Two molecular pathways initiate mitochondria-dependent dopaminergic neurodegeneration in experimental Parkinson's disease

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Dysfunction of mitochondrial complex I is associated with a wide spectrum of neurodegenerative disorders, including Parkinson's disease (PD). In rodents, inhibition of complex I leads to degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNpc), as seen in PD, through activation of mitochondria-dependent apoptotic molecular pathways. In this scenario, complex I blockade increases the soluble pool of cytochrome *c* in the mitochondrial intermembrane space through oxidative mechanisms, whereas activation of pro-cell death protein Bax is actually necessary to trigger neuronal death by permeabilizing the outer mitochondrial membrane and releasing cytochrome *c* into the cytosol. Activation of Bax after complex I inhibition relies on its transcriptional induction and translocation to the mitochondria. How complex I deficiency leads to Bax activation is currently unknown. Using gene-targeted mice, we show that the tumor suppressor p53 mediates Bax transcriptional induction after PD-related complex I blockade *in vivo*, but it does not participate in Bax mitochondrial translocation in this model, either by a transcription-independent mechanism or through the induction of BH3-only proteins Puma or Noxa. Instead, Bax mitochondrial translocation in this model relies mainly on the JNK-dependent activation of the BH3-only protein Bim. Targeting either Bax transcriptional induction or Bax mitochondrial translocation results in a marked attenuation of SNpc dopaminergic cell death caused by complex I inhibition. These results provide further insight into the pathogenesis of PD neurodegeneration and identify molecular targets of potential therapeutic significance for this disabling neurological illness.

apoptosis | Bax | Bim | complex I | 1-methyl-4-pheny-1,2,3,6-tetrahydropyridine

Complex I deficiency impairs mitochondrial respiration and is associated with a wide spectrum of neurodegenerative disorders, including Parkinson's disease (PD). Reduced complex I activity is found in both autopsy brain tissues and platelets of patients affected with sporadic PD (1–3). Furthermore, complex I inhibitors, such as 1-methyl-4-pheny-1,2,3,6-tetrahydropyridine (MPTP), reproduce some of the clinical and neuropathological hallmarks of PD in monkeys and humans, including degeneration of dopaminergic (DA) neurons of the substantia nigra pars compacta (SNpc) (4). Studies in rodents and human postmortem PD samples indicate that SNpc DA neurodegeneration linked to complex I deficiency occurs, at least in part, through activation of mitochondria-dependent apoptotic molecular pathways (5, 6). Complex I blockade, however, is not the actual executioner but rather sensitizes neurons to mitochondria-dependent apoptosis through oxidative damage and activation of the proapoptotic Bcl-2 family member Bax (6).

Activation of Bax relies, in most instances, not only on its transcriptional induction but also on its posttranslational modification. The latter results in Bax translocation and insertion into the mitochondrial outer membrane, thereby eliciting cytochrome *c* release and activation of the caspase cascade, which ultimately causes cell death (7). Both transcriptional and posttranslational activation of Bax have been observed in the SNpc of MPTP-intoxicated mice (6, 8) and PD patients (9, 10). Furthermore, genetic ablation of Bax in mutant mice prevents mitochondria-dependent apoptotic SNpc DA cell death caused by complex I inhibition with MPTP (6, 8). In contrast, both Bid and Bak, which cooperate with Bax to initiate mitochondria-dependent apoptosis in response to activation of cell-surface death receptors, are probably dispensable for MPTP-induced neuronal death (11, 12). Bax thus governs SNpc DA cell death linked to PD-related complex I deficiency. However, the molecular mechanisms of Bax activation after complex I blockade remain unknown.

Although Bax transcriptional induction associated with complex I blockade might be mediated by activation of the tumor suppressor p53 (13), the mechanisms driving its mitochondrial translocation, which is critical for the Bax pro-cell death effect, remain uncertain. Evidence indicates that the so-called "BH3-only" proteins, a proapoptotic subgroup of the Bcl-2 family that share with each other and the rest of the Bcl-2 family only the short (9–16 aa) BH3 region, play a crucial role in Bax posttranslational activation (14). Although at least eight different BH3-only molecules have been described so far in mammals, three of them, Puma (p53-up-regulated modulator of apoptosis), Noxa (the Greek word for "damage"), and Bim (Bcl-2-interacting mediator of cell death), might account for Bax mitochondrial translocation and subsequent SNpc DA cell death in PD-related complex I deficiency because: (*i*) Puma and Noxa can

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Abbreviations: DA, dopaminergic; DAT, dopamine transporter; PARP, poly(ADP-ribose) polymerase; PD, Parkinson's disease; SNpc, substantia nigra pars compacta; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-pheny-1,2,3,6-tetrahydropyridine; ROS, reactive oxygen species; TH, tyrosine hydroxylase.

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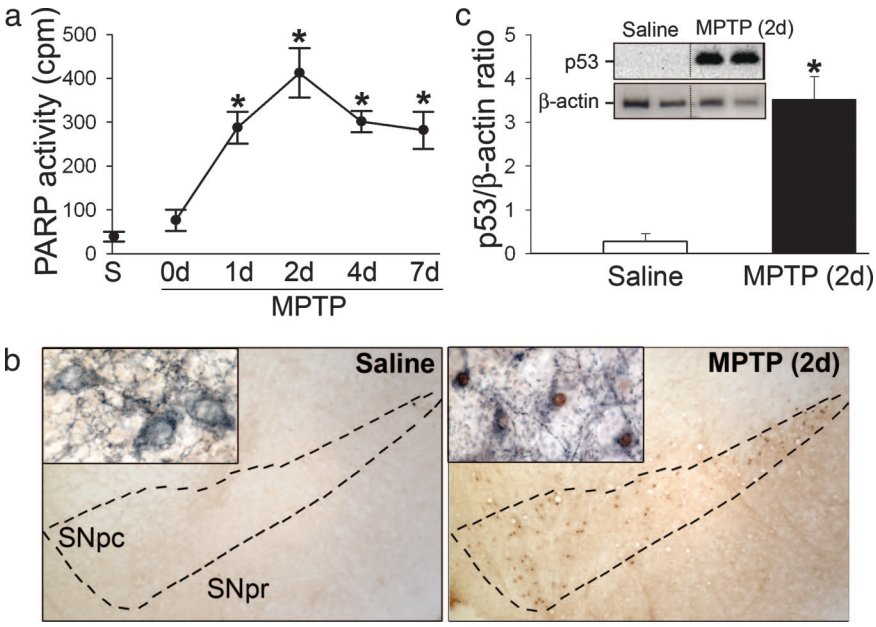

Fig. 1. MPTP-induced complex I inhibition causes DNA damage and p53 induction. (*a*) Ventral midbrain samples from MPTP-injected mice exhibit a time-dependent increase in PARP activity, evidenced by quantifying the formation of its product, ADP-ribose polymers. PARP activity starts to increase by 24 h and peaks at 48 h after MPTP injection. (*b*) At the peak of PARP activation, *in situ* PARP histochemistry (brown signal) combined with immunohistochemistry for DAT (blue-gray signal) reveals PARP activation only in DAT-positive SNpc cells, which exhibit a definite neuronal morphology. (*c*) At the peak of PARP activation, *p53* mRNA levels are markedly increased in the ventral midbrain of MPTP-injected mice as determined by RT–PCR. *, $P < 0.05$ compared with saline-injected mice.

be induced by the transcription factor p53 (15, 16), whereas activation of Bim has been associated with the JNK pathway (17, 18); and (*ii*) both p53 and JNK molecular pathways have been shown to participate in MPTP-induced SNpc DA neurodegeneration (13, 19). In addition, p53 has been shown to directly induce posttranslational activation of Bax in some cellular settings by transcription-independent mechanisms requiring translocation of p53 to the mitochondria (20–23).

Here, by using gene-targeted mice, we show that p53 mediates Bax transcriptional induction after complex I blockade with MPTP, but it does not participate in Bax mitochondrial translocation in this model, either by a transcription-independent mechanism or through induction of the BH3-only proteins Puma or Noxa. In this context, posttranslational activation of Bax mostly relies on JNK-dependent activation of the BH3-only protein Bim. Targeting either Bax transcriptional induction or Bax posttranslational activation results in a marked attenuation of mitochondria-dependent apoptotic SNpc DA cell death caused by complex I inhibition with MPTP.

Results

DNA Damage and p53 Activation After Complex I Inhibition. One of the most potent activators of p53 is DNA damage. To confirm the occurrence of DNA damage in PD-related complex I deficiency, we injected mice with MPTP and assessed at different time points the activity of poly(ADP-ribose) polymerase (PARP), a DNA-repair and protein-modifying enzyme that is activated by DNA damage in many cell types, including neurons (24, 25). In MPTP-injected mice, ventral midbrain PARP activity, evidenced by quantifying the formation of its product, ADP-ribose polymers, was markedly increased, beginning by 24 h and peaking at 48 h (Fig. 1*a*). PARP catalytic activity in the hippocampus was low and was unaffected by MPTP injection (data not shown). To determine whether PARP was actually activated in SNpc DA neurons, we performed *in situ* PARP histochemistry combined with immunohistochemistry for the dopamine transporter (DAT) at the peak of PARP activation. This double-staining procedure revealed PARP activation only in DAT-positive cells, which exhibited a definite neuronal morphology (Fig. 1*b*). Coinciding with the peak of PARP activation, p53 was markedly up-regulated in the ventral midbrain of MPTP-intoxicated mice, as determined by RT-PCR (Fig. 1*c*). No up-regulation of p53 was detected in the cerebellum or striatum, two regions devoid of MPTP-induced cell body loss (data not shown). These results confirmed the occurrence of DNA damage and p53 activation after complex I blockade. These changes occurred in a region- and time-specific manner that paralleled Bax induction and preceded Bax mitochondrial translocation and apoptotic SNpc DA cell death in this model (6, 8).

Loss of p53 Prevents Bax Up-Regulation but Not Bax Mitochondrial Translocation After Complex I Inhibition. We have previously shown that Bax is up-regulated and translocated into the mitochondria after MPTP-induced complex I inhibition in a region- and time-specific manner that parallels SNpc DA cell death in this model (6, 8). The tumor suppressor p53 is among the factors known to transcriptionally up-regulate Bax expression (26, 27). In addition, p53 has also been reported to participate in Bax mitochondrial translocation by either transcription-dependent or -independent mechanisms. The former involves p53-dependent induction of the BH3-only proteins Puma and Noxa (15, 16), whereas the latter requires translocation of p53 to the mitochondrial outer membrane (20–23). Here, we assessed whether MPTP-related Bax induction and mitochondrial translocation are controlled by p53. Loss of p53 in gene-targeted mice prevented MPTP-induced Bax up-regulation in the ventral midbrain at both the mRNA (Fig. 2*a*) and protein (Fig. 2*b*) levels. In p53-deficient ventral midbrain samples, however, Bax was still able to translocate to the mitochondria after MPTP intoxication, although its levels were not increased (Fig. 2*c*). These results indicate that, although p53 is responsible for MPTP-induced transcriptional up-regulation of Bax, it does not participate in Bax posttranslational activation in this model of complex I inhibition. In agreement with this, (*i*) BH3-only proteins Puma and Noxa, which mediate p53-dependent Bax

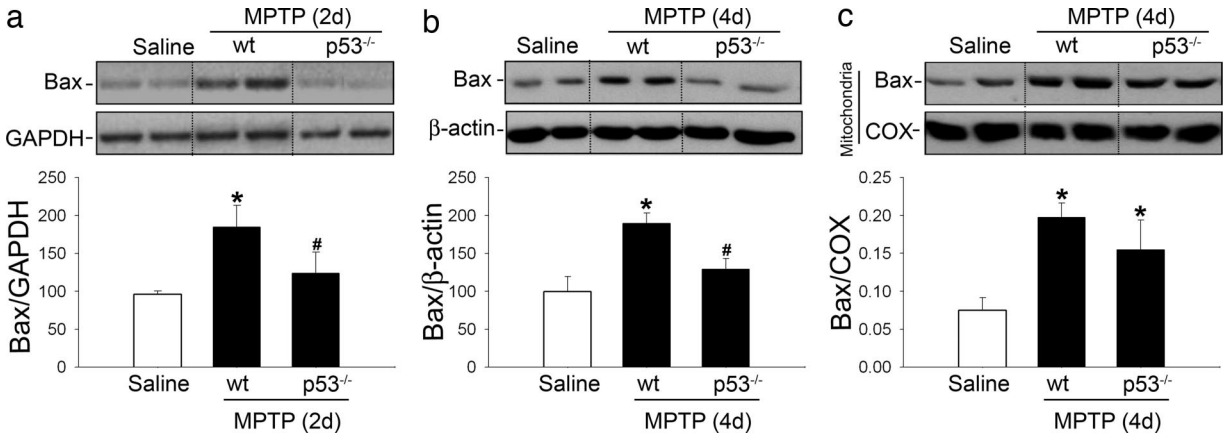

Fig. 2. Loss of p53 in gene-targeted mice inhibits MPTP-induced Bax up-regulation but not Bax mitochondrial translocation. (*a* and *b*) Loss of p53 prevents MPTP-induced ventral midbrain Bax mRNA (*a*) and protein (*b*) up-regulation at its peak (2 and 4 days after the last MPTP injection, respectively). (*c*) In p53-deficient mice injected with MPTP, constitutive Bax is still able to translocate to mitochondria, as assessed by Western blot analysis of ventral midbrain mitochondrial protein fractions. *, $P < 0.05$ compared with saline-injected mice; #, $P \leq 0.05$ compared with MPTP-injected wild-type mice.

mitochondrial translocation in certain cellular settings, were not induced after MPTP injections despite the strong up-regulation of p53 in this model [see supporting information (SI) Fig. 6], and (*ii*) we did not find any evidence of p53 being translocated to the mitochondria after MPTP intoxication (data not shown) as it has been shown in other cellular settings to directly induce Bax mitochondrial translocation (20–23). Our results therefore indicate that p53 mediates MPTP-induced Bax transcriptional up-regulation, but it does not participate in Bax mitochondrial translocation in this model, either by induction of BH3-only molecules Puma and Noxa or through transcription-independent mechanisms requiring translocation of p53 to the mitochondria.

Blockade of Bax Up-Regulation Inhibits Mitochondria-Dependent Apoptotic SNpc DA Cell Death Caused by Complex I Inhibition. Attenuation of MPTP-induced Bax up-regulation by genetic ablation of p53 in mutant mice resulted in a significant reduction of MPTP-induced mitochondrial cytochrome *c* release (Fig. 3*a*), caspase-3 activation (Fig. 3*b*), and apoptotic cell death (Fig. 3*c*). Consequently, p53-deficient mice exhibited significant protection against MPTP-induced SNpc DA cell death (Fig. 3*d*), which is in agreement with a previous report using a pharmacological p53 inhibitor, pifithrin-α (13). This protective effect is not due to altered metabolism of MPTP in the absence of p53 because the striatal levels of MPP^+, MPTP's active metabolite, did not differ between MPTP-intoxicated p53 mutant and wild-type mice (data not shown). Despite the significant preservation of SNpc DA cell bodies in p53-deficient mice, striatal dopamine levels, as measured by HPLC, were decreased to a similar extent in MPTP-injected mutant and wild-type mice (SI Fig. 7). These results indicate that loss of p53 prevents MPTP-induced Bax up-regulation and attenuates SNpc DA cell death in mice.

JNK-Dependent Activation of Bim After Complex I Inhibition. Because p53 does not participate in MPTP-induced posttranslational activation of Bax, other factors must account for Bax mitochondrial translocation in this model. The BH3-only protein Bim emerged as a major candidate. Bim is currently considered one of the most potent killers among the BH3-only proteins because it can bind to all Bcl-2 family prosurvival proteins with high affinity, thereby facilitating Bax activation and cytochrome *c* release (14, 28–31). Here, we show that the BH3-only protein Bim is up-regulated in the ventral midbrain of MPTP-intoxicated mice in a region-specific and time-dependent manner that precedes Bax mitochondrial translocation and SNpc DA cell death in this model (Fig. 4*a*). *Bim* mRNA induction started early after MPTP intoxication, peaked at 24 h, and was maintained for up to 4 days, thus covering the entire period of MPTP-induced SNpc DA apoptotic cell death (8); it then decreased to control levels by 7 days after MPTP intoxication (Fig. 4*a*).

Activation of Bim is tightly regulated by a multitude of transcriptional and posttranslational mechanisms that often

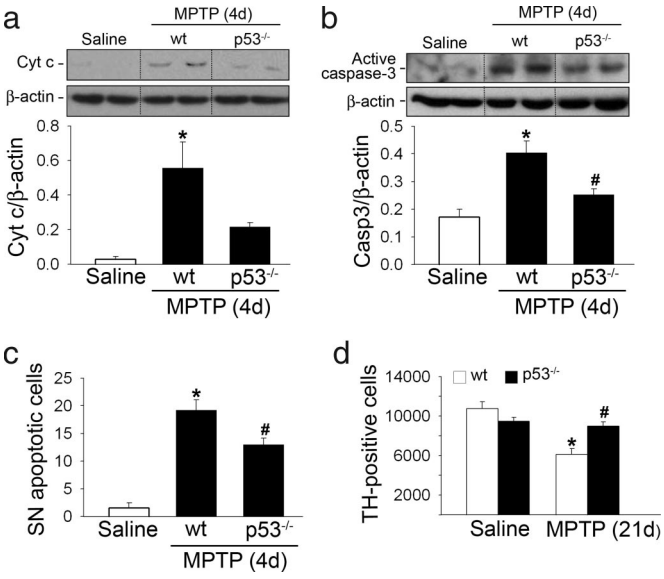

Fig. 3. Loss of p53 in gene-targeted mice attenuates MPTP-induced SNpc DA apoptotic cell death. (*a* and *b*) At the peak of MPTP-induced mitochondria-dependent apoptotic cell death (4 days after the last MPTP injection), cytochrome *c* release and caspase-3 activation are reduced in ventral midbrain cytosolic fractions of p53-deficient mice. (*c*) The number of MPTP-induced SNpc apoptotic neurons is decreased in the ventral midbrain of p53-deficient mice compared with similarly treated wild-type mice. Morphological criteria to identify apoptotic cells included shrinkage of the cell body, chromatin condensation, and the presence of distinct, round, well defined chromatin clumps, demonstrated on thionin staining, as in ref. 8. (*d*) p53-deficient mice exhibit a significant protection against MPTP-induced SNpc DA cell death, as determined by assessing the number of SNpc DA neurons at 21 days after the last MPTP injection. *, $P < 0.05$ compared with saline-injected mice; #, $P < 0.05$ compared with MPTP-injected wild-type mice.

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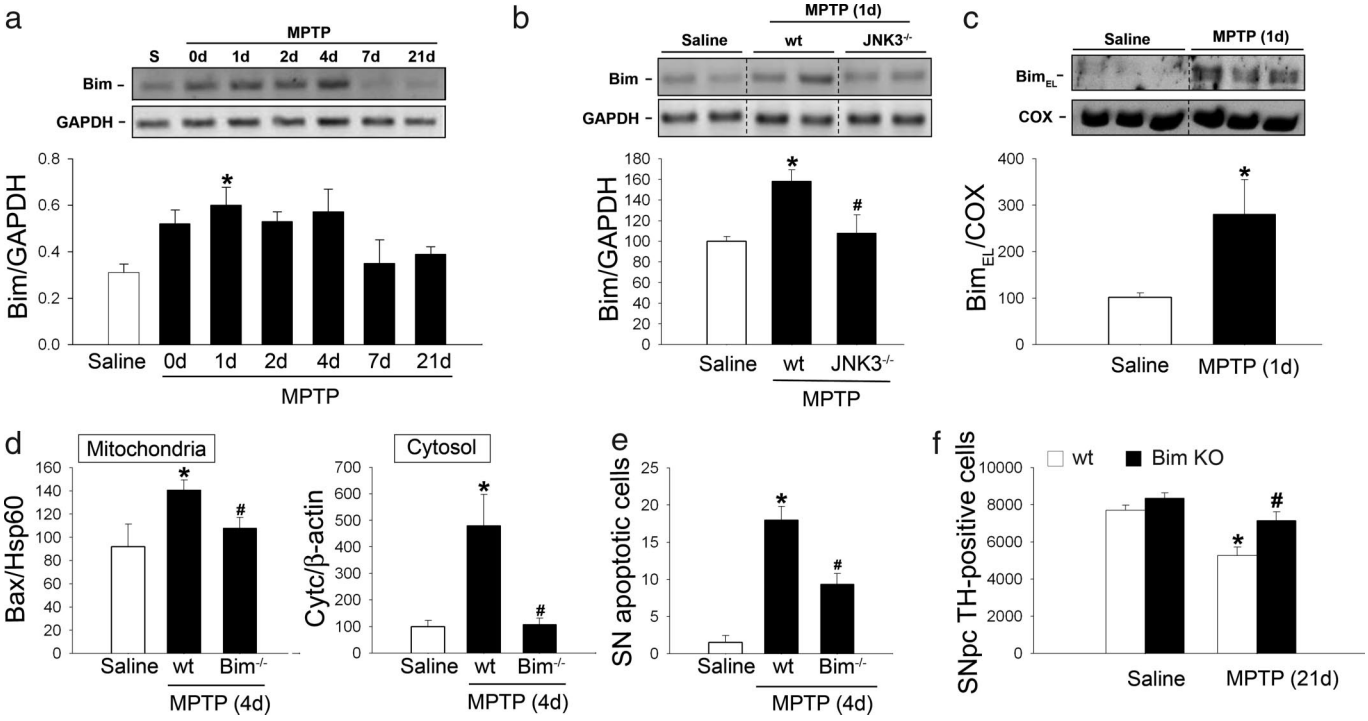

Fig. 4. Role of Bim in MPTP-induced SNpc DA neurodegeneration. (*a*) Ventral midbrain *bim* mRNA expression is induced in a time-dependent manner after MPTP intoxication, peaking at 24 h post-MPTP. (*b*) At its peak, MPTP-induced *bim* mRNA up-regulation is prevented in JNK-3-deficient mice. (*c*) At the peak of MPTP-induced *bim* mRNA up-regulation, mitochondrial Bim_{EL} protein levels are markedly increased in the ventral midbrain. (*d*) Loss of Bim in gene-targeted mice significantly attenuates MPTP-induced Bax mitochondrial translocation (*Left*) and cytochrome *c* release (*Right*), as assessed by Western blot analysis of mitochondrial and cytosolic fractions from ventral midbrain, respectively. (*e* and *f*) Bim-deficient mice exhibit a significant reduction in the number of MPTP-induced SNpc apoptotic neurons (*e*) and a significant protection from MPTP-induced SNpc DA cell death (*f*). *, $P < 0.05$ compared with saline-injected mice; #, $P < 0.05$ compared with MPTP-injected wild-type mice.

operate in a cell type- and/or stimulus-specific manner (32, 33). In neurons, Bim is subject to the transcriptional control of JNK (17, 18), a pathogenic pathway that has been implicated in MPTP-induced SNpc DA neurodegeneration (4, 19). Moreover, evidence from *in vitro* studies indicates that JNK activation is required for Bax mitochondrial translocation and activation of the mitochondrial apoptotic pathway elicited by DNA damage (34, 35). We assessed whether MPTP-induced up-regulation of Bim depended on JNK. In particular, we examined the involvement of JNK3, a JNK isoform selectively expressed in the nervous system that has been shown to play a critical role in stress-induced neuronal apoptosis and to transcriptionally regulate Bim in ischemic neuronal injury (36). Supporting a role for JNK3 in *bim* transcriptional induction caused by complex I inhibition, ablation of JNK3 in mutant mice prevented MPTP-induced *bim* mRNA up-regulation (Fig. 4*b*).

At the peak of MPTP-induced *bim* mRNA up-regulation (24 h after the last MPTP injection), levels of Bim_{EL}, the predominant and physiologically relevant Bim isoform in neurons (17, 37), were markedly increased in the ventral midbrain of MPTP-intoxicated mice (Fig. 4*c*), but not in regions unaffected by MPTP (data not shown). Bim_{EL} was detected exclusively in the mitochondrial fraction of ventral midbrain homogenates (Fig. 4*c*) in agreement with its known localization as an integral mitochondrial membrane protein in neurons (17). Overall, our results indicate that Bim is transcriptionally induced in a JNK-dependent manner after MPTP intoxication and that it is localized in mitochondrial fractions.

Loss of Bim Attenuates Bax Mitochondrial Translocation, Cytochrome *c* Release, and SNpc DA Apoptotic Cell Death Caused by Complex I Inhibition. Supporting a pathogenic role of Bim in SNpc DA cell death caused by complex I inhibition, loss of Bim in gene-targeted mice significantly attenuated MPTP-induced Bax mitochondrial translocation (Fig. 4*d*), mitochondrial release of cytochrome *c* (Fig. 4*d*), and apoptotic cell death (Fig. 4*e*). As a consequence, Bim-deficient mice exhibited significant protection against MPTP-induced SNpc DA cell death (Fig. 4*f*). This protective effect was not due to altered metabolism of MPTP in the absence of Bim because the striatal levels of MPP^+ did not differ between MPTP-intoxicated $bim^{-/-}$ and wild-type mice (data not shown). In contrast to SNpc DA cell bodies, striatal levels of dopamine, as measured by HPLC, were decreased to a similar extent in MPTP-injected $bim^{-/-}$ and wild-type mice (SI Fig. 7). Overall, our results indicate that the BH3-only protein Bim participates in MPTP-induced Bax mitochondrial translocation and SNpc DA cell death.

Discussion

Mitochondrial oxidative damage and Bax have both been shown to play critical roles in neuronal degeneration caused by complex I deficiency (6, 8). In particular, free radical production, secondary to complex I blockade, increases the "releasable" soluble pool of cytochrome *c* in the mitochondrial intermembrane space through peroxidation of the inner mitochondrial lipid cardiolipin, whereas activated Bax triggers neuronal death by permeabilizing the outer mitochondrial membrane, thereby releasing cytochrome *c* into the cytosol, where it promotes activation of the caspase cascade as part of the "apoptosome" (6). In agreement with this model, activation of Bax is required for mitochondria-dependent SNpc DA apoptotic cell death caused by MPTP-induced complex I inhibition (6, 8). Of note, although the mechanism by which Bax induces cytochrome *c* release is still a matter of intense debate, our preliminary data indicate that in the context of complex I deficiency, it seems to be independent

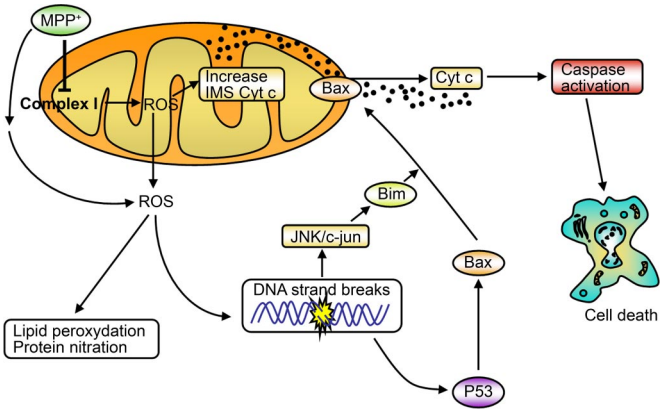

Fig. 5. Proposed pathogenic scenario induced by complex I deficiency with MPTP in which DA neuronal death results from a self-amplifying cascade of deleterious events that start at the mitochondria with the alteration of oxidative phosphorylation and finish at the mitochondria with the activation of the programmed cell death machinery. In this scenario, p53 transcriptionally up-regulates Bax, whereas JNK promotes Bax mitochondrial translocation through transcriptional activation of the BH3-only protein Bim.

of the mitochondrial permeability transition pore because (*i*) Bax-induced cytochrome *c* release in MPP^+-treated brain mitochondria is not responsive to the mitochondrial pore blocker cyclosporin A (SI Fig. 8*A*), and (*ii*) mice deficient for cyclophilin D, a critical component of the mitochondrial pore, do not exhibit reduced susceptibility to MPTP (SI Fig. 8 *B* and *C*).

On the basis of our results, we propose a pathogenic scenario in which neuronal death caused by complex I deficiency results from a self-amplifying cascade of deleterious events, starting at the mitochondria by the alteration of the oxidative phosphorylation and finishing also at the mitochondria by the activation of the programmed cell death machinery. In this scenario (Fig. 5), MPTP impairs mitochondrial respiration in SNpc DA neurons by inhibiting complex I of the electron transport chain. Inhibition of complex I blocks the flow of electrons along the mitochondrial electron transport chain, which results in increased production of reactive oxygen species (ROS). Mitochondrial ROS then increase the soluble pool of cytochrome *c* in the mitochondrial intermembrane space by a mechanism involving peroxidation of cardiolipin, whereas ROS outside the mitochondria, probably emanating also from sources other than complex I inhibition (38), damage different cellular elements, such as lipids, proteins, and DNA. DNA damage activates both p53 and JNK. p53 induces transcriptional up-regulation of Bax, whereas JNK participates in Bax mitochondrial translocation through transcriptional activation of the BH3-only protein Bim. Once localized to the mitochondrial outer membrane, Bax induces the release of cytochrome *c* into the cytosol and the ensuing caspase activation and cell death. Supporting the relevance of this scenario to SNpc DA neurodegeneration occurring in PD, several elements of this molecular cascade have been demonstrated in postmortem human brain samples from PD patients, including complex I deficiency (1–3); ROS production (39); oxidative damage to lipids (40, 41), proteins (42, 43), and DNA (44, 45); JNK activation (19, 46, 47); Bax activation (9, 10); and activation of caspase-9 (11) and caspase-3 (9, 48).

The complexity of this cascade, which probably includes other factors not depicted here, guarantees exquisite control over neuronal cell death and provides several targets of potential therapeutic significance. Approaches aimed at targeting different key elements of this cascade, especially those acting upstream of cytochrome *c* release, all result in a significant attenuation of MPTP-induced SNpc DA neurodegeneration (5). We have previously identified Bax as a potential therapeutic target for SNpc DA neurodegeneration linked to MPTP-induced complex I inhibition (6, 8). Here, we provide evidence that interfering upstream of Bax, by targeting molecular pathways involved in either Bax transcriptional or post-translation regulation, also provides significant neuroprotection in this model. However, probably because of the multiplicity of regulatory pathways converging on Bax activation, these strategies (at least singly) do not provide as much neuroprotection as targeting Bax itself (8), most notably at the level of striatal DA terminals. Because the symptoms of PD are caused by the loss of DA terminals in the striatum, preventing the death of SNpc DA cell bodies without preventing the degeneration of their axons is unlikely to be a helpful therapeutic strategy. Neurons might have two self-destruction programs, one for the cell body and a second one for the axon (49). So, a combination of antiapoptotic and axon-protective strategies might be required to obtain optimal clinical benefit from such neuroprotective approaches.

Materials and Methods

Animals and Treatment. The following lines of mutant mice were used here: (*i*) p53-deficient mice (50), (*ii*) JNK3-deficient mice (47), (*iii*) Bim-deficient mice (51), and (*iv*) cyclophilin D-deficient mice (52). The different lines of mutant mice were maintained in a C57BL/6 genetic background. Eight- to 10-week-old wild-type or mutant male mice received one i.p. injection of MPTP-HCl per day (30 $mg^{-1}{\cdot}kg^{-1}$day of free base; Sigma–Aldrich, St. Louis, MO) for 5 consecutive days and were killed at the indicated time points after the last MPTP injection; control mice received saline injections only. For all of the experiments, 3 to 10 mice per group were used.

Subcellular Fractionation. Protein extraction of mitochondrial and cytosolic fractions at the indicated time points was performed on fresh ventral midbrain tissue from saline- and MPTP-injected mice, as previously described (6).

PARP Activity and *in Situ* Histochemistry. Ventral midbrain, striatum, and cerebellum from mice killed at the indicated time points post-MPTP were homogenized. From each sample, 20 μg of the protein extract were used to determine PARP activity and assayed with a commercial kit (Trevigen, Gaithersburg, MD) using [^{32}P]NAD (PerkinElmer, Boston, MA) following the manufacturer's instructions. For PARP histochemistry, fresh cryostat-cut sections (14 μm) were fixed in ethanol and permeabilized with Triton X-100. The assay was performed using biotinylated NAD+ as described previously (53).

Antibodies. The following primary antibodies were used for Western blot analyses: active caspase-3 (BD Biosciences, San Jose, CA), β-actin (clone AC15; Sigma–Aldrich), Bax (B-9; Santa Cruz Biotechnology, Santa Cruz, CA), Bim (Bim/BOD, AAP-330; Stressgen Biotechnologies, San Diego, CA), COX-IV (Molecular Probes, Eugene, OR), cytochrome *c* (Pharmingen, San Diego, CA), DAT (Chemicon, Temecula, CA), and HSP60 (Santa Cruz Biotechnology).

RNA Extraction and RT-PCR. Total RNA was extracted at the indicated time points from midbrain, striatal, and cerebellar samples from saline and chronic MPTP-treated mice and used for RT-PCR analysis using the following primer sequences: Bax, 5′-CTGAGCTGACCTTGGAGC-3′ and 5′-GACTCCAGCCACAAAGATG-3′; p53, 5′-CACTGGAGTCTTCAAGTGTG-3′ and 5′-GTCTGGGACAGCCAAGTCTG-3′; Puma/Bbc3, 5′-TGCAGTCAGAGCAGCAACAAC-3′ and 5′-GGTCACCATGAGTCCTTCAGC-3′; Noxa, 5′-GGATCTAAGTCCCCTGTACGC-3′ and 5′-TTTCCCTCTCATCACAGTCCA-3′; Bim, 5′-GGTAATCCCGACGGCGAAGGGAC-3′ and 5′-AAGAGAAATACCCACTGGAGGACC-3′; β-actin, 5′-CTTTGATGTCACGCACGATTTC-3′ and 5′-GGGCCGCTCTAG-

GCACCAA-3′; and GAPDH, 5′-GTTTCTTACTCCTTG-GAGGCCAT-3′ and 5′-TGATGACATCAAGAAGTGGT-GAA-3′.

Quantification of DA Neurodegeneration. The total number of tyrosine hydroxylase (TH)-positive SNpc neurons was counted in the different groups of mice at 21 days after the last MPTP or saline injection by stereology using the optical fractionator method (StereoInvestigator; MBF Bioscience, Williston, VT). Immunostaining was performed with a polyclonal antibody to TH (Calbiochem, EMD Biosciences, San Diego, CA). Quantification of the number of apoptotic neurons in the SN of MPTP- and saline-injected mice was assessed as previously described (8). Because apoptotic neurons may have lost their TH expression as a result of the dying process (54), counts of apoptotic profiles refer to all cells in the substantia nigra.

HPLC Measurements. Striatal levels of dopamine and metabolites were measured by HPLC with electrochemical detection, as previously described (8). Striatal MPP^+ levels were determined by HPLC with UV detection ($\lambda = 295$ nm) 90 min after a single i.p. injection of MPTP (30 mg/kg).

Statistical Analysis. All values are expressed as the mean ± SEM. Differences among means were analyzed with one- or two-way ANOVA with treatment or genotype as the independent factors. When ANOVA showed significant differences, pairwise comparisons between means were tested by Student–Newman–Keuls post hoc testing. In all analyses, the null hypothesis was rejected at the 0.05 level.

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