Defining "Neuroinflammation"

Lessons from MPTP- and Methamphetamine-Induced Neurotoxicity

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Neuroinflammation is a hot topic in contemporary neuroscience. A relatively new open-access journal, the *Journal of Neuroinflammation*, focuses on this field. As another example, abstracts to the 2007 Annual Meeting of the Society for Neuroscience could be submitted in several subcategories of neuroinflammation, a strong signal of growth in this research area. While it is becoming clear that activation of microglia and astroglia and the attendant expression of proinflammatory cytokines and chemokines often are associated with disease-, trauma-, and toxicant-induced damage to the CNS, it is by no means clear that a cause-and-effect relationship exists between the presence of a neuroinflammatory process and neural damage. We have explored this issue with two models of dopaminergic neurotoxicity. We used a single low-dose regimen of MPTP or METH, a paradigm that causes selective degeneration of striatal dopaminergic nerve terminals without affecting the cell body in the substantia nigra. Both compounds increased the expression of the microglia-associated factors, Il-1α, Il6, Ccl2, and Tnf-α, and also elicited morphologic evidence of microglial activation prior to induction of astrogliosis. Pharmacologic antagonism of MPTP and METH neurotoxicity prevented these proinflammatory responses, findings suggestive of a link between neuroinflammation and the observed neurotoxic outcomes. Nevertheless, when we used minocycline to suppress the expression of all these mediators, with the exception of Tnf-α, we failed to see neuroprotection. Likewise, when we examined the effects of MPTP or METH in transgenic mice lacking Il6, Ccl2, or Tnfr1/2 genes, deficiency of either Il6 or Ccl2 did not alter neurotoxicity, whereas deficiency in Tnfr1/2 was neuroprotective. Although these observations pointed to a role of the proinflammatory cytokine, TNF-α, in the neurotoxic effects of MPTP and METH, other observations did not support this supposition. For example, activation of NF-κB or induction of iNOS, known components of inflammatory responses and free radical formation, were not observed. Moreover, immunosuppressive regimens of glucocorticoids failed to suppress TNF-α or attenuate neurotoxicity. Taken together, our observations suggest that MPTP and METH neurotoxicity are associated with the elaboration of a "neuroinflammatory" response, yet this response lacks key features of inflammation and, with the exception of TNF-α, neurotoxicity appears to be the cause rather than the consequence of proinflammatory signals.

Key words: **neuroinflammation; neurotoxicity; methamphetamine; MPTP; gliosis; microglia; astroglia; cytokines; chemokines; dopamine; dopaminergic**

Abbreviations: **AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; Ccl2, chemokine (C-C motif) ligand 2; CCL2 [in new nomenclature], monocyte ahemoattractant protein; CNS, central nervous system; CORT, corticosterone; CREB, cAMP**

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response element-binding; DA, dopamine; DHBA, 3,4-dihydroxybenzylamine hydrobromide; DOPAC, 3,4-dihydroxyphenylacetic acid; EDTA, ethylene-diamine-tetra-acetic acid; ERK 1/2, extracellular signal-regulated kinases; GFAP, glial fibrillary acidic protein; GLUT, glucose transporter; HSP, heat-shock protein; HVA, homovanillic acid; ICAM, intercellular cell-adhesion molecule; Il-1α, interleukin-1; Il6, interleukin-6; iNOS, inducible nitric oxide synthase; JNK, jun N-terminal kinase; LIF, leukemia inhibitory factor; MCP-1, monocyte chemotactic protein-1; MDMA, methylenedioxymethamphetamine; MEK 1/2, methyl ethyl ketone; METH, methamphetamine; MHC, major histocompatibility complex; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MRF, microglial response factor; MS, multiple sclerosis; NF-κB, nuclear factor kappa B; NGF, nerve growth factor; OSM, oncostatin M; PD, Parkinson's disease; qRT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; RT, room temperature; SAPK, stress-activated protein kinase; STAT3, signal transducer and activator of transcription 3; TBST, Tris-buffered saline Tween-20; TH, tyrosine hydroxylase; Tnfr, tumor necrosis factor receptor; TNF, tumor necrosis factor

Introduction

Inflammatory responses in the CNS have now been associated with many chronic neurodegenerative conditions (e.g., MS, AD, ALS, and PD).[1–7] Not surprisingly, therefore, the notion of neuroinflammation has become a dominant theme in contemporary neuroscience, and it has been used as a "catch-all" term to describe the role of inflammatory processes in the pathophysiology of most neurodegenerative diseases. Whether neuroinflammation is a cause or a consequence of neurologic disease remains unclear.[5,8–10] Moreover, whether inflammatory responses seen in brain diseases have relevance to chemically-induced injury of the CNS, including damage caused by drugs of abuse, remains even less obvious at this juncture.

Neuroinflammation is not easy to define. Traditionally, inflammation involves the synthesis/release of proinflammatory mediators, such as cytokines and chemokines. Outside the CNS, this typically involves monocytes, neurotophils, and macrophages, cells that elaborate complement cascades and generate reactive oxygen species at sites of infection or injury. More recently, this same "inflammation" concept has been extended to the role of activated microglia and astroglia, that is, the brain glia cells known to serve as both source and targets of proinflammatory mediators (Fig. 1). A dominant response to all types of CNS injuries (e.g., those of disease, trauma, chemicals, and drugs) is the activation of microglia and astroglia, often referred to as gliosis, at the sites of damage.[11–13] Although the mediators of gliosis have yet to be identified, proinflammatory cytokines and chemokines have long been implicated. Microglia and astroglia also are viewed as a source of inflammatory mediators and as reactive oxidant generators, the so-called "dark side" of glia,[14] because of the potential for these responses to damage neural targets (Fig. 1). These detrimental effects of glial activation can be achieved via aberrant or prolonged responses to the primary neural insult upstream, or even due to their activation as direct targets of disease, trauma, or toxic injuries of the CNS (Fig. 1). Despite the view of glia and glial signaling as a source of pathologic mediators, it is equally important to emphasize aspects of glia-derived signaling that may be far more in the physiological rather than the pathologic realm. Thus, resting or activated microglia and astrocytes have long been known to be sources of trophic factors, including proinflammatory cytokines, which likely are important in development, plasticity, and repair of the CNS.[8,10] Moreover, it is important not to overlook physiological roles for glia in pain, stress, and autonomic and immune responses key to maintenance of normal homeostasis.[8,10,15–17]

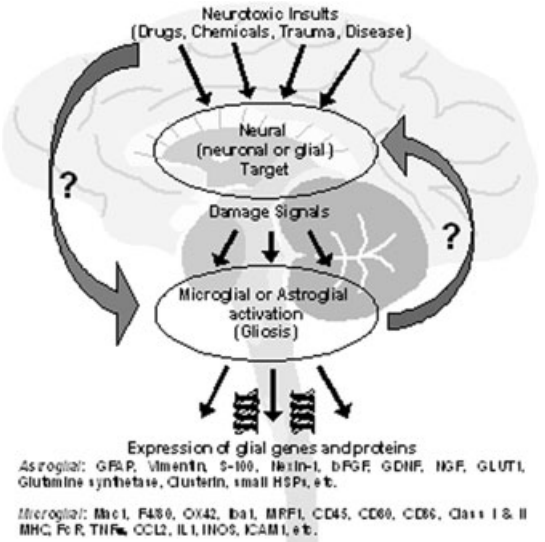

Figure 1. Cellular events associated with disease, injury, or toxicant-induced gliosis. Neural elements of the CNS (neurons or glia) serve as targets for neurotoxic insults. The damaged cells serve as a source of signals that activate microglia or astroglia, cellular responses collectively referred to as gliosis. Gliosis is characterized by the expression of a variety of glial genes and proteins. The possibility exists that neurotoxic exposures directly activate microglia and astroglia (*large downward arrow*), with the ensuing elaboration of glia-derived neuroinflammatory mediators (*large upward arrow*) causing damage to neural targets. bFGF: basic fibroblast growth factor; FcR: Fc receptor; GDNF: glia-derived neurotrophic factor; GFAP: glial fibrillary acidic protein; GLUT: glucose transporter; HSP: heat-shock protein; ICAM: intercellular cell-adhesion molecule; iNOS: inducible nitric oxide synthase: MCP-1: monocyte chemoattractant protein (CCL2 in new nomenclature); MHC: major histocompatibility complex; MRF: microglial response factor; NGF: nerve growth factor; TNF: tumor necrosis factor. (Adapted from O'Callaghan and Sriram.[13])

Proinflammatory mediators have been implicated in dopaminergic neurotoxicity initiated by the known dopaminergic neurotoxicant, MPTP,[5,18–21] and recent evidence extends their involvement to methamphetamine (METH) as well.[20,22] Dopaminergic neurons appear uniquely vulnerable to increases in oxidative stress as a result of their already elevated intracellular oxidative processes related to synthesis of dopamine.[6,23] Thus, these neurons would seem to be innately vulnerable targets to neuroinflammation-driven generation of ROS. Thus, investigation of a potential link between neuroinflammation and dopaminergic neurotoxicity seems warranted. Here, we summarize some of our efforts to define the role of selected cytokines and chemokines in the neurotoxic effects of MPTP and METH, as well as our efforts to block neurotoxic damage by ameliorating cytokine and chemokine expression with glucocorticoids and the tetracycline-like anti-inflammatory agent, minocycline.

In Vivo Approaches to the Assessment of Gliosis and the Role of Inflammatory Mediators in MPTP- and METH-Induced Neurotoxicity

Animals and Tissue Preparation

For several years now, we have continued to use mice, as opposed to rats, in our studies of dopaminergic neurotoxicity. As we noted in a recent review,[24] mice and rats respond differently to substituted amphetamines. In our hands, mice consistently display neuropathologic effects when given amphetamines, whereas short- or even-long term neurotransmitter deficits are seen in rats, but these effects are not always accompanied by evidence of neural damage.[24,25] As demonstrated widely for effects of amphetamines, mice also remain attractive subjects for this work because of the availability of a variety of mutants with which to dissect underlying mechanisms of neurotoxicity. Finally, we have focused on the use of the dopaminergic neurotoxicant, MPTP, to serve essentially as a positive control for our work with other dopaminergic-acting agents. MPTP has a rapid onset of action, it targets the nigrostriatal dopaminergic pathway, its active metabolite can readily be measured in brain tissue, its neurotoxic effects can be blocked by a variety of pharmacologic antagonists, and it

results in dopaminergic neurotoxicty after only a single s.c. dose.[26] All of these features make MPTP a useful denervation tool for proof-of-concept explorations, and we have used it as such. Of course, because MPTP is almost devoid of neurotoxic activity in rats and some strains of mice, one is limited to its use in susceptible strains of mice, making cross-species comparisons difficult. This latter limitation of the MPTP model has also influenced our propensity to limit our studies of dopaminergic neurotoxity to mice. Thus, in the present studies we used male and female C57Bl/6J mice or transgenic mice carrying homozygous mutant alleles for TNF receptors [B6;129S-*Tnfrsf1a*^{tm1Imx} *Tnfrsf1b*^{tm1Imx}/J; Tnfr1/2 (−/−)], Il-6 gene [B6.129S2-*Il6*^{tm1Kopf}/J; Il6 (−/−)] or Ccl2 gene [B6.129S4-*Ccl2*^{tm1Rol}/J], all purchased from Jackson Laboratories (Bar Harbor, ME, USA). Appropriate strain controls (B6.129S) and C57BL/6J also were procured from the same vendor (Jackson Laboratories). Male mice were given *d*-METH (Sigma Chemical Co., St. Louis, MO, USA) at a dosage of 20 mg/kg, s.c., whereas female mice were given 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrdidine (MPTP) (Aldrich, Milwaukee, WI, USA) at a dosage of 12.5 mg/kg (s.c.). Saline solution served as a vehicle control. These regimens were developed by trial and error to obtain single administrations (making it easy to track time course) that resulted in approximately 50% decrements in DA and/or TH (in order to stay away from "floor" or "ceiling" level effects so that we could evaluate pretreatments that alter damage in either direction). For the neuroglial protection experiments, mice were given corticosterone in the drinking water (400 μg/mL) 1 week prior to administration of MPTP or they were given minocycline, 100 mg/kg, 12 hours before, during, and 12 hours after MPTP or METH. Mice were sacrificed by decapitation for preparation of RNA and for dopamine analysis, or by focused microwave irradiation (Muromachi Kikai, Inc., Tokyo, Japan; Model TMW-4012C, 3.5 KW applied power, 0.90 sec) to preserve steady-state phosphorylation for subsequent analysis with phospho-state-specific antibodies.[27–29] Brain regions were dissected freehand, weighed, and homogenized in 10 volumes of hot 1% SDS or directly snap-frozen and then stored at −80°C until assayed. Total protein concentration of the SDS homogenates was assayed by the method of Smith *et al.*[30] with bovine serum albumin used as the standard.

GFAP, TH, and DA Analysis

As baseline measures to establish the time course of MPTP- or METH-related dopaminergic neurotoxicity, we have relied on the analysis of TH holoenzyme protein and/or DA. The astrocyte intermediate filament protein, GFAP, remains our key index for assessing the astroglial response to dopaminergic neurotoxicity. GFAP was assayed in accordance with a previously described enzyme-linked immunosorbent assay (ELISA).[31,32] TH was assayed by a sandwich fluorescent ELISA based on the GFAP ELISA protocol. In brief: a rabbit polyclonal antibody to GFAP was coated on the wells of Immulon-2 microtiter plates (Thermo Labsystems, Franklin, MA, USA). The detergent-denatured homogenates and standards were diluted in phosphate-buffered saline (pH 7.4) containing 0.5% Triton-X 100 solution; the wells of the plates were blocked with 5% non-fat dry milk, and 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). For the TH ELISA a mouse monoclonal antibody to TH was used as the plate capture antibody and a rabbit polyclonal antibody was used to "sandwich" TH protein.[19,33] 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, Rockford, IL, USA) that has excitation/emission maxima of 325/420 nm and was read on an Fmax Plate Reader (Molecular Devices). The amount of GFAP or TH in the samples was calculated as μg GFAP or TH/mg total protein.

Levels of DA in striatal homogenates from control and MPTP- or METH-treated mice were analyzed by high-performance liquid chromatography with electrochemical detection (HPLC-EC). Samples were homogenized in 0.3-mL standard volume of ice-cold 0.2 N perchloric acid, containing DHBA 1 μM as internal standard. After centrifugation at 10,000 × *g* for 10 min, an aliquot of supernatant (10 μL) was injected using a temperature-controlled (4°C) automatic sample injector (Waters 717 Plus Autosampler connected to a Waters 515 HPLC pump; Waters Corporation, Milford, MA, USA) into a C18 reversed-phase column (Waters SYMMETRY, 4.6 × 250 mm, 5 μm, 100 A). DA was electrochemically detected (Waters 464 Pulsed Electrochemical Detector; range 10 nA, potential +0.7 V) and analyzed using Millennium-32 Software. The mobile phase (pH 3.0) for isocratic separation of DA 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. DA, DOPAC, and HVA standards (0.5–25 pmol) were prepared in 0.2 M perchloric acid containing DHBA. Recovery of each analyte was adjusted with the internal standard and quantified from a standard curve. The levels of DA and its metabolites are expressed as μg/g wet tissue.

Phospho-State-Specific Antibodies as Reagents to Identify and Characterize Signaling Cascades Involved in MPTP and METH-Induced Neurotoxicity

With the widespread availability of phospho-state-specific antibodies, it became possible to map activation of specific signal transduction cascades on a substrate-by-substrate, kinase-by-kinase basis. We demonstrated that combining these specific signaling tools with application of focused microwave irradiation, to prevent both the rapid artifactual loss or gain in phosphorylation associated with traditional modes of sacrifice,[27–29] allowed us to screen multiple pathways and identify those affected by METH and MPTP.[33,34] After microwave sacrifice, tissue homogenates were resolved on 10% SDS gels and electrophoretically transferred to a 1.0-μM nitrocellulose membrane. Membranes were blocked with Blotto in TBST (TBS including 0.1% Tween-20), washed in TBST for 5 min (3×) and incubated overnight at 4°C with a polyclonal anti-rabbit antibody (1:1000, 5% bovine serum albumin in TBST). Primary antibodies (Cell Signaling Technology, Beverly, MA, USA) were either phosphorylation-state-specific, directed against the "activated" state of the phosphoproteins or the context-independent, MEK1/2 (S217/221), ERK 1/2 (T202/Y204), JNK/SAPK (T183/Y185), p70 S6 kinase (T389), CREB (S133), and STAT3 (Y705).[34] Only data for $STAT3^{tyr705}$ will be presented in this paper. After washing, the blots were incubated with HRP-conjugated anti-rabbit (1:2000) amplified with horseradish peroxidase (HRP)-biotin (1:1000) in blocking buffer for 1 hr at RT. Detection was accomplished using enhanced chemiluminescence. Exposed films were scanned into Personal Densitometer and quantified using ImageQuant software (Molecular Dynamics). All data were obtained from the linear portion of the densitometry curves.[35] The results of this approach led us to focus on the gp130/JAK2/STAT3 pathway and its putative proinflammatory upstream effectors.

Analysis of Inflammation-Related Cytokine and Chemokine Expression by Real-Time PCR Amplification

The evidence we obtained implicating downstream effectors in the JAK2-STAT3 pathway,[33,34] during the early stages of MPTP- and METH-induced neurotoxicity, implicated upstream ligands linked to microglial activation and inflammation. Therefore we screened several likely candidate cytokines and chemokines as well as mRNAs associated with activation of microglia. Total striatal RNA was isolated using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) and phase-lock heavy gel (Eppendorf AG, Hamburg, Germany). RNeasy mini spin columns (Qiagen, Valencia, CA, USA) were used as a further purification step. 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, MRF1, F4/80, Il-1α, Il-6, Ccl2 (Mcp-1) Tnf-α, Tnfrsf1a (Tnfr1), and Tnfrsf1b (Tnfr2) was performed in an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) in combination with TaqMan® chemistry. Specific primers and dual-labeled internal fluorogenic (FAM/TAMRA) probe sets (TaqMan Gene Expression Assays) for these genes were procured 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 of the specific primer/probe mix, and 25 μL of TaqMan Universal master mix (Applied Biosystems), respectively. Sequence detection software (version 1.7; Applied Biosystems) results were exported as tab-delimited text files and imported into Microsoft Excel for further analysis. Relative quantification of gene expression was performed using the comparative threshold (C_T) method as described by the manufacturer (Applied Biosystems, User Bulletin 2). Changes in mRNA expression level were calculated after normalization to GAPDH. The ratios obtained following normalization are expressed as fold change over corresponding saline-treated controls.

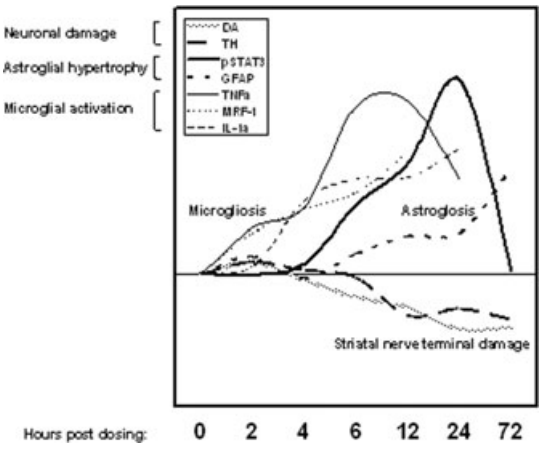

Figure 2. Time course of MPTP- or METH-induced striatal dopaminergic neurotoxicity and ensuing gliosis as assessed by multiple markers of neurotoxicity and glial activation. DA: dopamine; TH: tyrosine hydroxylase (protein); pSTAT3: phosphoSTAT3^{tyr705}; GFAP: glial fibrillary acidic protein; TNFa: tumor necrosis factor alpha mRNA; MRF-1: miroglial response factor-1 mRNA; IL-1a: interleukin-1 alpha mRNA.

Statistical Analysis

All analyses were performed using SigmaStat (version 2.03) or JMP (version 6.0.3) statistical analysis software packages. The test of significance for individual variables was performed using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test or Tukey–Kramer HSD test (alpha level set at .05). Graphic representations are mean ± SEM.

Results and Discussion

The administration of either MPTP or METH to the C57BL6J mouse results in dopaminergic damage-related events that are of the same magnitude and follow the same time course (Fig. 2). The data for METH reflect the pattern of effects seen after a single dose of

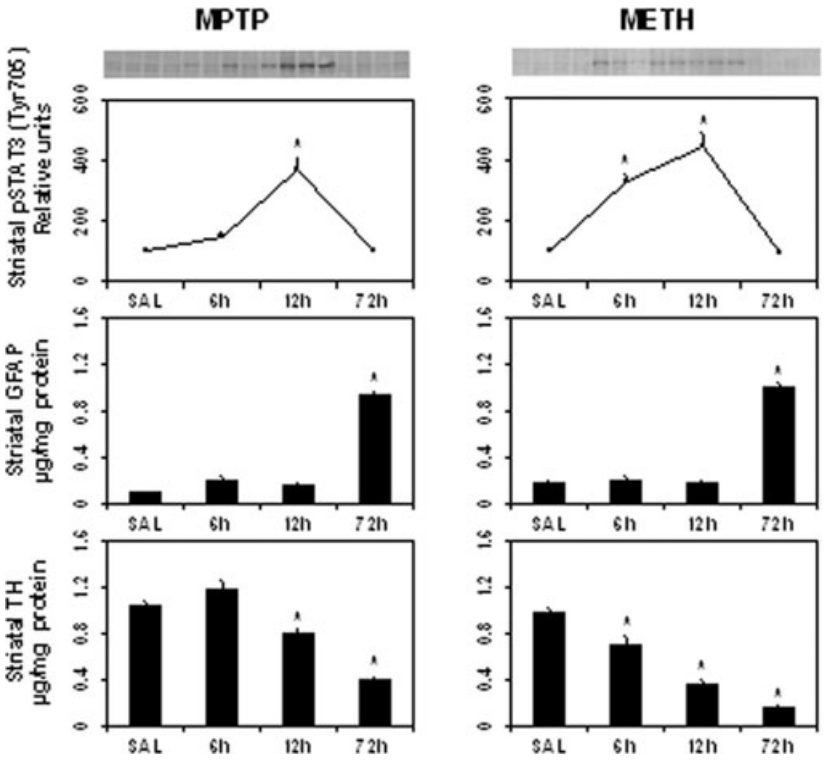

Figure 3. Time course of the MPTP- or METH-induced activation of pSTAT3 (phosphorylation on tyr705) and the subsequent enhanced expression of GFAP in comparison to the decrease in dopamine nerve terminal marker, tyrosine hydroxylase (TH)(protein). Both immunoblots for pSTAT3^{tyr705} and corresponding densitometry are shown. GFAP and TH values were determined by ELISA. Each value represents the mean ± SEM of at least four independent determinations. *Significantly different from corresponding control, $P < 0.05$.

20 mg/kg to male mice, but in general this same time course applies to the 3–4 × 10 mg/kg dosing regimen of METH (given over a 6-hour period) seen in female mice.[36] A rapid decline in DA or TH, reflective of dopaminergic nerve terminal damage, takes place over a period of 72 hours. That the DA and TH decreases are reflective of damage, and are not regulatory in nature[24,25,37] are indicated by the rapid upregulation of multiple markers associated with microglia and astroglial activation. These include both a number of proinflammatory mediators associated with microgliosis as well as a key signal transducer associated with astrocytic hypertrophy (pSTAT3)[33] and the subsequent increase in the astrocytic intermediate filament protein, GFAP. This temporal pattern of events involving several mediators implicated in proinflammatory responses makes it easy to label the overall damage response as "neuroinflammation." However, evidence can be marshaled to support either a "yes" or "no" to the neuroinflammation issue. The path of reasoning that led us to investigate the role of inflammatory responses in MPTP and METH neurotoxicity, and the approaches we used to address the role of neuroinflammation in neurotoxic damage responses, are presented below.

In our initial screening of phosphorylation pathways that might be involved in the early phases of MPTP or METH dopaminergic neurotoxicity, we identified STAT3, the downstream effector in the gp130/JAK2/STAT3 pathway, as a key element in early responses to administration of these dopaminergic neurotoxicants.[34] Thus, as shown in Figure 3, activation of STAT3 via phosphorylation on Y705 *in vivo* precedes the onset of astrogliosis and

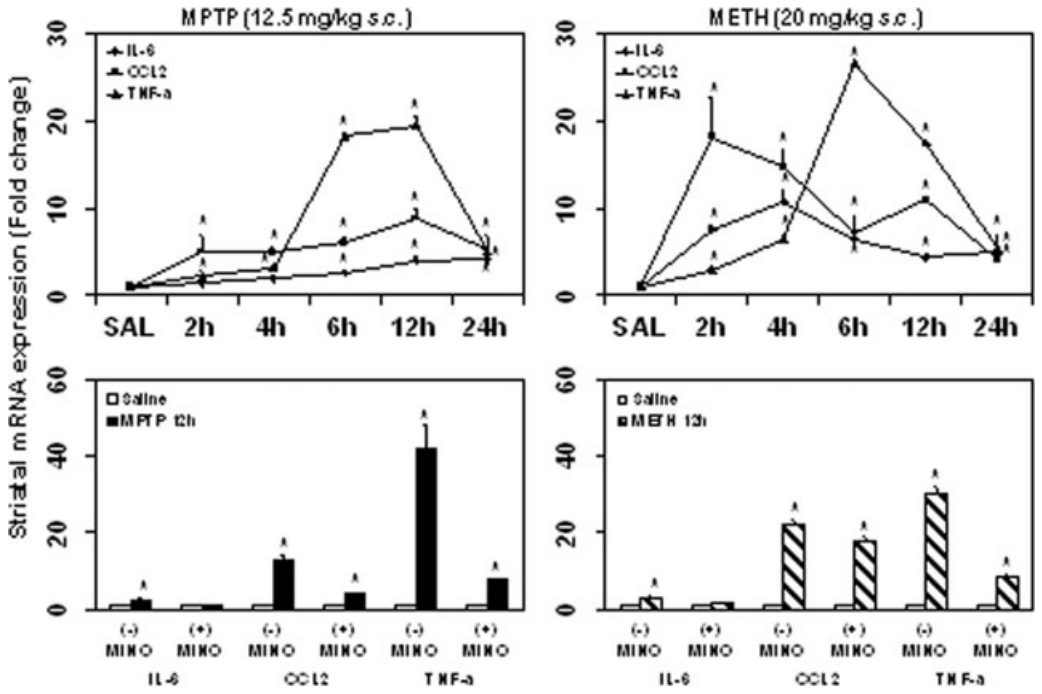

Figure 4. Proinflammatory responses in mouse striatum after MPTP and METH are attenuated by minocycline. Striatal expression of mRNA for the proinflammatory cytokines/chemokines, IL-6, CCL2, and TNF-a was assessed by real-time PCR at different post-dosing time points after MPTP or METH administration (*upper panels*). Pretreatment with minocycline (100 mg/kg) attenuated, but did not block, the expression of IL-6, CCL2, and TNF-a. Each value represents the mean ± SEM of at least four independent determinations. *Significantly different from corresponding control, $P < 0.05$. (Adapted from Sriram *et al*.[20] and Berman *et al*.[42])

the maximal loss of TH or DA. Our subsequent work indicates that pSTAT3^{tyr705} appears to be broadly involved in neurotoxicity-related glial signaling, specifically, astrocytic hypertrophy.[38] A linkage between the activation of STAT3 and damage was further established by the fact that pharmacologic or physiological neuroprotection of neurotoxicity (e.g., by dopamine uptake inhibitors for MPTP, and lowered core temperature for METH) also completely blocked the phosphorylation of STAT3^{tyr705}.[33,38] Because ligands that activate the JAK/STAT3 pathway often are considered to be proinflammatory in nature (e.g., IL-6, OSM, LIF), our observations were indicative of a potential upstream involvement of these STAT3 effectors. In other words, our findings for STAT3 were suggestive of a vote "for" involvement of "neuroinflammation" in dopaminergic and other types of neurotoxic responses. Nevertheless, our findings also suggested that if this was the case, activation of a glial-localized JAK/STAT3 pathway did not initiate neurotoxicity because, at least for MPTP, neuroprotective agents acting at the level of the dopamine transporter protected not only the dopaminergic nerve terminal, but also prevented the activation of STAT3.[33] Therefore, activation of STAT3 in glia is downstream of the damaged target.

We next pursued the leads we had: knowledge that the upstream effectors of STAT3 should be expressed, knowledge that these cytokines are associated with microglial activation, and knowledge that microglia are activated after MPTP.[19,23,39] Using qRT-PCR, we indeed found several-fold enhanced expression of IL6, CCL2, and TNF mRNA (Fig. 4, top panels), proinflammatory cytokines and chemokines directly upstream of STAT3

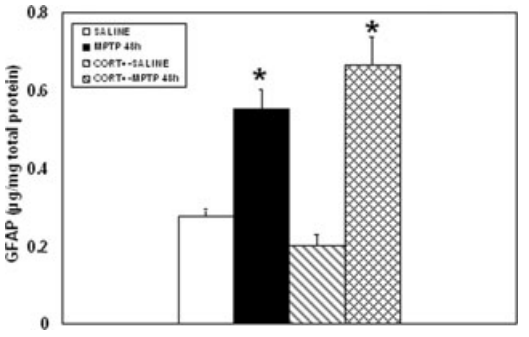

Figure 5. Suppression of cytokines/chemokines by minocycline is not neuroprotective against MPTP or METH and does not block neurotoxicity-associated astrogliosis. GFAP and TH values were determined by ELISA. Each value represents the mean ± SEM of at least four independent determinations. *Significantly different from corresponding control, $P < 0.05$. (Adapted from Sriram *et al.*[20])

(IL6) or involved in cross-talk pathways feeding into STAT3, and/or other downstream inflammatory pathways. The fact that these signaling events coincided with morphologic evidence for microglial activation, preceded STAT3 activation and astrogliosis, and were transient in nature were suggestive of their mediatory or modulatory role in dopaminergic neurotoxiciy. Because activated microglia and proinflammatory responses have been associated with dopaminergic neurodegeneration, we reasoned that suppression of these factors might be neuroprotective against MPTP and/or METH. Therefore, we pretreated mice with the semisynthetic tetracycline derivative, minocycline. This agent has been reported to have broad anti-inflammatory properties and to be protective against a variety of neurotoxic insults, including MPTP.[21] Pretreatment with minocycline was very effective in suppressing the expression of IL6, CCL2, and TNF mRNA (Fig. 4, lower panels). Although these findings suggested that minocycline suppresses MPTP- and METH-related activation of microglia, or at least proinflammatory cytokines and chemokines associated with activated microglia, minocycline failed to afford protection against MPTP- or METH-induced dopaminergic neurotoxicity and astrogliosis (Fig. 5). In addition to minocycline, we also evaluated a more classic anti-inflammatory agent, corticosterone (CORT), a glucocorticoid, as a potential neuroprotectant against MPTP. Maintenance of mice on CORT in the drinking water, at a concentration sufficient to involute the thymus (a sign of the potent immune suppressive actions of this steroid), failed to alter striatal dopamine depletion (data not shown) or the increase in GFAP seen after MPTP (Fig. 6). This failure of immunosuppressive therapy to affect MPTP-induced neurotoxicity is consistent with our previous findings for MDMA-induced dopaminergic neurotoxicity in striatum[40] and TMT-induced hippocampal neuronal loss,[41] where high-dose CORT also was not protective. Together these data indicate that immunosuppressive anti-inflammatory therapy is sufficient to downregulate some proinflammatory cytokines and chemokines, but is not neuroprotective in the multiple neurotoxicity models we have examined.

In addition to the pharmacologic approach we took to modulate inflammatory-like responses, we also pursued a genetic approach with selected knockout mice. When mice deficient in IL-6, Ccl2, or Tnf1/2 receptors were given either MPTP or METH, only the mice lacking the Tnf1/2 receptors showed attenuated neurotoxic effects (based on analysis of TH and GFAP) (Fig. 7). These findings left open the possibility that TNF could play some role in the dopaminergic neurotoxicity of MPTP and METH.

As one final avenue of investigation aimed at determining the potential role of TNF-α in dopaminergic neurotoxicity, we evaluated the activation of the NF-κB after administration of MPTP to wild-type mice and mice lacking both TNF receptors (Fig. 8). NF-κB can be considered the downstream "inflammatory" transcription factor for TNFα, in that it mediates cellular inflammatory responses initiated by this cytokine, including the production of reactive oxygen species often implicated in dopaminergic neurotoxicity.[6] We examined phosphorylation of both

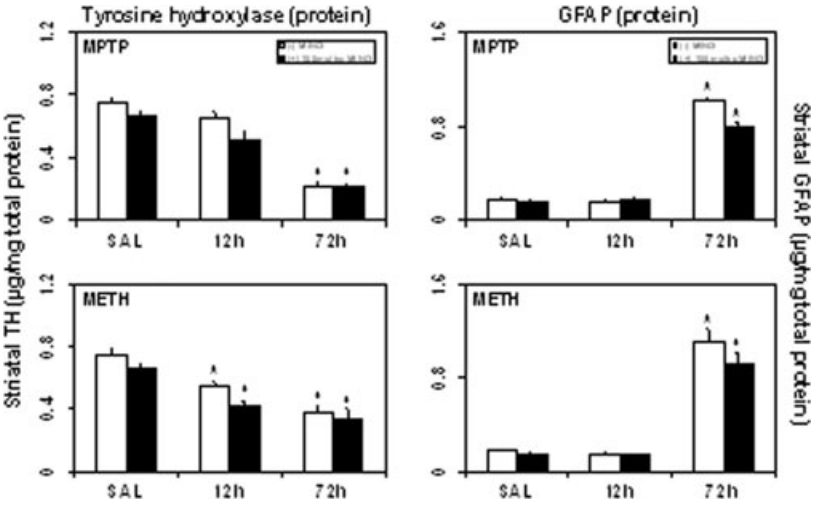

Figure 6. High physiological levels of corticosterone fail to suppress MPTP neurotoxicity-associated striatal astrogliosis at 48 hours post dosing (MPTP 48 h or CORT + MPTP 48 h). Pretreatment of mice with immunosuppressive levels of corticosterone in the drinking water for 1 week prior to administration of MPTP did not block the subsequent induction of astrogliosis as assessed by GFAP ELISA. Each value represents the mean ± SEM of at least four independent determinations. *Significantly different from corresponding control, $P < 0.05$.

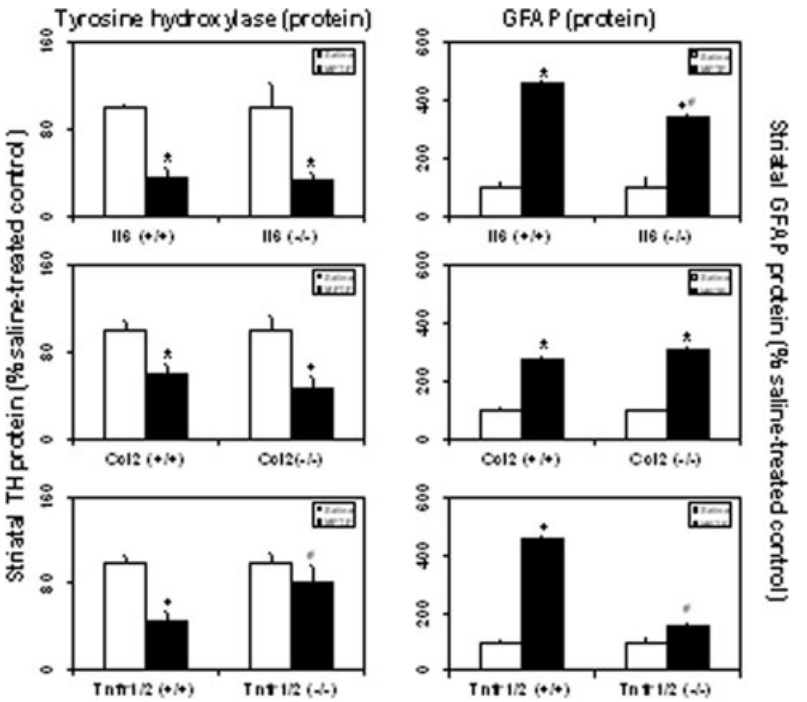

Figure 7. Deficiency of TNF receptors protects against MPTP neurotoxicity. Striatal neurotoxicity due to MPTP and METH was assessed by TH and GFAP ELISA using tissue prepared from wild-type (+/+) or mutant (−/−) mice lacking Il-6, Ccl2, or Tnfr1/2 genes. Mice were killed at 72 hours post dosing. Each value represents the mean ± SEM of at least four independent determinations. *Significantly different from corresponding control, $P < 0.05$. #Significantly different from corresponding MPTP or METH-treated group, $P < 0.05$. (Adapted from Sriram *et al*.[20])

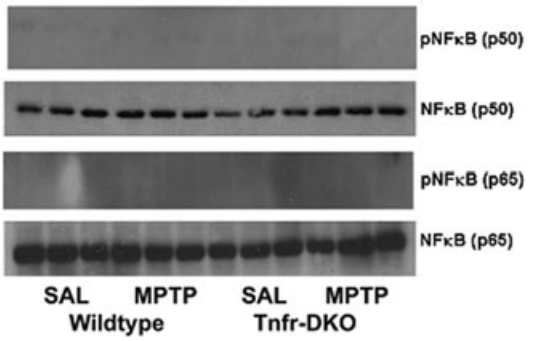

Figure 8. Activation (phosphorylation) of NFκB is not affected in striatum of wild-type or Tnfr double-knockout mice 48 hours after administration of MPTP. Both phosphorylated and unphosphorylated forms of NFκB (p50 and p65) were assessed in striatal samples prepared from mice sacrificed by focused microwave irradiation to preserve steady-state protein phosphorylation. (Adapted from Sriram *et al*.[19])

the p50 and p65 subunits of NF-κB and did not find evidence for activation in either the wild-type or double-knockout mice. We also failed to see an induction in iNOS mRNA as would be expected for an inflammation-related production of ROS (data not shown).[19] While hardly definitive, these data tend to suggest that the role of TNF-α in MPTP-induced dopaminergic neurotoxicity may involve actions outside the traditional proinflammatory pathway usually attributed to effects of this cytokine.

So where do these data leave us with respect to answering the question: "Is neuroinflammation involved in dopaminergic neurotoxicity?" In general, on the basis of the limited data available, neuroinflammatory responses do not appear to play a role in instigating the damage. While we now have obtained evidence for participation of TNF-α in MPTP neurotoxicity, because its increase is blocked by the neuroprotective action of nomifensine, dopaminergic damage is not initiated by TNF-α. Moreover, expression of this and most proinflammatory cytokines and chemokines after MPTP or METH appears largely to be associational and not causal for neurotoxic outcomes because pharmacologic and most genetic manipulations of these mediators don't block evidence of damage. What seems critical at this juncture is to more carefully define the role of neuroinflammation with respect to its involvement in a given neurotoxic response. This is not just an issue of semantics, as a presumed role of inflammation can influence the field without being on substantial scientific footing. One need only remember how many mechanisms were ascribed to amphetamine neurotoxicity before the dominant role of body temperature was established by Bowyer and colleagues.[37] Addressing concerns now regarding the basis of the "neuroinflammatory hypothesis" of neurotoxicity will become important in the future because links are beginning to emerge among neuroAIDS, drug abuse, and inflammation.[42] Sorting out the key aspects of neuroinflammatory responses that do or do not contribute to dopaminergic neurotoxicity will be critical if we are to achieve a clear understanding of the fundamental mechanisms that contribute to specific neurotoxic effects of drugs of abuse.

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Conflicts of Interest

The authors declare no conflicts of interest.

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