Neurobiology of Disease

Regulation of Dopaminergic Loss by Fas in a 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Model of Parkinson's Disease

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Accumulating evidence suggests that apoptotic and inflammatory factors contribute to the demise of dopaminergic neurons. In this respect, Fas, a member of the tumor necrosis factor receptor family with proapoptotic and inflammatory functions, was reported to be elevated within the striatum and substantia nigra pars compacta (SNc) of Parkinson's disease (PD) patients. Accordingly, the present investigation evaluated the function of Fas in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD. Injection of MPTP increased nigral Fas expression, and mice lacking Fas displayed attenuated MPTP-induced SNc dopaminergic loss and microglial activation. In addition, Fas induction was blocked by expression of a dominant-negative c-Jun adenovirus that also protected dopamine neurons from MPTP-induced damage. Together, these data suggest the critical nature of the c-Jun–Fas signaling pathway in MPTP-induced neuronal loss. Although critical for degeneration of the soma, Fas deficiency did not significantly prevent the reduction of dopaminergic terminal fibers within the striatum or normalize the activation of striatal microglia and elevation of the postsynaptic activity marker ΔFosB induced by denervation. Interestingly, Fas-deficient mice displayed a pre-existing reduction in striatal dopamine levels and locomotor behavior when compared with wild-type mice. Despite the reduced terminals, dopamine levels were not further suppressed by MPTP treatment in mutant mice, raising the possibility of a compensatory response in basal ganglia function in Fas-deficient mice.

Key words: **Fas; neurodegeneration; cytokine; kinase; stress; dopamine**

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the selective loss of dopaminergic neurons of the substantia nigra pars compacta (SNc) resulting in reduced striatal dopamine (DA) and the cardinal clinical features of PD (Hirsch et al., 1988). The majority of PD cases are idiopathic, and environmental factors, including pesticides and immunological insults, have been implicated (Trimmer et al., 1996; Herdegen et al., 1998; Morishima et al., 2001; Vila et al., 2001; Xia et al., 2001; Antolin et al., 2002). Along these lines, systemic exposure to the mitochondrial complex 1 toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) provokes many of the hallmarks of PD, including selective dopaminergic loss, attenuation of striatal DA, and behavioral deficits (Langston et al., 1983; Blum et al., 2001).

Although the mechanisms governing dopaminergic loss after exposure to MPTP are not fully understood, a number of general processes may be involved, including ATP depletion, generation of free radicals, disruption of Ca^{2+} homeostasis, secondary excitotoxicity, and oxidative cellular damage related to mitochondrial dysfunction (Nicklas et al., 1985; Greenamyre et al., 1999; Blum et al., 2001). In turn, these stresses likely regulate specific death-related effectors that are critical for dopaminergic loss, such as the tumor suppressor p53 and the B-cell leukemia-2 family member Bcl-2-associated X protein (Bax), both of which have been shown to participate in MPTP-induced neuron loss (Hirsch et al., 1988; Blum et al., 2001; Vila et al., 2001). Recent evidence has also implicated the c-Jun–JNK (c-Jun N-terminal kinase) stress-activated pathway in multiple *in vivo* models of death, including MPTP and medial forebrain axotomy (Crocker et al., 2001, 2003; Xia et al., 2001). Although c-Jun, a transcription factor of the activator protein-1 (AP-1) complex, has been implicated in a wide variety of processes, including cell-cycle regulation and death, the mechanism by which c-Jun mediates death is unclear.

One potentially important mediator of c-Jun-induced neuro-

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nal death is the tumor necrosis factor-α (TNF-α) receptor superfamily member Fas (Herdegen et al., 1998; Morishima et al., 2001; Lee and Ferguson, 2003). Although Fas is best characterized for its ability to trigger apoptosis through its intracellular Fas-associated death domain (FADD) and activation of caspases (Lee and Ferguson, 2003), it also has important modulatory actions on inflammatory processes (Park et al., 2003). Mice with a leaky mutation of the Fas gene (*lpr*, resulting in reduced endogenous Fas levels) are resistant to cell death and to the behavioral deficits ordinarily observed in animal models of ischemia and multiple sclerosis (Dittel et al., 1999; Sabelko-Downes et al., 1999). Intriguingly, the soluble form of Fas is increased in tissue from the nigrostriatal region of PD brains (Mogi et al., 1996). Furthermore, Fas–Fas ligand (FasL) as well as FADD were reduced in DA neurons of PD patients, raising the possibility that Fas signaling may render these neurons vulnerable to PD-related pathology (Ferrer et al., 2001; Hartmann et al., 2002). Because the functional role of Fas in dopaminergic loss is unknown, we investigated its role in dopaminergic function and degeneration in the MPTP model of PD and assessed the contribution of the c-Jun–JNK pathway to Fas regulation in this paradigm.

Materials and Methods

Animals. Male and female Fas knock-out (KO) mice (homozygous for targeted mutations of the Fas receptor) were obtained from The Jackson Laboratory (Bar Harbor, ME). Importantly, all mice were back-crossed to a C57BL/6J background for at least 8–13 generations (The Jackson Laboratory, personal communication), thereby ensuring a standard C57BL/6J genetic background. Animals were maintained on a 12 hr light/dark cycle with lights on at 6:00 A.M., room temperature was kept at 21°C, and animals were permitted an *ad libitum* diet of Purina (Richmond, IN) mouse chow. All experimental procedures were approved by the University of Ottawa Committee for Animal Care and met the guidelines set by the Canadian Council on Animal Care.

MPTP administration. MPTP (25 mg/kg, i.p., measured as free base; Sigma, St. Louis, MO) was administered to mice (8–10 weeks of age) intraperitoneally once per day for 5 consecutive days (Tatton and Kish, 1997; Xia et al., 2001; Crocker et al., 2003). Mice used as controls received once-daily injections of an equivalent volume of saline (0.9%).

Adenoviral gene delivery. Wild-type C57BL/6 mice received an intrastriatal infusion of adenoviral vectors (1×10^7 particles/μl) expressing either the dominant-negative (DN) form of c-Jun or the bacterial reporter gene (control expression marker) lacZ (Ad.lacZ). Adenoviruses were stereotaxically infused into the dorsolateral striatum (0.5 mm rostral, 2.2 mm to the right of bregma, and 3.4 mm below the skull surface) at an infusion rate of 0.5 μl/min using a syringe pump (PHD2000; Harvard Apparatus, St. Laurent, Quebec, Canada) until a total volume of 2.0 μl was delivered. At 1 week after adenovirus injection, mice received either MPTP or saline as described in the aforementioned section.

Immunohistochemistry. After overnight postfixation in 4% paraformaldehyde, brains were cryoprotected in sucrose, and free-floating sections were obtained as described previously (Crocker et al., 2001, 2003). Immunostaining was performed using mouse anti-tyrosine hydroxylase (TH) (1:10,000; ImmunoStar, Hudson, WI), rat anti-cd11b (microglia marker) (1:100; Serotec, Indianapolis, IN), or rabbit anti-ΔFosB (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies and visualized using diaminobenzidine. Alternatively, brains were flash frozen in isopentane, and sections were mounted onto superfrost plus slides. These sections were preincubated with 10% normal donkey serum (30 min at room temperature) and then incubated with mouse anti-Fas or anti-FasL monoclonal antibodies (1:400; Transduction Laboratories, Lexington, KY) overnight at 4°C. Immunostaining was then visualized after incubation with donkey anti-mouse carbocyanine 3 (Cy3)-conjugated antiserum (1:200; The Jackson Laboratory). Double labeling involved the use of primary antibodies for rabbit anti-Fas (1:400; Santa Cruz Biotechnology) together with mouse anti-TH (1:10,000; ImmunoStar) or rat anti-GFAP (1:500; Dako, Carpinteria, CA). The secondary antibody anti-rabbit biotin (1:200; The Jackson Laboratory) was applied for 1 hr at room temperature, and then sections were visualized using streptavidin-conjugated Cy3 (1:200; The Jackson Laboratory) together with either anti-mouse or anti-rat Alexa 488 nm-conjugated antiserum (1:200; Molecular Probes, Eugene, OR).

Assessment of neuronal loss. Loss of neurons in the SNc of Fas null mice was determined by serial section analysis of the total number of TH-positive (TH+) neurons as described previously (Crocker et al., 2001, 2003) at 14 d after MPTP treatment. Adjacent tissue was also stained for cresyl violet to validate survival as assessed by TH staining. Estimates of total TH+-stained and cresyl violet-stained neurons in the SNc were calculated using Abercrombie's correction (Abercrombie, 1946). Briefly, the Abercrombie correction considers the number of sections collected, the interval between sections, and the thickness of each section to estimate the total number of neurons (cresyl violet) or TH+ cells within the entire SNc. The Abercrombie values are calculated by multiplying the total neuronal count for each of these factors. For analysis of animals expressing lacZ or DN c-Jun, only the level of the medial terminal nucleus (the level of highest adenoviral-mediated expression) was examined.

Quantification of immunohistochemistry. Quantification of striatal dopaminergic fibrous staining and striatal FosB-positive nuclei were analyzed as described previously (Crocker et al., 2003). In all cases of immunohistochemical quantification, analyses were performed by an individual unaware of the experimental treatments. Additionally, the relative density of cd11b staining was evaluated within the SNc and striatum of digitized images using the same computer software (Northern Eclipse; Empix, Mississauga, Ontario, Canada) and associated subroutine that was used for striatal TH+ and FosB quantification. Briefly, the same predetermined threshold level was used on all sections (ensuring that only labeling within this intensity range was evaluated), and background staining (outside the SNc or striatum) was subtracted to yield a value for the area of positive staining.

N-methyl-4-phenylpyridinium ion measurements. *N*-methyl-4-phenylpyridinium ion (MPP+) measurements were performed as described previously. Briefly, striatal concentrations of MPP+ were measured 90 min after a single dose of MPTP using HPLC measurements as described previously (Crocker et al., 2003).

Brain microdissection. After decapitation, brains were flash frozen in isopentane and sectioned into a series of coronal slices using a plastic dissecting block with adjacent slots spaced 1.0 mm apart. The SNc and striatum were obtained by micropunch using 1- and 2-mm-diameter biopsy needles, respectively. Brain punches were taken according to the mouse brain atlas of Franklin and Paxinos (1997).

Neurochemical analyses. HPLC analyses were performed 14 d after MPTP treatment. Levels of DA, DOPAC, and homovanillic acid (HVA) were determined by HPLC using a modification of methods described previously (Seegal et al., 1986). Tissue punches were sonicated in a homogenizing solution that was composed of 14.17 gm of monochloroacetic acid, 0.0186 gm of disodium EDTA, 5.0 ml of methanol, and 500 ml of H_2O. After centrifugation, the supernatants were passed through the system at a flow rate of 1.5 ml/min (1400–1600 psi). The mobile phase used for the separation was a modification of that used by Chiueh et al. (1983). Each liter consisted of 1.3 gm of heptane sulfonic acid, 0.1 gm of disodium EDTA, 6.5 ml of triethylamine, and 35 ml of acetonitrile. The area and height of the peaks were determined using a Hewlett-Packard (Palo Alto, CA) integrator. The protein content of each sample was determined using bicinchoninic acid with a protein analysis kit (Pierce Scientific, Brockville, Ontario, Canada).

Western blot. SNc tissue punches were diluted in 250 μl of extraction buffer (0.5 M HEPES, pH 7.5, 5.0 M NaCl, 0.5 M EDTA, 100 mM EGTA, 1 M DTT, 50% glycerol, 0.1 M PMSF, 10 mg/ml leupeptin, 5 μg/ml aprotinin, 1 M β-glycerophosphate, 0.5 M NaF, and 100 mM Na-orthovanadate), respectively. Tissues were then sonicated and centrifuged at 14,000 rpm (15 min), and supernatant was assessed for protein concentration using a Bio-Rad (Mississauga, Ontario, Canada) protein assay. Thereafter, 30 μg of protein was analyzed by SDS-PAGE and Western blot analyses as described previously (Crocker et al., 2001). The primary antibod-

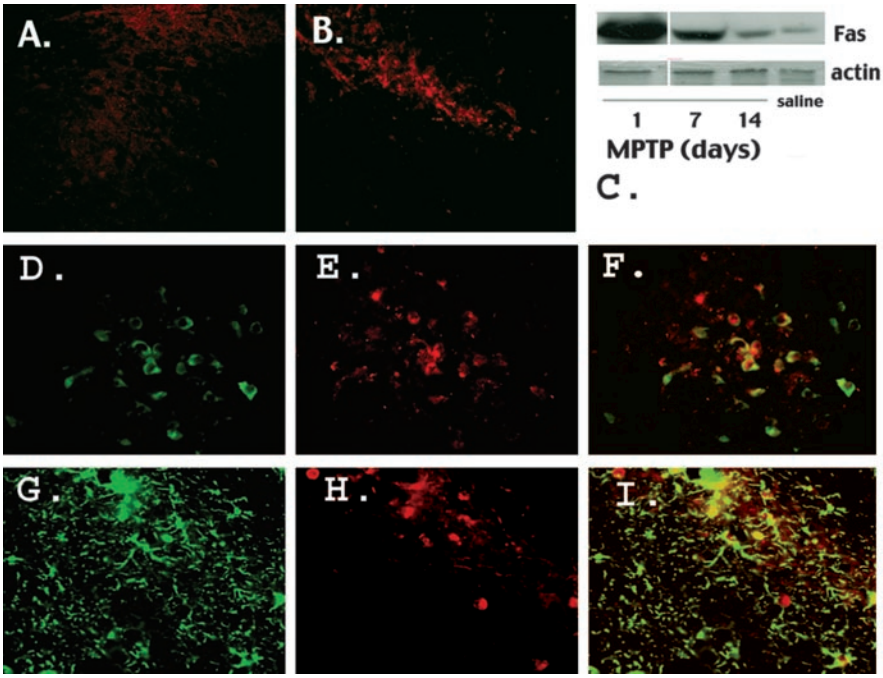

Figure 1. Increased Fas expression in the SNc after MPTP. Representative photomicrographs indicate enhanced immunohistochemical detection of Fas in the SNc 1 d after MPTP treatment (*B*) relative to animals receiving saline (*A*). A time course for Fas expression after MPTP is presented using Western blot analysis (*C*). Double-label immunofluorescent analyses were conducted for TH (*D*) and Fas (*E*) as well as GFAP (*G*) and Fas (*H*) within the SNc. The colocalization of Fas together with TH and GFAP is depicted in *F* and *I*, respectively.

ies used were Fas (1:2000; Santa Cruz Biotechnology), FasL (1:400; Santa Cruz Biotechnology), and β-actin (1:20,000; Sigma).

Behavioral analyses. The home-cage activity of mice was monitored using a micromax beam-break apparatus equipped with 16 infrared sensors (Accuscan , Columbus, OH). Briefly, singly housed mice were placed in the apparatus, and the number of interruptions of the infrared beams provided an index of total activity (horizontal, vertical). Behavior was monitored for 24 hr on day 14 after the initial MPTP injection.

To assess the functioning of surviving dopaminergic neurons in response to a challenge stimulus, separate groups of mice were subjected to a brief swim stressor before being placed in the beam-break apparatus. The swim stress comprised placing the animal in a 4 l beaker containing room-temperature water for 60 sec, after which the mouse was placed in the apparatus, and behavior was monitored for 120 min. Importantly, our findings indicate that MPTP-induced activity deficits are augmented in response to this mild stressor.

Statistical analysis. The histochemical, behavioral, and monoamine data were analyzed by a 2 (MPTP vs saline injection) × 2 (Fas knock-out vs wild-type strain) factorial design. In the study involving adenoviral injection, histochemical data were analyzed by one-way ANOVA. Significant interactions were followed by Tukey's honestly significant difference test ($\alpha = 0.05$) of the simple effects comprising the interaction.

Results

MPTP induction of Fas within the SNc

Initial experiments assessed the impact of MPTP administration on Fas–FasL expression. As shown in Figure 1, immunofluorescence analyses revealed that saline-treated wild-type mice displayed minimal Fas immunoreactivity within the SNc, whereas Fas labeling was evident within the SNc 1 d after MPTP injection (Fig. 1). Moreover, double-label immunofluorescence analyses revealed that ~40% of the TH-positive neurons within the SNc colocalized the Fas protein. However, it was clear that Fas immunoreactivity was also apparent in many nondopaminergic neurons. Indeed, additional immunofluorescence indicated that Fas expression was colocalized with numerous GFAP-positive cells within the SNc (Fig. 1), whereas there was virtually no colocalization of Fas together with cd11b (data not shown). The induction of Fas, which was particularly marked at 1 and 7 d after MPTP, was further confirmed by Western blot analyses of SNc extracts (Fig. 1). In contrast, using immunohistochemistry or Western blot detection procedures, FasL expression was not altered at any time after MPTP treatment (data not shown).

Regulation of Fas induction and MPTP-provoked DA neuronal death by c-Jun

The fact that previous reports indicated that Fas induction may be mediated by c-Jun (Lasham et al., 2000), coupled with the observation that the c-Jun–JNK pathway may be required for dopaminergic neuronal death, led us to examine whether expression of a DN c-Jun may block neuronal death associated with MPTP treatment and inhibit Fas induction. As shown in Figure 2, the FLAG-tagged DN c-Jun adenovirus was coexpressed in TH+ neurons within the SNc, and expression of the adenovirus attenuated MPTP-provoked DA neuronal death. Indeed, at the level of the medial terminal nucleus (−3.16 relative to bregma), MPTP-treated mice that were infused previously with the lacZ control adenovirus displayed significantly less TH+ neurons (29.1 ± 5.1 neurons per section) than saline-treated controls (52.3 ± 7.9 neurons per section). However, MPTP treatment had little effect on mice that received the DN c-Jun adenovirus (45.8 ± 8.1 neurons per section). Importantly, DN c-Jun expression also inhibited MPTP-induced Fas expression, as determined using immunofluorescence and Western blot analyses (Fig. 2). Thus, Fas is a downstream effector of the c-Jun–JNK pathway.

Fas-deficient mice are resistant to MPTP-induced SNc DA neuron death

Given the observed c-Jun-dependent upregulation of Fas after MPTP, we next assessed the requirement of Fas for dopaminergic loss by using Fas-deficient mice. As shown in Figure 3, the number of TH-positive neurons did not differ between saline-treated Fas null or wild-type mice, indicating that the basal number of SNc TH neurons was not altered by Fas deficiency. After MPTP treatment of wild-type animals, there was a clear reduction in the number of TH-labeled cells across several levels of the SNc (Fig. 3). In particular, the reduction of TH+ neurons was most prominent at medial levels of the SNc around the medial terminal nucleus ($p < 0.05$). In contrast, MPTP provoked only a small and nonsignificant reduction of TH labeling in Fas-deficient mice compared with saline controls at any level of the SNc. Using the Abercrombie correction, estimation of the total number of TH-positive SNc neurons revealed an overall reduction in dopaminergic neurons in wild-type animals exposed to MPTP; however, this reduction was significantly attenuated in Fas null mice (Table 1).

To address the possibility that the differences in TH-positive neurons after MPTP may stem from altered phenotypic expression of the DA marker, the overall number of neurons within the

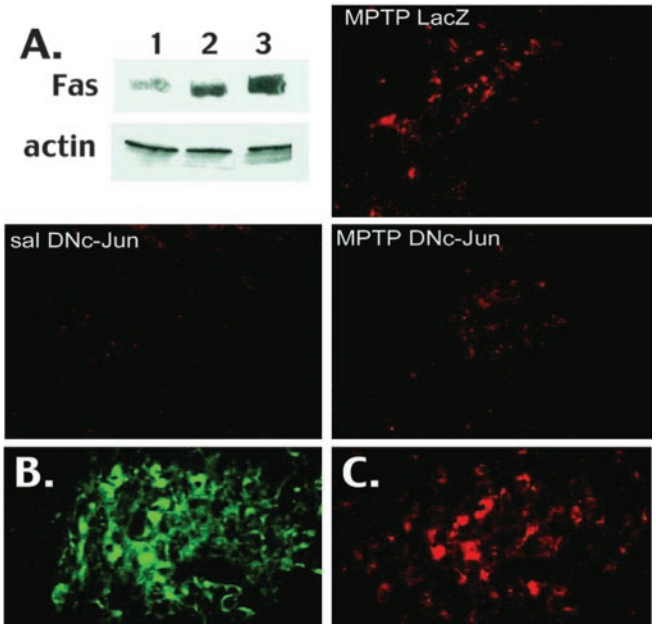

Figure 2. Adenoviral-mediated inhibition of c-Jun attenuates MPTP-induced Fas expression and dopaminergic neuronal death. The Western blot depicts Fas expression 1 d after intraperitoneal saline or MPTP treatment in mice that had received intrastriatal adenoviral infusion 7 d earlier (*A*). Lane 1 shows saline-treated mice that received the lacZ control adenovirus previously, and lanes 2 and 3 depict MPTP-treated mice that had been pretreated with either the dominant-negative c-Jun or the lacZ adenoviruses, respectively. Immunohistochemical photomicrographs revealed Fas staining within the SNc of MPTP-treated mice previously administered the lacZ (MPTP LacZ) or dominant-negative c-Jun (MPTP DNc-Jun) adenoviruses or animals that received saline injection and the adenovirus (sal DNc-Jun). Double labeling indicated that expression of the FLAG-tagged adenovirus (*C*) was observed within TH+ SNc neurons (*B*).

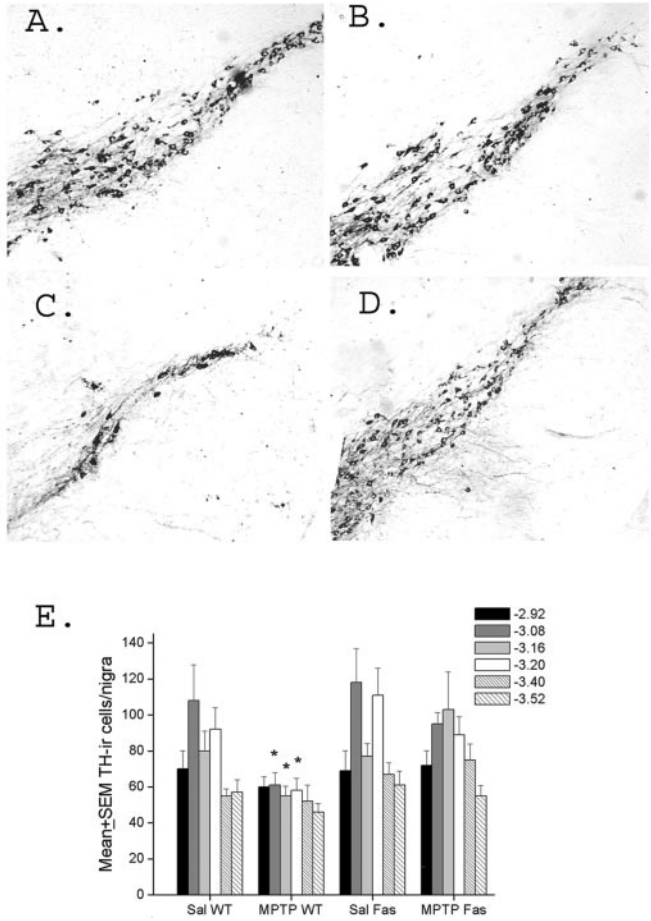

Figure 3. Fas-deficient mice are resistant to the death-inducing effects of MPTP on dopaminergic SNc neurons. The photomicrographs reveal that MPTP treatment provoked a loss of TH+ neurons within the SNc of wild-type mice (*C*) but not Fas-deficient mice (*D*) relative to saline-treated wild-type mice (Sal WT) (*A*) or saline-treated Fas null mice (Sal Fas) (*B*). Quantitative analyses across multiple levels of the SNc confirmed the MPTP-induced TH+ loss in wild-type mice (MPTP WT) but not Fas mice (MPTP Fas) (*E*). *$p < 0.05$ relative to Sal WT. Error bars represent SEM.

SNc of all groups was determined using cresyl violet staining as reported previously (Crocker et al., 2003). Consistent with the TH staining, in response to the MPTP treatment, the number of cresyl violet-labeled cells within the SNc was reduced in wild-type mice (Table 1). This reduction was again attenuated significantly in Fas null mice ($p < 0.05$), indicating that the observed differences were not attributable to phenotypic suppression.

MPTP metabolism is not affected by Fas deficiency

The neurotoxic effects of MPTP on DA neurons require the metabolism of MPTP to MPP+ by monoamine oxidase (Heikkila et al., 1984). Therefore, the protective effects of Fas deficiency may be attributable to altered MPTP metabolism and reduced MPP+ production. To examine this possibility, striatal tissues were evaluated from mice 90 min after a single injection of MPTP (30 mg/kg), and levels of MPP+ were determined using HPLC. It was observed that MPP+ levels did not differ significantly between wild-type and Fas-deficient animals (data not shown), suggesting that the protection afforded by Fas deficiency was not attributable to impairment of MPTP metabolism.

Fas-deficient mice show attenuated microglial activation in the SNc in response to MPTP

Because potent glial activation has been reported within the SNc after MPTP treatment (Czlonkowska et al., 2002), we determined whether this effect was altered in Fas-deficient mice. As shown in Figure 4, cd11b immunoreactivity was minimal within the SNc and striatum of saline-treated, wild-type, and Fas null mice. However, robust microglia (cd11b-positive) activation was evident throughout the SNc and striatum of MPTP-treated wild-type mice. Interestingly, Fas null mice displayed increased cd11b staining within the striatum but not the SNc after the MPTP regimen (Fig. 4). In terms of morphological characteristics, the cd11b-positive cells appeared more compact, rounded, and with obvious cellular thickening, indicative of an activated state (Soltys et al., 2001), when compared with saline-treated control animals that displayed a more ramified state with thin processes. Importantly, microglial activation was attenuated in the Fas-deficient mice (Fig. 4). Quantification of cd11b relative density confirmed the qualitative observations. Indeed, microglial relative density was augmented in response to MPTP within the striatum of wild-type and Fas null mice; however, only wild-type animals showed increased cd11b staining within the SNc after MPTP (Fig. 4). Thus, it appears that Fas deficiency inhibits the

Table 1. Neuronal survival within the SNc among saline-treated or MPTP-treated wild-type (WT) or Fas-deficient (Fas KO) mice as determined using Abercrombie's correction for total TH+ neurons and cresyl violet staining (neurons per section)

	Saline WT	Saline Fas KO	MPTP WT	MPTP Fas KO
Abercrombie	7998 ± 805	7109 ± 607	4832 ± 519*	6552 ± 781
Cresyl violet	89 ± 8	92 ± 11	61 ± 15*	82 ± 7

*$p < 0.05$ relative to saline WT.

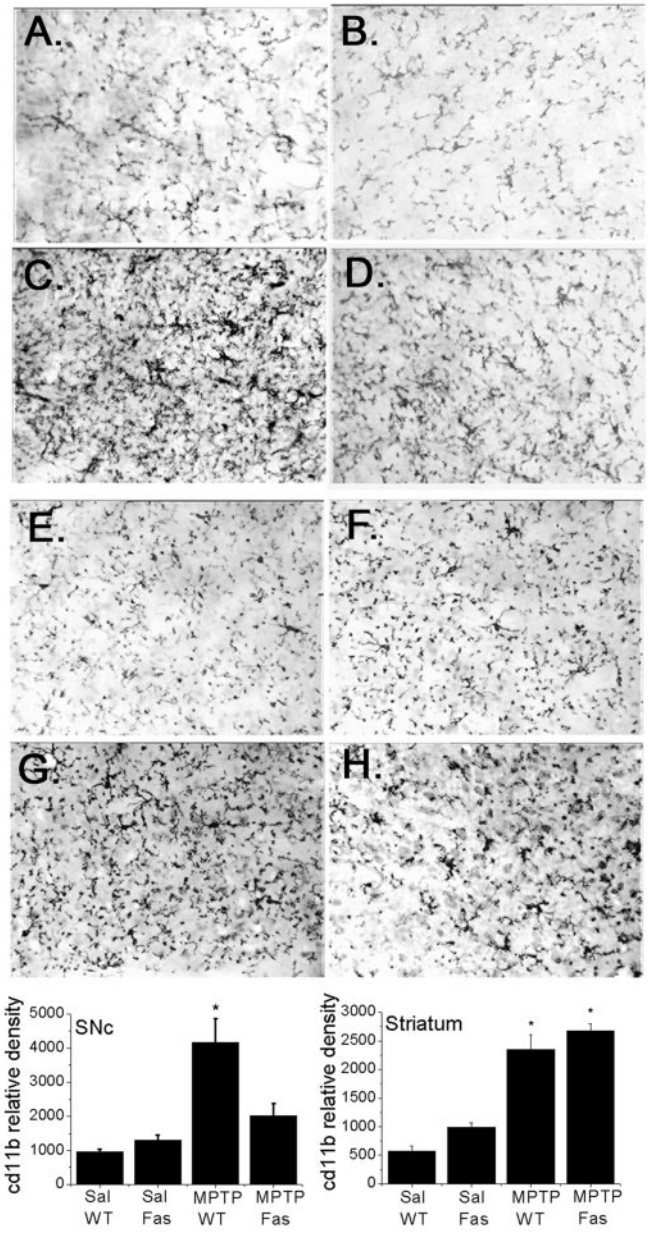

Figure 4. MPTP-induced microglial activation is attenuated within the SNc but not the striatum in Fas-deficient mice. Photomicrographs display immunoreactivity for the microglial marker cd11b within the SNc of wild-type mice that received saline (*A*) or MPTP (*C*) as well as Fas null animals treated with saline (*B*) or MPTP (*D*). Correspondingly, cd11b immunoreactivity within the striatum is displayed for wild-type mice administered saline (*E*) or MPTP (*G*) as well as Fas null animals receiving saline (*F*) or MPTP (*H*) injections. Quantitative analysis of the relative density of cd11b within the SNc (bottom left) and striatum (bottom right) confirmed that microglial recruitment–activation after MPTP was attenuated in Fas null mice only within the SNc. $*p < 0.05$ relative to Sal WT or Sal Fas. Error bars represent SEM.

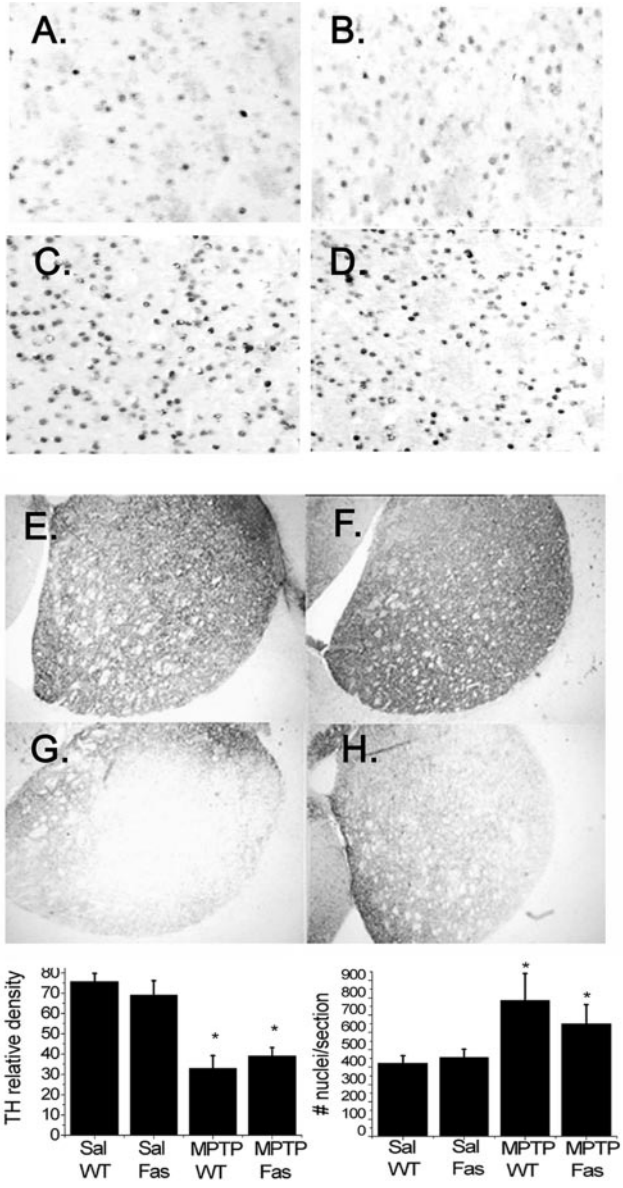

Figure 5. MPTP reduced striatal TH density and augmented FosB staining in wild-type and Fas null mice. Photomicrographs depict ΔFosB staining within the striatum of wild-type mice after saline (*A*) or MPTP (*C*) treatments as well as that of Fas null mice after saline (*B*) or MPTP (*D*) injections. The immunohistochemical analyses also display striatal TH+ labeling in wild-type mice administered saline (*E*) or MPTP (*G*) and the corresponding Fas null mice injected with either saline (*F*) or MPTP (*H*). Observations were confirmed by quantification of striatal TH+ density (bottom left) and number of FosB-positive nuclei per striatal section (bottom right) (*I*). $*p < 0.05$ relative to Sal WT or Sal Fas. Error bars represent SEM.

loss of dopaminergic cell bodies as well as the associated gliosis within the SNc but not the striatum.

Effect of Fas deletion on terminal fibers in the striatum

The major although not exclusive pathway leading from the dopaminergic neurons in the SNc is the nigrostriatal tract. Accordingly, we examined whether protection of dopaminergic cell bodies by Fas deficiency would also be accompanied by preservation of the distal terminal fibers in the striatum. As revealed by densitometric analysis (Fig. 5), TH staining within the striatum was reduced by MPTP treatment in wild-type animals compared with saline-treated controls ($p < 0.05$). In contrast to the neuroprotective effect within the SNc, there was only a modest nonsignificant attenuation of MPTP-provoked reduction of striatal TH staining in Fas null mice (Fig. 5), suggesting that the striatal terminals were not protected by Fas deficiency.

We and others have demonstrated previously that MPTP treatment provoked relatively long-term expression of the transcription factor ΔFosB within the striatum, which is believed to reflect changes in postsynaptic signaling (Crocker et al., 2003). In addition, ΔFosB has been suggested to mediate the supersensitivity of striatal dopamine receptors after denervation (Dragunow et al., 1995). Consistent with previous findings, the present study

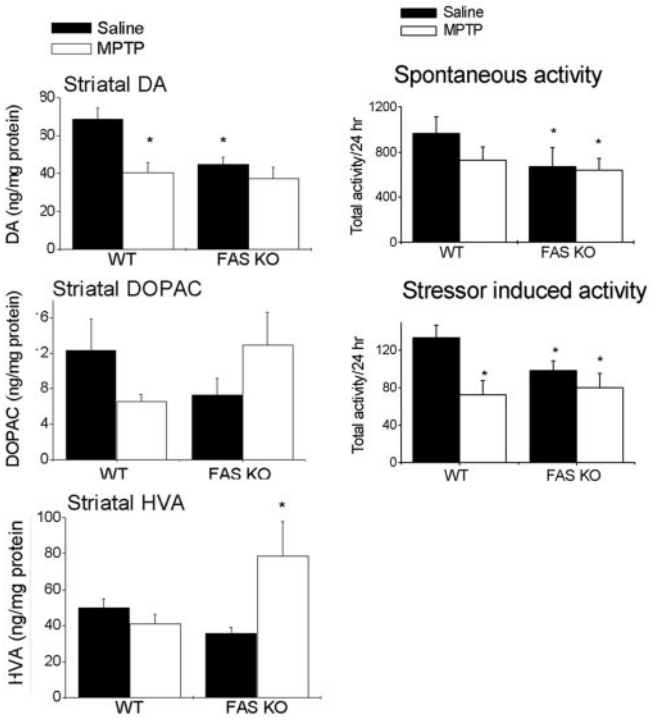

Figure 6. Neurochemical and behavioral effects of MPTP are modified in Fas-deficient mice. All measurements were performed 14 d after the initial MPTP or saline injection. The left panels depict striatal DA (top), DOPAC (middle), and HVA (bottom) concentrations of wild-type (WT) and Fas null (FAS KO) mice that received saline (black bars) or MPTP (white bars). The right panels display home-cage activity in WT and FAS KO mice exposed to the MPTP or saline regimen. Both spontaneous (basal; top) and stressor-induced (bottom) activity levels were monitored for a 24 hr period. *$p < 0.05$ relative to saline-treated WT mice. Error bars represent SEM.

demonstrated that MPTP increased labeling for ΔFosB within the striatum of wild-type and Fas null mice (Fig. 5). The lack of an effect of Fas deficiency on expression of the transcription factor is in keeping with the lack of protection of distal dopaminergic striatal fibers.

Because terminals were not preserved by Fas deficiency, we were interested in examining whether microglial activation in the striatum would be attenuated after MPTP treatment. Consistent with the lack of terminal preservation in Fas-deficient mice, MPTP increased cd11b labeling within the striatum of both wild-type and Fas-deficient animals 14 d after the initial MPTP injection (Fig. 4). Together, it appears that although Fas deficiency protects dopaminergic soma in the SNc, denervation of striatal dopaminergic fibers, glial reactions, and postsynaptic activation still occurs in Fas-deficient mice treated with MPTP.

Effect of Fas deletion on striatal DA levels

To further evaluate the impact of Fas deletion on DA neuronal functioning, we examined the effects of Fas deletion on striatal levels of DA and its metabolites DOPAC and HVA using HPLC. In agreement with previous reports, at 14 d after initiation of MPTP treatment, a profound reduction in DA and DOPAC levels was evident within the striatum of wild-type mice relative to those treated with saline ($p < 0.005$). Interestingly, as shown in Figure 6, striatal DA levels of Fas-deficient mice that received either the saline or MPTP treatments were not significantly different. However, levels of DA in saline-treated Fas-deficient mice were already significantly reduced relative to saline-treated wild-type animals. Fas-deficient mice also displayed a trend (albeit not significant) toward reduced basal striatal levels of DOPAC and HVA. Moreover, striatal DOPAC and HVA levels were elevated in Fas null mice in response to MPTP compared with their saline-injected counterparts ($p < 0.05$) (Fig. 6), suggesting that DA turnover is increased in Fas-deficient animals in response to MPTP. Thus, Fas null mice displayed enhanced dopaminergic utilization in the absence of any decline of DA levels in response to MPTP.

Behavioral variations of MPTP-treated Fas and wild-type mice

To ascertain any functional implications of the observed neurochemical changes, behavioral responses were evaluated after the MPTP regimen. Numerous studies, including our own, have reported that MPTP reduces locomotor activity (Crocker et al., 2003). Accordingly, 14 d after MPTP injection, wild-type mice showed a modest (20%) reduction of home-cage locomotor behavior (24 hr in the beam-break apparatus that served as the home cage) relative to saline-treated animals. Interestingly, exposure to the brief mild swim stressor greatly exacerbated the behavioral deficit such that MPTP-treated mice now displayed almost a 50% reduction of activity during a 2 hr period after the stressor. Paralleling the DA changes within the striatum, activity levels of Fas-deficient mice treated with MPTP did not differ from those that received saline under basal or stressor conditions (Fig. 6). However, again consistent with DA levels, activity of saline-treated Fas-deficient mice was significantly reduced when compared with saline-treated wild-type animals. Together, the monoamine and behavioral analyses indicate that Fas-deficient mice display a basal reduction of DA and related activity levels. Moreover, these animals also appear to display a compensatory response to MPTP, thereby preventing additional reduction of DA and locomotor behavior.

Discussion

Fas requirement in death of dopaminergic cell bodies in the SNc

We found that MPTP provoked a time-dependent elevation of Fas expression, which plays a role in modulating dopaminergic neurodegeneration. Mice lacking the Fas gene were protected to a significant degree from loss of SNc dopaminergic soma associated with MPTP treatment. Although altered expression of the Fas signaling system has been associated with PD (Nagatsu et al., 2000; Hartmann et al., 2002), the present investigation represents the first report that disruption of the Fas–FasL signaling system may be neuroprotective in an animal model of dopaminergic neurodegeneration.

The manner through which Fas participates in MPTP-induced dopaminergic death may involve several different nonmutually exclusive mechanisms. Upregulation of the Fas pathway may directly activate apoptotic signals through the Fas–death complex. Evidence in favor of apoptotic consequences of MPTP is apparent in several studies reporting apoptotic-like profiles, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling, and induction of p53, Bax, caspase-3, and caspase-8 (Jackson-Lewis et al., 1995; Hartmann et al., 2001; Vila et al., 2001; Nicotra et al., 2002). Although some studies did not detect apoptotic factors within PD postmortem tissue (Banati et al., 1998; Jellinger, 2002), others demonstrated apoptotic nuclei using sensitive confocal and electron microscopy (Anglade et al., 1997; Tatton et al., 1998).

Fas may also modulate cellular death through proinflammatory or anti-inflammatory factors, depending on the state of the

local microenvironment. In fact, Fas has proinflammatory actions attributable to the release of chemoattractant chemokines, including interleukin-8 (IL-8) and proinflammatory cytokines, including IL-1β, IL-6, and TNF-α from astrocytes, monocytes, and macrophages (Miwa et al., 1998; Choi et al., 2001, 2002; Hohlbaum et al., 2001; Park et al., 2003). In addition, cross talk between intracellular Fas and TNF-α receptor components (e.g., FADD, TNF receptor-associated death domain, receptor-interacting protein, and TNF receptor-associated factor-2) (Wallach et al., 1996) has been proposed to provoke inflammatory cascades. Consistent with Fas contributing to degeneration through inflammatory processes, we observed a marked increase in microglial activation within the SNc and striatum after MPTP treatment that was reduced within the SNc but not the striatum of Fas-deficient animals. It is difficult to distinguish whether this reduction in gliosis is a cause or a consequence of the limited neuronal loss observed in Fas-deficient animals after MPTP treatment. Because Fas deletion did not reduce microglial activation within the striatum, it is possible that distinct microglial populations (e.g., SNc vs striatum) may respond differently to the effects of MPTP lesioning. Surprisingly, GFAP but not cd11b labeling was colocalized modestly on Fas-expressing SNc cells. Thus, MPTP-induced microglial activation may occur through mechanisms independent of Fas. It remains to be determined whether Fas expression on SNc astrocytes contributes to neurodegeneration.

Regulation of Fas induction

Although c-Jun and its upstream regulators [e.g., cdc42 (rho family GTPase), MKK4 (mitogen-activated protein kinase kinase 4), and JNKs] are important in various models of neuronal death, their exact mechanisms are unclear. One proposed mechanism of c-Jun action has been through the upregulation of Fas. For instance, c-Jun has been shown to be a potent transcriptional activator of Fas (Nagatsu et al., 2000), and Fas ligand synthesis may be regulated by a novel AP-1 (c-Jun–c-Fos complex) site in the FasL promoter (Nicotra and Parvez, 2002). Importantly, the JNK–c-Jun–Fas pathway has been implicated in ischemia-induced and β-amyloid-induced neuronal death (Herdegen et al., 1998; Martin-Villalba et al., 1999; Morishima et al., 2001). However, a clear functional link between c-Jun, Fas–FasL, and neuronal death has not been established previously using *in vivo* models of neurodegeneration. The present results indicate that a dominant-negative form of c-Jun was protective against neuronal death induced by MPTP exposure, a finding consistent with reports that c-Jun and JNKs are involved in other models of dopaminergic degeneration (Crocker et al., 2001, 2003; Xia et al., 2001). Our present findings that inhibition of c-Jun attenuates MPTP-induced Fas induction and that Fas deficiency promotes survival provide compelling evidence for involvement of the c-Jun–Fas pathway in dopaminergic loss.

Functional effects of Fas depletion

In contrast to the protective effects observed in the SNc, Fas deficiency had little effect on dopaminergic terminals within the striatum. Indeed, Fas-deficient animals treated with MPTP showed only a modest attenuation of striatal fiber loss coupled with a similar induction of postsynaptic markers and microglial activation. Consistent with previous reports indicating that SNc protection does not always translate into fiber terminal preservation (Przedborski et al., 1996; Eberhardt and Schulz, 2003), the present data suggest that Fas deficiency does not substantially protect striatal fibers from MPTP-induced loss. However, the possibility should be considered that TH staining may have been reduced in the striatum, and that terminals were indeed preserved in Fas null mice. Although our cresyl violet staining ruled out the possibility of a phenotypic suppression of TH within the SNc, such a conclusion cannot be applied to the striatum. Nevertheless, it is interesting that Fas deletion certainly preserved TH expression within the SNc without any such effect at the striatum.

The neurochemical determinations revealed a reduction of basal striatal DA and DOPAC levels in Fas-deficient animals, suggesting a role for Fas in the normal functioning of the basal ganglia dopaminergic system. Correspondingly, reduced basal behavioral activity (either resting or stimulated home cage) was also evident in Fas-deficient animals. This finding is consistent with a previous report indicating reduced activity in Fas-reduced MRL–*lpr* mice (Sakic et al., 1992). In contrast, untreated Fas-deficient mice did not show any difference in the number of dopaminergic cell bodies or striatal TH fiber density relative to wild-type animals. Thus, it appears that Fas deficiency results in a functional defect of dopaminergic activity in the absence of any drastic changes in nigrostriatal architecture. Although it is possible that the Fas mutation influenced the development or morphology of alternate brain regions important for dopaminergic functioning, to our knowledge, no such evidence exists, and in the present investigation, we did not observe any morphological variations in regions near the SNc or striatum. However, MRL–*lpr* (Fas-reduced strain) mice displayed disturbed hippocampal morphology and cognitive functioning (Petitto et al., 2002).

In response to MPTP, a dramatic reduction of striatal TH fiber density as well as DA and DOPAC levels was coupled with attenuated locomotor behavior in wild-type mice. However, no significant decrease in striatal DA levels was observed in MPTP-treated Fas-deficient animals. In addition, MPTP increased DOPAC and HVA metabolite levels and the DOPAC/DA ratio in Fas-deficient mice, suggesting increased DA turnover. Such enhanced dopaminergic turnover may indicate that compensatory responses are being engendered in Fas null mice. Indeed, others have suggested that as a result of the extensive plasticity of brain monoaminergic systems, extensive loss of dopaminergic neurons may provoke increased synthesis and release of dopamine from the remaining cells (Zigmond et al., 1990). The surviving dopaminergic terminals may be working at a faster rate, leading to increased metabolite accumulation in the absence of any loss of the parent amine dopamine. Such compensatory systems undoubtedly function to attempt to maintain stable dopamine stores. Alternatively, the lack of germ-line Fas may have served to activate an alternate transmitter system that impacts on nigrostriatal circuitry after challenge. Consistent with these observations, basal and stressor-stimulated locomotor activity did not decrease with MPTP treatment in Fas-deficient animals, and in fact, stressor-induced locomotor activity of MPTP-treated Fas null mice was even greater than that of their wild-type counterparts.

Although the nature of the potential compensatory response(s) is not clear, several alternatives may be suggested. First, surviving terminals may increase DA turnover, limiting behavioral deficits associated with MPTP treatment, as observed in the Fas null mice of the present investigation. Indeed, preserved SNc soma may augment DA activity by increasing the rate of synthesis or metabolism at the striatal terminals. An additional compensatory mechanism involves the potential impact of SNc preservation on basal ganglia function independent of striatal DA, possibly involving the substantia nigra reticulata or subthalamic nucleus. For instance, our own work indicated that preservation of the SNc, through inhibition of calpains, attenuated behavioral

deficits without recovering striatal dopamine levels (Crocker et al., 2003). Additionally, others reported that direct SNc administration of glial cell line-derived neurotrophic factor-modulated behavioral deficits after 6-OHDA without affecting striatal dopamine levels (Choi-Lundberg et al., 1998). These observations are significant, because it is now recognized that nigral dopaminergic neurons release dopamine not only from their axons projecting to the striatum but also from their dendrites (Bjorklund and Lindvall, 1975; Cheramy et al., 1981). We hypothesize that preservation of the SNc by Fas deficiency may contribute to improved functional outcomes through stimulation of DA turnover in the striatum and extrastriatal regulation of the basal ganglia.

In conclusion, the present findings support a required role for c-Jun-mediated induction of Fas in neurodegeneration and impairment of dopaminergic functions in a model of Parkinson's disease. In addition, it appears that selective preservation of soma may trigger compensatory dopaminergic responses at the striatum or alternate regions. This is significant, because motor dysfunction in PD is likely regulated through an array of abnormal basal ganglia circuits and not just a simple consequence of striatal dopamine loss.

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