($p<0.001$), as well as from TUDCA plus MPTP-treated mice, in both midbrain ($p<0.001$) and striatum ($p<0.01$). We had previously demonstrated that MPTP-induced JNK phosphorylation in GSTP null mice was fast and transient since control levels were restored 6 h post-MPTP administration [14]. The results presented herein reveal that treatment with TUDCA prior to MPTP administration completely abrogates MPTP-induced JNK phosphorylation.

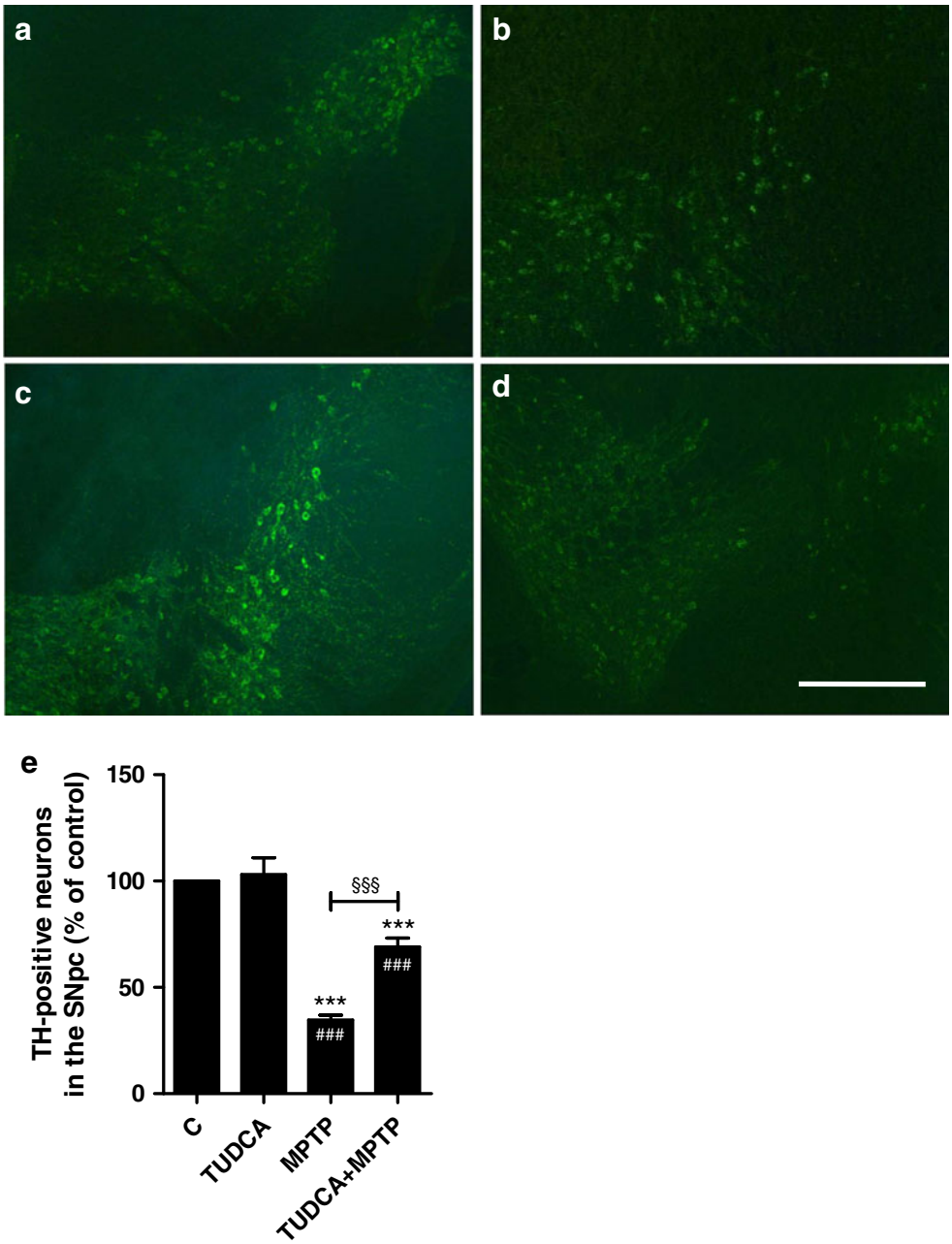

Fig. 2 TUDCA protects from MPTP-induced neurodegeneration of the nigral dopaminergic neurons. Immunohistological analysis of coronal sections at the level of SNpc (Bregma −3.20) from C57BL/6 GSTP null mice stained with the dopaminergic neuronal marker TH: **a** saline-treated (control, C); **b** MPTP treated; **c** TUDCA treated; **d** TUDCA+MPTP treated. All groups of mice were sacrificed 30 days after MPTP administration. **e** The values shown are the averaged number of TH positive neurons in the *zona compacta* of the SN counted in four adjacent sections±SEM of three independent experiments, ***$p<0.001$ vs. control, $^{###}p<0.001$ vs. TUDCA and $^{§§§}p<0.001$ vs. MPTP. Microphotographs shown are representative of three independent experiments. *Scale bar*=100 μm

Effect of TUDCA on Intracellular ROS Levels

MPTP toxicity is associated with increased ROS production, which in turn may activate cell death pathways, such as JNK-mediated apoptosis and, in parallel, survival compensatory pathways, such as GSTP- [36] and Hsp-induced expression [37]. In this context, and to further explore the anti-oxidative role of TUDCA, we investigated whether this bile acid modulates the levels of ROS in the presence of MPTP, using the fluorescent ROS dye DCF-DA (Fig. 5). MPTP treatment significantly increased the intracellular ROS formation in both midbrain ($p<0.01$) and striatum ($p<0.01$ at 3 h and $p<0.05$ at 6 h). Pre-treatment of GSTP null mice with TUDCA before MPTP administration abrogated the ability of MPTP to generate ROS, as fluorescence was similar to control in both midbrain and striatum. These results demonstrate the anti-oxidant role of TUDCA in vivo and suggest that TUDCA is modulating the intracellular oxidative environment by interfering with the cellular redox threshold.

Effect of TUDCA on Hsp27 Expression

Hsp27 has been described as a potent neuroprotective factor against several neuronal insults [38]. Here, we also investigated the in vivo expression of Hsp27 in the brain of mice under

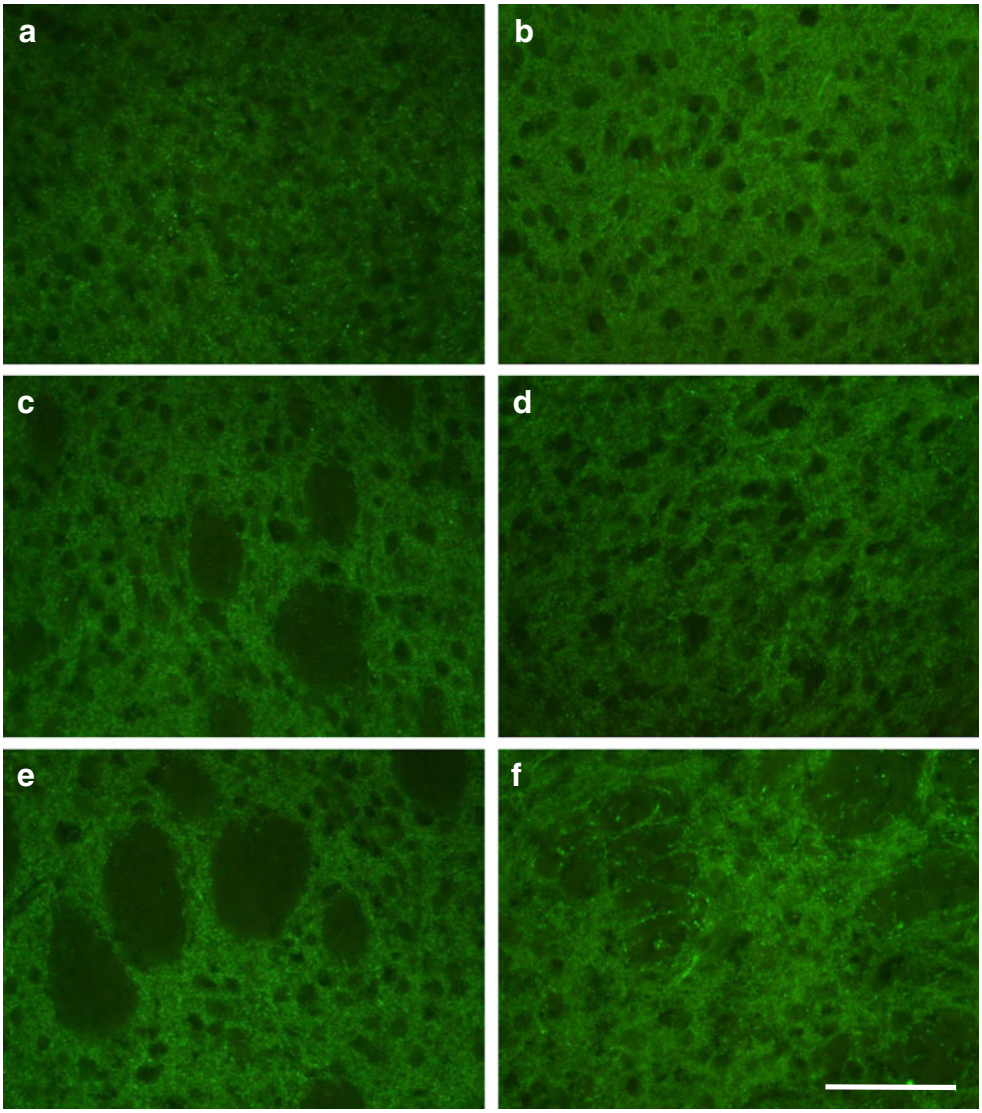

Fig. 3 TUDCA protects from MPTP-induced neurodegeneration of striatal dopaminergic fibers. Coronal sections at the level of striatum (Bregma 1.00) from C57BL/6 GSTP null mice were immunostained with the dopaminergic neuronal marker TH: **a** saline-treated (control); **b** TUDCA treated; **c**, **e** MPTP treated; **d**, **f** TUDCA+MPTP treated. Mice were sacrificed 7 days (**c**, **d**) or 30 days (**e**, **f**) post-MPTP administration. Microphotographs shown are representative of three independent experiments. *Scale bar*= 100 μm

TUDCA and/or MPTP administration. Neither TUDCA alone nor the combination of TUDCA plus MPTP altered Hsp27 protein levels (Fig. 6). Interestingly, single-dose administration of MPTP alone induced a significant increase of Hsp27 expression in both midbrain ($p<0.05$) and striatum ($p<0.01$) as early as 3 h post-neurotoxin injection. Moreover, the MPTP-induced increase of Hsp27 expression was significantly reduced in the presence of TUDCA in both midbrain ($p<0.01$) and striatum ($p<0.001$ at 3 h and $p<0.05$ at 6 h).

Effect of TUDCA on Bad Phosphorylation and NF-κB Activation

To further elucidate how the presence of TUDCA may protect from the neurotoxic effects following MPTP treatment, we investigated the ability of TUDCA to modulate the Akt intracellular survival pathway. We have already shown that TUDCA activates Akt signaling using other experimental paradigms [26, 39]. Thus, we conducted Western blot analysis using an antibody that recognizes phosphorylated Akt (p-Akt) at Ser 473, the active form of this kinase [40]. At the time points evaluated, the levels of p-Akt were not significantly different from controls in both midbrain and striatum (data not shown). This may be explained by the fact that Akt phosphorylation is usually a very fast and transient event, perhaps undetected in brain extracts at the selected time points. Nevertheless, the presence of Akt phosphorylated downstream targets can be indicative of prior Akt phosphorylation. In fact, TUDCA increased p-Bad in the midbrain 3 h after MPTP administration ($p<0.05$) (Fig. 7). Interestingly, p-Bad in the striatum was also significantly increased in the samples obtained from mice treated only with TUDCA ($p<0.05$). In contrast, treatment only with MPTP did not change p-Bad levels at the indicated time points neither in midbrain nor in striatum. Akt may also associate with IκB kinase (IKK) and induce its activation. IKK phosporylates IκB-α, the endogenous repressor of NF-κB, targeting it for degradation by proteosome 26S [41, 42].

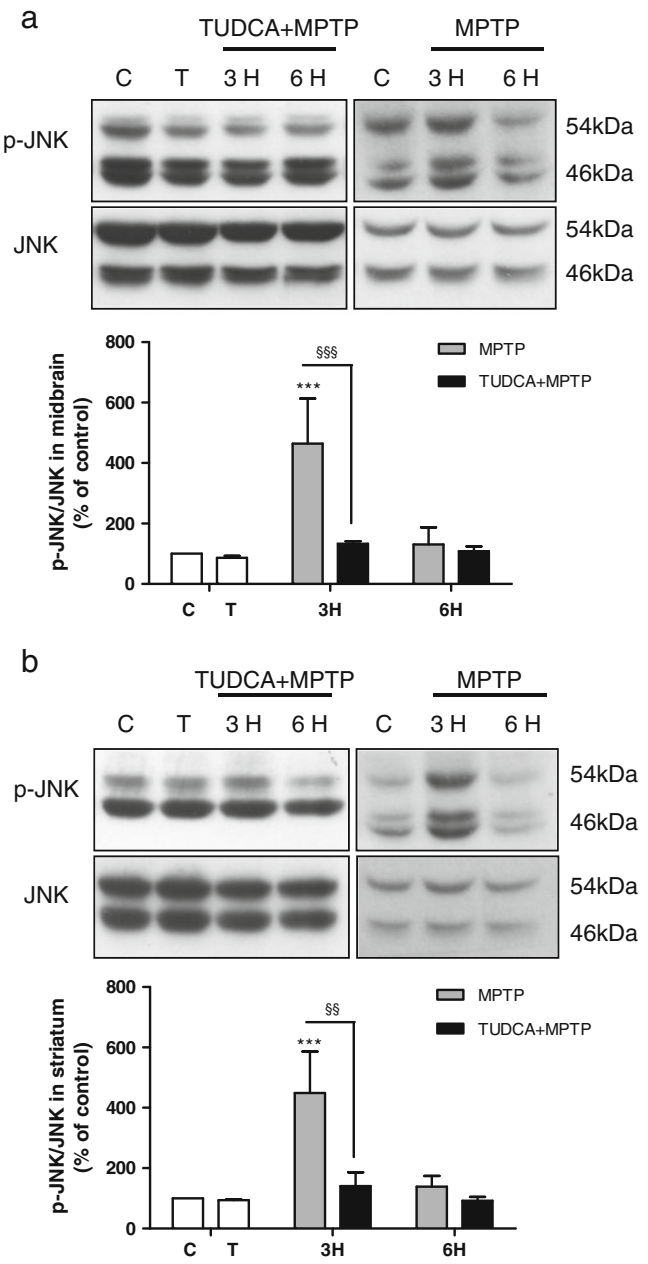

Fig. 4 TUDCA modulates MPTP-induced JNK phosphorylation. C57BL/6 GSTP null mice were treated with saline (control, *C*), TUDCA (*T*), MPTP or with TUDCA+MPTP and sacrificed 3 h or 6 h after MPTP administration. Tissue extracts from mice midbrain (**a**) and striata (**b**) were prepared, subjected to 12% SDS–PAGE, and the corresponding blots were probed with antibodies to p-JNK and JNK. Representative immunoblots from mice treated with TUDCA+MPTP are shown in the *left panels*, while immunoblots from mice treated only with MPTP are presented in the *right panels*. All immunoblots include the respective control samples analyzed in parallel. Data are expressed as the mean±SEM of three independent experiments, indicated as percentage of the respective controls. ***$p<0.001$ vs. control; $^{§§}p<0.01$ vs. MPTP and $^{§§§}p<0.001$ vs. MPTP

TUDCA resulted in significant decreases of IκB-α concentration in midbrain either in the absence ($p<0.05$) or in the presence ($p<0.05$) of MPTP (Fig. 8). However, treatment

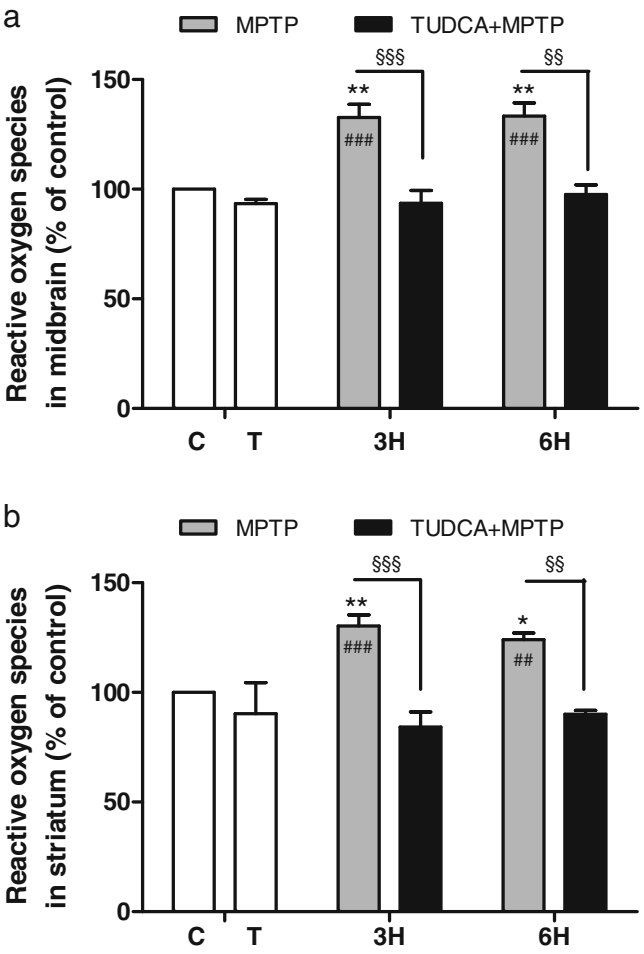

Fig. 5 TUDCA modulates MPTP-induced ROS generation. ROS generation was determined by a fluorimetric assay using 10 μM of DCF-DA ROS dye. This assay was performed using 50 μg of midbrain (**a**) and striatum (**b**) tissue extracts from saline-treated control (*C*), TUDCA treated (*T*), MPTP treated or TUDCA+MPTP treated C57BL/6 GSTP null mice, sacrificed 3 h or 6 h after MPTP administration. Fluorescence intensity of DCF was measured in a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 528 nm, and results are presented as a percentage of control. Data shown are the mean values±SEM of three independent experiments. *$p<0.05$ and **$p<0.01$ vs. control; $^{§§}p<0.01$ and $^{§§§}p<0.001$vs. MPTP; $^{##}p<0.01$ and $^{###}p<0.001$ vs. TUDCA

with TUDCA and/or MPTP did not induce IκB-α degradation in striatum. These results further corroborate the effect of TUDCA on AKT pathway and indicate that TUDCA activates the NF-κB pathway in GSTP null mice midbrain.

Discussion

Both genetic predisposition and environmental exposures (e.g., toxins and pesticides) have been implicated in the etiology of PD. In addition, it is now well accepted that increased oxidative stress, mitochondrial dysfunction, and protein aggregation are common features of PD [4–6]. Here,

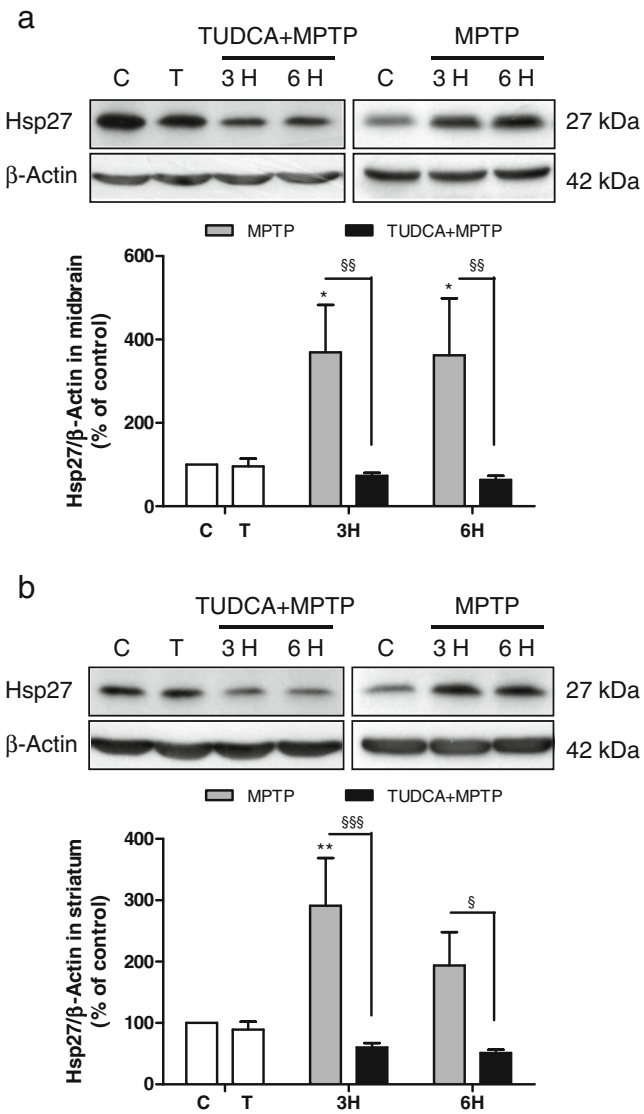

Fig. 6 Modulation of Hsp27 expression following TUDCA and MPTP administration. C57BL/6 GSTP null mice were treated with saline (control, *C*), TUDCA (*T*), MPTP or TUDCA+MPTP and sacrificed 3 h or 6 h after MPTP administration. Tissue extracts from mice midbrain (**a**) and striata (**b**) were prepared, subjected to 12% SDS–PAGE, and the corresponding blots were probed with antibodies to Hsp27 and β-actin (as a loading control). Representative immunoblots from mice treated with TUDCA+MPTP are shown in the *left panels*, while immunoblots from mice treated only with MPTP are represented in the *right panels*. All immunoblots included the respective control samples analyzed in parallel. Data are expressed as the mean±SEM of three independent experiments, indicated as percentage of the respective controls. *$p<0.05$ and **$p<0.01$ vs. control; $^{§}p<0.05$, $^{§§}p<0.01$, and $^{§§§}p<0.001$ vs. MPTP

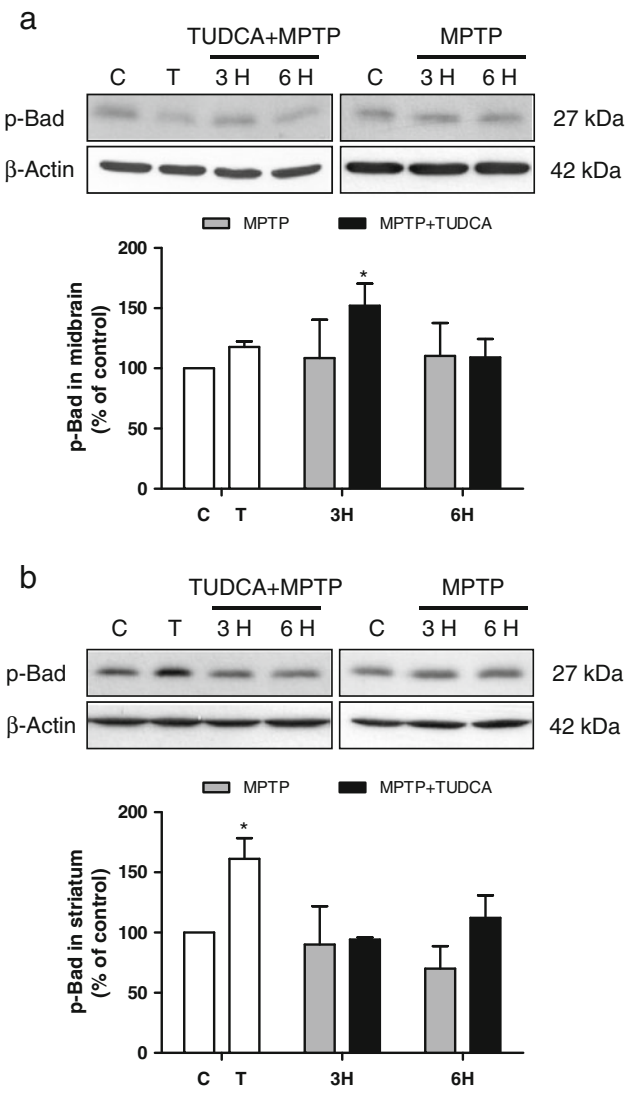

Fig. 7 TUDCA induces the phosphorylation of Bad. C57BL/6 GSTP null mice were treated with saline (control, *C*), TUDCA (*T*), MPTP or TUDCA+MPTP and sacrificed 3 h or 6 h after MPTP administration. Tissue extracts from mice midbrain (**a**) and striata (**b**) were prepared, subjected to 12% SDS–PAGE, and the corresponding blots were probed with antibodies to p-Bad and β-actin (as a loading control). Representative immunoblots from mice treated with TUDCA+MPTP are shown in the *left panels*, while immunoblots from mice treated only with MPTP are represented in the *right panels*. All immunoblots included the respective control samples analyzed in parallel. Data are expressed as the mean±SEM of three independent experiments, indicated as percentage of the respective controls, *$p<0.05$ vs. control

we investigated the putative protective role of TUDCA, a potent anti-apoptotic and mitochondrial stabilizer, against MPTP neurotoxicity in GSTP null mice.

We have previously demonstrated that the C57BL/6 GSTP null mice strain is more susceptible to MPTP neurotoxicity, showing earlier dopaminergic neuronal degeneration accompanied by exacerbated phosphorylation and activation status of JNK, when compared with C57BL/6 wild-type mice [14]. Moreover, *GSTP1* polymorphisms are associated with increased risk of incidence for PD, indicating a neuroprotective role for native GSTP1 [43, 44]. Therefore, GSTP null mice seem to be a suitable model to highlight the beneficial role of TUDCA treatment against MPTP neuronal toxicity and to

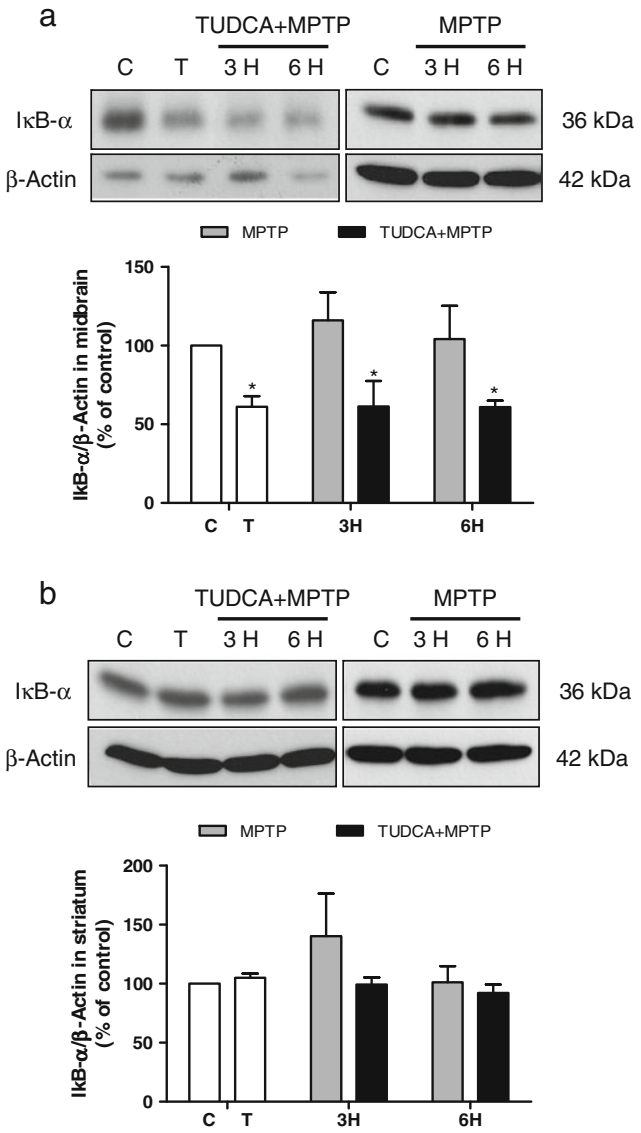

Fig. 8 TUDCA decreases the levels of IκB-α. C57BL/6 GSTP null mice were treated with saline (control, *C*), TUDCA (*T*), MPTP or TUDCA+MPTP and sacrificed 3 h or 6 h after MPTP administration. Tissue extracts from mice midbrain (**a**) and striata (**b**) were prepared, subjected to 12% SDS–PAGE, and the corresponding blots were probed with antibodies to IκB-α and β-actin (as a loading control). Representative immunoblots from mice treated with TUDCA+MPTP are shown in the *left panels*, while immunoblots from mice treated only with MPTP are represented in the *right panels*. All immunoblots included the respective control samples analyzed in parallel. Data are expressed as the mean±SEM of three independent experiments, indicated as percentage of the respective controls, *$p<0.05$ vs. control

evaluate whether the neuroprotective properties of TUDCA can overcome a genetic deficiency in the cellular endogenous anti-oxidative pathway.

We show that pre-treatment with TUDCA negatively modulates JNK phosphorylation induced by MPTP in mouse brain. Hence, in the presence of TUDCA, treatment with MPTP no longer triggers the activation of the JNK signaling pathway, resulting in reduced cellular toxicity of MPTP. This is particularly evident when comparing the degeneration rate of nigral dopaminergic neurons and striatal fibers triggered by MPTP, in the presence or absence of TUDCA. As previously described, the number of TH positive cells significantly decreases after sub-acute dose of MPTP [36]. However, administration of TUDCA delayed the degeneration of dopaminergic cell bodies, as well as striatal fibers induced by MPTP, clearly indicating an in vivo neuroprotective end-point effect of TUDCA.

ROS are thought to serve as second messengers to control a broad range of physiological and pathological processes. Excessive ROS levels beyond the antioxidant-buffering capacity results in potentially cytotoxic effects, leading to irreversible damage in DNA, proteins, and lipids, as well as to dysfunction of the energy metabolism [45]. To further explore the mechanism of TUDCA-induced neuroprotection, we looked at modulation of ROS production by TUDCA in the presence of MPTP. Importantly, in the presence of TUDCA, ROS levels in mice midbrains and striata were unaltered even after single-dose administration of MPTP. Our results point to a regulatory role of TUDCA against MPTP neurotoxicity, which may involve upstream modulation of the cellular redox status.

The precise balance between the ROS levels and the activity of endogenous antioxidants is of utmost importance in the maintenance of the cellular redox state. Thus, the coordinated induction of proteins possessing radical-scavenging capabilities in response to elevated concentration of ROS is required to maintain cellular homeostasis. One such ROS-activated protein is heat shock transcription factor 1 (HSF1), which binds to heat shock elements in the promoter of heat shock genes, and is responsible for the transcriptional regulation of Hsp genes, namely Hsp27 [37, 46]. Notably, we show that treatment with MPTP induces a significant increase in Hsp27 expression levels in midbrain and striatum from GSTP null mice. However, in mice pre-treated with TUDCA, MPTP administration no longer triggers the induction of Hsp27 expression. These results suggest that TUDCA is modulating the threshold of oxidative stress so that the redox environment even in the presence of MPTP is not sufficient to elicit up-regulation of Hsp27 expression.

The regulation of JNK activity is complex and under tight control of endogenous repressors, which are activated before or after cellular stress. In GSTP null mice, the constitutive activation of JNK pathway is higher [47, 48]. However, we have recently shown that when GSTP null mice are treated with MPTP, the levels of p-JNK are only transiently increased [14], indicating that in the absence of GSTP other cellular repressors must compensate for the lack of this important JNK regulator. Taken in consideration with the results presented here, Hsp27 appears as a

good candidate. In fact, it has been previously shown that synthesis of Hsp27 in SH-SY5Y cells after MPP^+ exposure was increased owing to a self-protection mechanism [37]. Importantly, Hsp27 is protective against oxidative stress through its ability to raise the pool of reduced glutathione, which decreases the intracellular ROS levels [49], therefore inhibiting the stimulus eliciting JNK activation. Moreover, Hsp27 is a direct inhibitor of the upstream kinase of JNK, ASK1, via physical association with the ASK1 kinase domain [46].

UDCA was shown to increase liver GST catalytic activity in response to toxic insults [50]. Therefore, we investigated whether TUDCA could be modulating total GST activity, in the brain, as part of its anti-oxidative role. However, we found that in the presence of TUDCA, total GST catalytic activity in midbrains and striata was not increased either before or after MPTP administration (data not shown). These results indicate that TUDCA is not modulating the cellular redox threshold by increasing GST activity and/or expression in GSTP null mice.

Akt, also referred to as PKB or Rac, plays a critical role in controlling cell survival and apoptosis. This kinase may be activated by phosphorylation within the carboxy terminus at Ser 473 [40]. Once phosphorylated, Akt promotes cell survival by several different mechanisms. For example, p-Akt enhances cell survival by inhibiting the function of pro-apoptotic proteins, namely the Bcl-2 family member Bad [39, 51]. Pro-apoptotic Bad forms heterodimers with the anti-apoptotic proteins Bcl-xL or Bcl-2, antagonizing their anti-apoptotic activity [52]. Active Akt directly phosphorylates Bad on Ser 136, triggering the release of Bad from its mitochondrial targets [53]. On the other hand, Akt may be a signal intermediate upstream of survival gene expression by the transcription factor NF-κB. Akt has been shown to activate IKKα, which directly phosphorylates IκB-α on the two serine residues that are critical for IκB ubiquitination and degradation, leading to the induction of NF-κB activity [41, 42]. Interestingly, TUDCA induces Akt activation and the phosphorylation of the aforementioned downstream effectors, Bad and IκB-α, in other experimental paradigms [26, 32, 39]. Here, we were unable to detect significantly altered levels of the phosphorylated form of Akt, either in the midbrain or striatum, in any of the experimental conditions tested. Still, we were able to show that the levels of p-Bad at Ser 136 were significantly increased in midbrains and striata from mice treated with TUDCA and MPTP, or with TUDCA alone, respectively. Moreover, TUDCA alone or in the presence of MPTP induced the decrease of IκB-α concentration in midbrain, indicating degradation of this protein and activation of the NF-κB pathway. Taken together, these results suggest that, in vivo, TUDCA activates the pro-survival Akt pathway, modulating the activity of its downstream effectors, Bad and IκB-α, as part of its neuroprotective action.

In summary, we provide evidence that pre-treatment with TUDCA modifies the cellular environment so that the deleterious events of MPTP are attenuated. The major targets of TUDCA against MPTP neurotoxicity seem to be the blockage of ROS production and JNK activation, together with the activation of pro-survival mediators such as phosphorylated Bad and NF-κB. MPTP itself, in parallel with neurodegeneration mediated by ROS and JNK, activates survival mechanisms, for example Hsp27 expression. Interestingly, the compensatory adaptive responses that occur in the presence of MPTP are also shut-down by TUDCA. These observations further confirm that TUDCA is "buffering" the intracellular milieu, diminishing MPTP toxicity.

The ability of TUDCA to modulate cell death in stroke, Huntington's disease, and Alzheimer's disease is now well recognized [24–29, 31, 32]. Moreover, UDCA is FDA-approved to treat liver diseases [17] and its administration is also safe and well tolerated in patients with amyotrophic lateral sclerosis [54]. In PD, approximately 50–70% of the dopaminergic neurons are already lost at the time of clinical diagnosis [55]. Thus, the challenge is to induce neuroprotection or neurorescue by therapeutics that respectively prevent or stop the progressive neurodegenerative process. Further studies are needed to explore the exact intracellular pathways that mediate the neuroprotective effects of TUDCA against MPTP toxicity. However, this work provides new perspectives for the use of TUDCA as a potential therapeutic modulator of nigral cell death in PD.

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