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Glutathione *S*-Transferase pi Mediates MPTP-Induced c-Jun N-Terminal Kinase Activation in the Nigrostriatal Pathway

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Abstract Parkinson's disease (PD) is a progressive movement disorder resulting from the death of dopaminergic neurons in the substantia nigra. Neurotoxin-based models of PD using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) recapitulate the neurological features of the disease, triggering a cascade of deleterious events through the activation of the c-Jun N-terminal kinase (JNK). The molecular mechanisms underlying the regulation of JNK activity under cellular stress conditions involve the activation of several upstream kinases along with the fine-tuning of different endogenous JNK repressors. Glutathione *S*-transferase pi (GSTP), a phase II detoxifying enzyme, has been shown to inhibit JNK-activated signaling by protein–protein interactions, preventing c-Jun phosphorylation and the subsequent trigger of the cell death cascade. Here, we use C57BL/6 wild-type and GSTP knockout mice treated with MPTP to evaluate the regulation of JNK signaling by GSTP in both the substantia nigra and the striatum. The results presented herein show that GSTP knockout mice are more susceptible to the neurotoxic effects of MPTP than their wild-type counterparts. Indeed, the administration of MPTP induces a progressive demise of nigral dopaminergic neurons together with the degeneration of striatal fibers at an earlier time-point in the GSTP knockout mice when compared to the wild-type mice. Also, MPTP treatment leads to increased p-JNK levels and JNK catalytic activity in both wild-type and GSTP knockout mice midbrain and striatum. Moreover, our results demonstrate that in vivo GSTP acts as an endogenous

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regulator of the MPTP-induced cellular stress response by controlling JNK activity through protein–protein interactions.

Keywords Glutathione *S*-transferase pi · c-Jun N-terminal kinase · Parkinson's disease · MPTP · Neurodegeneration · Detoxification

Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, characterized by a progressive degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and the presence of intracytoplasmic inclusions of aggregated proteins (Lewy bodies, LB; [1]). The cellular mechanisms underlying DA cell death in PD are still not fully understood, but several lines of evidence indicate that environmental toxins, genetic factors, mitochondrial dysfunction, oxidative stress and neuro-inflammation are implicated in both familial and sporadic forms of PD [1–4]. Mitochondrial inhibitors, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induce a cascade of deleterious events that recapitulate the neuropathological features of idiopathic PD and culminate with nigral DA neuron degeneration [1, 5, 6]. After its systemic administration, MPTP rapidly crosses the blood–brain barrier [7]. Once in the brain, MPTP is metabolized in glial cells to 1-methyl-4-phenylpyridinium (MPP+), the active toxic compound. MPP+has high affinity for plasma membrane dopamine transporter, and once inside DA neurons, MPP+accumulates in the mitochondria where it potently inhibits complex I of the electron transport system, leading to ATP depletion, loss of mitochondrial membrane potential and the formation of reactive oxygen species (ROS; [8–10]).

Several studies have demonstrated that c-Jun N-terminal kinase (JNK), a stress-activated protein kinase (SAPK), is a key mediator of MPTP/MPP+-induced neuronal apoptosis in animal and cellular models of PD [7, 11–13], and evidence also points to a role of JNK in the neurodegenerative process that occurs in sporadic PD [14, 15].

The activation of JNK is mediated through a sequential kinase cascade that includes mitogen-activated protein kinase (MAPK) kinase (MAPKK) and MAPK kinase kinase (MAPKKK). JNK is activated by dual phosphorylation of the Thr-Pro-Tyr motif located in the activation loop by MKK4 or MKK7 [12, 16]. The downstream events of JNK activation leading to apoptosis-mediated cell death involve both transcriptional and non-transcriptional or mitochondrial mechanisms. In the transcriptional mechanism c-Jun is the critical mediator of the pro-apoptotic effects of JNK. Once phosphorylated by JNK on two sites within the activation domain (Ser63 and Ser73; [17]), c-Jun has increased transcriptional activity leading to up-regulation of a number of genes involved in the control of cell survival and apoptosis [12, 15, 18]. The mitochondrial mechanism of JNK signaling involves direct phosphorylation and modulation of pro- and anti-apoptotic activity of Bcl-2 family proteins [10, 12, 16, 18, 19].

The assumption that the JNK signaling pathway is an important checkpoint in MPTP/MPP^+-induced neurotoxicity comes from the observation that increased levels of phosphorylated MKK4, JNK, and c-Jun precede and are implicated in the degeneration of DA neurons from MPTP-treated mice SNpc [7, 10, 12, 15, 18]. Selective JNK inhibitors, such as CEP-1347/KT-7515, SP600125 and small inhibitor peptides also protect against MPTP-induced apoptosis in the nigrostriatal DA neurons in vivo [20–22]. Moreover, gene-targeting studies showed that JNK-null mice have increased resistance to MPTP-induced neuronal cell death in the SNpc [14].

Glutathione *S*-transferases (GST) are phase II drug metabolizing enzymes that catalyze the conjugation of reduced glutathione to electrophilic groups on substrate molecules, namely by-products of oxidative stress rendering them more soluble and thus more easily eliminated from the cell. There are seven cytosolic subunit classes of GSTs (alpha, mu, pi, theta, omega, sigma, and zeta), three microsomal and one mitochondrial form [23–25]. GSTs have generally a ubiquitous localization, while some isoforms show a tissue-specific distribution. In mammals, only alpha, mu, and pi isoforms are expressed in the central nervous system [26, 27]; the most highly expressed is GSTP, which in addition to detoxification reactions also acts as a ligand-binding protein controlling the catalytic activity of JNK [28–30]. The interaction between GSTP and JNK involves the C-terminal region of domain 2 of GSTP and the C-terminal domain of JNK, and requires the monomeric [28, 31] or, as recently suggested, dimeric forms of GSTP [32]. Under conditions of oxidative stress, GSTP dissociates from JNK which may then be phosphorylated and phosphorylate its downstream substrates. Therefore, GSTP may serve as an endogenous regulator of the cellular stress response, eliciting protection against cell death induced by ROS by controlling JNK activity [28, 30].

We and others have reported that in the mouse brain, GSTP is constitutively and predominantly expressed in glial cells (namely oligodendrocytes and astrocytes) and also in DA neurons from SNpc [5, 26, 33–36]. In a previous study, a single dose of MPTP in C57BL/6 mice induced the transient overexpression of GSTP particularly in glial cells from both midbrain and striatum [5]. Moreover, we have also demonstrated that in SH-SY5Y cells, GSTP interacts with JNK, suggesting that in neuronal cells GSTP directly modulates JNK catalytic activity and cell death [37].

An association between *GSTP1* genotypes and sporadic forms of PD was described in different groups of patients

exposed to pesticides [38], and GST polymorphisms have been shown to increase DA neuron loss in a parkin mutant *Drosophila* model [39]. Importantly, data from our group has shown that subjects who carry the GSTP1*B allele may be at a significantly increased PD risk, strongly suggesting a neuroprotective role for native *GSTP1* [40].

In this work, we used MPTP-treated C57BL/6 wild-type and GSTP knockout (GSTP ko) mice to further evaluate the role of GSTP in this in vivo model of PD. We show that GSTP ko mice are more susceptible to MPTP neurotoxicity than wild-type mice, and suggest that GSTP exerts its neuroprotective role through direct regulation of JNK catalytic activity.

Materials and Methods

Materials

MPTP, mouse anti-tyrosine hydroxylase (TH) (T2928) antibody, Hoechst 33258, amido black and the GST assay kit were purchased from Sigma Chemical Co (St Louis, MO, USA). Mouse anti-GSTP antibody (G59720) was from BD Biosciences Pharmingen (San Jose, CA, USA). The Dako-Cytomation Fluorescent Mounting Medium was from Dako (Copenhagen, Denmark). The non-radioactive SAPK/JNK assay kit (#9810), the rabbit anti-JNK (#9258), and mouse anti-p-JNK (Thr183/Tyr185) (#9255) antibodies, the horseradish peroxidase (HRP)-conjugated anti-mouse IgG (#7076) and the anti-c-Jun antibodies (#9260) were from Cell Signaling Technology (Beverly, MA, USA). The Tyramide Signal Amplification Kit was purchased from Invitrogen/Molecular Probes (Eugene, OR, USA), the Protein A/G PLUS-Agarose beads and the anti-α-tubulin antibody (B-7) (sc-5286) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-rabbit secondary antibody (W401B) was from Promega (Madison, USA). Immobilon P was from Millipore (Bedford, MA, USA). ECL and Hyperfilm ECL were purchased from Amersham Biosciences (Piscataway, NJ, USA). Other chemical 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 National Institutes of Health guidelines for the care and use of animals and methods were approved by the local Institutional Animal Care and Use Committee.

Twelve-week-old male wild-type C57BL/6 mice were purchased from the Gulbenkian Institute of Science Animal House (Oeiras, Portugal). C57BL/6 *Gstp1/p2* null mice from Cancer Research UK were kindly provided by C. Roland Wolf and this null lineage was re-derived and also maintained at the same Animal House. 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 [41]. Throughout the text and figures, this knockout mice line will be referred as GSTP knockout (GSTP ko). All animals were housed under standardized conditions on a 12-h light–dark cycle with free access to a standard diet and water ad libitum.

MPTP was administered intra-peritoneally (i.p.) at a single dose of 40 mg/kg [5, 7]. Control mice received saline alone. At the indicated times after neurotoxin or vehicle administration, mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), decapitated, and brains were quickly removed and placed in fresh PBS. Mice brains were then placed on their ventral surface onto a mouse brain matrice (Agar Scientific), and a slice between Bregma −2.5 and Bregma −3.8 was isolated. This removed brain slab was placed flat and the entire midbrain region, containing the SNpc was dissected as previously described [42]. The remaining brain piece was then cut between Bregma –1.0 and 1.5, and whole striatum was isolated after discarding the cerebral cortex, the corpus callosum and the region of the septum (including septohippocampal bundle, lateral septal nucleus, medial septal nucleus, and diagonal band). The specific pieces of interest were flash frozen under liquid nitrogen and kept at −80°C until further use.

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

Immunohistochemistry

After MPTP treatment mice were anesthetized, transcardially perfused and processed for cryostat sectioning. Brain sections at the level of SNpc (Bregma −3.20) and at the level of midstriatum (Bregma 1.00) were obtained, as previously described [5]. TH immunolocalization was performed with the Tyramide Signal Amplification Kit according to the manufacturer's instructions. Briefly, 14-μm-thick coronal sections were permeabilized with 0.2 % Triton X-100 in PBS, and then pre-treated with blocking solution (2 % bovine serum albumin, 0.05 % Tween-20 in PBS). Incubation with the anti-TH antibody (1:250) was performed overnight at 4°C, followed by incubation with the HRP-conjugated goat anti-mouse IgG for 30 min, and subsequently with the Alexa Fluor 488-labeled tyramide for 10 min at room temperature. Sections were 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 antibody used was previously confirmed by Western blot analysis. Control experiments for

non-specific binding were performed in parallel by omission of the primary and secondary antibodies.

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

Western Blot Analysis

Tissue extracts from mice midbrains and striata were prepared as indicated elsewhere [5], and were resolved on 12 % SDS-PAGE, and electro-transferred onto Immobilon P. The membrane was blocked with 5 % non-fat dry milk in Tris-buffered saline with 0.1 % Tween-20 and further incubated with the anti-phospho-JNK antibody (1:500), anti-phospho-c-Jun (1:1,000), overnight at 4°C, followed by incubation with HRP-conjugated anti-mouse and anti-rabbit antibodies. The immunocomplexes were detected by the ECL chemiluminescent method and visualized with Hyperfilm ECL. Analysis of total JNK (1:1,000) and α-tubulin (1:15,000) expression was performed in stripped membranes 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 JNK Catalytic Activity

JNK catalytic activity was determined as previously described [29] by measuring the levels of c-Jun phosphorylation using the non-radioactive SAPK/JNK assay kit, according to the manufacturer's instructions. Briefly, the kinase reaction was performed in the presence of excess ATP and was optimized for both time (30 min) and protein concentration (1 mg of total protein tissue extract). Samples were electrophoresed on 12 % SDS-PAGE and electroblotted onto Immobilon P. c-Jun phosphorylation was selectively measured using an anti-phospho-c-Jun antibody (1:1,000), which specifically measures JNK-catalysed phosphorylation of c-Jun at Ser-63. To demonstrate equivalent protein loading, the membranes were stripped and then incubated with anti-c-Jun antibody (1:5,000).

Co-Immunoprecipitation Assays

Co-immunoprecipitations were carried out using 1 mg of total protein extracts from striatum or midbrain incubated with 4 μl of the anti-JNK antibody, overnight at 4°C. Then, 20 μl of protein A/G PLUS-Agarose beads were added for 2 h, at 4°C. Co-immunoprecipitation complexes were ressuspended in washing buffer (50 mM Tris–HCl pH 7.4, 180 mM NaCl, 1 mM EDTA, 1 % Triton-X 100) and recovered by centrifugation at 2,000×*g* for 10 min at 4°C. The washing process was repeated three times. Samples were boiled in denaturating buffer (0.25 mM Tris-HCl, pH 6.8, 4 % SDS, 40 % glicerol, 0.2 % bromophenol blue, 1 % β-mercaptoethanol). The presence of the protein immuno-complexes was evaluated by 12 % SDS-PAGE followed by immunoblotting using the anti-GSTP antibody (1:1,000). As a control, JNK was detected in the same membrane after stripping off the immune complex for the detection of GSTP. Immunoblot analysis showed an absence of nonspecific binding of the JNK antibody to GSTP. In addition, control experiments for non-specific binding were performed by immunoblot analysis of immunoprecipitation assays using an antibody reactive to Bcl-2.

Statistical Analysis

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

Results

MPTP-Induced DA Cell Loss in the Nigrostriatal Pathway

To determine whether endogenous GSTP has a role in MPTP-dependent nigrostriatal DA cell loss, we examined the effects of the neurotoxin on SNpc and striatal TH immunopositive cells and fibers, respectively, in wild-type and in GSTP ko mice.

We have previously shown that MPTP induces an evident depletion of DA fibers and cells of the nigrostriatal pathway from wild-type C57BL/6 animals by a "dying back" mechanism. Results presented in Fig. 1 show that the loss of DA cells in the SNpc from wild-type mice was evident and significant 30 days after MPTP administration, whereas MPTP induces a significant decrease of about 60 % in the number of nigral DA neurons from C57BL/6 GSTP ko mice, 7 days after i.p. injection (one-way ANOVA with Tukey post-hoc test: F=251.1 df=2, p<0.001). An equivalent DA cell loss was detected in GSTP ko mice sacrificed 30 days after MPTP administration (one-way ANOVA with Tukey post-hoc test: F=251.1 df=2, p<0.001).

Representative sections from the midstriatum, presented in Fig. 2, show that MPTP administration resulted in a dramatic reduction of TH positive fibers density as early as 7 days after the neurotoxic insult in GSTP ko mice, relatively to its own control (compare panel d with panel b) and also to the corresponding results from wild-type animals (compare panel d with panel c).

DA fiber degeneration in striata from GSTP ko, as well as wild-type mice was significantly evident 30 days after

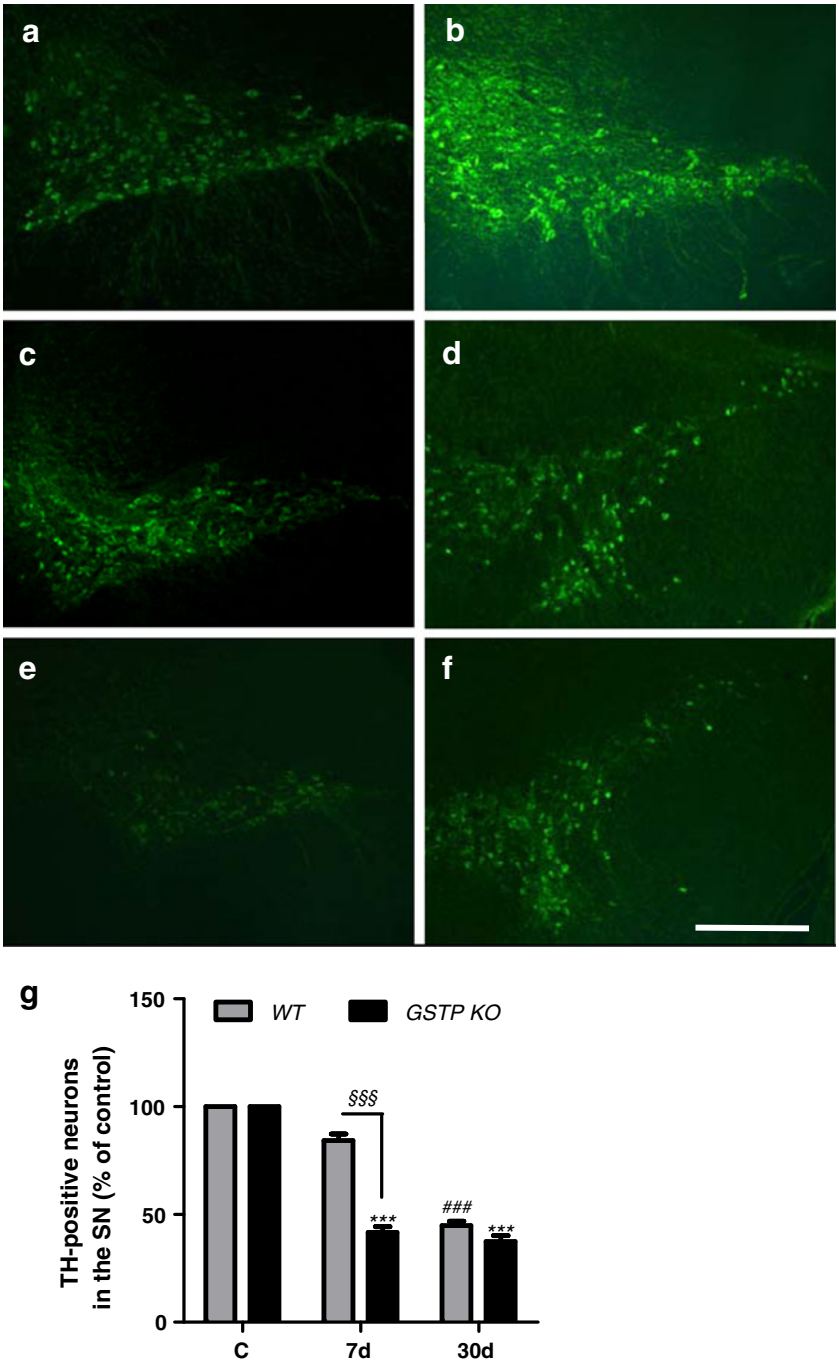

Fig. 1 Nigral dopaminergic neurons degeneration after MPTP administration. Dopaminergic neurons were identified by TH immunostaining in coronal sections at the level of SNpc (Bregma −3.20) from C57BL/6 wild-type (WT) (**a**, **c** and **e**) and GSTP knockout (KO) (**b**, **d** and **f**) mice. **a** and **b** Saline-treated (control); **c** and **d** 7 days and (**e** and **f**) 30 days post-MPTP administration; **g** The values shown are the averaged number of TH positive neurons in the *zona compacta* of the SN counted in 4 adjacent sections± SEM of three independent experiments, $^{###}p<0.001$ vs WT control; $***p<0.001$ vs KO control; $^{§§§}p<0.001$ vs WT (7 days). Microphotographs shown are representative of three independent experiments. *Scale bar* 100 μm

MPTP administration. By that time point, TH-positive striatal fibers density and immunofluorescence intensity showed a similar pattern in striata from both wild-type and GSTP ko mice (Fig. 2: panels e and f).

No significant difference in the number and density of TH positive cells and fibers was observed in the brain slices from mice sacrificed before day 7 post-MPTP administration (data not shown). Moreover, these results also indicate that 30 days after MPTP administration the level of DA cell death is equivalent in both wild-type and GSTP ko mice.

Taken together, these results show that in GSTP ko mice the MPTP-induced DA cell loss occurs earlier, indicating that these mice are more susceptible to MPTP toxicity.

MPTP-Induced JNK Phosphorylation and Activation

To determine whether endogenous GSTP affects the profile of JNK activation in the presence of MPTP, the phosphorylation status as well as the catalytic activity of JNK was evaluated in striata and midbrains from wild-type and GSTP ko mice treated with MPTP.

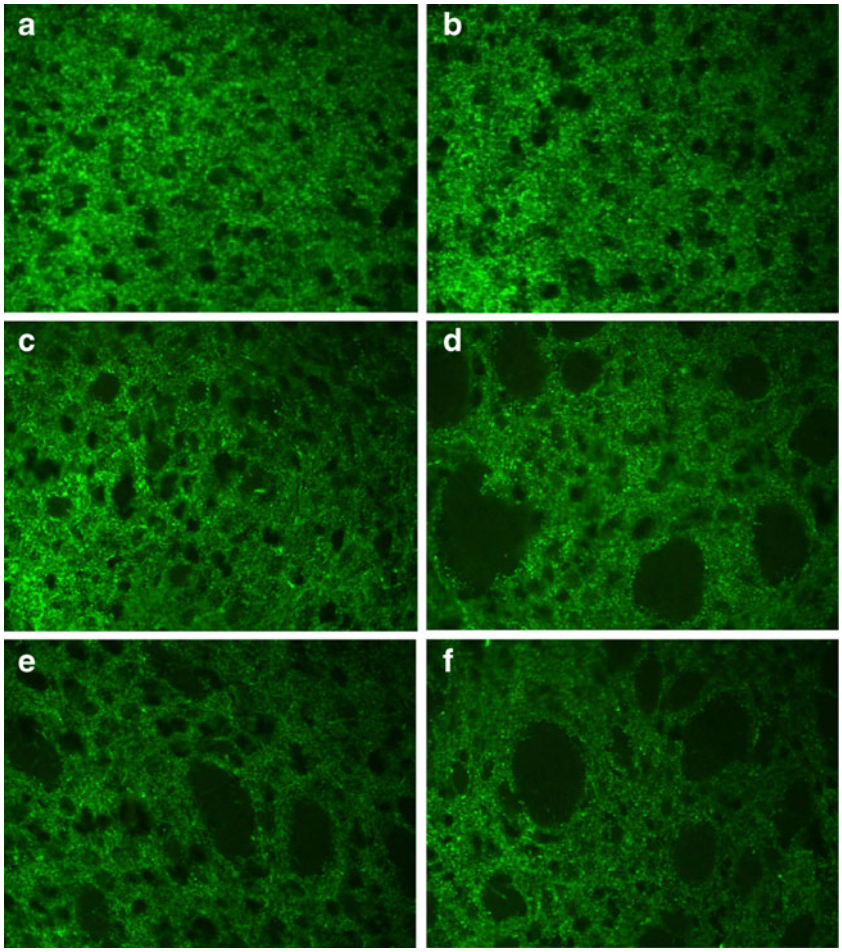

Fig. 2 Striatal dopaminergic fibers degeneration after MPTP administration. Dopaminergic fibers were identified by TH immunostaining in coronal sections at the level of midstriatum (Bregma 1.00) from C57BL/6 wild-type (**a**, **c**, and **e**) and GSTP knock-out (**b**, **d**, and **f**) mice. **a** and **b** Saline-treated (control); **c** and **d** 7 days; **e** and **f** 30 days post-MPTP administration, respectively. Microphotographs shown are representative of three independent experiments. *Scale bar* 100 μm

JNK phosphorylation was evaluated by Western blot assay using a specific antibody that recognizes dual phosphorylation of JNK (Thr183/Tyr85). Figure 3 shows that 3 h post MPTP administration, the levels of phosphorylated JNK are significantly increased in striata (Fig. 3a) and midbrains (Fig. 3b) from wild-type and GSTP ko animals. The observed increases of the JNK phosphorylation status were shown to be both fast and transient since p-JNK control levels are restored 6 h after MPTP administration, in both brain regions and mouse lines.

Interestingly, the phosphorylation level of JNK 3 h after MPTP treatment was more evident and statistically different in striata from GSTP ko mice as compared with both controls and the corresponding sample from wild-type mice (Fig. 3c; $p<0.05$ and $p<0.001$, one-way ANOVA with Tukey post-hoc test).

To further elucidate the effect of endogenous GSTP on the JNK pathway, a non-radioactive kinase assay for measuring JNK activity was performed. In this assay, phosphorylation of the downstream substrate c-Jun was utilized to determine JNK enzymatic activity in the studied brain regions from wild-type and GSTP ko mice. There was a transient increase in the amount of in vitro phosphorylated c-Jun, reaching a peak 3 h or 6 h following MPTP administration, in striata (Fig. 4a and c), and 3 h or 6 h in midbrains (Fig. 4b and d) from GSTP ko and wild-type mice, respectively. Although the results from wild-type and GSTP ko mice, shown in Fig. 4, are presented as a percentage of their respective controls, it is worth noticing that the ability of JNK to in vitro phosphorylate c-Jun is significantly higher in the striatum and midbrain control samples from GSTP ko mice.

In vivo phosphorylation of c-Jun was also assessed by Western blot assay using a specific antibody that recognizes phosphorylation of c-Jun (Ser73). Results presented in Fig. 5 show that in the evaluated time points no significant increase in the levels of p-c-Jun were detected in both midbrain and striata from wild-type mice. However, the endogenous c-Jun is significantly activated in the midbrain of GSTP ko mice at 6 h following MPTP administration.

GSTP Modulation of JNK Activity by Physical Interaction In Vivo

GSTP was characterized as a JNK-associated protein exerting its inhibitory effects on the kinase activation by physical protein–protein interaction [28].

To elucidate the role of GSTP on the regulation of JNK activity upon MPTP administration we performed co-

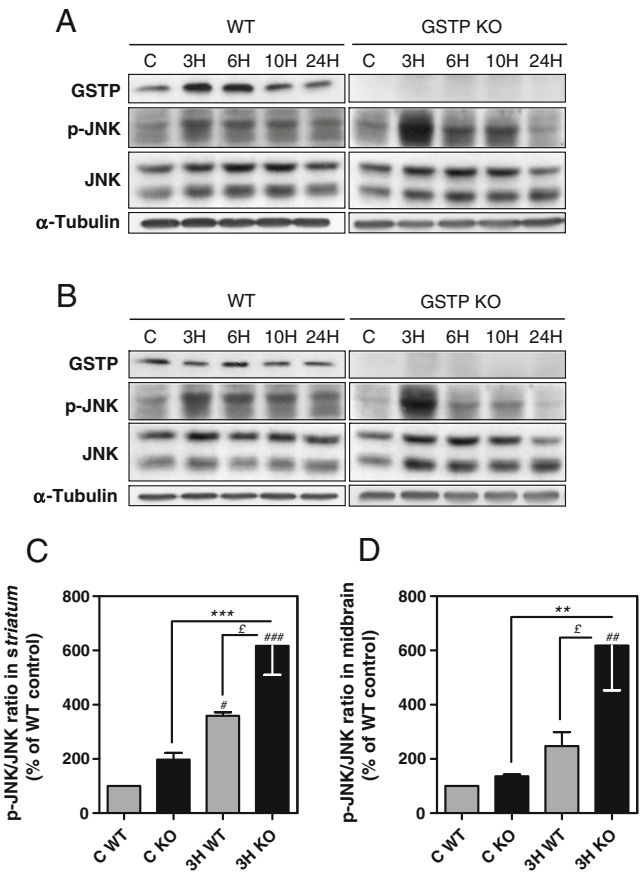

Fig. 3 JNK phosphorylation levels in response to MPTP administration in wild-type and GSTP knockout mice. Tissue extracts from C57BL/6 wild-type (WT) and GSTP knockout (GSTP KO) mice striatum (**a**) and midbrain (**b**) were prepared after saline (control, **c**) or MPTP single dose (40 mg/kg body weight) i.p. injection. Mice were sacrificed 3, 6, 10, and 24 h after MPTP administration. Tissue extracts were subjected to SDS/PAGE, and the corresponding blots were probed with antibodies to GSTP, p-JNK, JNK, and α-tubulin. The phospho-JNK/ JNK ratios from striatum (**c**) and from midbrain (**d**) at 3 h time-point from WT and GSTP KO mice were plotted as the mean± SEM of three independent experiments, indicated as percentage of controls from wild-type animal samples, $^{\#}p<0.05$, $^{\#\#}p<0.01$, and $^{\#\#\#}p<0.001$ vs WT control; $^{**}p<0.01$ and $^{***}p<0.001$ vs KO control; $^{£}p<0.05$ vs WT (3 h)

immunoprecipitation assays to evaluate putative alterations of GSTP-JNK protein–protein complex in striatal and midbrain samples from wild-type mice.

The results of JNK-GSTP pull-down assays, presented in Fig. 6, show that these two proteins physically interact in vivo, and that this complex is present in both tissue extracts from control mice. Interestingly, treatment with MPTP induces the dissociation of this complex 3 h post-treatment in both striatum and midbrain. The protein complex dissociation seems to be transient since 10 h post-MPTP administration GSTP-JNK association levels return to a value comparable to the control. Remarkably, the time points in which the transient increase of JNK enzymatic activity occurs correlate with the dissociation of GSTP-JNK complex, indicating that in the absence of GSTP, JNK is free to be phosphorylated and to phosphorylate its downstream substrates. Moreover, the re-association between GSTP and JNK occurs at time points when we have previously described an increased expression of GSTP following MPTP administration [5], suggesting that the increase in the pool of GSTP restores JNK to an inactive state by protein–protein interaction.

Discussion

GSTP belongs to the phase II family of detoxification enzymes and plays a critical role in protection against oxidative stress by either conjugation of glutathione (GSH) to electrophilic substrates or by direct regulation of the stress kinase JNK pathway [24, 43, 44]. Our present data are novel in that they provide experimental evidence that, in vivo, one mechanism by which GSTP exerts its neuroprotective actions is by physical association with JNK leading to down-regulation of kinase activity.

Results presented herein show that administration of MPTP induces a progressive demise of nigral DA neurons in parallel with the degeneration of striatal terminals in both wild-type and GSTP ko mice. However, while in GSTP ko mice DA cell death in SNpc is significant 7 days post-MPTP, in wild-type animals it only reaches significance 30 days post-MPTP administration as we had previously demonstrated [5]. In accordance, the degeneration of striatal fibers also occurs at an earlier time point in GSTP ko mice as compared with wild-type mice. These results indicate that GSTP ko mice are more susceptible to MPTP neurotoxic effects than wild-type mice. Since 30 days after MPTP both groups of animals show equivalent DA neurodegeneration, we hypothesize that endogenous GSTP exerts its neuroprotective effects against MPTP toxicity through the modulation of upstream events in the degenerative cascade.

MPTP administration causes the inhibition of mitochondrial respiration which results in rapid secondary events, including depletion in intracellular ATP and increased levels of oxidative stress [8, 9]. We previously confirmed the presence of increased markers of oxidative stress following MPTP treatment in both wild-type and GSTP ko mice (data not shown). Oxidative stress is also an important activator of the JNK signaling cascade. In fact, we have shown that MPTP administration induces a fast and transient increase in the levels of phosphorylated JNK in striata and midbrains from wild-type and GSTP ko mice. Moreover, we demonstrate that upon MPTP treatment JNK is transiently activated and capable of phosphorylating its downstream substrate c-Jun.

Constitutively increased JNK activity has been previously described in the liver, lung, and fibroblasts from

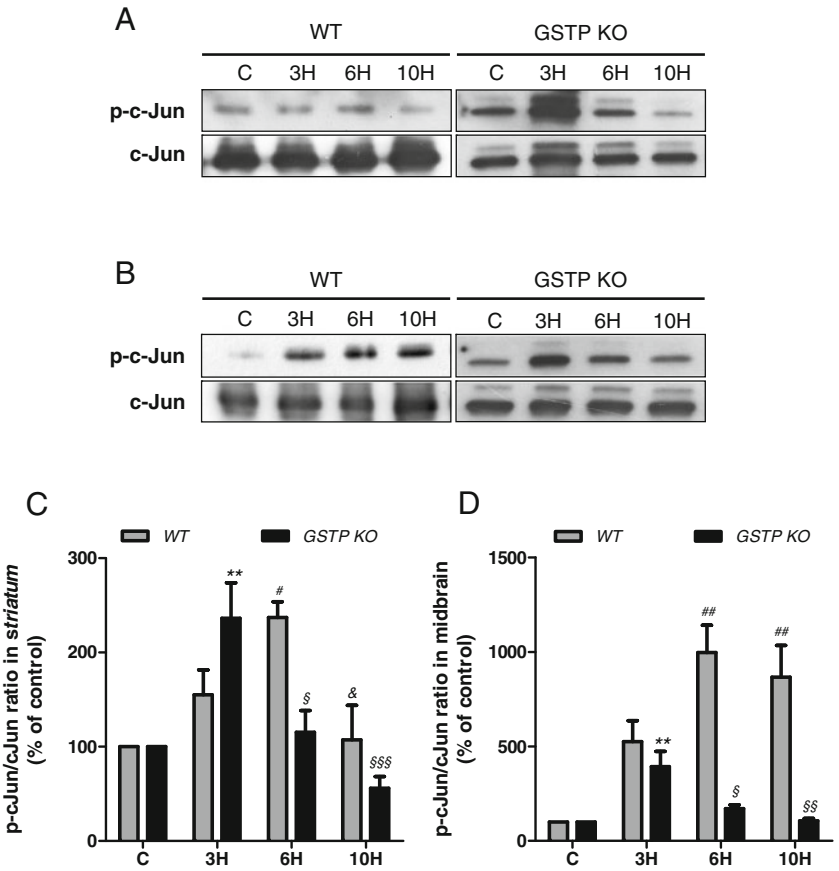

Fig. 4 JNK activation after MPTP administration in wild-type and GSTP knockout mice. Tissue extracts from C57BL/6 wild-type (WT) and GSTP knockout (GSTP KO) mice striatum (**a**) and midbrain (**b**) were prepared after saline (control, **c**) or MPTP single dose (40 mg/kg body weight) i.p. injection. Mice were sacrificed 3, 6, and 10 h after MPTP administration. JNK enzymatic activity was evaluated with a non-radioactive kinase assay: tissue extracts were incubated with c-Jun fusion beads to pull-down JNK. Kinase reactions were then performed, in vitro and the phosphorylation of c-Jun was assessed by immunoblotting. The blots were probed with antibodies to phospho-c-Jun (p-c-Jun) and c-Jun. The immunoblots presented are representative of three independent experiments. The p-c-Jun/c-Jun ratios from striatum (**c**) and from midbrain (**d**) are given as mean±SEM of three independent experiments, $^{**}p<0.01$ vs KO control; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs WT control; $^{\S}p<0.05$, $^{\S\S}p<0.01$, and $^{\S\S\S}p<0.001$ vs KO (3 h); $^{\&}p<0.05$ vs WT (6 h)

GSTP ko mice [28, 29]. In agreement, our results show that the basal levels of phosphorylated JNK are higher in GSTP ko mice. Importantly, following MPTP treatment the levels of phosphorylated JNK are significantly higher in GSTP ko mice as compared with the wild-type counterparts.

Given the extensive experimental evidence showing that JNK signaling pathway mediates part of the cellular mechanisms operating in neurodegenerescence induced by MPTP [14, 20–22], we believe that the significant difference in the in vivo activity of JNK, between GSTP ko and wild-type mice, might explain their different susceptibility to MPTP.

It is now becoming evident that GSTP may have important cellular actions that do not solely involve its primary catalytic functions. GSTP regulates intracellular signaling pathways as an endogenous inhibitor of JNK and can have a non-enzymatic regulatory role in controlling cellular response to external stimuli [45].

Interestingly, by co-immunoprecipitation assays, we demonstrate that GSTP physically interacts with JNK in mice brain extracts from striatum and midbrain. Moreover, these assays also indicate that the kinetics of GSTP-JNK dissociation in response to MPTP reflects the time course of JNK activation and the up-regulation of GSTP expression following MPTP intoxication. In untreated control mice, GSTP is bound to JNK, keeping the kinase in an inactive state. MPTP administration induces a significant transient increase in JNK phosphorylation and activation, which is accompanied by the release of GSTP. In parallel with the increased activation of JNK, we have previously shown that GSTP expression is induced by MPTP [5]. This explains, at least in part, the transient activation of JNK, since 10 h after MPTP administration GSTP-JNK complexes return to levels comparable to the control. GSTP has been shown to directly bind to JNK in several cell types and to negatively regulate JNK pathway under different stress conditions [28,

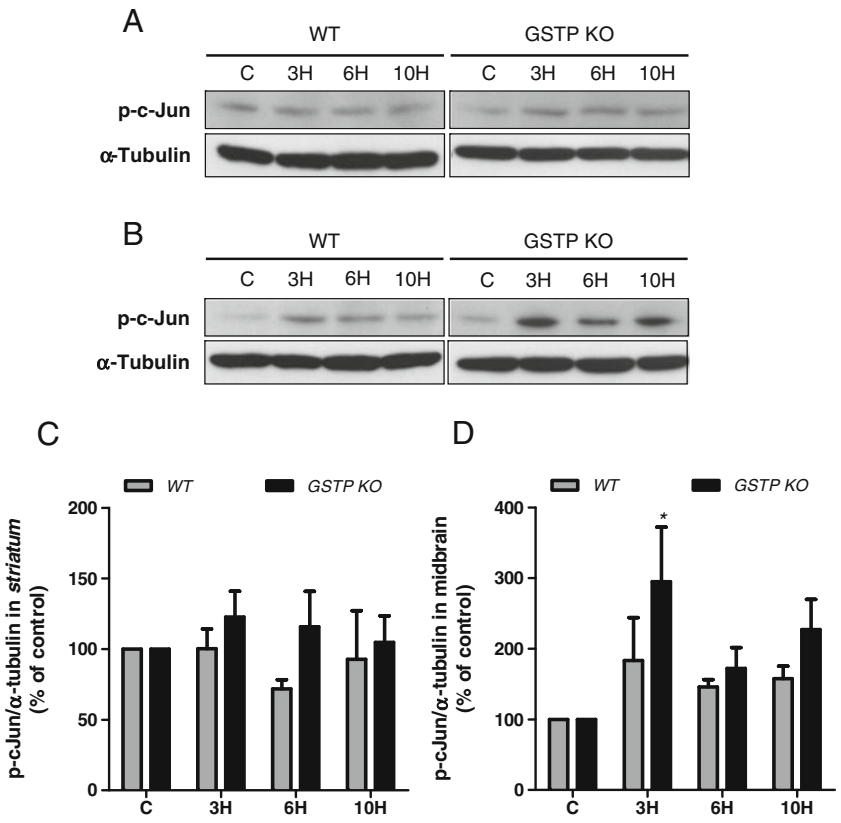

Fig. 5 p-c-Jun protein expression in wild-type and GSTP knockout mice following MPTP treatment. Tissue extracts from C57BL/6 wild-type (WT) and GSTP knockout (GSTP KO) mice striatum (**a**) and midbrain (**b**) were prepared after saline (control, **c**) or MPTP single dose (40 mg/ Kg body weight) i.p. injection. Mice were sacrificed 3, 6 and 10 h after MPTP administration. Tissue extracts were subjected to SDS/PAGE, and the corresponding blots were probed with antibodies to phospho-c-Jun (p-c-Jun) and α-tubulin. The immunoblots presented are representative of three independent experiments. The p-c-Jun/α-tubulin ratios from striatum (**c**) and from midbrain (**d**) are given as mean±SEM of three independent experiments, *$p<0.05$ vs KO control

31, 32]. For example, using human SH-SY5Y neuroblastoma cell cultures, we have previously shown that, in vitro, GSTP forms a complex with JNK, therefore maintaining the catalytic kinase activity low in the absence of a stress stimulus. UV light triggered the dissociation of the complex liberating JNK to be phosphorylated and to phosphorylate c-Jun [37]. However, as far as we know, this is the first time that the interaction between GSTP and JNK is demonstrated to occur in vivo.

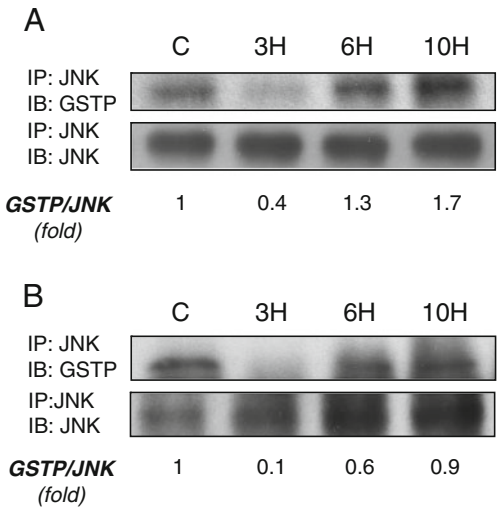

Fig. 6 Determination of protein-protein interaction between GSTP and JNK after MPTP treatment. Tissue extracts from C57BL/6 wild-type mice striatum (**a**) and midbrain (**b**), before (control, **c**) or 3, 6, and 10 h after MPTP administration were prepared. Protein–protein interactions between GSTP and JNK were evaluated by co-immunoprecipitation assays (IP) using the anti-JNK antibody followed by immunoblotting (IB) with the antibody to GSTP. The immunoblots presented are representative of three independent experiments

Previously, we have identified DA neurons, but mainly astrocytes and oligodendrocytes, as GSTP-positive cells, in midbrain and striatum from C57BL/6 wild-type mice [5]. Interestingly, up-regulation of the JNK pathway has been demonstrated not only in DA neurons following MPTP administration, but also in glial cells in a neurodegenerative environment.

There is now increasing recognition for the role of astrogliosis and activated microglia as major factors in the pathogenesis of PD, and also in the neurodegenerative process that occurs in the presence of MPTP/MPP + [2, 46, 47]. Neuronal inflammation is characterized by glial morphological changes, increased expression of surface molecules, secretion of soluble pro-inflammatory mediators, up-regulation of cyclooxygenase-2 and nitric oxide synthase and phagocytosis of degenerating neurons [2, 47, 48]. Thus, once becoming activated by injured neurons, glia can turn into a source of damaging toxic factors to inflict harm to other neurons and glial cells in the vicinity. For example, oligodendrocytes are extremely vulnerable to increases in ROS and pro-inflammatory mediators produced by activated

microglia and astrocytes. Oligodendrocyte cell death results in extensive demyelination which further contributes to neuron degeneration [49]. On the other hand, astrogliosis and microgliosis have a parallel paracrine effect, inducing cell death in astrocytes and microglia, playing a role in a self-amplifying cycle of degeneration. Glial death induced by neuroinflammation requires the activation of JNK intracellular signaling pathway [50, 51].

To further characterize the neuroprotection elicited by endogenous GSTP against MPTP intoxication, we evaluated total GST catalytic activity (Online Ressource 1). We found no differences between total GST specific activity in striata and midbrains from wild-type and GSTP ko mice, neither before nor after MPTP administration. These results indicate that there probably is a compensatory increase in other brain GST isoforms (alpha and mu) in GSTP ko mice; however the protective role of endogenous GSTP in wild-type mice does not seem to depend on its catalytic activity but rather on its ability to inhibit JNK activation.

Taken together, the findings presented in this work indicate that the neuroprotective role of GSTP involves the direct regulation of JNK pathway, which is an early event in the degenerative cascade triggered by MPTP. It is likely that the increase in GSTP previously observed in wild-type mice is an adaptive mechanism in response to the neurotoxic and oxidative insults. This hypothesis is corroborated by previous studies showing that an increase in GSTP expression occurs in response to ROS-generating agents and is mediated via the JNK/c-Jun cascade, in a feedback regulatory loop [30, 52, 53].

It is worth noting that even in GSTP ko mice the phosphorylation and activation of JNK in response to MPTP is also transient, indicating that other factors besides GSTP regulate the catalytic kinase activity. In fact, it has also been shown that the heat shock protein, Hsp70, is another intracellular protein that inhibits JNK signaling in vitro and in vivo [54]. Interestingly, preliminary results from our work point to a transient increase in heat shock proteins expression in mice brain under MPTP treatment (data not shown). Therefore, Hsp70 appears as a putative candidate that, in the absence of GSTP, may contribute to the negative regulation of JNK activation in the GSTP ko mice.

Finally, GSTP may also exert neuroprotective effects against MPTP-triggered oxidative stress by *S*-glutathionylation of several proteins. This constitutes a reversible mechanism by which a disulfide bond is established between a protein cysteinyl residue and GSH, protecting proteins from oxidative damages [55]. Protein *S*-glutathionylation following MPTP systemic administration is currently under investigation in our laboratory.

In this present study, we provide evidence that GSTP has a neuroprotective role through a non-catalytic, ligand-binding activity with JNK. We consider that drug targeting of the cellular GSTP levels is a potential therapeutic approach for PD intervention.

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Conflict of Interest The authors declare that they have no conflict of interest.

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