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Minocycline attenuates microglial activation but fails to mitigate striatal dopaminergic neurotoxicity: role of tumor necrosis factor-α

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Abstract

Activated microglia are implicated in the pathogenesis of disease-, trauma- and toxicant-induced damage to the CNS, and strategies to modulate microglial activation are gaining impetus. A novel action of the tetracycline derivative minocycline is the ability to inhibit inflammation and free radical formation, factors that influence microglial activation. Minocycline is therefore being tested as a neuroprotective agent to alleviate CNS damage, although findings so far have yielded mixed results. Here, we showed that administration of a single low dose of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or methamphetamine (METH), a paradigm that causes selective degeneration of striatal dopaminergic nerve terminals without affecting the cell body in substantia nigra, increased the expression of mRNAs encoding microglia-associated factors F4/80, interleukin (IL)-1α, IL-6, monocyte chemoattractant protein-1 (MCP-1, CCL2) and tumor necrosis factor (TNF)-α. Minocycline treatment attenuated MPTP- or METH-mediated microglial activation, but failed to afford neuroprotection. Lack of neuroprotection was shown to be due to the inability of minocycline to abolish the induction of TNF-α and its receptors, thereby failing to modulate TNF signaling. Thus, TNF-α appeared to be an obligatory component of dopaminergic neurotoxicity. To address this possibility, we examined the effects of MPTP or METH in mice lacking genes encoding IL-6, CCL2 or TNF receptor (TNFR)1/2. Deficiency of either IL-6 or CCL2 did not alter MPTP neurotoxicity. However, deficiency of both TNFRs protected against the dopaminergic neurotoxicity of MPTP. Taken together, our findings suggest that attenuation of microglial activation is insufficient to modulate neurotoxicity as transient activation of microglia may suffice to initiate neurodegeneration. These findings support the hypothesis that TNF-α may play a role in the selective vulnerability of the nigrostriatal pathway associated with dopaminergic neurotoxicity and perhaps Parkinson's disease.

Keywords: brain, microglia, minocycline, neurodegeneration, neuroprotection, Parkinson's disease, tumor necrosis factor.
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Any insult to the CNS engenders 'reactive gliosis', a cellular response characterized by activation of microglia and astroglia at the sites of injury (O'Callaghan 1993; Kreutzberg 1996; Streit *et al*. 1999; Norenberg 2005; Sriram and O'Callaghan 2005). Microglial activation occurs very early in the injury process and activated microglia exhibit morphological, immunological, molecular and functional changes in response to a given injury. Microglial activation occurs in association with various types of brain injury (Kato *et al*. 2000), including damage associated with neurological disease states (De Simone *et al*. 1995; Banati *et al*. 1998; Versijpt *et al*. 2003), as well as with the damage caused by neurotoxic chemicals often used to model disease (Jorgensen *et al*. 1993; Scali *et al*. 1999; Fiedorowicz *et al*. 2001). For example, microglial activation is associated with dopaminergic neurodegeneration seen in Parkinson's disease (PD) and in rodent models of the disease (Francis *et al*. 1995; Czlonkowska *et al*. 1996; Langston *et al*. 1999).

Although predominantly viewed as scavenger cells, microglia also are known to initiate tissue repair and regeneration through secretion of growth and neurotrophic

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Abbreviations: CCL2, monocyte chemoattractant protein-1; COX2, cyclo-oxygenase-2; DOPAC, dihydroxyphenylacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HVA, homovanillic acid; IL, interleukin; METH, methamphetamine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NOS2, (iNOS), inducible nitric oxide synthase; PD, Parkinson's disease; SDS, sodium dodecyl sulfate; TH, tyrosine hydroxylase; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor.

factors, thereby exerting a beneficial/neuroprotective role (Kreutzberg 1996; Gonzalez-Scarano and Baltuch 1999; Stoll and Jander 1999; Streit *et al*. 1999). On the other hand, activation of microglia may also initiate inflammation and exacerbate degeneration owing to release of cytotoxic products, such as reactive oxygen and nitrogen species, pro-inflammatory cytokines and proteases (Colton and Gilbert 1987; Boje and Arora 1992). Thus, microglial activation may exert neurotrophic and/or cytotoxic effects, observations indicative of a dual role for this cell type in response to neural injury. The conditions dictating whether microglia exhibit a beneficial or deleterious role are not completely understood. Nevertheless, certain features that seem to influence their role include the density, distribution and the morphological or functional state of these cells across various brain regions (Lawson *et al*. 1990; Ren *et al*. 1999).

Given the above considerations, interventions designed to modulate microglial responses could be viewed as a means of achieving neuroprotection against diverse injuries of the CNS. To address this possibility, several studies have attempted to use candidate drugs, including antioxidants and anti-inflammatory agents, to demonstrate that inhibition of microglial activation can afford protection against neuronal injuries (Bruce-Keller *et al*. 2000; Li *et al*. 2001; Asanuma *et al*. 2003; Liu *et al*. 2003; Yan *et al*. 2003; Zawadzka and Kaminska 2005). Among the compounds investigated is the antimicrobial agent minocycline, a broad-spectrum semisynthetic tetracycline derivative with anti-inflammatory properties that are distinct from its antimicrobial actions (Yrjanheikki *et al*. 1999; Wu *et al*. 2002).

Minocycline has been shown to afford protection against brain ischemia (Yrjanheikki *et al*. 1998, 1999), excitotoxicity (Tikka and Koistinaho 2001; Tikka *et al*. 2001), β-amyloid neurotoxicity (Ryu *et al*. 2004) and spinal cord injury (Stirling *et al*. 2004), and to delay disease onset in a murine model of amyotrophic lateral sclerosis (Kriz *et al*. 2002; Van Den Bosch *et al*. 2002). Similarly, it has been reported to be neuroprotective against dopaminergic neurotoxicity caused by 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Du *et al*. 2001; He *et al*. 2001; Wu *et al*. 2002). The neuroprotection afforded by minocycline is thought to be associated with its ability to inhibit microglial activation, thereby reducing the levels of cytotoxic factors released by microglia. However, recent studies have demonstrated that minocycline treatment worsened or exacerbated brain injury (Yang *et al*. 2003; Diguet *et al*. 2004; Tsuji *et al*. 2004), indicating that minocycline may have actions that depend on the specific brain injury in question. The possible causes for such differential effects exhibited by minocycline remain unclear. In this study, we evaluated the effects of minocycline on the neurotoxicity of two dopaminergic neurotoxicants, MPTP and methamphetamine (METH). We show that, although minocycline treatment attenuates microglial activation, it fails to afford protection against dopaminergic neurotoxicity. Furthermore, we present evidence to demonstrate that this lack of neuroprotection results from the inability of minocycline to completely abolish the induction of tumor necrosis factor (TNF)-α and its receptors (TNFRs), thereby failing to modulate TNF signaling.

Materials and methods

Chemicals and reagents

MPTP-HCl was obtained from Aldrich (Milwaukee, WI, USA). (+)METH-HCl and minocycline-HCl were purchased from Sigma (St Louis, MO, USA). Mouse anti-rat tyrosine hydroxylase (TH) monoclonal antibody and rabbit anti-rat TH polyclonal antibody were from Calbiochem-Novabiochem Corporation (San Diego, CA, USA). Mouse anti-glial fibrillary acidic protein (GFAP) monoclonal antibody was obtained from Chemicon International, Inc. (Temecula, CA, USA). Rabbit anti-cow GFAP polyclonal antibody was obtained from Dako Corporation (Carpenteria, CA, USA). Horseradish peroxidase-conjugated anti-rabbit IgG and enhanced chemiluminescence immunoblotting substrate were purchased from Amersham Biosciences (Piscataway, NJ, USA). Alkaline phosphatase-conjugated anti-mouse IgG was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). *p*-Nitrophenyl phosphate, was from Bio-Rad (Hercules, CA, USA). Horseradish peroxidase-conjugated anti-mouse IgG and the fluorogenic peroxidase substrate Quantablu were from Pierce (Rockford, IL, USA). Superscript™ II RNase H^- reverse transcriptase and oligo $(dT)_{12-18}$ primers were from Invitrogen (Carlsbad, CA, USA). TaqMan® Universal PCR master mix, MicroAmp 96-well plates and optical caps for real-time PCR analysis were purchased from Applied Biosystems (Foster City, CA, USA). Nitrocellulose membranes (0.1 μm) were purchased from Schleicher & Schuell (Keene, NH, USA). All other chemicals and reagents were of analytical grade and were purchased from Sigma.

Animals

Male mice (4–6 months; 28–35 g) were used in all experiments. Mice carrying homozygous mutant alleles for TNFRs [B6; 129S-*Tnfrsf1a*^{tm1Imx} *Tnfrsf1b*^{tm1Imx}/J; *Tnfr1/2*(–/–)], interleukin (IL)-6 (*Il-6*) gene [B6.129S2-*Il6*^{tm1Kopf}/J; *Il-6*(–/–)] or monocyte chemoattractant protein-1 (*Ccl2*) gene [B6.129S4-*Ccl2*^{tm1Rol}/J; *Ccl2*(–/–)] were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Appropriate strain controls (B6.129S) and C57BL/6J mice were procured from the same source. The animals were housed in a temperature (22 ± 2°C)- and humidity (30–40%)-controlled colony room maintained on a 12-h light–dark cycle. Animals were allowed free access to chow and water. All animal experiments were carried out in accordance with Centers for Disease Control Guidelines for Care and Use of Laboratory Animals. All procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health. The National Institute for Occupational Safety and Health animal facility is accredited by the American Association for Accreditation of Laboratory Animal Care.

Drug treatment and dissections

Solutions of minocycline, MPTP and METH, with dosages calculated as free base, were prepared freshly in 0.9% sodium chloride. Two dosing regimens of minocycline were employed to determine the protective effects, if any, on dopaminergic neurotoxicity. Although minocycline readily crosses the blood–brain barrier (Aronson 1980), brain levels of the compound required to afford neuroprotection have not been fully evaluated. We therefore used a low-dose regimen that approximated a clinical dose of minocycline and, to maximize the possibility of neuroprotection, we also used a high-dose regimen. The latter regimen exceeded the dosage used in previous studies in which neuroprotection was achieved (Wu *et al*. 2002), but it remained far below the known LD_{50} of 3600 mg/kg (Hockly *et al*. 2003). In the low-dose regimen (10 mg/kg, s.c.), minocycline was administered at 24-h intervals commencing 2 days before MPTP or METH treatment and continued at 24-h intervals until death. In the high-dose regimen (100 mg/kg, s.c.), mice received minocycline 12 h before, during and 12 h after MPTP or METH treatment. Appropriate groups of mice then were given either vehicle (0.9% saline) or a single dose of MPTP (12.5 mg/kg, s.c.) or METH (20 mg/kg, s.c.) and killed by decapitation at 12 or 72 h thereafter. The low-dose MPTP regimen employed limits the damage and subsequent glial response to the striatum by causing degeneration of a portion of the dopaminergic terminals without affecting the cell body in the substantia nigra (Sriram *et al*. 2004). The METH dosing regimen used also limits the damage to dopaminergic terminals as shown by the fact that a higher-dose regimen still spares the substantia nigra from damage (O'Callaghan and Miller 1994). Rectal temperature was taken at 12 and 18 h before administration of minocycline, and 12 and 24 h after administration of METH. Temperature was then taken at 1, 2, 4, 8, 12 and 24 h after METH and minocycline alone or in combination. The striata and substantia nigra from both hemispheres was dissected by hand and used for estimation of dopamine and metabolites, isolation of total RNA or analysis of specific proteins.

RNA isolation, cDNA synthesis and real-time PCR amplification

Total RNA from the striatum or substantia nigra was isolated using Trizol® reagent (Invitrogen) and Phase-lock heavy gel (Eppendorf AG, Hamburg, Germany). The RNA was subsequently cleaned using a RNeasy mini spin column (Qiagen, Valencia, CA, USA). Total RNA (1 μg) was reverse transcribed to cDNA using SuperScript™ II RNase H^- and oligo $(dT)_{12-18}$ primers (Invitrogen) in a 20-μL reaction. Real-time PCR analysis of *Gapdh*, *Gfap*, vimentin, *F4/80*, *Il-1α*, *Il-6*, *Ccl2*, *Cox-2*, *Nos-1*, *Nos-2*, *Tnf-α*, *Tnfr1* and *Tnfr2* mRNAs was performed in an ABI PRISM 7700 sequence detection system (Applied Biosystems) in combination with TaqMan® chemistry. Specific primers and dual-labeled internal fluorogenic [carboxyfluorescein/carboxytetramethylrhodamine (FAM/TAMRA)] probe sets (TaqMan® Gene Expression Assays) for these genes were from Applied Biosystems and used according to the manufacturer's recommendation. All PCR amplifications (40 cycles) were performed in a total volume of 50 μL, containing 1 μL cDNA, 2.5 μL specific primer/probe mix and 25 μL TaqMan® Universal master mix (Applied Biosystems). Sequence detection software (version 1.7; Applied Biosystems) results were exported as tab-delimited text files and imported into Excel (Microsoft, Redmond, WA, USA) for further analysis. Relative quantification of gene expression was performed using the comparative threshold (C_T) method as described by the manufacturer (Applied Biosystems). Changes in mRNA expression level were calculated following normalization to *Gapdh* mRNA. The ratios obtained following normalization are expressed as fold change over corresponding saline-treated controls.

Tissue preparation for total and specific protein analysis

Tissues for protein analysis were homogenized in 10 volumes of hot (85–95°C) 1% sodium dodecyl sulfate (SDS) and stored at −75°C until use. Total protein was determined by the bicinchoninic acid method (Smith *et al*. 1985), with bovine serum albumin as standard.

GFAP and TH assays

GFAP was assayed in accordance with a previously described procedure (O'Callaghan 1991; O'Callaghan 2002). In brief, a rabbit polyclonal antibody to GFAP was coated on the wells of Immulon-2 microtiter plates (Thermo Labsystems, Franklin, MA, USA). The SDS homogenates and standards were diluted in phosphate-buffered saline (pH 7.4) containing 0.5% Triton X-100. After blocking non-specific binding with 5% non-fat dry milk, aliquots of the homogenate and standards were added to the wells and incubated. Following washes, a mouse monoclonal antibody to GFAP was added to 'sandwich' the GFAP between the two antibodies. An alkaline phosphatase-conjugated antibody directed against mouse IgG was then added and a colored reaction product was obtained by subsequent addition of the enzyme substrate *p*-nitrophenol. Quantification was achieved by spectrophotometry of the colored reaction product at 405 nm in a microplate reader, Spectra Max Plus and analyzed with Soft Max Pro Plus software (Molecular Devices, Sunnyvale, CA, USA). The amount of GFAP in the samples was calculated as micrograms GFAP per milligram total protein.

TH holoenzyme protein was assessed by a fluorescence-based ELISA developed in the laboratory (J. P. O'Callaghan, unpublished). The protocol was essentially similar to that for the GFAP assay except that a mouse monoclonal antibody to TH was used as the plate capture antibody and a rabbit polyclonal antibody was used to 'sandwich' TH protein. The amount of sandwich antibody bound to TH was then detected using a peroxidase-labeled antibody directed against rabbit IgG. Peroxidase activity was detected using the fluorogenic substrate Quantablu (Pierce), which has excitation and emission maxima of 325 and 420 nm respectively (read at 320/405 nm). The amount of TH in the samples was calculated and expressed as micrograms TH per milligram total protein.

HPLC determination of dopamine and its metabolites

Dopamine and its metabolites were quantified by high-performance liquid chromatography with electrochemical detection (HPLC-EC; Waters, Milford, MA, USA). Tissues were homogenized in 300 μL ice-cold 0.2 M perchloric acid, containing 1 μM dihydroxybenzylamine as internal standard, and centrifuged at 10 000 *g* for 10 min at 4°C. The supernatant was filtered through a 0.2-μm membrane and an aliquot (10 μL) was injected from a temperature-controlled (4°C) automatic sample injector (Waters 717plus Autosampler) connected to a Waters 515 HPLC pump. Catecholamines were separated on a C18 reverse-phase column (LC-18 RP; Waters SYMMETRY, 25 cm × 4.6 mm; 5 μm), electrochemically detected (Waters 464 Pulsed Electrochemical Detector; range 10 nA,

potential + 0.7 V) and analyzed using Millennium software (Waters, Milford, MA, USA). The mobile phase (pH 3.0) for isocratic separation of dopamine consisted of dibasic sodium phosphate (75 mM), octane sulfonic acid (1.7 mM), acetonitrile (10% v/v) and EDTA (25 μm). Flow rate was maintained at 1 mL/min. Dopamine, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) standards (0.5–25 pmol) were prepared in 0.2 M perchloric acid-containing dihydroxybenzylamine. Recovery of each analyte was adjusted with respect to the internal standard and quantified from a standard curve. The levels of dopamine and its metabolites were expressed as micrograms per gram of wet tissue.

Statistical analysis

All analyses were performed using SigmaStat 3.1 (Systat Software Inc., Point Richmond, CA, USA) statistical software. Significance was tested using two-way ANOVA and *post hoc* group comparisons were made by applying the Student–Newman–Keuls test. Differences were considered statistically significant at the 5% level ($p < 0.05$). Mean ± SEM values are presented in graphs.

Results

Minocycline fails to protect against MPTP- or METH-mediated loss of striatal TH and dopamine

The single-dose MPTP or METH regimen employed in this study caused a significant decrease in striatal TH protein (Fig. 1), consistent with a loss of dopaminergic nerve terminals known to be caused by these agents (O'Callaghan *et al*. 1990, 1998; O'Callaghan and Miller 1994; Sriram *et al*. 2002, 2004). By 72 h after dosing, striatal TH protein levels had decreased by 60–70% ($p < 0.01$) in mice administered MPTP alone (Figs 1a and c). Similarly, a significant decrease in striatal TH protein (25–35%; $p < 0.01$) was observed within 12 h in mice given METH alone (Figs 1b and d). The TH levels further decreased (45–50%; $p < 0.01$) by 72 h of METH treatment (Figs 1b and d). Neither the low- nor high-dose minocycline treatment altered the striatal TH loss caused by MPTP (Fig. 1a and c) or METH (Figs 1b and d). The decreases in striatal TH levels were more or less comparable to those in treatment groups that did not receive minocycline. Furthermore, the loss of striatal dopamine caused by MPTP or METH was not altered by even the high-dose minocycline regimen (Fig. 2). MPTP and METH alone decreased striatal dopamine levels by 67% ($p < 0.001$) and 68% ($p < 0.01$) by 72 h after dosing (Fig. 2a). Correspondingly, the loss of striatal dopamine caused by MPTP or METH in the groups treated with high-dose minocycline was 63% ($p < 0.01$) and 60% ($p < 0.01$) respectively (Fig. 2a), findings consistent with a lack of protection. In contrast to the effects of a multiple-dose METH regimen (O'Callaghan and Miller 1994; Miller and

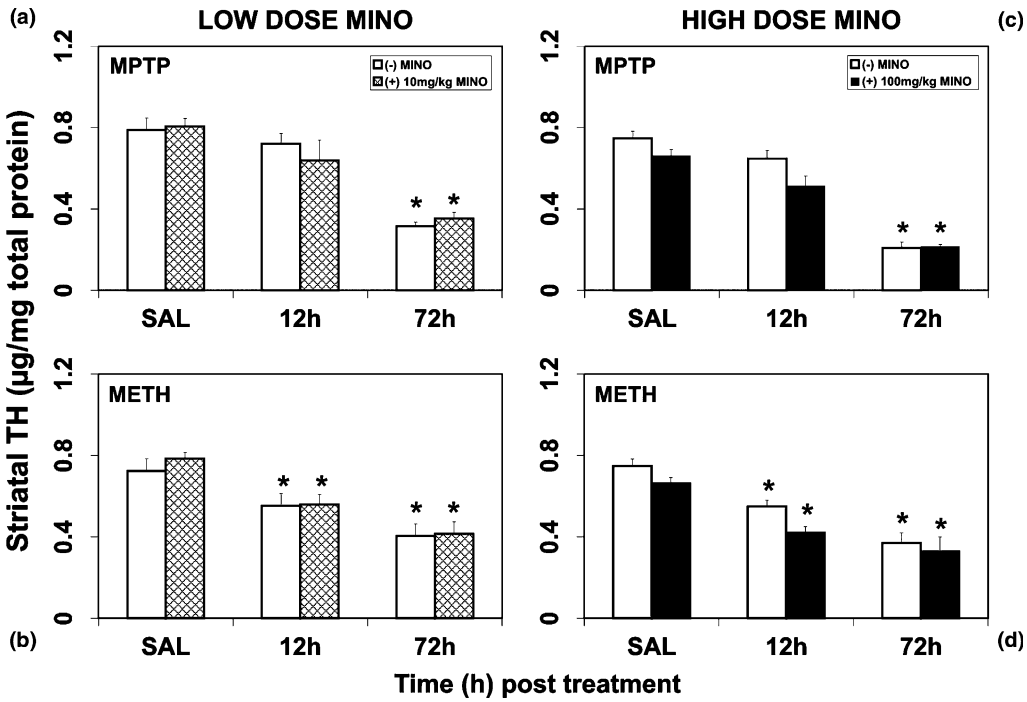

Fig. 1 Minocycline fails to protect against striatal TH loss caused by MPTP or METH. Male C57BL/6J mice were administered minocycline (MINO) according to the scheme described in the methods. Appropriate groups of mice were then administered a single dose of either saline (SAL), MPTP (12.5 mg/kg, s.c.) or METH (20 mg/kg, s.c.) and the animals were killed 12 or 72 h later. Effect of (a, b) low-dose and (c, d) high-dose minocycline treatment on striatal TH protein loss mediated by MPTP or METH. Values are mean ± SEM ($n = 5$ animals per group). *$p < 0.01$ versus saline-treated controls. Newman–Keuls pairwise comparisons were used for *post hoc* statistical analysis.

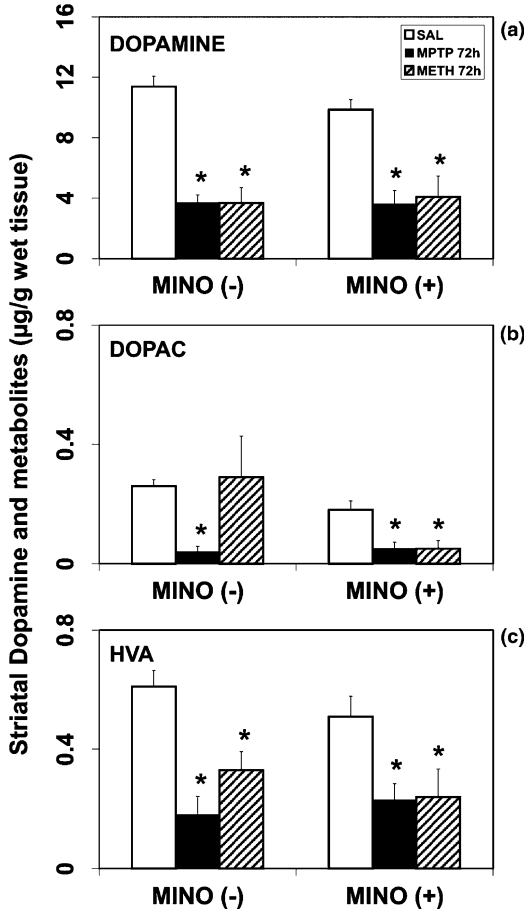

Fig. 2 Minocycline fails to protect against striatal dopamine loss caused by MPTP or METH. Male C57BL/6J mice were administered a high dose of minocycline (MINO). Appropriate groups of mice were then administered a single dose of either saline (SAL), MPTP (12.5 mg/kg, s.c.) or METH (20 mg/kg, s.c.) and the animals were killed 72 h later. Levels of (a) dopamine, (b) DOPAC and (c) HVA were measured in the striatum. Values are mean ± SEM ($n = 5$ animals per group). *$p < 0.001$ versus saline-treated controls. Newman–Keuls pairwise comparisons were used for *post hoc* statistical analysis.

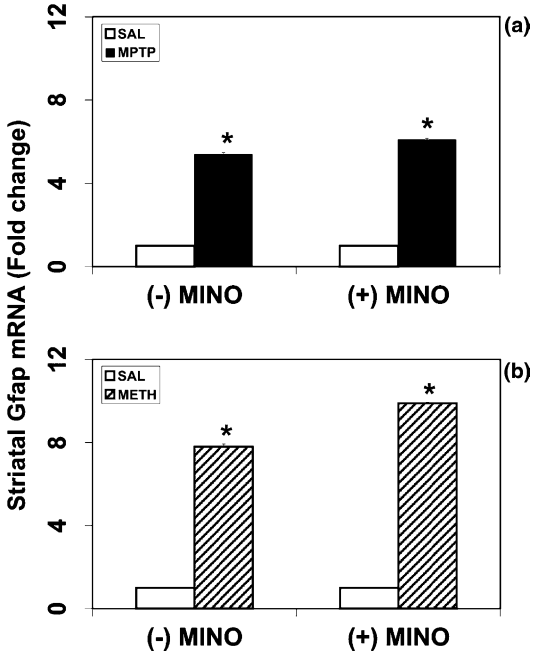

Fig. 3 Minocycline does not alter astroglial responses associated with MPTP or METH neurotoxicity: analysis of *Gfap* mRNA. Male C57BL/6J mice were administered a high dose of minocycline (MINO). Appropriate groups of mice were then administered a single dose of either saline (SAL), MPTP (12.5 mg/kg, s.c.) or METH (20 mg/kg, s.c.) and the animals were killed 12 h later. (a) Levels of striatal *Gfap* mRNA in (–)MINO and (+)MINO groups treated with MPTP. (b) Levels of striatal *Gfap* mRNA in groups treated with METH. *Gfap* mRNA levels are expressed as fold change over corresponding saline-treated control values. Values are mean ± SEM ($n = 5$ animals per group). *$p < 0.01$ versus saline-treated controls. Newman–Keuls pairwise comparisons were used for *post hoc* statistical analysis.

O'Callaghan 1994), the single-dose regimen used in the present study did not affect core temperature, nor was core temperature affected by minocycline when administered in combination with METH (data not shown).

Minocycline fails to alter astroglial responses associated with striatal dopaminergic neurotoxicity due to MPTP or METH

Chemically induced damage to the CNS, including damage caused by MPTP or METH (Miller and O'Callaghan 1994; O'Callaghan and Miller 1994; Sriram *et al*. 2002, 2004), results in astrogliosis at the site of damage, the hallmark of which is an increase in GFAP. Consistent with these previous observations, the single-dose regimen of MPTP or METH caused significant up-regulation of striatal *Gfap* mRNA, as measured by TaqMan® real-time PCR (Fig. 3). By 12 h after dosing with MPTP or METH, striatal *Gfap* mRNA levels had increased 4.4-fold ($p < 0.01$) and 6.8-fold ($p < 0.01$) respectively (Figs 3a and b). The high-dose minocycline treatment did not significantly alter the up-regulation of striatal *Gfap* mRNA levels induced by either MPTP or METH (Fig. 3a and b). Consistent with the increases in *Gfap* mRNA, MPTP or METH treatment caused significant increases in striatal GFAP protein. Within 72 h of treatment with MPTP alone, levels of striatal GFAP protein content had increased 3–5.5-fold ($p < 0.01$) (Figs 4a and c). Similarly, treatment with METH alone increased striatal GFAP protein content by 4–6-fold ($p < 0.01$; Figs 4b and d). Neither low- nor high-dose minocycline treatment altered MPTP- or METH-mediated induction of striatal GFAP protein (Figs 4a–d). The striatal GFAP protein content in MPTP-treated (Figs 4a and c) or METH-treated (Figs 4b and d) minocycline groups was 3.5–6-fold ($p < 0.01$) over that in respective saline-treated controls, levels comparable to that of MPTP or METH alone groups (Fig. 4).

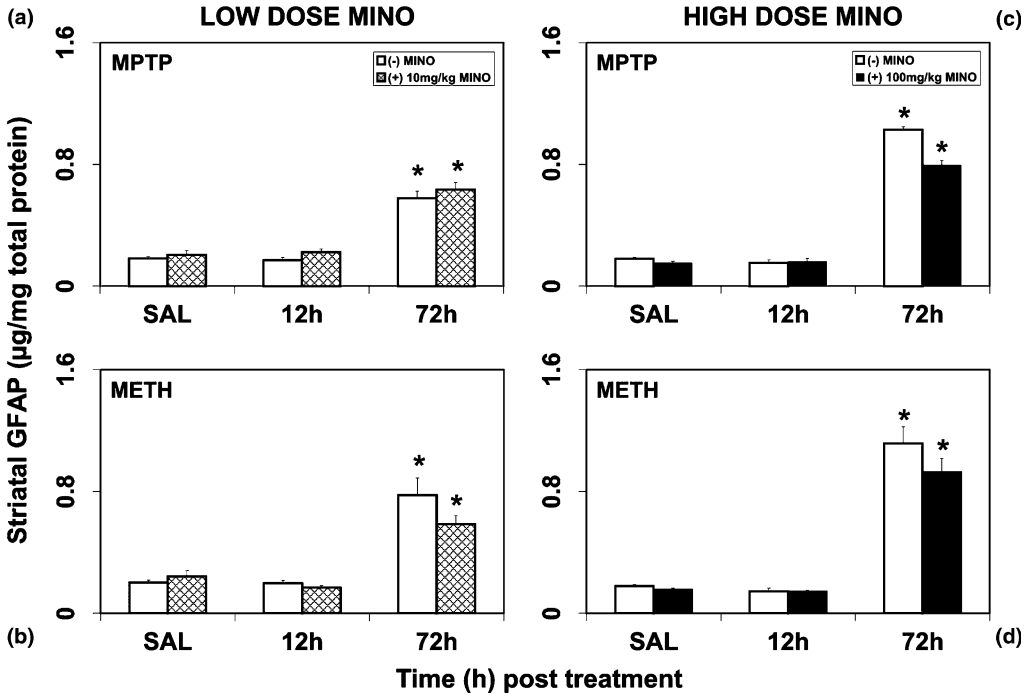

Fig. 4 Minocycline does not alter astroglial responses associated with MPTP or METH neurotoxicity: analysis of GFAP protein. Male C57BL/6J mice were administered either a low or high dose of minocycline (MINO). Appropriate groups of mice were then administered a single dose of either saline (SAL), MPTP (12.5 mg/kg, s.c.) or METH (20 mg/kg, s.c.) and the animals were killed 12 h later. Effect of (a, b) low-dose and (c, d) high-dose minocycline treatment on striatal GFAP protein induction by MPTP or METH. Values are mean ± SEM ($n = 5$ animals per group). *$p < 0.01$ versus saline-treated controls. Newman–Keuls pairwise comparisons were used for *post hoc* statistical analysis.

Minocycline attenuates MPTP- or METH-induced expression of microglial markers

Administration of MPTP or METH resulted in enhanced expression of several markers known to be associated with activated microglia. This included increased striatal expression of mRNAs encoding the microglial marker F4/80, and cytokines or chemokines typically expressed by microglia, such as IL-1α, IL-6, CCL2 (Fig. 5a–h) and TNF-α (Figs 6a–d). The expression of these microglial-associated mRNAs was quantified by TaqMan® real-time PCR analysis. Within 12 h of dosing with MPTP, striatal *F4/80*, *Il-1α*, *Il-6* and *Ccl2* mRNA levels had increased by nearly 2–20-fold (Figs 5a–d). Although minocycline treatment completely abolished MPTP-mediated induction of *F4/80* and *Il-6* mRNA expression (Figs 5a and c) it only attenuated the expression of *Il-1α* or *Ccl2* mRNAs (Figs 5b and d). Similarly, METH treatment caused a 2–30-fold increase in the levels of striatal *F4/80*, *Il-1α*, *Il-6* and *Ccl2* mRNA levels within 12 h of dosing (Figs 5e–h). As with MPTP, minocycline treatment abolished METH-mediated induction of *F4/80* mRNA (Fig. 5e), but it only attenuated the expression of *Il-1α*, *Il-6* and *Ccl2* mRNAs (Fig. 5f–h).

Minocycline attenuates MPTP- or METH-induced up-regulation of striatal TNF-α but fails to modulate the expression of TNFRs

Consistent with the up-regulation of microglial markers noted above, MPTP or METH treatment also induced the expression of the pro-inflammatory cytokine TNF-α, a microglia-derived cytokine known to play a significant role in dopaminergic neurotoxicity (Fig. 6). By 12 h after dosing with MPTP or METH, striatal *Tnf-α* mRNA levels had increased by 40-fold ($p < 0.001$) and 30-fold ($p < 0.01$) respectively (Figs 6a and d). High-dose minocycline treatment attenuated (~7-fold; $p < 0.01$) but did not block MPTP- or METH-mediated induction of *Tnf-α* mRNA (Figs 6a and d). Thus, sufficient TNF-α potentially remained to initiate striatal dopaminergic degeneration. Consistent with this view, MPTP or METH treatment caused a significant increase in striatal levels of mRNAs encoding Tnfr1 and Tnfr2 (Figs 6b, c, e and f). Striatal *Tnfr1* mRNA levels increased by 3–5-fold ($p < 0.05$) within 12 h of dosing with either MPTP (Fig. 6b) or METH (Fig. 6e). Similarly, a small but significant (~2-fold; $p < 0.05$) increase in striatal *Tnfr2* mRNA was observed 12 h after treatment with MPTP or METH (Figs 6c and d).

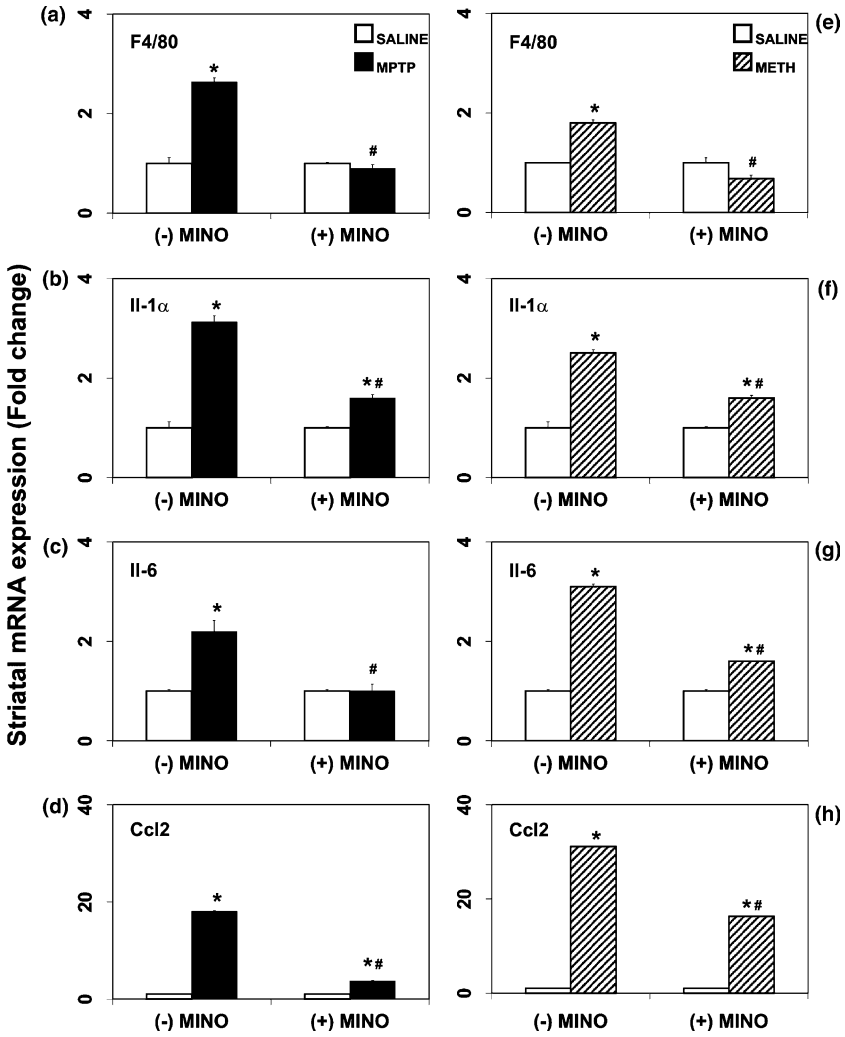

Fig. 5 Minocycline attenuates microglial activation associated with MPTP or METH neurotoxicity. Male C57BL/6J mice were administered a high dose of minocycline (MINO). Appropriate groups of mice were then administered a single dose of either saline, MPTP (12.5 mg/kg, s.c.) or METH (20 mg/kg, s.c.) and the animals were killed 12 h later. (a–d) Levels of mRNA encoding the microglial marker F4/80 and microglia-derived inflammatory factors IL-1α, IL-6 and CCL2 were measured in (–)MINO and (+)MINO groups treated with MPTP. (e–h) mRNA levels in groups treated with METH. The mRNA levels are expressed as fold change over corresponding saline-treated control values. Values are mean ± SEM ($n = 5$ animals per group). *$p < 0.05$–0.01 versus saline-treated controls; #$p < 0.05$ versus (–)MINO group. Newman–Keuls pairwise comparisons were used for *post hoc* statistical analysis.

Neither *Tnfr1* nor *Tnfr2* mRNA content was altered by minocycline treatment (Figs 6b, c, e and f) indicating that available TNF-α would still be capable of mediating its toxic effects in the striatum. The single low dose of MPTP or METH did not alter the expression of several markers of reactive gliosis (microglial and astroglial) in the substantia nigra (Fig. 6g), consistent with our previous observations (O'Callaghan and Miller 1994) of lack of dopaminergic cell body loss with this dosing regimen.

Striatal dopaminergic neurotoxicity due to MPTP can be abolished by modulating the TNF signaling pathway, but not that of IL-6 or CCL2

We previously demonstrated that enhanced expression of TNF-α is associated with the earliest stages of damage in the MPTP model of dopaminergic neurotoxicity and deficiency of TNFRs protects against MPTP-induced neurotoxicity (Sriram *et al.* 2002). As MPTP treatment also causes the induction of other pro-inflammatory cytokines and chemokines, such as IL-6 and CCL2, it was critical to determine the specific cytokine or chemokine that may be associated with dopaminergic neurotoxicity. We therefore evaluated MPTP-mediated striatal dopaminergic neurotoxicity in mice lacking *Il-6*, *Ccl2* or *Tnfr1/2* genes. Administration of MPTP to mice deficient in *Il-6*, *Ccl2* or *Tnfr1/2* genes resulted in significant loss (45–55%; $p < 0.01$) of striatal TH protein by 72 h after dosing (Figs 7a–c) and a corresponding increase (200–350%; $p < 0.01$) in striatal GFAP protein (Figs 7d–f). Deficiency of *Il-6* or *Ccl2* genes did not alter these neurotoxic effects of MPTP. The loss of striatal TH in *Il-6*- or *Ccl2*-deficient mice was 65 and 55% respectively (Figs 7a and b), and the increase in GFAP was 200–250% ($p < 0.01$) (Figs 7d and e), indicating a lack of neuroprotection. However, deficiency of both TNFRs afforded protection against these indicators of MPTP-mediated striatal neurotoxicity (Figs 7c and f). These findings further strengthen our hypothesis that the pro-inflammatory cytokine TNF-α is an obligatory component in dopaminergic neurotoxicity and, perhaps, in the neurodegenerative processes underlying PD.

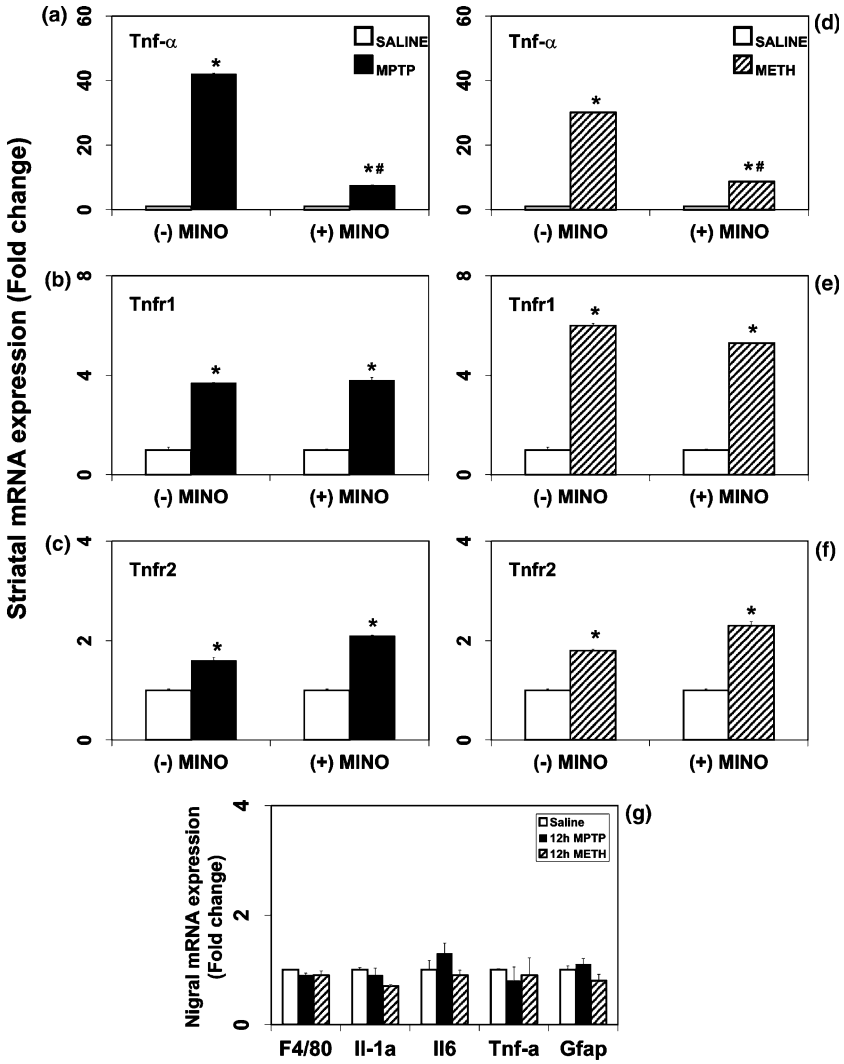

Fig. 6 Minocycline attenuates MPTP- or METH-mediated up-regulation of striatal TNF-α but fails to alter TNFR activation. Male C57BL/6J mice were administered a high dose of minocycline (MINO). Appropriate groups of mice were then administered a single dose of either saline, MPTP (12.5 mg/kg, s.c.) or METH (20 mg/kg, s.c.) and the animals were killed 12 h later. Levels of mRNA encoding Tnf-α and its receptors Tnfr1 and Tnfr2 were measured in (–)MINO and (+)MINO groups treated with (a–c) MPTP and (d–f) METH. (g) Levels of mRNA encoding microglial and astroglial markers in substantia nigra of MPTP- or METH-treated mice. The mRNA levels are expressed as fold change over corresponding saline-treated controls. Values are mean ± SEM ($n = 4$–5 animals per group). *$p < 0.05$–0.01 versus saline-treated controls. #$p < 0.05$ versus (–)MINO group. Newman–Keuls pairwise comparisons were used for *post hoc* statistical analysis.

Striatal dopaminergic neurotoxicity due to MPTP or METH is not associated with induction of cyclooxygenase-2 (COX2) and inducible nitric oxide synthase (NOS2, iNOS)

Enhanced expression of the microglia-associated inflammatory mediators COX2 and NOS2 has specifically been linked to dopaminergic neurotoxicity caused by MPTP and METH (Itzhak *et al*. 1999; Liberatore *et al*. 1999; Thomas and Kuhn 2005). Therefore, we assessed the striatal levels of the mRNA for these mediators in our MPTP and METH dosing models. The single low dose of MPTP or METH did not cause up-regulation of *Cox2*, *Nos1* or *Nos2* mRNAs (data not shown). Furthermore, minocycline alone did not alter the expression of these factors; however, minocycline in the presence of both MPTP and METH appeared to down-regulate *Nos2* expression (data not shown).

Discussion

Microglia and astrocytes play a major role in brain inflammatory responses (Raivich *et al*. 1996; Ransohoff *et al*. 1996). Brain injury-related activation of microglia, in particular, is associated with enhanced expression of cytokines, chemokines and growth factors (De Bock *et al*. 1996; Botchkina *et al*. 1997; Sriram *et al*. 2002; Sriram and O'Callaghan 2005). Many of these factors have been implicated in the pathogenesis of neurodegenerative disorders such as PD (Boka *et al*. 1994; Mogi *et al*. 1996), Alzheimer's disease (Bauer *et al*. 1991), multiple sclerosis (Merrill 1992) and stroke (Sairanen *et al*. 2001). Pharmacological suppression of microglial activation therefore represents a potential intervention strategy to modulate or alleviate a variety of CNS injuries. In the present study, we demonstrated that suppression of multiple indices of

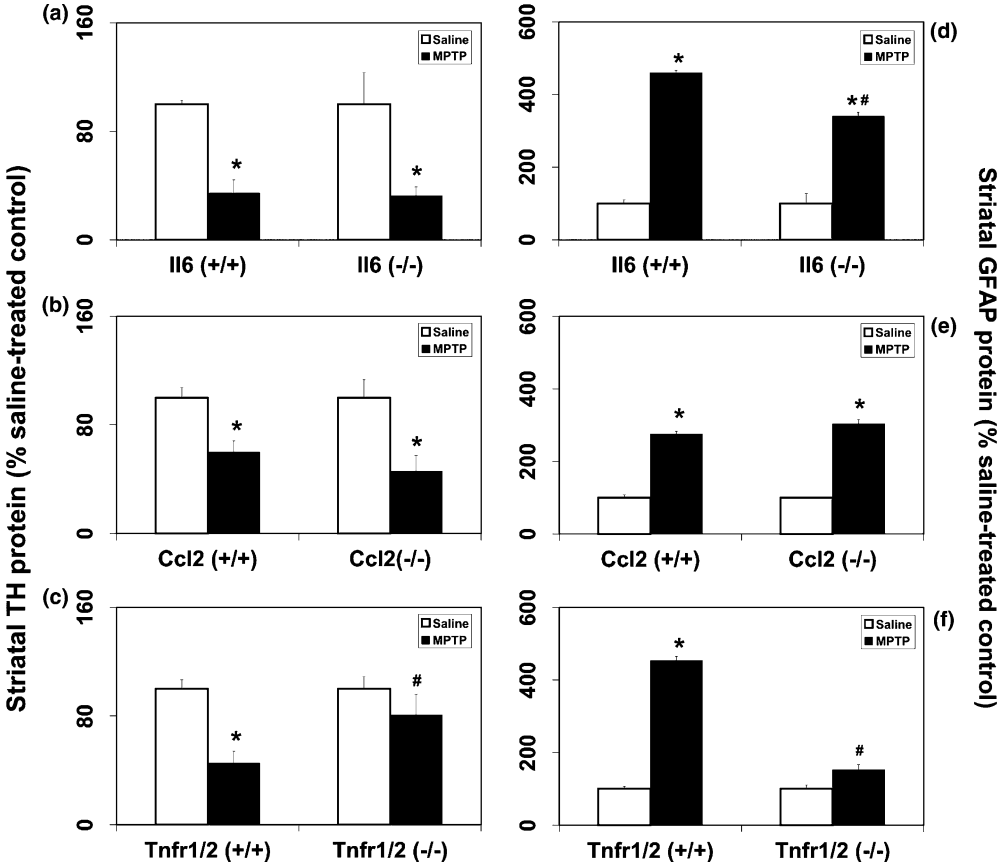

Fig. 7 Striatal dopaminergic neurotoxicity due to MPTP is abolished in *Tnfr*-deficient mice, but not in mice lacking *Il-6* or *Ccl2* genes. Wild-type (+/+) or mutant (–/–) mice lacking *Il-6*, *Ccl2* or *Tnfr1/2* genes were administered a single dose of either saline or MPTP (12.5 mg/kg, s.c.) and killed 72 h later. (a–c) TH and (d–f) GFAP protein levels were measured in the striatum by ELISA. The striatal levels of TH and GFAP protein were expressed as a percentage of those in saline-treated controls. Values are mean ± SEM ($n = 5$ animals per group). $^*p < 0.01$ versus saline-treated controls; $\#p < 0.05$ versus MPTP-treated (+/+) group. Newman–Keuls pairwise comparisons were used for *post hoc* statistical analysis.

microglial activation with the tetracycline antibiotic minocycline fails to protect against the striatal dopaminergic neurotoxicity caused by MPTP or METH. Data obtained using mice deficient in specific cytokines, chemokines or their receptors, combined with our previous observations on the role of TNF-α in striatal dopaminergic neurotoxicity, suggest that a complete blockade of the striatal effects of microglial-associated TNF-α may be required to achieve dopaminergic neuroprotection with minocycline or related interventions designed to inhibit microglial activation.

Although minocycline treatment significantly attenuated F4/80, a marker of microglial activation, it did not completely block MPTP- or METH-mediated induction of mRNAs encoding pro-inflammatory cytokines and chemokines such as TNF-α, IL-1α, IL-6 and CCL2. Thus, neurotoxicologically significant levels of these cytokines and chemokines were potentially available to initiate dopaminergic neurodegeneration. These findings suggest that even transient activation of microglia and/or elaboration of microglia-derived factors may be sufficient to elicit neurodegeneration in the striatum. To determine which of these microglia-derived factors play a key role in the neurodegenerative process, we examined the effects of MPTP in transgenic mice lacking *IL-6*, *Ccl2* or *Tnfr1/2* genes. MPTP neurotoxicity was not altered in mice lacking either the *Il-6* or *Ccl2* gene, but deficiency of both *Tnfr* genes protected against the dopaminergic neurotoxicity of MPTP. TNF-α is known to be associated with degenerating dopaminergic neurons in patients with PD (Boka *et al*. 1994) and our demonstration of its early induction in our MPTP-dosing model (Sriram *et al*. 2002) is suggestive of a participatory role for this cytokine in damage to the nigrostriatal dopaminergic pathway. Furthermore, TNFRs have been found to be expressed on dopaminergic neurons in PD (Boka *et al*. 1994). Their localization on the very cell type selectively vulnerable to damage in PD is consistent with the region-specific nature of the detrimental effects of TNF-α. Thus, consistent with our previous findings (Sriram *et al*. 2002),

TNF-α appears to be a critical factor in striatal dopaminergic neurotoxicity. However, contrasting findings on the involvement of TNFRs in dopaminergic neurotoxicity have been reported. Rousselet *et al*. (2002) failed to observe neuroprotection of nigral dopaminergic neurons in TNFR-deficient mice, following a multiple-dose MPTP regimen. Similarly, Leng *et al*. (2005) reported absence of neuroprotection in mice lacking TNFRs, following chronic dosing with MPTP. An earlier report from the same group (Ferger *et al*. 2004), however, indicated that mice lacking the *Tnf*-α gene exhibited significant reductions in the loss of dopaminergic markers in the striatum, but not in the nigra. Thus, TNF-α appears to exhibit region-specific actions in the CNS. Indeed, we have recently observed such regional heterogeneity in the action of TNF-α in response to MPTP (K. Sriram, unpublished observations). Another important point to note is that neither of the above studies evaluated the effects of multiple or chronic doses of MPTP on glial cells. It is well known that high doses of MPTP can damage astrocytes (Di Monte *et al*. 1992; Wu *et al*. 1992), which might contribute to alterations in glial–neuronal signaling. Such an effect would modulate cellular responses of glial cells and alter the pattern of pro-inflammatory cytokine expression. The repertoire of pro-inflammatory cytokines expressed following different dosing paradigms may vary and the outcome of such varied responses could have very different or even opposing effects. We cannot, however, rule out the contribution of IL-1α, as we did not examine the role of this cytokine using genetic manipulations.

Our observations on the effect of minocycline on MPTP or METH neurotoxicity are at least partially inconsistent with those of earlier studies that reported neuroprotective effects of minocycline against the dopaminergic neurotoxicity caused by MPTP (Du *et al*. 2001; Wu *et al*. 2002). The disparity between the results of the present study and those reported previously is probably due to differences in the dosing regimen employed. The previous studies (Du *et al*. 2001; Wu *et al*. 2002) employed a multiple-dose MPTP regimen and varying doses of minocycline (ranging from 1.4 to 120 mg/kg) administered before, along with or after MPTP, to demonstrate neuroprotective effects on dopaminergic cell loss in the substantia nigra. Although the minocycline regimens used in the present study were similar, in that low- and high-dose paradigms were used before, during and after administration of MPTP or METH, we administered only a single low dose of MPTP or METH that does not cause dopaminergic cell body loss (O'Callaghan and Miller 1994; Sriram *et al*. 2004) or microglial and astroglial activation in the substantia nigra (shown in this study). Our intention was to evaluate very early changes associated with dopaminergic neurotoxicity in the striatum, the onset of which precedes the degeneration of dopaminergic cell bodies in the substantia nigra (Borit *et al*. 1975; Bradbury *et al*. 1986; Ichitani *et al*. 1991; Sauer and Oertel 1994; Calabresi *et al*. 2000). The single-dose MPTP or METH regimens used in this study have the advantage of making post-dosing time points easier to establish and analyze than multidose regimens. Finally, our single-dose regimens of MPTP or METH were designed to achieve partial denervation of dopaminergic nerve terminals, which decreases morphological and neurochemical markers of neurotoxicity in the striatum by approximately 50%. By doing so, we could readily evaluate the potential neuroprotective as well as neurodegenerative effects of a given intervention by the convenient evaluation of multiple markers of dopaminergic neurotoxicity and the associated microglial and astroglial responses.

Because our results clearly demonstrated dose-dependent albeit incomplete suppression of microglial markers by minocycline, without achieving neuroprotection against MPTP or METH neurotoxicity in the striatum, it seemed likely that other key factors distinguish the neurotoxic effects of the single- versus multiple-dosing models of these compounds. Prostanoids and nitric oxide represent two likely candidates because increased COX2 (Teismann *et al*. 2003; Feng *et al*. 2003) and iNOS (Itzhak *et al*. 1999; Liberatore *et al*. 1999; Dehmer *et al*. 2000) have been reported following a multiple-dosing regimen of MPTP or METH, effects that may not be associated with our single-dose model. Indeed, neither MPTP nor METH altered the striatal expression of *Cox2*, *Nos1* or *Nos2* (*iNos*) mRNAs. These data stand in contrast to those of other studies in which a multiple-dose MPTP regimen was shown to cause robust expression of iNOS in the substantia nigra linked to dopaminergic cell loss in this region. Furthermore, mice deficient in the *iNos* gene were more resistant to MPTP (Liberatore *et al*. 1999). However, in agreement with our findings, no induction of iNOS was observed in the striatum and iNOS deficiency did not protect against striatal dopaminergic neurotoxicity caused by MPTP (Liberatore *et al*. 1999; Dehmer *et al*. 2000). Thus, regional differences in the expression of microglia and their elaboration of cytotoxic factors may determine the susceptibility of brain regions to neurotoxicity, and such differences may serve as the basis for dopaminergic neurotoxicity models resulting in nigral cell loss versus those that damage striatal dopaminergic nerve terminals while sparing the cell bodies in the nigra. Indeed, we (K. Sriram, unpublished observations) and others (Lawson *et al*. 1990; Ren *et al*. 1999) have observed that the levels of microglial markers and microglia-derived factors are differentially expressed across brain regions. Therefore, factors produced by activated microglia that may be specific to various brain regions may influence the fate of the neurons and/or nerve terminals in that particular region, and the effects may vary depending on the dose or dosing paradigm followed.

In summary, attenuation of microglial activation is insufficient to protect against striatal dopaminergic neurotoxicity.

We attribute the failure of minocycline to afford neuroprotection against MPTP- and METH-induced dopaminergic neurotoxicity in striatum to its inability to completely suppress the induction of TNF-α and its receptors, leaving signaling via TNFRs at least partially active. As TNF-α is an obligatory component of dopaminergic neurotoxicity (Sriram *et al*. 2002), the failure to abolish TNF signaling reflects the lack of efficacy of minocycline in completely abolishing microglial activation. On the other hand, deficiency of TNFRs afforded neuroprotection against striatal dopaminergic neurotoxicity, suggesting that TNF-α signaling must be completely abolished to achieve protection against dopaminergic neurotoxicity in the striatum. The present observations support our earlier hypothesis that TNF-α is a key player in striatal dopaminergic neurotoxicity. Finally, our findings also strengthen the notion that TNF-α may be responsible for the selective vulnerability of the nigrostriatal pathway to degenerative changes associated with dopaminergic neurotoxicity and perhaps PD.

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Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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