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GSTpi Expression in MPTP-Induced Dopaminergic Neurodegeneration of C57BL/6 Mouse Midbrain and Striatum

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Abstract MPTP-induced dopaminergic neurotoxicity involves major biochemical processes such as oxidative stress and impaired energy metabolism, leading to a significant reduction in the number of nigrostriatal dopaminergic neurons. Glutathione *S*-transferase pi (GSTpi) is a phase II detoxifying enzyme that provides protection of cells from injury by toxic chemicals and products of oxidative stress. In humans, polymorphisms of GSTP1 affect substrate selectivity and stability increasing the susceptibility to parkinsonism-inducing effects of environmental toxins. Given the ability of MPTP to increase the levels of reactive oxygen species and the link between altered redox potential and the expression and activity of GSTpi, we investigated the effect of MPTP on GSTpi cellular concentration in an in vivo model of Parkinson's disease. The present study demonstrates that GSTpi is actively expressed in both substantia nigra pars compacta and striatum of C57BL/6 mice brain, mostly in oligodendrocytes and astrocytes. After systemic administration of MPTP, GSTpi expression is significantly increased in glial cells in the vicinity of dopaminergic neurons cell bodies and fibers. The results suggest that GSTpi expression may be part of the mechanism underlying the ability of glial cells to elicit protection against the mechanisms involved in MPTP-induced neuronal death.

Keywords Glutathione *S*-transferase pi · Parkinson's disease · MPTP · Neurodegeneration · Detoxification

Introduction

Idiopathic Parkinson's disease (PD), the second most common neurodegenerative disease, is characterized by a progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc), resulting in a severe loss of striatal dopaminergic fibers and consequent dopamine depletion in the striatum (Hornykiewicz and Kish 1987). The multifactorial etiology of idiopathic PD, although not fully understood, is known to involve aging, genetic predisposition, and environmental exposure to compounds that generate oxidative stress (Riedl et al. 1999; Riess and Kruger 1999; Grunblatt et al. 2004). Several experimental models of parkinsonism have been developed, including the systemic treatment of experimental animals with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Dauer and Przedborski 2003). The C57BL/6 mouse strain was found to be the most sensitive to MPTP and the more selective in terms of targeting the nigrostriatal dopaminergic neurons;

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consequently, MPTP-treated C57BL/6 mouse is an established useful model of PD (Shimohama et al. 2003).

Current evidence suggests that oxidative mechanisms and increased levels of reactive oxygen species (ROS) are key components of the processes underlying nigral cell degeneration in PD (Beal 2003; Jenner 2003). Importantly, neurons in the SNpc are thought to be particularly sensitive to oxidative stress because they contain elevated levels of iron, alpha-synuclein, and dopamine (Zucca et al. 2006). On the other hand, several in vivo studies also provide support for the importance of ROS in MPTP-induced neurodegeneration (Cassarino et al. 1997; Sriram et al. 1997).

Studies on the cellular and molecular mechanisms of MPTP-induced dopaminergic neurotoxicity have revealed that the neurotoxic process is made up of a cascade of deleterious events that include major biochemical processes such as oxidative stress and impaired energy metabolism, leading to a significant reduction in the number of nigral dopaminergic neurons (Zhang et al. 2000).

Cell protection from external damage largely depends on the availability and activity of antioxidative enzymes, which maintain homeostatic control of ROS (Yin et al. 2000). Mechanisms of protection from oxidative free radical damage include scavenging enzyme systems which detoxify ROS such as superoxide dismutase, catalase, or glutathione peroxidase (Park et al. 2004). Thus, antioxidants, as scavengers of ROS and free radicals, may play an important role in the prevention of PD (Seaton et al. 1997; Wang et al. 2006).

Glutathione *S*-transferases (GSTs) are a multigene family of isoenzymes responsible for the detoxification of electrophiles by conjugation with the nucleophilic thiol-reduced glutathione (GSH; Mannervik and Danielson, 1988) and play critical roles in providing protection of cells from injury by toxic chemicals (drugs, pesticides, carcinogens) and products of oxidative stress (Menegon et al. 1998; Hayes et al. 2005). In mammals, eight different isoforms of cytosolic–soluble GSTs, namely alpha, kappa, mu, pi, sigma, theta, zeta, and omega have been identified (Mannervik et al. 2005), although only the isoforms alpha, mu, and pi have been described in the central nervous system (Awasthi et al. 1994; Beiswanger et al. 1995). Among these three, GSTpi has been implicated in protection of cells from ROS-inducing agents due to its ability to alter levels of cellular glutathione in response to production of ROS (Baez et al. 1997; Tew and Ronai 1999). Taken together, these evidences suggest that the level of GSTpi expression is crucial in determining cell sensitivity to a broad spectrum of toxic chemicals (Hayes and Pulford 1995), therefore ameliorating the oxidative milieu especially prevalent in dopaminergic neurons. Recent epidemiological studies from our own group (Vilar et al. 2007) and others (Golbe et al. 2007) indicate that *GSTP* wild-type allele is an individual protective genetic trait in idiopathic PD. In fact, there is good evidence that the *GSTP* polymorphisms affect substrate selectivity and stability increasing the susceptibility to parkinsonism-inducing effects of environmental toxins (Hayes and Strange 2000).

Given the ability of MPTP to increase the levels of ROS and the link between altered redox potential and the expression and activity of GSTpi, we investigated the effect of MPTP on GSTpi cellular concentration in an in vivo model of PD. The present study demonstrates that GSTpi is actively expressed in both SNpc and striatum of C57BL/6 mice brain, mostly in glial cells. GSTpi expression is significantly increased after systemic MPTP treatment, probably as part of the mechanism underlying its ability to elicit protection against the mechanisms involved in MPTP-induced cell death.

Materials and Methods

Materials

MPTP, mouse and rabbit anti-tyrosine-hydroxylase (TH), rabbit anti-glial-fibrillary-acidic protein (GFAP), fluorescein isothiocyanate (FITC)- and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat antirabbit and goat antimouse immunoglobulin G (IgG) secondary antibodies, Hoescht 33258, and amido black were purchased from Sigma Chemical Co. (St Louis, MO, USA). Mouse anti-GSTpi antibody was from BD Biosciences Pharmingen (San Jose, CA, USA). Mouse anti-Bcl-2 (C-2), mouse anti-Bax (delta 21), rabbit anti-integrin-alpha-M (H-61), and anti-α-tubulin (B-7) antibodies and TRITC-conjugated goat antimouse IgM were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse IgM antioligodendrocyte marker O4 antibody was purchased from Chemicon (Temecula, CA, USA). 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). Horseradish-peroxidase-conjugated antimouse and antirabbit secondary antibodies were from Cell Signaling Technology (Beverly, MA, USA) and Promega (Madison, USA), respectively. Immobilon P, polyvinylidene fluoride (PVDF) membrane was from Millipore (Bedford, MA, USA). ECL and Hyperfilm ECL were purchased from Amersham Biosciences (Piscataway, NJ, USA). DEVD-pNA was purchased from Bachem AG (Switzerland). 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 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.

Ten-week-old male C57BL/6 mice purchased from the Gulbenkian Institute of Science Animal House (Oeiras, Portugal) were used throughout these experiments. 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.

MPTP was administered intraperitoneally (i.p.) at a single dose of 40 mg/kg (Saporito et al. 2000). Control mice received saline alone. Mice were sacrificed 3, 6, 10, 24, or 48 h and 7, 14, or 30 days after neurotoxin or vehicle administration.

After being anesthetized with sodium pentobarbital (50 mg/kg, i.p.), mice were decapitated and brains were quickly removed and placed in fresh phosphate buffer saline (PBS). Brains were then placed on their ventral surface onto a mouse brain matrix (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 (Smeyne and Smeyne 2002). The remaining brain piece was then cut between Bregma −1.0 and 1.5, and the 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 until further use.

Preliminary studies have shown that in the saline control samples the evaluated parameters did not change through the time course; therefore, collection of control tissues was carried out at injection day 1, reducing the number of animals needed in these studies. Groups of three and six mice were used for immunohistochemistry and for western blot analysis, respectively. The time course study was carried out in three independent experiments.

Western Blot Analysis

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 at 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% sodium dodecyl sulfate (SDS), 40% glycerol, 0.2% bromophenol blue, 1% β-mercaptoethanol), boiled for 5 min, resolved on 12% SDS-polyacrylamide gel electrophoresis, and electrotransfered to PVDF membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20, for 1 h at room temperature and further incubated with the primary antibodies (anti-GSTpi, anti-Bcl-2, and anti-Bax), overnight at 4°C, followed by incubation with horseradish-peroxidase-conjugated antimouse or antirabbit secondary antibodies, for 1 h at room temperature. The immunocomplexes were detected by the electrogenerated chemiluminescent (ECL) method and visualized with Hyperfilm ECL. Analysis of α-tubulin expression was done in parallel as a loading control. The relative intensities of protein bands were analyzed using the Eagle-eye (Stratagene, CA, USA) densitometric analysis software.

Caspase-3 Activity

Caspase-3 activity was measured in midbrain and striatum tissue extracts by using the specific colorimetric tetrapeptide substrate DEVD-pNA. Briefly, 100 μg of total cell extracts were incubated with DEVD-pNA for 1 h at 37°C in a reaction buffer as described in the manufacturer's instructions. Samples were then read on a multiwell plate reader at a wavelength of 405 nm (reference wavelength of 620 nm).

Immunohistochemistry

Mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and transcardially perfused with ice-cold PBS, followed by 4% paraformaldehyde–PBS, pH 7.4. 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 pretreated with blocking solution (2% bovine serum albumin, 0.05% Tween-20 in PBS), for 1 h at room temperature. Incubation with primary antibodies (anti-TH, anti-GSTpi, anti-GFAP, anti-integrin-alpha-M, and antioligodendrocyte marker O4) was performed overnight at 4°C. After extensive rinsing in PBS, the sections were incubated with either FITC-conjugated goat antirabbit or goat antimouse IgG or TRITC-conjugated goat antimouse IgM secondary antibodies, according to the primary antibody used, for 1 h at room temperature. Finally, sections were rinsed with PBS, mounted in fluorescent mounting medium containing 5 μg/ml Hoescht 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 nonspecific binding were performed in parallel by omission of the primary antibody.

Immunohistochemical Double Staining

Simultaneous staining for two antigens within the same sections was carried out by incubating the sections simultaneously with the selected pair of primary antibodies (GSTpi and TH; GSTpi and GFAP; GSTpi and integrin alpha-M), followed by incubation with TRITC-conjugated goat antimouse plus FITC-conjugated goat antirabbit secondary antibodies. Double staining of GSTpi and oligodendrocyte marker O4 was performed by incubating slides simultaneously with mouse anti-GSTpi IgG and mouse antioligodendrocyte marker O4 IgM primary antibodies, followed by incubation with TRITC-conjugated goat antimouse IgM and then, after extensive rinsing, with FITC-conjugated goat antimouse IgG. Control experiments for nonspecific binding of secondary antibodies were performed in parallel by incubating with TRITC-conjugated goat antimouse IgM or FITC-conjugated goat antimouse IgG after preincubation with the primary antibodies mouse IgG anti-GSTpi or mouse IgM antioligodendrocyte marker O4, respectively. The remaining experimental procedures were performed as described above for the detection of a single antigen.

Statistical Analysis

All results are expressed as mean±SEM values. Data were analyzed by the one-way analysis of variance (ANOVA), and differences between groups were determined by post hoc Bonferroni's test (Prism 2.01; Graphpad, USA). Comparison of data from two groups was made by Student's two-tailed unpaired *t* test. Means were considered statistically significant at a p value below 0.05.

Results

1. Nigrostriatal TH protein expression after MPTP treatment

As a measure of dopaminergic cell death, TH protein expression was evaluated by immunohistochemistry in saline- and MPTP-treated mice. Representative sections through the midstriatum (Bregma 1.00) are shown in Fig. 1. It can be observed that TH-positive fiber density as well as immunofluorescence intensity in the striatum decline significantly 7 days after MPTP treatment. This result is further evidenced in striatal sections from mice sacrificed 14 and 30 days after MPTP administration, strongly suggesting the progressive degenerescence of dopaminergic terminals. No significant difference in the density of striatal TH-positive fibers was observed in brains from mice sacrificed before day 7 post-MPTP administration (data not shown).

The degree of nigral cell loss following MPTP administration was also determined in brain sections performed at the level of SNpc (Bregma −3.20). Nigral dopaminergic neurons were immunostained for TH, and the number of TH-positive cells in the zona compacta of the SN were counted in four adjacent sections of this area and averaged. Results presented in Fig. 2 show that a single dose of MPTP caused a decline of nearly 67% in the number of SNpc dopaminergic neurons after 30 days postneurotoxin administration. A decrease in the number of SNpc TH-positive cells was observed 7 and 14 days after MPTP treatment (data not shown); however, it only reached significance 30 days after MPTP administration.

Our results suggest that the decrease in TH-positive fiber intensity observed at the level of striatum caused by MPTP precedes the evident dying of SNpc TH-positive neurons.

2. Bcl-2 and Bax protein expression and caspase-3 activity after MPTP treatment

In order to characterize the involvement of the apoptotic cascade on the MPTP-induced neurotoxicity in C57BL/6 mice, the cellular levels of the antiapoptotic Bcl-2 and proapoptotic Bax proteins were evaluated by western blot analysis, using tissue extracts prepared from dissected midbrain and striatum. Caspase-3 activity was determined on equivalent samples, using DEVD-pNA as an exogenous substrate.

Results presented in Fig. 3 show that Bax expression and caspase-3 activity were not affected by MPTP treatment, in both SN and striatum. Moreover, MPTP treatment did not affect protein levels of Bcl-2 in the SN. However, our results show that a single 40-mg/kg dose of MPTP caused a significant reduction of the antiapoptotic protein Bcl-2 cellular content in the striatum. In mice sacrificed 6 h post-MPTP, the relative amount of striatal Bcl-2 protein declined to 66.3±10.1% of saline-treated mice ($p<0.05$), further declining to 61.3±14.4% and 47.7±9.3% of control values at 24 and 48 h after MPTP administration, respectively ($p<0.01$; Fig. 3A2).

3. Nigrostriatal GSTpi protein expression after MPTP treatment

The effect of MPTP on the GSTpi protein pattern of expression in SNpc and striatum was determined by using both western blot and immunohistochemistry assays. Western immunoblotting results show that a single-dose administration of MPTP caused a significant transient increase of the GSTpi levels in isolated SN (Fig. 4A) and striatum (Fig. 4B). At 6 h post-MPTP treatment, GSTpi protein expression increased significantly to 220±45.6%

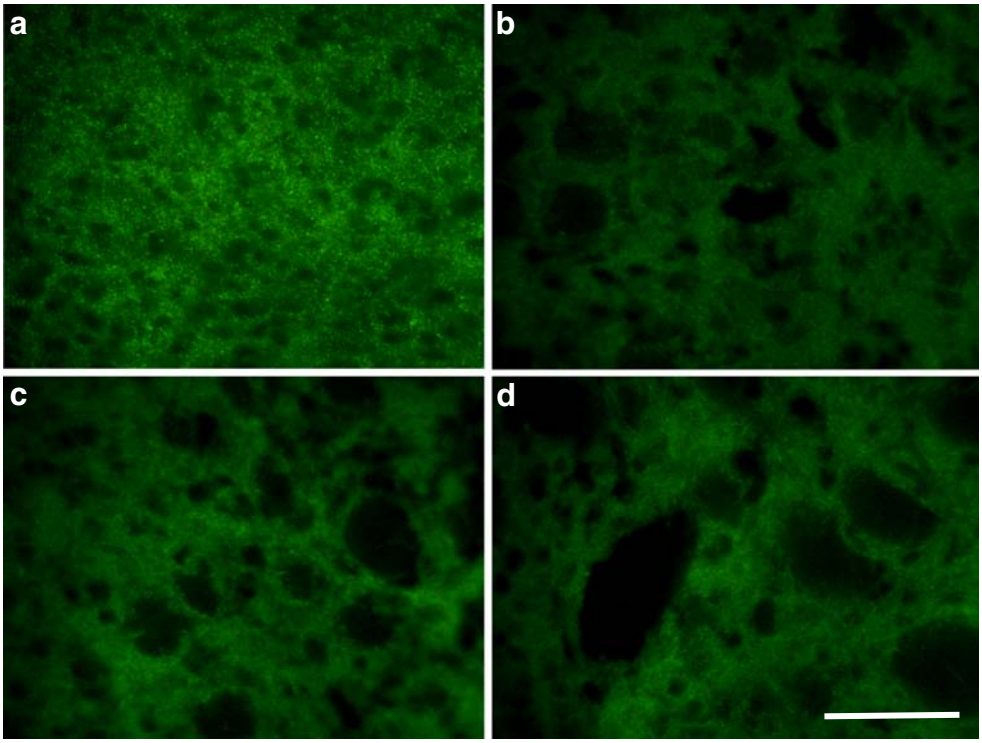

Figure 1 Loss of striatal TH-immunoreactive fibers in C57BL/6 mouse brain after MPTP treatment. Coronal sections at the level of midstriatum (Bregma 1.00) were immunostained for TH protein: **a** Saline-treated (control); **b** 7 days; **c** 14 days; and **d** 30 days post-MPTP administration, respectively. Microphotographs shown are representative of three independent experiments. *Scale bar*=100 μm

and 189.9±22.8% of the control values in the SN ($p<0.01$) and in the striatum ($p<0.05$), respectively. Twenty-four hours after administration of MPTP, the levels of GSTpi protein were similar to those found in control animals, in both SN and striatum.

Immunohistochemistry was used to evaluate the cellular distribution of GSTpi. The number of GSTpi-positive cells at the level of SNpc (Bregma −3.20) and midstriatum (Bregma 1.00) was counted in four adjacent sections of both areas and averaged for each region separately.

Results presented in Figs. 5 and 6 show that GSTpi is actively expressed in the SNpc and striatum of the C57BL/6 control mice. Interestingly, the number of GSTpi-positive cells is substantially higher in the SNpc than in the striatum. However, 6 h after MPTP treatment, the number of GSTpi-positive cells significantly increased in the SNpc (about 314% of control) and whole midbrain, as well as in the striatum (about 343% of control). This is a transient increase since 24 h after MPTP administration the number of GSTpi-immunoreactive cells, in both SNpc and striatum, although apparently higher, are not significantly different from those observed in saline-treated mice. Moreover, 30 days post-MPTP injection, the number of GSTpi-positive cells returned to basal levels, confirming our immunoblotting data.

4. Characterization of GSTpi-immunoreactive cells

To get further insights into the characterization of GSTpi-positive cells, we performed double immunofluorescent staining assays on SNpc (Bregma −3.20) mice brain sections using specific antibodies for both GSTpi and TH (Fig. 7A and B). The number of nigral dopaminergic (TH-positive) cells expressing GSTpi was counted in consecutive sections of the entire region and in sections from three different animals for each time point. Results indicate that in brain sections from control animals colocalization of TH and GSTpi occurred in less then 10% of SNpc dopaminergic neurons. Treatment with MPTP did not induce changes in the number of TH cells expressing GSTpi. We also observed that the majority of GSTpi staining was found in morphologically smaller cells surrounding the TH-positive neurons. In order to identify these cells, double immunolabeling using specific antibodies for both glial cell markers (GFAP, oligodendrocyte marker O4, and Integrin alpha-M) and GSTpi was performed.

Results presented in Fig. 7 show that GSTpi protein was found in 22.3±6% of GFAP-positive astrocytes and was also abundantly expressed in oligodendrocyte-marker-O4-positive cells (62.5±19%), in brain sections from saline-treated mice. Interestingly, 6 h after MPTP administration, GSTpi expression was detected in 52±9% of GFAP-positive astrocytes in the vicinity of dopaminergic cell bodies in the SNpc. On the other hand, the number of oligodendrocytes expressing GSTpi (73±9%) did not significantly changed after MPTP administration. Moreover, a general increase in the fluorescent staining intensity for GSTpi in glial cells was observed, reflecting an increase of GSTpi cellular content 6 h after MPTP treatment (Fig. 5

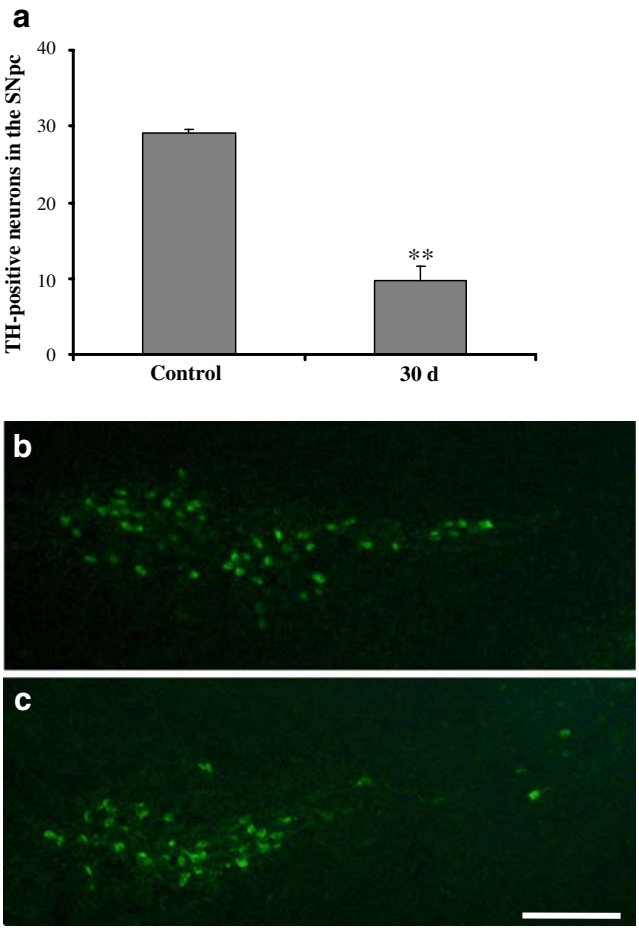

Figure 2 Loss of SNpc TH-immunoreactive neurons in C57BL/6 mouse brain after MPTP treatment. Coronal sections at the level of SNpc (Bregma −3.20) were immunostained for TH protein: **a** The values shown are the averaged number of TH-positive neurons in the SNpc counted in four adjacent sections±SEM of three independent experiments (**$p<0.01$, Student's two-tailed unpaired t test); **b** saline-treated (control); and **c** 30 days post-MPTP administration. Microphotographs shown are representative of three independent experiments. *Scale bar*=100 μm

C2 and Fig. 6C2). Equivalent results were obtained with the immunolabeling assays performed in midstriatum sections (Bregma 1.00; data not shown). No colocalization of GSTpi and microglial marker was observed (data not shown).

Taken together, our results indicate that basal level and MPTP-induced expression of GSTpi is predominant in nonneuronal cells, namely astrocytes and oligodendrocytes in both SNpc and striatum regions.

Discussion

MPTP-selective dopaminergic neuronal toxicity is known to involve mitochondrial complex I activity impairment, increased production of ROS, and consequent degeneration of nigrostriatal dopamine neurons, with the subsequent loss of striatal dopamine (Przedborski et al. 2004). Indeed, in the present study, we observed that a single dose of MPTP administration to C57BL/6 mice lead to the loss of about 67% of TH-positive nigral dopaminergic cells and to a significant decrease of striatal dopaminergic fibers. The MPTP-induced depletion of dopaminergic nerve endings in the striatum occurred before significant loss of nigral dopaminergic cell bodies. This evidence is in agreement with previous reports, suggesting that striatal dopaminergic nerve terminals are the primary target of the degenerative process and that neuronal cell death in the MPTP model of PD may result from a "dying back process" (Jackson-Lewis et al. 1995; Cochiolo et al. 2000; Faherty et al. 2005).

It is generally accepted that the dosage schedule of MPTP administration in mice determines the mode of neuronal cell death occurring in the SNpc (Schmidt and Ferger 2001; Nicotra and Parvez 2002).

In this work, we have checked specific apoptotic markers at several time points after MPTP administration. A significant decrease of the antiapoptotic protein Bcl-2 concentration was observed in the striatum after MPTP treatment. This may reflect a decrease in the endogenous defense mechanisms since Bcl-2 is an antiapoptotic protein, and some reports also suggest that Bcl-2 may inhibit necrosis (Hockenbery et al. 1993; Kane et al. 1995; Lawrence et al. 1996). On the other hand, no changes of Bcl-2 levels in the SNpc were observed, neither caspase-3 activation nor in situ end labeling with terminal deoxynucleotidyl transferase assays were shown to be positive in both SNpc and striatum in response to MPTP (data not shown). Using our paradigm of MPTP treatment, we found no morphological or biochemical evidence to support the concept that 40 mg/kg MPTP induces cell death by apoptosis in vivo. Apoptosis is an active process and such a high dose of MPTP might induce severe ATP depletion and ROS overproduction soon after intraperitoneal injection, subjecting the intoxicated cells to an energy crisis and oxidative stress (Kuhn et al. 2003; Tatton and Kish 1997). At all time points examined, we did not observe apoptosis, but we cannot exclude that programmed cell death could be induced in the mouse brain by another paradigm of MPTP injection. Necrosis is accompanied by an inflammatory infiltration of phagocytic cells. The microglial reaction, which is also quite strong in the MPTP mouse model, was previously observed to occur much earlier than that of astrocytes and more importantly reaches a maximum before the peak of dopaminergic neurodegeneration (Czlonkowska et al. 1996). The response of both astrocytes and microglial cells in the striatum and SNpc clearly occurs within a timeframe allowing these glial cells to participate in the demise of dopaminergic neurons in the MPTP mouse model (Teismann et al. 2003). In this study, we observed severe lesion of TH-positive nerve fibers in striatum days before

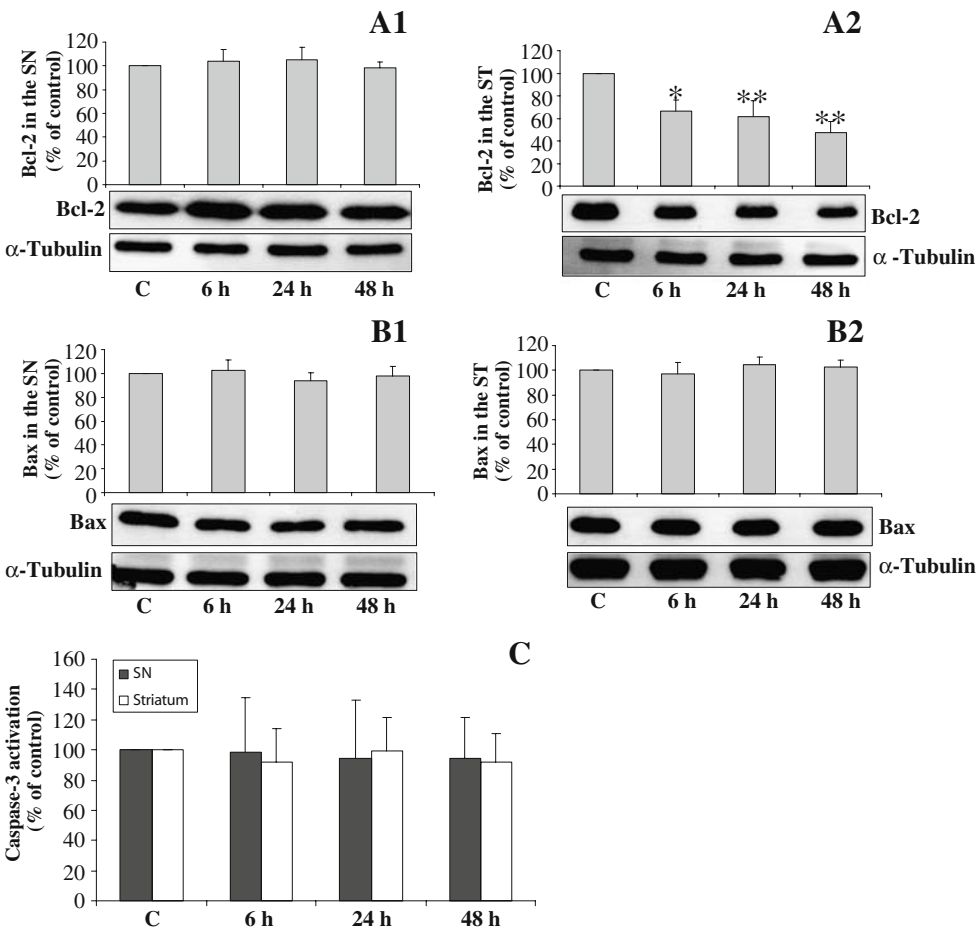

Figure 3 Bcl-2 and Bax protein expression and caspase-3 activity in SN and striatum. Effect of MPTP treatment. Western blot assays were conducted for SN and striata tissue extracts as described under "Materials and Methods" using mouse monoclonal anti-Bcl-2 (*A1* and *A2*) and anti-Bax (*B1* and *B2*) antibodies. Analysis of α-tubulin was done in parallel as a loading control. The relative levels of the studied proteins in saline-treated control mice was arbitrarily set as 100%, and their level in MPTP-treated samples was calculated and plotted as a percentage of this value. *C* Caspase-3 activity was determined on SN and striatal samples using DEVD-pNA as substrate. C-saline-treated (control); time points after MPTP administration: 6, 24, and 48 h. Data shown are mean values±SEM of three independent experiments (*$p<0.05$ and ** $p<0.01$, one-way ANOVA—Bonferroni's posttest)

the evident dying of dopaminergic neurons in the SNpc. Our results suggest that MPTP produces a "hit-and-run" kind of damage suggesting that single acute insult in the nigrostriatal pathway could set in motion a self-sustaining cascade of events with long-lasting deleterious effects.

In the present study, we show that GSTpi is actively expressed in the mouse brain and that administration of a single dose of MPTP significantly increases its expression at the level of both midbrain SNpc and striatum. The observed GSTpi over expression at 6 h post-MPTP

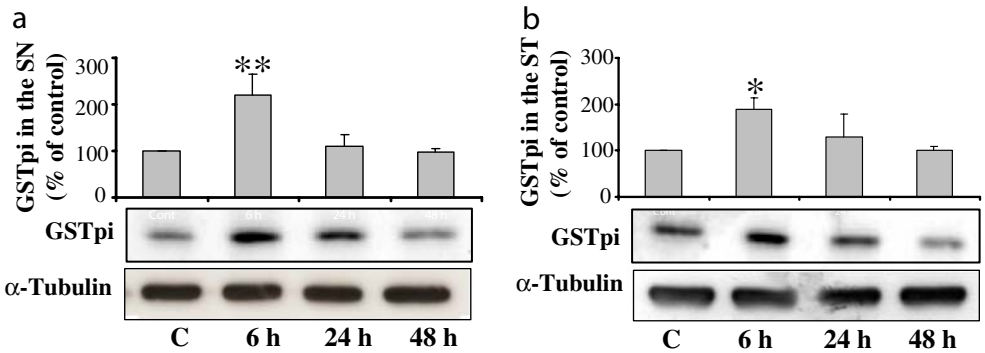

Figure 4 GSTpi protein expression in SN and striatum. Effect of MPTP treatment. Western blot assays were conducted for SN (**a**) and striatum (**b**) tissue extracts, as described under "Materials and Methods" using a mouse monoclonal anti-GSTpi antibody. Analysis of α-tubulin was done in parallel as a loading control. The relative levels of GSTpi protein in nigral and striatal tissue homogenates from saline-treated control mice was arbitrarily set as 100%, and GSTpi levels in MPTP-treated samples was calculated and plotted as a percentage of this value. Data shown are mean values±SEM of three independent experiments (*$p<0.05$ and **$p<0.01$, one-way ANOVA—Bonferroni's posttest)

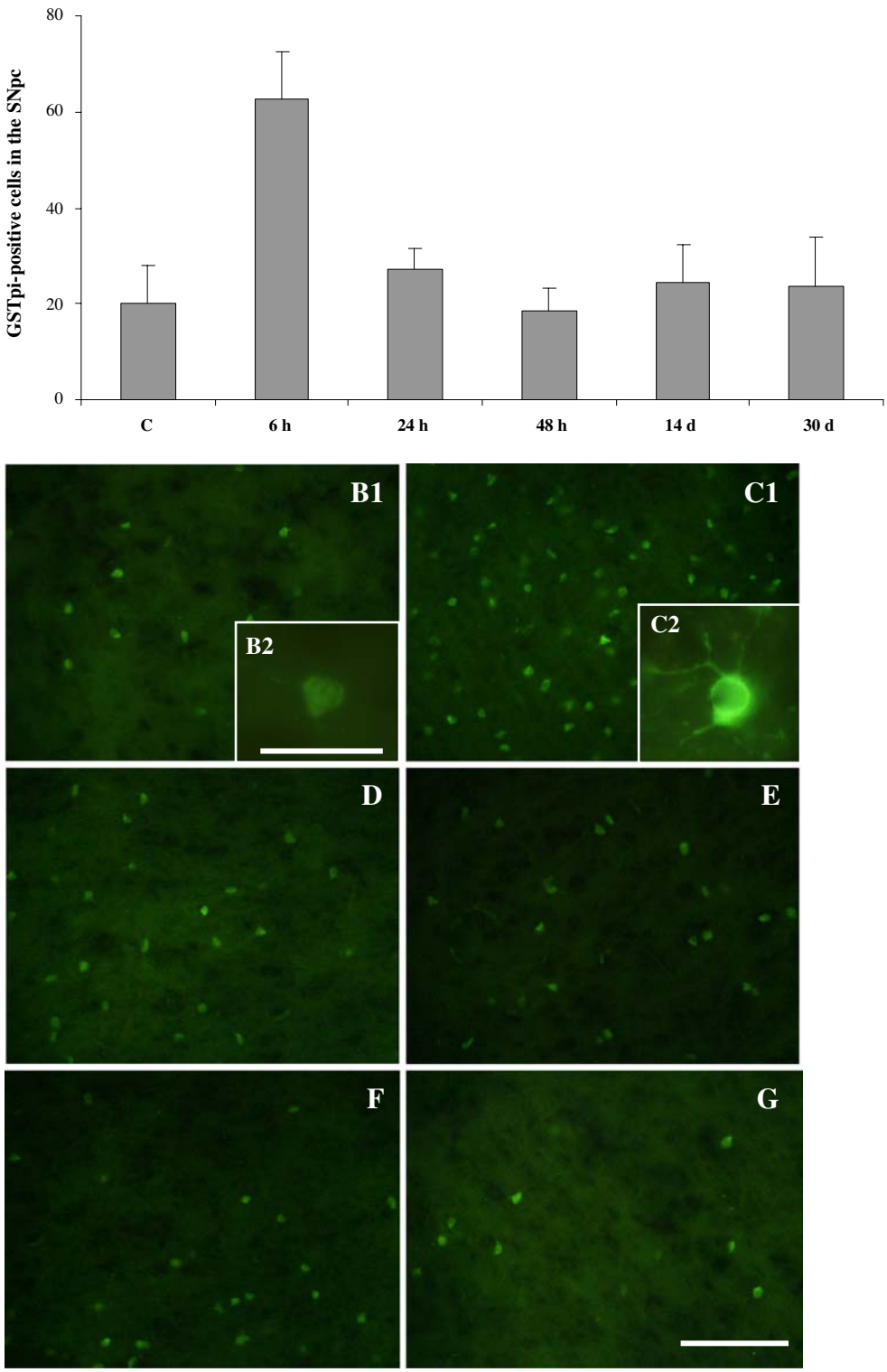

Figure 5 Effect of MPTP on the number of nigral GSTpi-immunoreactive cells. Coronal sections at the level of SNpc (Bregma −3.20) were stained for GSTpi protein. *A* Number of GSTpi-positive cells in the SN. The values shown are the averaged number of GSTpi-positive cells in the SNpc counted in four adjacent sections±SEM (**$p<$ 0.01, one-way ANOVA—Bonferroni's posttest); *B1* and *B2* Saline-treated (control); time points after MPTP administration: *C1* and *C2* 6 h; *D* 24 h; *E* 48 h; *F* 14 days; and *G* 30 days. Microphotographs shown are representative of three independent experiments. *Scale bar*= 100 μm; *Insert*=50 μm

treatment may reflect the involvement of this phase II detoxifying enzyme in an adaptive or compensatory cytoprotective response by the cell in response to ROS-mediated toxic and oxidative insults. The involvement of oxidative stress in PD is closely related to the specific vulnerability of nigrostriatal dopaminergic neurons to degeneration (Jenner 2003; Yamamoto et al. 2007). Oxidative stress may be sensed by the NF-E2-related factor 2 (Nrf2)-Keap1 system, which in response regulates the expression of antioxidant response proteins and phase II drug metabolizing enzymes, namely GSTpi through the antioxidant responsive element (ARE) leading to cellular

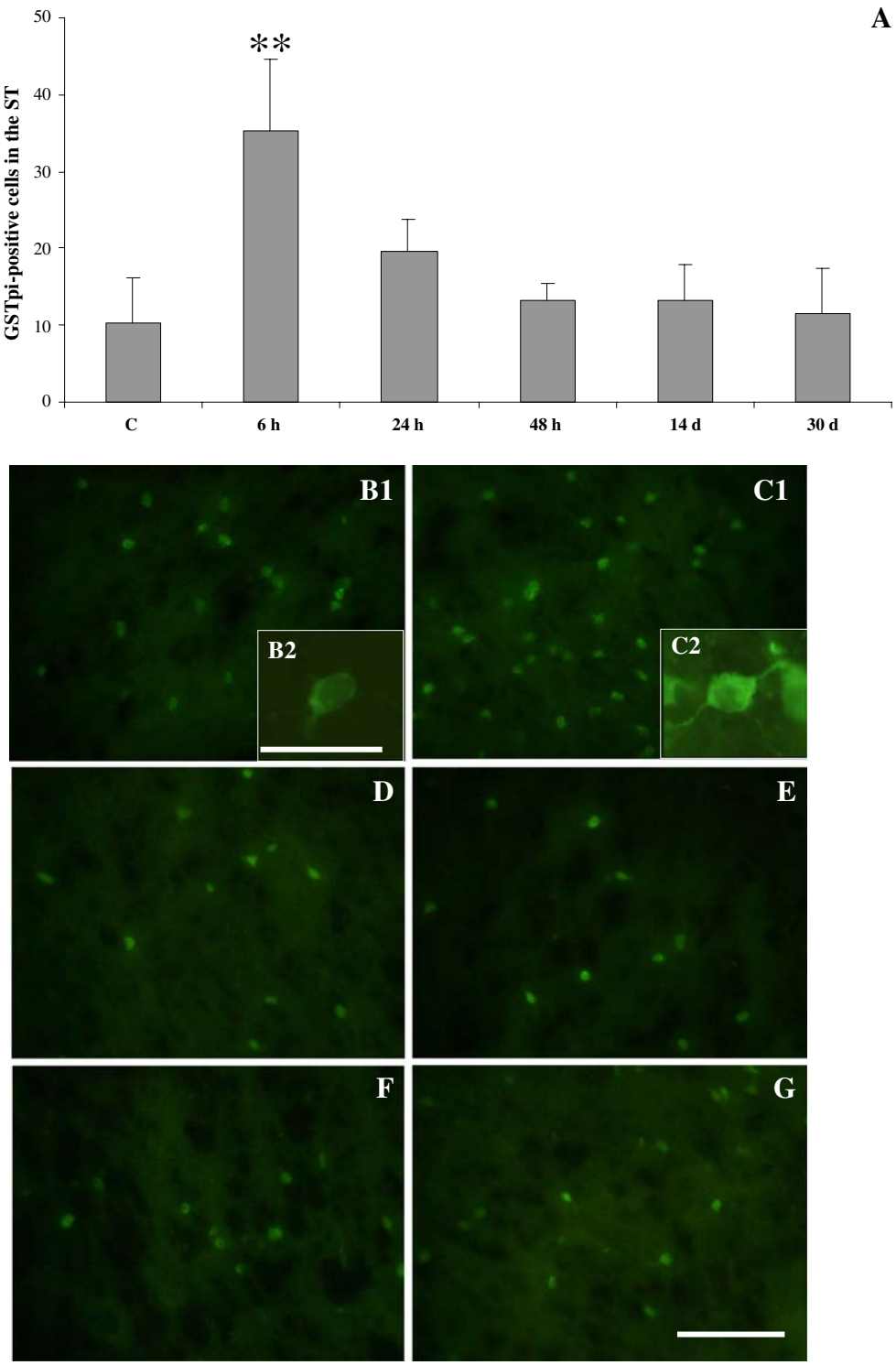

Figure 6 Effect of MPTP on the number of striatal GSTpi-immunoreactive cells. Coronal sections at the level of midstriatum (Bregma 1.00) were stained for GSTpi protein. *A* Number of GSTpi-positive cells in the striatum. The values shown are the averaged number of TH-positive neurons in the striatum counted in four adjacent sections±SEM (**$p<0.01$, one-way ANOVA—Bonferroni's posttest); *B1* and *B2* Saline-treated (control); time points after MPTP administration: *C1* and *C2* 6 h; *D* 24 h; *E* 48 h; *F* 14 days; and *G* 30 days. Microphotographs shown are representative of three independent experiments. *Scale bar*= 100 μm; *Insert*=50 μm

detoxification of ROS (Nguyen et al. 2003; Lee and Johnson 2004; Kobayashi et al. 2006). Although dopaminergic neuronal dysfunction is central in PD, the activation of glial cells is associated to many processes possibly involved in the pathogenesis of PD (Jenner 2003; Teismann et al. 2003). Glial cells have a higher antioxidant potential than neurons, an advantage resulting in part from the activation of Nrf2–ARE pathway. In fact, Nrf2 overexpression increases the resistance of neurons to oxidative stress (Shih et al. 2003).

Furthermore, a link between redox active components of GSTpi and the c-Jun N-terminal kinase has been redefined

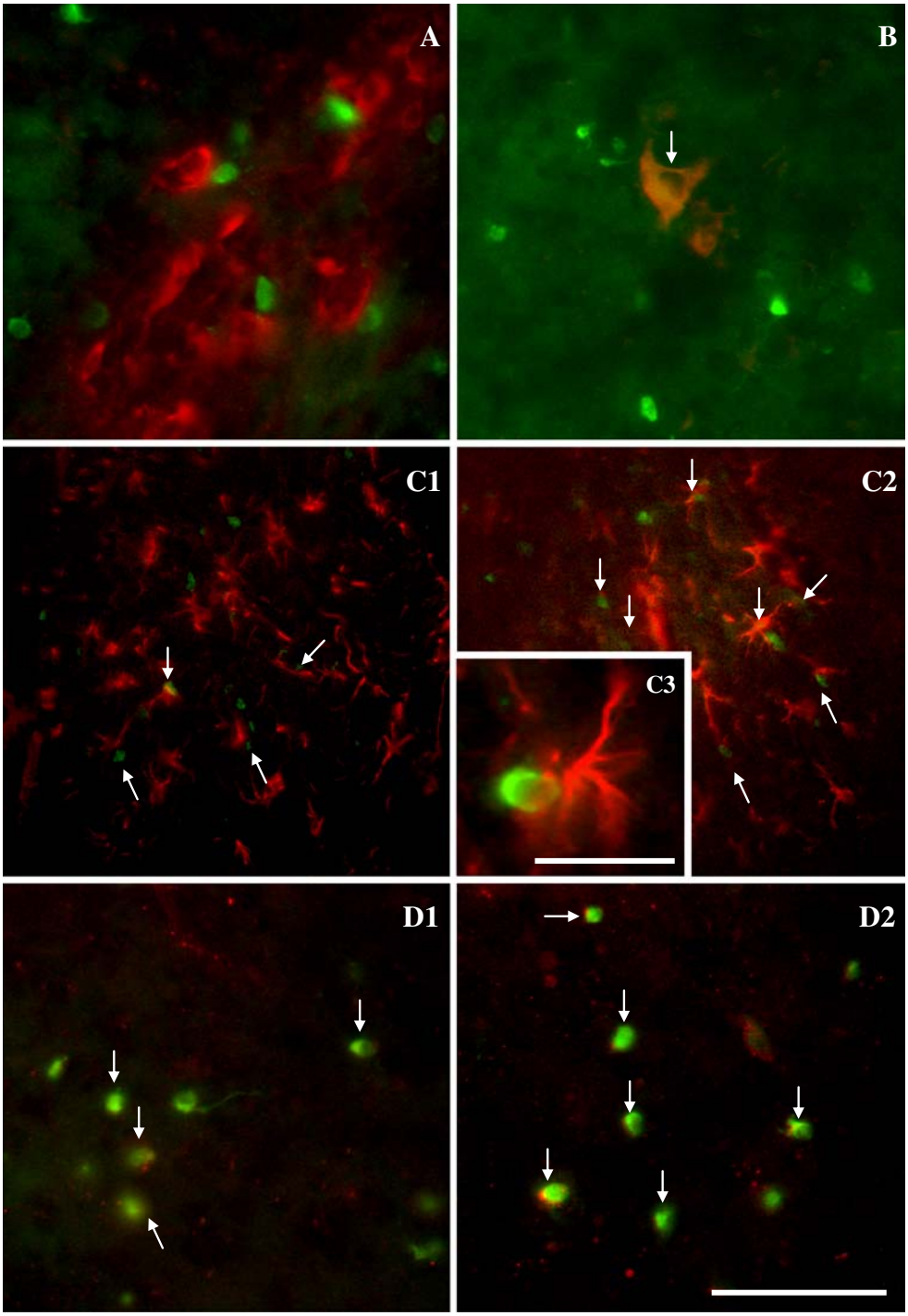

Figure 7 Characterization of nigral GSTpi-immunoreactive cells. Coronal sections at the level of SNpc (Bregma −3.20) from saline-treated mice (*C1* and *D1*) or from MPTP-treated mice collected 6 h after neurotoxin administration (*A*, *B*, *C2*, *C3*, and D2) were double-immunostained for GSTpi protein and TH, and for GSTpi protein and different glial cell markers. *A* and *B*: GSTpi (green) + TH (red); *C1*, *C2*, and *C3*: GSTpi (green) + GFAP (red); *D1* and *D2*: GSTpi (green) + oligodendrocyte marker O4 (*red*). The number of nigral dopaminergic (TH-positive) cells expressing GSTpi was counted in consecutive sections of the entire region and in sections from three different animals for each time point. *Arrows* indicate colocalization. Microphotographs shown are representative of three independent experiments. *Scale bar*= 100 μm; *Insert*=50 μm

as a noncatalytic activity that mediates both stress and apoptotic responses (Adler et al. 1999). The association between GSTpi and JNK has also been demonstrated in other studies that confirmed the importance of GSTpi as an antiapoptotic factor since regulation of its expression can modulate the JNK activity (Ishisaki et al. 2001). Therefore, GSTpi can elicit protection against cell death induced by MPTP by controlling the balance of ROS vs JNK activity (Adler et al. 1999; Yin et al. 2000).

Data from our laboratory have shown that GSTP1 wild-type allele is an individual protective genetic trait in idiopathic PD (Golbe et al. 2007; Vilar et al. 2007) and GSTpi polymorphism has been shown to enhance dopaminergic neuron loss in a parkin transgenic *Drosophila* model (Whitworth et al. 2005) demonstrating the putative protective role of GSTpi in sporadic as well as in familiar PD.

Recently, it has been shown that acute administration of MPTP induced a transient reduction of nigral GSTpi

protein expression in both the MPTP-sensitive C57BL/6 and the MPTP-resistant Swiss-Webster mouse strains immediately after the insult. This depletion was followed by a recovery to basal levels of GSTpi, indicative of de novo synthesis of the enzyme in response to xenobiotic exposure (Smeyne et al. 2007). Our results are also corroborated by previous studies that show an increase in the expression of GSTpi in response to ROS-generating agents, which is mediated via the c-Jun N-terminal kinase–Jun cascade, in a possible feedback regulatory loop for regulation of stress kinases (Elsby et al. 2003; Zucca et al. 2006).

GSTpi may serve as a sensor of intracellular changes in redox potential that are elicited by various forms of stress (Adler et al. 1999) and is a good candidate for mediating the detoxification of ROS, protecting dopaminergic neurons from MPTP toxicity. Although the significant changes of GSTpi cellular expression occur after 6 h of MPTP administration, we cannot exclude the possibility that the catalytic activity of the GSTpi is altered after administration of MPTP. Increased catalytic activity of GSTpi would also reflect a protective mechanism against MPTP intoxication.

Data from the present study show that GSTpi is constitutively expressed in mouse brain and upregulated by MPTP, in glial cells, namely oligodendrocytes and astrocytes, and only in a scarce number of dopaminergic neurons. Several studies have shown that GSTpi is localized primarily in oligodendrocytes (Tansey and Cammer 1991b; Tamura et al. 2007) and associated with myelin (Tansey and Cammer 1991a), but it has also been shown to be present in astrocytes (Martinez-Lara et al. 2003; Smeyne et al. 2007). Controversy still exists as to whether neurons, and in particular SNpc neurons, express GSTpi. Some studies, including the present one, indicate positive results, whereas others suggest that mouse brain neurons are not immunoreactive for antibodies against GSTpi (Tansey and Cammer 1991b; Smeyne et al. 2007; Tamura et al. 2007). The differences in reports of cell-specific GST distribution in the nervous system may result from differences in the methodology used, e.g., fixed versus frozen tissue, tissue specificity of the antigenic source, or species and strain differences in GST expression.

The interaction of neurons and glia has attracted considerable interest in the pathogenesis of PD. Microglia and astrocytes have been extensively described as participating in the propagation of the neurodegenerative process (Teismann et al. 2003) or in the survival of neurons following exposure to oxidative stress (McGeer and McGeer 2004; Smeyne et al. 2005), respectively. Aside from the dramatic loss of dopaminergic neurons, the SNpc is also the site of a glial reaction in both PD and experimental models of PD (Imamura et al. 2003; Teismann et al. 2003; Yasuda et al. 2007). In fact, several findings evidence the important role of glia in protecting the functioning of the nigrostriatal dopaminergic system from MPTP toxicity (Tomac et al. 1995). Astrocytes attenuate neurodegeneration and elicit cellular protection during oxidative stress through several mechanisms including their ability to buffer the cellular milieu, the production of neurotrophic factors some of which have been shown to support dopaminergic neurons (Teismann et al. 2003), and detoxifying ROS through the enzyme glutathione peroxidase (Hirsch et al. 1999), GSH (Dringen and Hirrlinger 2003), and phase II detoxifying enzymes (Riedl et al. 1999), namely GSTpi (present work). In this study, we show that GSTpi-positive cells are astroglia but also oligodendroglia. Oligodendrocytes, which are involved in the process of myelination, have not been implicated in PD (Wilkin and Knott 1999). However, it is now becoming clear that there are a number of in vivo insults associated with oxidative stress where oligodendrocytes are preferentially affected (Juurlink et al. 1998). In fact, oligodendrocytes appear to be as vulnerable, if not more vulnerable, as neurons, and even astrocytes, to insults that cause oxidation or ischemia (Osterhout et al. 2002; Sypecka 2003). Firstly, the majority of brain iron is associated with oligodendrocytes and myelin sheaths. Moreover, oligodendrocytes are low in GSH and in glutathione peroxidase (Juurlink et al. 1998), as well as in mitochondrial manganese superoxide dismutase (Pinteaux et al. 1998) which make these cells more sensitive to oxidative stress. The observed elevated number of oligodendrocytes expressing GSTpi, together with the increase of GSTpi expression by these cells, in response to MPTP treatment, may reflect an attempt to overcome the deficiencies in their ability to scavenge ROS. In addition, the glial reaction observed in early time points after MPTP administration suggest that these cells participate in the early response to MPTP neurotoxic processes.

Several reports using rodent and primate models show that exogenous administration of glial cell-derived neurotrophic factor (GDNF) protects nigrostriatal dopaminergic neurons from different neurotoxic insults and induces fiber outgrowth. Very recently, it has also been unequivocally demonstrated that GDNF is indispensable for adult catecholaminergic neuron survival (Pascual et al. 2008). In this work, we have not addressed the study of GDNF expression by glial cells after MPTP administration but, based on the literature (McGeer and McGeer 2008), we would predict that endogenous GDNF would also be a protective factor in our experimental model of PD.

The ability of dopaminergic neurons in the SNpc to survive to the neurotoxic insult may in part be dependent upon the intrinsic capacity of glial cells to protect against ROS by increasing the expression of GSTpi. Thus,

cellular GSTpi levels may be a useful target for risk assessment or therapeutic intervention of PD.

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