

Activation of p38 MAPK in the substantia nigra leads to nuclear translocation of NF- κ B in MPTP-treated mice: implication in Parkinson's disease

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Abstract

Activation and translocation of the transcription factor nuclear factor kappa B (NF- κ B) from cytoplasm to the nucleus has been reported in models of Parkinson's disease (PD). Our focus was to discern the upstream events which ultimately lead to NF- κ B nuclear translocation using animal model of PD. We demonstrate that p38 activation results in downstream phosphorylation of NF- κ B and accumulation of p65 subunit of NF- κ B selectively in ventral midbrain but not in striatum. Treatment with p38 inhibitor, SB239063, prevented downstream phosphorylation of I κ B α and p65 translocation to the nucleus in the ventral midbrain. Phosphorylation of anti-apoptotic Bcl2, an NF- κ B target gene by p38 to inactive pBcl2ser87 was also attenuated by SB239063. Increased

staining of p65 in the nuclei of cells in the substantia nigra but not in the ventral tegmental area of MPTP-treated mice further suggests a role for NF- κ B in PD. In agreement with the above, sustained caspase activation is seen in the ventral midbrain but not in striatum. We demonstrate the region specific p38-mediated activation of NF- κ B following MPTP treatment demonstrating the role of p38/NF- κ B signaling in the pathogenesis and progression of the disease. Selective inhibitors of p38 may therefore, help preserve the surviving neurons in PD and slow down the disease progression.

Keywords: Bcl2, caspase 3, mitogen-activated protein kinase, neurodegeneration, NF- κ B p65.

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Degeneration of the dopaminergic neurons in substantia nigra pars compacta (SNpc) and their terminals in striatum is the pathological hallmark of Parkinson's disease (PD), a movement disorder that afflicts up to 1% of the growing world population. The mechanisms underlying the selective vulnerability of the dopaminergic neurons in SNpc are not clearly understood. Discovery of disease-modifying therapies that can slow down the progression of PD would require better understanding of the cell death mechanisms that selectively affect the dopaminergic neurons in SNpc.

One of the pathways implicated in the death of the dopaminergic neurons of SNpc in PD is the nuclear factor kappa B (NF- κ B)-mediated transcription of pro-apoptotic genes such as p53 (Liang *et al.* 2007). NF- κ B is a transcription factor that is present in the cytosol as dimer of RelA (p65) and I κ B α . Upon phosphorylation I κ B α dissociates from RelA, the p65 subunit of NF- κ B and is rapidly degraded by the proteasome. The p65 subunit of NF- κ B can then translocate to the nucleus and initiate the transcription of genes. Several important genes involved in the apoptotic pathway, both pro and anti-apoptotic, such as p53 and Bcl2 are transcribed through NF- κ B (de Moissac

et al. 1999; Zhang *et al.* 2001). Therefore, NF- κ B is often referred as a 'double-edged sword' that can be neuroprotective or promote neurodegeneration depending on the context and consequence of its transactivation. Increased amounts of nuclear p65 have been detected in SNpc of PD brains (Hunot *et al.* 1997) and in animal models (Cao *et al.* 2008; Aoki *et al.* 2009) and inhibition of NF- κ B activation prevents nuclear translocation of p65 and protects the SNpc neurons in animals dosed with MPTP (Dehmer *et al.* 2004; Ghosh *et al.* 2007). It has been proposed that preventing the activation of NF- κ B could be potentially considered as a drug target for PD therapy (Li *et al.* 2008).

The upstream mediator(s) of NF- κ B activation in PD are not clearly understood although inflammatory responses

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Abbreviations used: MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor kappa B; PD, Parkinson's disease; SNpc, substantia nigra pars compacta; TNF, tumor necrosis factor; TNFR, TNF receptor.

including the production of proinflammatory cytokines such as tumor necrosis factor (TNF)- α and interleukin-6 have been considered as potential upstream targets (Craig *et al.* 2000). However, the role of inflammatory cytokines in the pathogenesis of PD is questionable. Several animal models of PD including MPTP (Członkowska *et al.* 2002; Koprach *et al.* 2008) show inflammatory response including activated microglia. Furthermore, inactivation of the genes involved in the synthesis of proinflammatory molecules such as cyclooxygenase 2 (Feng *et al.* 2003) and NADPH oxidase (Wu *et al.* 2002) were shown to protect dopaminergic neurons against MPTP-induced neurotoxicity indicating that inflammation plays an important role in MPTP-mediated neurodegeneration. However, mice with knock-out of both TNF- α (Ferber *et al.* 2004) and its receptors (Rousselet *et al.* 2002; Sriram *et al.* 2002) failed to show attenuation of cell loss in SNpc although the terminals in the striatum showed moderate protection implying that inflammatory responses may be more damaging in the striatal terminals. Given the complexity of diverse cell types and signaling *in vivo* in the brain, it is possible that other players such as the mitogen-activated protein kinases (MAPKs) could participate in the initiation of the NF- κ B cascade (Wilms *et al.* 2003). p38 MAPK has been shown to play an important role in the activation of NF- κ B through several mechanisms involving phosphorylation of I κ B α , nuclear translocation of p65, and interference with p65-mediated transcription in the nucleus (Tsai *et al.* 2003).

We have recently shown the selective activation of p38 MAPK in the SNpc neurons of mice treated with MPTP. The activation of p38 results in downstream phosphorylation of p53 and increased p53-mediated transcription of Bax and Puma. Further, treatment with p38 inhibitor, SB239063, prevented the downstream phosphorylation of p53 and its translocation to the nucleus *in vivo* in the ventral midbrain (Karunakaran *et al.* 2008). In the present study we examined the potential role of p38 as an upstream mediator of NF- κ B activation.

Materials and methods

Materials

Antibodies to I κ B α (C-21, polyclonal), p38 (C-20, polyclonal, alpha p38), Bcl2 (C-2, monoclonal), and pBcl2 (Ser 87; polyclonal) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibody to β -tubulin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies to p-p38 MAPK (Thr180/Tyr182), pI κ B α (Ser32), NF- κ Bp65, and lamin A/C were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-histone H3 was obtained from Upstate Cell Signaling Solutions (Lake Placid, NY, USA). p38 inhibitor, SB239063, was obtained from Calbiochem (Darmstadt, Germany). All other chemicals and reagents were of analytical grade and were obtained from Sigma Aldrich or Qualigens (Mumbai, India).

Animals

All animal experiments were carried out as per the institutional guidelines for the use and care of animals. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques if available. Male C57BL6J (2–3 months, 25–30 g) were obtained from Central Animal Research Facility of National Brain Research Centre (NBRC). Male C57BL6J mice were administered MPTP (30 mg/kg body weight/day, s.c.). Control animals received saline alone. Animals were treated with a single dose of MPTP and killed 1, 4, 12, and 24 h later. Some animals also received the above dose of MPTP daily for 8 days and were killed 24 h after the last dose. Control animals received vehicle alone. Animals had access to pelleted diet and water *ad libitum*. At the end of the experimental period, mice were anesthetized with ether and perfused transcardially with ice-cold normal saline before decapitation. Ventral midbrain and striatum were dissected as described earlier (Karunakaran *et al.* 2007) and frozen in liquid nitrogen for immunoblotting. In some experiments, animals were perfused transcardially with buffered paraformaldehyde (4% w/v) and the brain was dissected out and processed for immunohistochemistry. For examining the effect of p38 kinase inhibitor *in vivo*, mice were divided into four groups. Two groups received the vehicle dimethylsulfoxide (3% in normal saline; 100 μ L) intrathecally and this was followed by normal saline or MPTP (30 mg/kg body weight; s.c.) 1 h later. The other two groups received SB239063 (92 μ g dissolved in 100 μ L of 3% dimethylsulfoxide) intrathecally prior to MPTP or saline treatment. Mice were killed 12 h following MPTP treatment and the ventral midbrain was dissected out.

Processing of tissue

Tissue was homogenized in 0.25 M sucrose and centrifuged at 1000 g for 10 min to obtain post-nuclear supernatant. The post-nuclear supernatant was used for immunoblotting. In some experiments the cytosol was isolated from the post-nuclear supernatant by centrifugation at 100 000 g for 1 h. Protein concentration was estimated by a dye-binding method (Bradford 1976). Nuclear extracts were prepared as described (Korner *et al.* 1989).

Immunohistochemistry

Male C57BL6J mice were administered MPTP (30 mg/kg body weight/day, s.c.) once daily for 1 or 8 days. Control animals received saline. Animals were anesthetized with ether 24 h after the last injection and perfused transcardially with phosphate-buffered saline followed by paraformaldehyde (4% w/v) in phosphate-buffered saline. Coronal sections (30- μ m thick) were cut throughout the entire midbrain using a cryostat. Immunostaining was visualized using FITC-labeled secondary antibody and counter-stained with 4-6-diamidino-2-phenylindole dihydrochloride or horseradish peroxidase-labeled secondary antibody followed by staining with Nova Red.

Immunoblotting

The post-nuclear supernatant prepared from ventral midbrain or striatum (20 μ g protein) of vehicle and MPTP-treated mice were resolved on 10% sodium dodecyl sulfate polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Towbin *et al.* 1979), incubated with primary antibody (1 : 1000) followed by secondary antibody (1 : 2000) labeled with alkaline phosphatase or secondary antibody (1 : 5000) labeled with horseradish peroxidase. Immuno-

stained bands were detected using nitroblue tetrazolium and 5-bromo 4-chloro 3-indolyl phosphate as chromogens (Roche, Mannheim, Germany) or using ECL kit (Amersham Pharmacia Biotech, Les Ulis, France). Blots were normalized with β -tubulin, lamin/histone as appropriate.

Statistical analysis

Statistical analysis of the data was performed using ANOVA or repeated measures of ANOVA followed by *post hoc* tests (Student–Newman–Keuls or Dunnett’s test). Student’s *t*-test or paired *t*-test were used when two groups were compared. Values of $p < 0.05$ were taken as being statistically significant.

Results

MPTP stimulates p38 MAPK phosphorylation and nuclear translocation in the ventral midbrain

Mitogen-activated protein kinase activation was assessed as the ratio of the phospho-MAPK to the total MAPK signal in each sample following normalization with β -tubulin/lamin as described earlier (Guan *et al.* 2003). In the ventral midbrain increased phosphorylation of p38 MAPK was observed 12 h after a single dose of MPTP (Fig. 1). Phosphorylation was 2.4-fold in the extranuclear compartment (Fig. 1a) and 1.6-fold (Fig. 1b) in the nuclear compartment.

MPTP induces NF- κ B activation in the ventral midbrain not in the striatum following MPTP treatment

We observed the phosphorylation state of I κ B α following single dose of MPTP in the ventral midbrain and striatum. In

the ventral midbrain, sustained phosphorylation of I κ B α was observed at 12 h (1.5-fold) after a single dose of MPTP (Fig. 2a). Total I κ B α levels decreased 24 h following MPTP in the midbrain and not in the striatum. Accordingly, the ratio pI κ B α /I κ B α increased 24 h after MPTP, which may be caused by decreased levels of I κ B α and not an absolute increase of pI κ B α . However, phosphorylation state of I κ B α was not altered in the striatum (Fig. 2b). Phospho-I κ B α levels were normalized to the total I κ B α level in each sample to assess NF- κ B activation. Pearson’s correlation analysis also showed a negative correlation between the expression levels of I κ B α and pI κ B α (Fig. 2c; $r = -0.961$). Further, we examined the effect of pre-treatment with SB239063, a selective p38 inhibitor on the phosphorylation state of I κ B α following MPTP exposure. Pre-treatment with SB239063 down-regulated the levels of both total and phospho-I κ B α in the post-nuclear supernatant. Co-administration of SB239063 and MPTP attenuated the levels of pI κ B α and led to the accumulation of total I κ B α in the post-nuclear supernatant such that it was significantly higher than the control levels (Fig. 2d).

SB239063, a p38 inhibitor, attenuates the accumulation of NF- κ Bp65 in the nucleus *in vivo*

NF- κ Bp65 translocated to the nucleus as early as 4 h and increased nuclear NF- κ Bp65 were seen up to 24 h post-MPTP treatment (Fig. 3a), while the cytosolic NF- κ Bp65 levels decreased (Fig. 3b). However, the levels of NF- κ Bp65 did not alter in the striatum (Fig. 3c). We further localized NF- κ Bp65 in the midbrain following subchronic exposure to

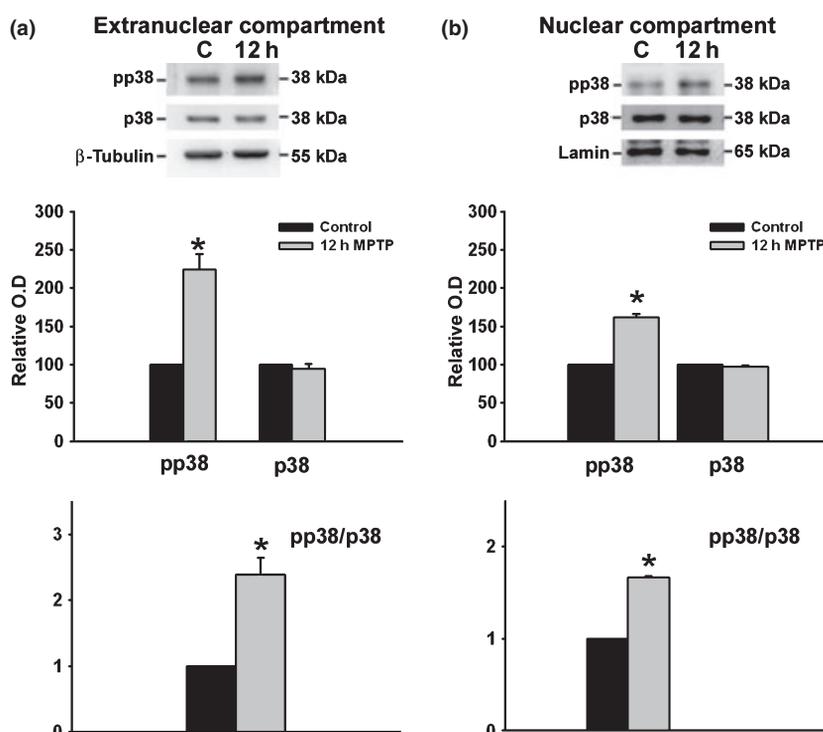


Fig. 1 MPTP-induced activation of p38 in ventral midbrain *in vivo*. (a) Animals were treated with a single dose of vehicle or MPTP and killed 12 h later. Representative immunoblots from ventral midbrain of animals treated with saline (C) and MPTP (lanes, 12 h) depicting the levels of phospho-p38 (pp38) and p38 in the extranuclear (a) and nuclear compartment (b). β -Tubulin levels were measured as loading controls. Activation of pp38 is indicated by their respective pp38 : p38 ratio. The increase in ratio is expressed as fold increase with respect to the control ratio (1.0). β -Tubulin or lamin levels were measured as loading control as appropriate. Values are mean \pm SD ($n = 3$ animals). Asterisks (*) indicate values significantly different from corresponding control ($p < 0.05$). Student’s *t*-test was performed for a and b.

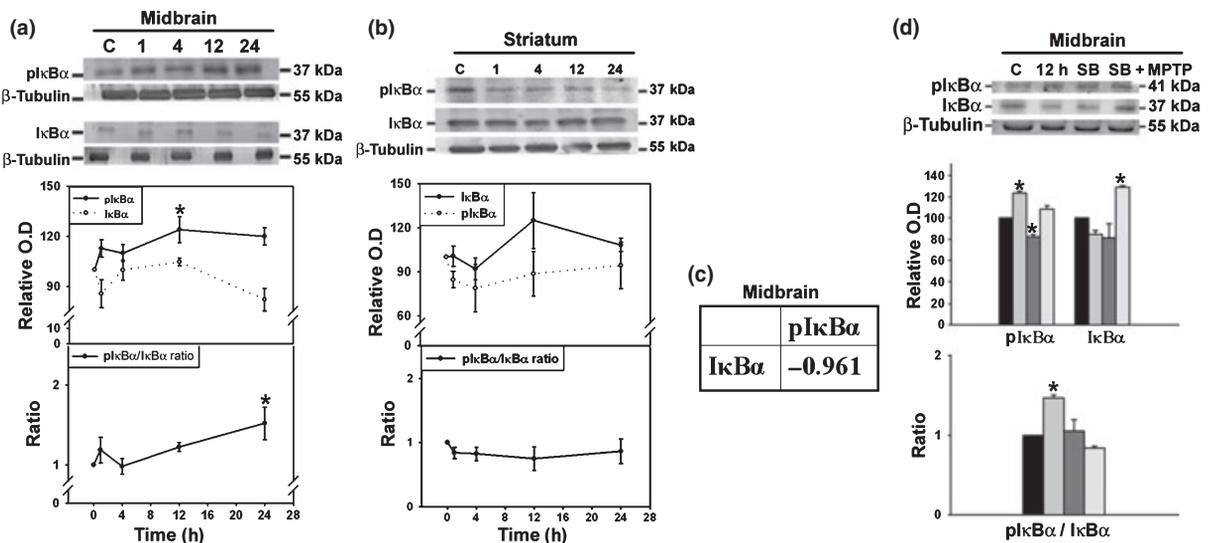


Fig. 2 MPTP-induced activation of NF- κ B in the ventral midbrain but not striatum. Animals were treated with a single dose of vehicle or MPTP and killed 1, 4, 12, and 24 h later. Representative immunoblots from ventral midbrain (a) and striatum (b) of animals treated with saline (c) and MPTP (lanes 1, 4, 12, and 24 h) depicting the levels of pI κ B α and I κ B α . Densitometric analyses of the immunoblots representing the relative intensity of the immunoreactive bands from midbrain and striatum are ($n = 3$) shown. They are represented as solid line (—) for pI κ B α , while I κ B α is depicted as dotted line (.....). (c) Pearson's correlation analysis showed negative correlation between the expression levels of pI κ B α and I κ B α in the ventral midbrain. (d) Animals were treated with a single dose of vehicle [3% dimethylsulfoxide

(DMSO)] or MPTP and killed 12 h later. Some animals also received a single dose of SB239063 intrathecally. Representative blots from ventral midbrain of animals treated with DMSO (lane 1), MPTP (lane 2), SB239063 (lane 3), and SB239063 + MPTP (lane 4) depicting the protein levels of phospho-I κ B α and I κ B α in the extranuclear compartment. β -Tubulin levels were measured as loading control. Activation of NF- κ B is indicated by their respective pI κ B α : I κ B α ratio. The increase in ratio is expressed as fold increase with respect to the control ratio (1.0). Values are mean \pm SD ($n = 3$ animals). Asterisks (*) indicate values significantly different from corresponding control ($p < 0.05$). Repeated measures of ANOVA followed by Dunnet's test were performed for a, b, and d.

MPTP for 8 days. Increased nuclear accumulation of NF- κ Bp65 was observed in the nucleus of the surviving cell in SNpc but not in the dopaminergic neurons of the ventral tegmental area. Vehicle-treated animals showed sparse staining for NF- κ Bp65 (Fig. 3d). Pearson's correlation analysis showed a high degree of positive correlation between NF- κ Bp65 and its target gene Bcl2 ($r = 0.986$; Fig. 3e). Co-localization of NF- κ Bp65 and tyrosine hydroxylase in the ventral midbrain showed nuclear translocation of NF- κ Bp65 in the dopaminergic neurons of SNpc (Fig. 4a) but not in the ventral tegmental area. The nuclear translocation of NF- κ Bp65 was not seen in the reticulata neurons. We further studied the effect of SB239063 on the translocation of NF- κ Bp65 to the nucleus. Surprisingly, SB239063 attenuated the accumulation of NF- κ Bp65 in the nuclear compartment (Fig. 4b).

Inactivation of Bcl2 following MPTP is abolished by p38 inhibitor, SB239063, in ventral midbrain

Activation and nuclear translocation of NF- κ B is known to induce the synthesis of anti-apoptotic proteins such as Bcl2. We observed that MPTP caused small but significant up-regulation of total Bcl2 at 4 h which was sustained up to 24 h after a single dose of MPTP in the ventral midbrain (Fig. 5a).

Phosphorylation of Bcl2 at Ser87 by p38 MAPK (De Chiara *et al.* 2006) resulted in loss of its anti-apoptotic function. We observed increase in the phosphorylated Bcl2 (Ser87) levels 12 to 24 h after MPTP indicating the loss of its anti-apoptotic activity. Pre-treatment with SB239063, the p38 inhibitor, not only prevented the increase in pBcl2 but also resulted in down-regulation of the increase in total Bcl2 levels seen after MPTP. This presumably indicates that the p38 inhibitor prevented both the activation of NF- κ B and the phosphorylation of Bcl2 (Fig. 5c). The level of Bcl2 was not altered in the striatum following MPTP treatment (Fig. 5b). Further, caspase 3 (p17) was also activated in a sustained manner in the midbrain but not in the striatum following single dose of MPTP (Fig. 5d). Pearson's correlation analysis showed a high degree of positive correlation between the levels of pBcl2Ser87, active caspase 3, and pp38 (Fig. 5e).

Discussion

In the present study we demonstrate that phosphorylation of p38 MAPK is an important event upstream of NF- κ B activation. Inhibitors of p38 MAPK effectively abolished phosphorylation of I κ B α and nuclear translocation of the RelA-p65 subunit of NF- κ B thus preventing the transactiva-

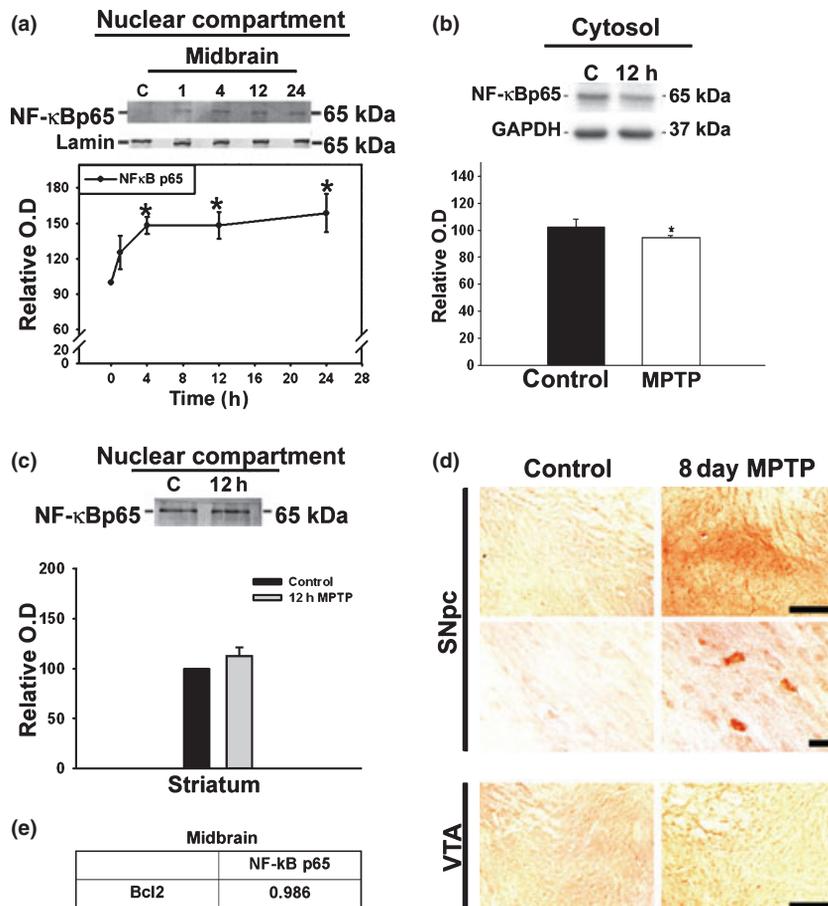


Fig. 3 NF-κBp65 translocates to the nucleus following MPTP treatment *in vivo*. Animals were treated with a single dose of vehicle or MPTP and killed 1, 4, 12, and 24 h later. Representative immunoblots from ventral midbrain (a) of animals treated with saline (C) and MPTP (lanes 1, 4, 12, and 24 h) depicting the levels of NF-κBp65 in the nuclear compartment. Lamin levels were measured as loading control. (b) Animals were treated with a single dose of vehicle or MPTP and killed 12 h later. The level of NF-κBp65 is decreased specifically in the cytosolic fraction. The blot was normalized with GAPDH for cytosol. (c) Representative blot from striatum of animals depicting the total NF-κBp65. (d) Animals were treated with a daily dose of MPTP for 8 days and killed on the ninth day. Immunohistochemical localization of NF-κBp65 revealed the accumulation of NF-κBp65 in the nucleus of surviving substantia nigra pars compacta (SNpc) neurons (first row).

The magnified images of SNpc (second row) show that NF-κBp65 is not present in the nucleus of control animals, while it is present in the nucleus in the surviving neurons after subchronic exposure to MPTP for 8 days. NF-κBp65 is not activated in the neurons of the ventral tegmental area (VTA) following MPTP (third row). Scale Bar = 25 μm. Scale bar represents 10 μm for the magnified images. Values are mean ± SD ($n = 3$ animals). Asterisks indicate values significantly different from corresponding control ($p < 0.05$). Repeated measures of ANOVA followed by Dunnett's test were performed for a. While Student's *t*-test was carried out for b and c. (e) Pearson's correlation analysis showed high degree of correlation between the expression levels of NF-κBp65 and Bcl2. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

tion mediated by NF-κB. p38 MAPK can potentially impact on NF-κB pathway through several mechanisms as shown in *in vitro* studies using cultured cells (Baeza-Raja and Muñoz-Cánoves 2004). For example, p38 is known to phosphorylate IκBα leading to its dissociation from the p65 subunit thus facilitating the translocation of p65 to the nucleus (Calleros *et al.* 2006). Phosphorylated IκBα was rapidly degraded by the proteasome (Finco and Baldwin 1995). In our study, treatment of mice with p38 inhibitor inhibited the phosphorylation of pIκBα (Fig. 2d) and importantly in the presence of

MPTP, the levels of IκBα increased significantly over controls indicating that the non-phosphorylated form of the protein was rapidly accumulating in the cell. SB239063 alone induced a decrease in basal plkBα levels. When SB239063 was given with MPTP it attenuated the increase in plkBα seen with MPTP alone. However, this decrease could also be because of the primary effect of the p38 inhibitor on plkBα levels *per se*. This may suggest that although p38 inhibitor is able to decrease the phosphorylated form of IκBα in basal condition, it may have nominal action on the

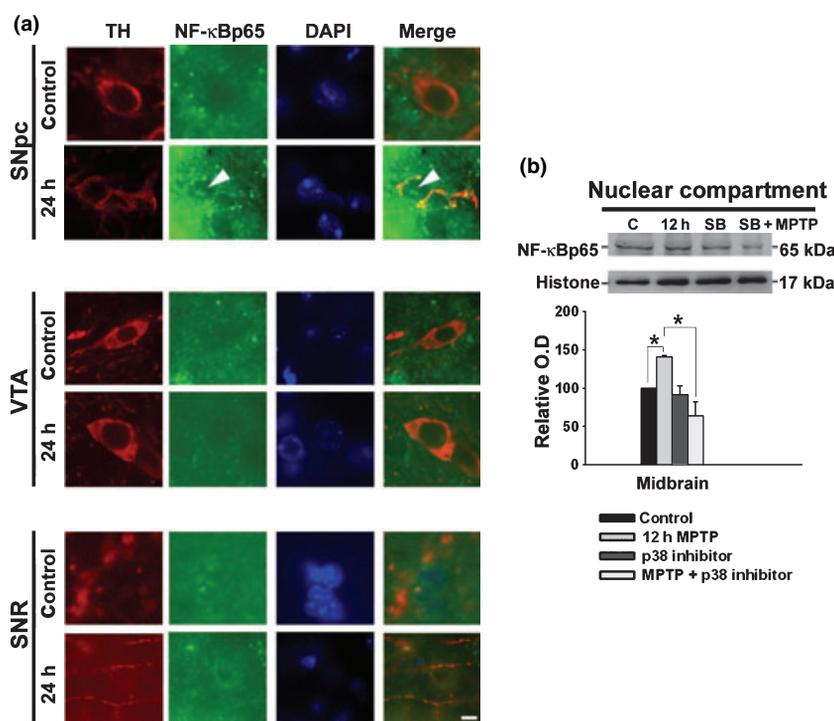


Fig. 4 MPTP-mediated translocation of NF-κBp65 to the nucleus is abolished by p38 inhibitor SB239063 *in vivo* in mice. Animals were treated with a single dose of MPTP and killed 24 h later. (a) Immunohistochemical co-localization revealed the presence of NF-κBp65 (arrowhead; green) in the nucleus of tyrosine hydroxylase (TH)-positive neurons of the SNpc (red) but not in the neurons of ventral tegmental area (VTA) or reticulata neurons (SNR). Scale Bar: 10 μm. (b) Animals were treated with a single dose of vehicle [3% dimethylsulfoxide (DMSO)] or MPTP and killed 12 h later. Some

animals also received a single dose of SB239063 intrathecally. Representative blots from ventral midbrain of animals treated with DMSO (lane 1), MPTP (lane 2), SB239063 (lane 3), and SB239063 + MPTP (lane 4) depicting the protein levels of NF-κBp65 in the nuclear compartment. Histone levels were measured as loading control. Values are mean ± SD ($n = 3$ animals). Asterisks (*) indicate values significantly different from corresponding control ($p < 0.05$). Repeated measures of ANOVA followed by Dunnett's test were performed for b. DAPI, 4-6-diamidino-2-phenylindole dihydrochloride.

phosphorylation of IκBα because of MPTP, which could presumably occur through other mechanisms. However, the co-treatment of MPTP and SB239063 increased the level of total IκBα clearly indicating its accumulation in the cell because of the fact that only the phosphorylated form was degraded by proteasome.

The TNF receptor (TNFR) superfamily also includes other prominent receptors like Fas and p75 nerve growth factor receptor among others. Upon activation, the TNFRs interact with an array of intracellular adaptor proteins to mediate downstream cell signaling. Particularly, TNFR1 and TNFR2 associate with TNFR-associated factors, which mediate activation of the NF-κB family of transcription factors. TNFR-associated factor 2 mediates NF-κB activation via NF-κB inducing kinase, which leads to IκB degradation and release of NF-κB (Pomerantz and Baltimore 1999).

It therefore, appears that p38 impacts the NF-κB pathway at the initiation of the cascade that is the phosphorylation of IκBα facilitating the dissociation and translocation of the p65 subunit. Further, p38 is also known to phosphorylate the p65 subunit of NF-κB and this could also be a potential

mechanism of action (Olson *et al.* 2007). We would like to add that while these mechanisms have been identified in cultured cells, very few studies have examined these pathways, *in vivo*. Thus we demonstrate for the first time that p38 MAPK is involved in NF-κB activation, *in vivo*, in the SNpc in animal model of PD.

We studied early events in MPTP toxicity by examining the signaling cascades following a single dose of MPTP. This strategy helped us to identify primary mechanisms underlying the neurotoxicity and we then examined if these responses were sustained after chronic administration of MPTP for 8 days. Thus we found that the NF-κBp65 levels were enhanced in the nuclear compartment of the ventral midbrain 4 h after a single dose of MPTP and this was sustained for up to 24 h. No such effect was seen in the striatum (Fig. 3c). Further, mice treated with MPTP for 8 days or a single dose for 24 h showed higher levels of p65 in the nucleus of cells in SNpc but not in the reticulata as seen by immunohistochemistry (Figs. 3d and 4a).

Activation of NF-κB can either enhance neuroprotection or promote neurodegeneration (Dehmer *et al.* 2004). For

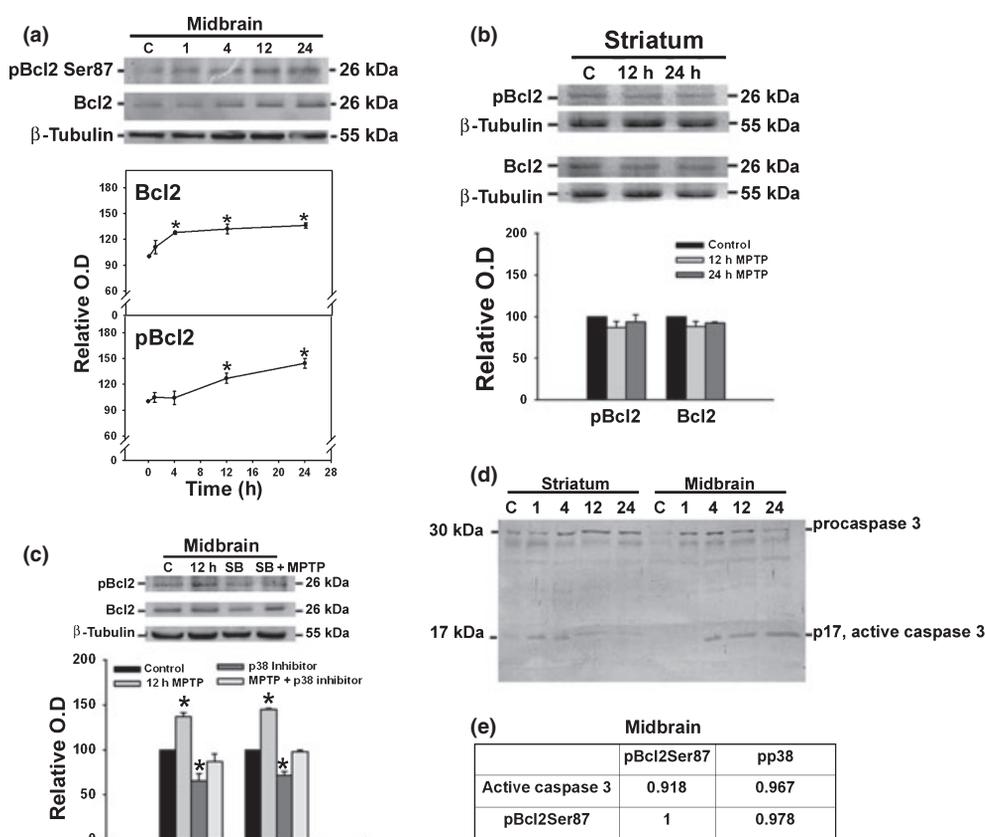


Fig. 5 Phosphorylation at Ser87 by p38MAPK inactivates anti-apoptotic Bcl2. (a) Animals were treated with a single dose of vehicle or MPTP and killed 1, 4, 12, and 24 h later. Representative immunoblots from ventral midbrain of animals treated with saline (C) and MPTP (lanes 1, 4, 12, and 24 h) depicting the levels of phospho-Bcl2 (pBcl2) and Bcl2. (b) Representative blots from striatum of animals treated with saline (C) and MPTP (12 and 24 h) depicting the protein levels of phospho-Bcl2 and Bcl2 in the extranuclear compartment. (c) Animals were treated with a single dose of vehicle [3% dimethylsulfoxide (DMSO)] or MPTP and killed 12 h later. Some animals also received a single dose of SB239063 intrathecally. Representative blots from ventral midbrain of animals treated with DMSO (lane 1), MPTP (lane

2), SB239063 (lane 3), and SB239063 + MPTP (lane 4) depicting the protein levels of phospho-Bcl2 and Bcl2 in the extranuclear compartment. (d) Representative blots from ventral midbrain and striatum depicting the protein levels of active caspase 3 (p17) and procaspase 3 in the extranuclear compartment. β -Tubulin levels were measured as loading control. Values are mean \pm SD ($n = 3$ animals). Asterisks (*) indicate values significantly different from corresponding control ($p < 0.05$). Repeated measures of ANOVA followed by Dunnett's test were performed for a, b, and c. (e) Pearson's correlation analysis showed high degree of correlation between the expression levels of pp38, pBcl2Ser87, and active caspase 3.

example, Bcl2, the anti-apoptotic molecule and p53 the pro-apoptotic gene are transcribed through NF- κ B. Previous studies have shown that p53 expression is increased following MPTP treatment (Karunakaran *et al.* 2008) and inhibition (Duan *et al.* 2002) or knock down (Trimmer *et al.* 1996) of p53 affords neuroprotection. In the present study we show that Bcl2 expression is increased in the ventral midbrain (Fig. 5a) but not in striatum where NF- κ B activation does not occur (Fig. 5b). However, the anti-apoptotic effects of Bcl2 were negated by its phosphorylation by p38 MAPK (Fig. 5c). Phosphorylation of Bcl2 by p38 to pBcl2ser87 resulted in decrease of the anti-apoptotic potential, and pBcl2 is an important player in the initiation of the apoptotic signaling cascade (De Chiara *et al.* 2006)

including release of cytochrome *c* and caspase activation. Thus, the present study also points out an important role of p38 in pro-apoptotic signaling through phosphorylation of Bcl2. The activation of p38 promotes neurodegeneration through multiple mechanisms involving phosphorylation of p53 and Bcl2 and activation of NF- κ B (Fig. 6b). Thus activation of p38 MAPK can potentially play a pro-survival role by enhancing the expression of genes such as Bcl2 through NF- κ B activation while at the same time it inactivates Bcl2 by phosphorylating it at Ser87. Thus, inhibition of p38 MAPK could potentially lead to both enhanced neuroprotection/neurodegeneration as it impacts both cell survival and cell death cascade. The impact of p38 on these pathways needs to be assessed independently

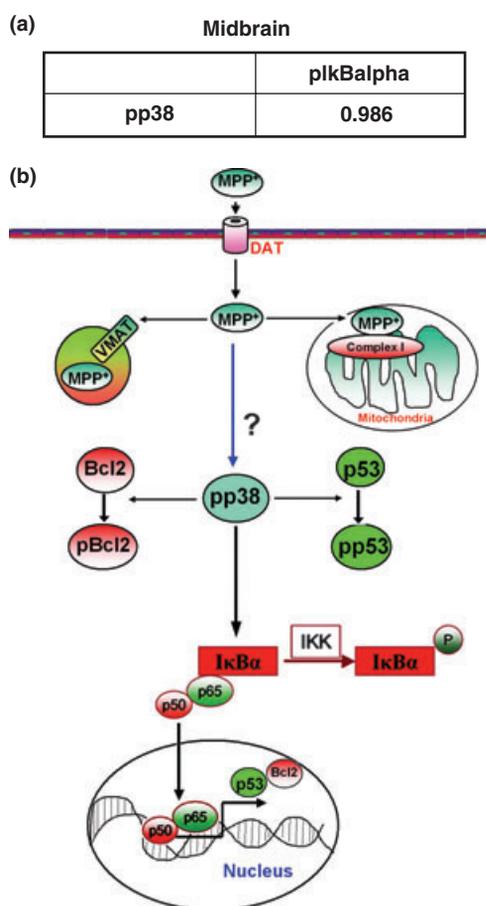


Fig. 6 Involvement of p38 and NF- κ B activation in MPTP-mediated toxicity: (a) Pearson's correlation analysis showed significant correlation between the expression levels of pp38 and amounts of plkB α formed ($r = 0.986$). (b) MPP $^{+}$, the toxic metabolite of MPTP causes mitochondrial dysfunction in dopaminergic neurons by inhibiting complex I of the electron transport chain. MPP $^{+}$ also activates the MAPK by phosphorylating p38. Activated p38 then phosphorylates I κ B α and p53. NF- κ B and p53 translocate to the nucleus where they transactivate the expression of anti-apoptotic and pro-apoptotic genes. Phospho-p38 MAPK also phosphorylates Bcl2 leading to inactivation of its anti-apoptotic function. Approaches aimed at inhibiting p38 could potentially prevent the initiation of the death-signaling cascade and offer neuroprotection. DAT, dopamine transporter; VMAT, vesicular monoamine transporter.

to determine which of these is influenced most by p38 MAPK.

It is interesting to note that activation of p38 (Fig. 1), phosphorylation of I κ B α , and nuclear translocation of p65 (Figs. 2 and 3) is seen only in the ventral midbrain but not in the striatum indicating a central role for p38 activation. In agreement with the above, the caspase activation is also limited to the ventral midbrain and not striatum (Fig. 5d).

Nuclear factor κ B activation has been implicated to be downstream to the inflammatory reaction seen in animal

models of PD and autopsy tissue from PD patients (Mogi *et al.* 2007). It is yet to be demonstrated if the inflammatory response is the cause or effect of the neurodegenerative process. The knock down of TNF- α and its receptors have failed to afford neuroprotection in mice and this argues against the role of inflammation as primary mediator of the degenerative process. In our model, we observed the activation of microglia (Karunakaran *et al.* 2008) but were unable to detect substantial increase in levels of cytokines such as TNF- α and interleukin-6 in the midbrain at early time periods after a single dose of MPTP (data not shown)~although sustained activation of MAPK was seen (Karunakaran *et al.* 2008). This observation shows that the inflammatory response are unlikely to be the triggering event and that activation of MAPK occurring possibly downstream of redox perturbation may be the one of the initiators of the death signaling cascade(s).

In conclusion, our studies demonstrate that activation of p38 is upstream of the activation of NF- κ B driven transcription which can potentially promote neurodegeneration. Thus, inhibition of p38 MAPK offers an attractive target for drug discovery. The cross-talk between p38, NF- κ B, and Bcl2 pathways demonstrated in the present study also pointed out the involvement of multiple pathways and redundancy that existed in the complex milieu, *in vivo*, in the mammalian brain, which presumably was far more intricate in the human brain in disease states. Therefore, disease-modifying therapies in humans may need to target multiple pathways through combinatorial approaches.

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