

Deficiency of TNF receptors suppresses microglial activation and alters the susceptibility of brain regions to MPTP-induced neurotoxicity: role of TNF- α ¹

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ABSTRACT Enhanced expression of tumor necrosis factor (TNF) - α , is associated with the neuropathological effects underlying disease-, trauma- and chemically induced neurodegeneration. Previously, we have shown that deficiency of TNF receptors protects against MPTP-induced striatal dopaminergic neurotoxicity, findings suggestive of a role for TNF- α in neurodegeneration. Here, we demonstrate that deficiency of TNF receptors suppresses microglial activation and alters the susceptibility of brain regions to MPTP. MPTP-induced expression of microglia-derived factors, TNF- α , MCP-1, and IL-1 α , preceded the degeneration of striatal dopaminergic nerve terminals and astrogliosis, as assessed by loss of striatal dopamine and TH, and an increase in striatal GFAP. Pharmacological neuroprotection with the dopamine reuptake inhibitor, nomifensine, abolished striatal dopaminergic neurotoxicity and associated microglial activation. Similarly, in mice lacking TNF receptors, microglial activation was suppressed, findings consistent with a role for TNF- α in striatal MPTP neurotoxicity. In the hippocampus, however, TNF receptor-deficient mice showed exacerbated neuronal damage after MPTP, as evidenced by Fluoro Jade-B staining (to identify degenerating neurons) and decreased microtubule-associated protein-2 (MAP-2) immunoreactivity. These effects were not accompanied by microglial activation, but were associated with increased oxidative stress (nitrosylation of tyrosine residues). These findings suggest that TNF- α exerts a neurotrophic/neuroprotective effect in hippocampus. The marked differences we observed in the regional density, distribution and/or activity of microglia and microglia-derived factors may influence the region-specific role for this cell type. Taken together, our results are indicative of a region-specific and dual role for TNF- α in the brain: a promoter of neurodegeneration in striatum and a protector against neurodegeneration in hippocampus.—Sriram, K., Matheson, J. M., Benkovic, S. A., Miller, D. B., Luster, M. I., O'Callaghan, J. P. Deficiency of TNF receptors suppresses microglial activation and alters the susceptibility of brain regions to MPTP-induced neurotoxicity: role of TNF- α . *FASEB J.* 20, 670–682 (2006)

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PROINFLAMMATORY CYTOKINES and chemokines (e.g., TNF- α , IL-1, and MCP-1) have been implicated as etiological factors in a variety of neurological disease states including Alzheimer's disease (1, 2), multiple sclerosis (3) and Parkinson's disease (PD) (4–6). Microglia and astrocytes are thought to be the primary sources of cytokines and chemokines that play a role in brain inflammatory responses (7–9). Both of these cell types exhibit a reactive phenotype in association with neurodegenerative diseases (10–13) as well as in response to neurotoxic insults (14–17). These observations suggest that conversion of glial cells to their “reactive” state, and the associated increase in expression of cytokines and chemokines, may play a role in neurodegeneration.

TNF- α is prominent among proinflammatory cytokines known to be associated with neuropathological effects underlying several neurodegenerative disorders (2, 3, 5, 18). TNF- α mediates its biological effects via its receptors, TNFRSF1A (TNFR1; p55) and TNFRSF1B (TNFR2; p75). Within the central nervous system, TNF- α is expressed predominantly by glial cells, especially microglia (19, 20). Expression of TNF- α in activated microglia has been reported in the substantia nigra of patients with PD (21, 22). Consistent with a role for TNF- α in dopaminergic neurodegeneration, we previously demonstrated that the effects of the dopaminergic neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), on multiple indices of damage to striatal dopaminergic nerve terminals, can

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be blocked in mice lacking both TNF receptors [*Tnfrsf1a*(-/-)/*Tnfrsf1b*(-/-); TNFR-DKO], but not in mice lacking the individual receptors (16). Based on these previous reports and our prior findings of neuroprotection in TNF receptor-deficient mice, we speculated that activated microglia may be involved in the early stages of a cascade of events leading to striatal dopaminergic nerve terminal degeneration and the accompanying astroglial reaction (16).

The neurotoxicity profiles of various MPTP dosing models have been established by assessing multiple indices of dopaminergic neuronal damage and, to a lesser extent, indices of the accompanying astroglial response (16, 17, 23–28) and microglial activation. Given the limited understanding of microglial involvement in MPTP neurotoxicity, in general, and our prior speculation that microglia-derived TNF- α may play an early role in disease- or toxicant-related dopaminergic neurodegeneration, the purpose of the present investigation was to examine the microglial response to dopaminergic nerve terminal damage after administration of MPTP. To extend our earlier findings, we examined proinflammatory cytokines and chemokines in addition to TNF- α and we also examined histological and biochemical markers of microglial activation. While our intent was to characterize the microglial reaction in striatum, the only known target of MPTP in our dosing model (e.g., see Materials and Methods and ref 17), we examined the hippocampus, a non-target region for MPTP effects, as a negative control. The hippocampus was examined because previous reports suggested that microglia-derived TNF- α may play a neuroprotective role against excitotoxic injury in this structure (29, 30). Our findings did, indeed, implicate activated microglia as participants in dopaminergic neurodegeneration caused by MPTP, but we found that TNF- α plays a neuroprotective role in hippocampus. These apparent region-selective effects associated with activated microglia suggest that TNF- α and other proinflammatory cytokines and chemokines play a dual role in the brain: cytotoxic to some brain regions and neurotrophic in others.

MATERIALS AND METHODS

Chemicals and reagents

MPTP-HCl was obtained from Aldrich (Milwaukee, WI, USA). Nomifensine maleate was purchased from Research Biochemicals, Inc. (Natick, MA, USA). Mouse anti-rat tyrosine hydroxylase (TH) monoclonal antibody and rabbit anti-rat TH polyclonal antibody were procured from Calbiochem-Novabiochem Corporation (San Diego, CA, USA). Horseradish peroxidase conjugated anti-rabbit IgG and ECL immunoblotting substrate were purchased from Amersham Biosciences (Piscataway, NJ, USA). Horseradish peroxidase conjugated anti-mouse IgG and the fluorogenic peroxidase substrate Quantablu were purchased from Pierce (Rockford, IL, USA). Mouse monoclonal antibody to MAP-2 was procured from Chemicon International (Temecula, CA, USA). Rabbit polyclonal antiserum to nitrotyrosine was purchased from Alpha

Diagnostic International (San Antonio, TX, USA). Rabbit polyclonal antibodies to NF κ B p50 and p65 subunits were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies to phospho-NF κ B p50 (Ser529) and phospho-NF κ B p65 (Ser276) were obtained from Abcam, Inc. (Cambridge, MA, USA). Fluoro-Jade B was a kind gift from Dr. Larry C. Schmued, NCTR/FDA, Jefferson, AR. FITC-conjugated anti-rabbit IgG was purchased from Vector Laboratories (Burlingame, CA, USA). Biotinylated GSA B₄-isolectin from *Griffonia simplicifolia* (GSA-IB₄) and ABC kit were obtained from Vector Laboratories. SuperscriptTM II RNase H⁻ reverse transcriptase and oligo (dT)₁₂₋₁₈ primers were procured from Invitrogen (Carlsbad, CA, USA). TaqMan[®] Universal PCR master mix, SYBR green 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 were purchased from Schleicher and Schuell (Keene, NH, USA). All other chemicals and reagents were of analytical grade and were purchased from Sigma (St. Louis, MO, USA).

Animals

Male mice aged 4–6 months (28–35 g) were used in all experiments. Mice carrying homozygous mutant alleles for both TNF receptors [*Tnfrsf1a*(-/-)/*Tnfrsf1b*(-/-); TNFR-DKO] were a kind gift from Dr. Larry Schook, University of Minnesota and were maintained on a C57BL/6J background (>10 backcross generations). Similarly, appropriate controls were maintained on a C57BL/6J background. In addition, to rule out any variations due to strain differences, another set of the knockouts (B6;129S-*Tnfrsf1a*^{tm11mx} *Tnfrsf1b*^{tm11mx}/J), appropriate strain controls (B6;129SF2/J) and wild-type C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and a comparative experiment of the strains was performed. All the animals were housed in a temperature (22±2°C) and humidity-controlled (30–40%) colony room maintained on a 12 h light:12 h dark cycle. Animals were allowed *ad libitum* access to chow and water. All animal experiments were carried out in accordance with CDC guidelines for Care and Use of Laboratory Animals. All procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of CDC-NIOSH. The NIOSH animal facility is accredited by the American Association for Accreditation of Laboratory Animal Care.

Drug treatment and dissections

Solutions of MPTP, calculated as free-base, were prepared fresh in 0.9% sodium chloride. Mice were administered a single dose of MPTP (12.5 mg/kg, s.c., as the base compound) or vehicle (0.9% saline) alone and killed by decapitation at various intervals of time (2–48 h) post-dosing. Nomifensine (25 mg/kg, s.c.) pretreatment was done 30 min prior to treatment with MPTP. For the experiments examining the activation of NF κ B, mice were killed by focused microwave irradiation to preserve steady-state protein phosphorylation, as described previously (31). The low-dose MPTP regimen we 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 (e.g., 17). By doing so, our intent was to evaluate very early changes associated with dopaminergic neurodegeneration in the striatum (e.g., see refs 32–36). The striatum and hippocampus from both hemispheres were dissected free-hand and used for isolation of total RNA or analysis of specific proteins. For histopatholog-

ical assessment, brains were post-perfusion fixed in 4% paraformaldehyde prior to cutting sections.

RNA isolation, cDNA synthesis, and real-time PCR amplification

Total RNA from striatum or hippocampus was isolated using Trizol[®] reagent (Invitrogen) and Phase-lock heavy gel (Eppendorf AG, Hamburg, Germany). The RNA was further cleaned using 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, TNF-α, IL-6, MCP-1 (CCL-2), IL-1α, MAC-1, and F4/80 was performed in an ABI PRISM 7700 sequence detection system (Applied Biosystems) 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. Real-time PCR analysis of TNFR1 and TNFR2 were performed using SYBR green PCR master mix and template-specific primers (400 nM) in an ABI PRISM 7700 sequence detection system (Applied Biosystems). Primers for murine TNFR1 and TNFR2 were procured from Invitrogen; the sequences are as follows: TNFR1 (X59238, 326bp), 5'-TCA AAG AGG AGA AGG CTG G-3' (FP) and 5'-CAC CAC AGC ATA CAG AAT CG-3' (RP); TNFR2 (NM_011610, 293bp), TGT AGC ATC CTG GCT ATT CC-3' (FP) and 5'-ATG AAG CAG TTC ACC AGT CC-3' (RP). All PCR amplifications (40 cycles) were performed in a total volume of 50 µL, containing 1 µL cDNA, 2.5 µL of the specific Assay on Demand primer/probe mix or 400 nM of custom primer set and 25 µL of either TaqMan[®] Universal master mix or SYBR green master mix, 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 after 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% SDS and stored at -75°C until use. Total protein was determined by bicinchoninic acid method (37) using bovine serum albumin as standard.

Tyrosine Hydroxylase (TH) ELISA

TH holoenzyme protein was assessed by a fluorescence-based ELISA developed in the laboratory. In brief, a mouse monoclonal antibody to TH was coated on the wells of Immulon-4 microtiter plates (Thermo Labsystems, Franklin, MA, USA). The SDS homogenates and standards (homogenates prepared from control mouse striatum) were diluted in phosphate-buffered saline (pH 7.4) containing 0.5% Triton-X 100 solution (PBS-T). After blocking nonspecific binding with 5% non-fat dry milk in PBS, aliquots of the homogenate and standards were added to the wells and incubated. After washes, a rabbit polyclonal antibody to TH was added to "sandwich" the TH protein between the two antibodies. 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 (read at 320/405). The amount of TH in the samples was calculated and expressed as µg TH/mg total protein.

Immunoblot analysis of TH

Changes in TH protein expression were analyzed from immunoblots. A linear range for the protein load in the immunoblot analysis of these proteins was established in our laboratory earlier (38). Aliquots of brain homogenates (3 µg) were diluted in sample buffer, boiled and loaded on 10% SDS-polyacrylamide gels (39). Proteins then were electrophoretically resolved and transferred to 0.1 µm nitrocellulose membranes (40). After transfer, immunoblot analysis for TH was performed. All steps were carried out at room temperature. Briefly, membranes were blocked for 1 h in 5% non-fat dry milk prepared in PBS-T, washed (1×15 min; 2×5 min) with PBS-T and incubated with antibodies to TH (rabbit polyclonal, 1:1000) for 2 h. After incubation with primary antibodies, blots were washed with PBS-T (1×15 min; 2×5 min) and subsequently incubated with anti-rabbit IgG-HRP conjugate (1:2500) for 1 h. Membranes were washed (1×15 min; 4×5 min) in PBS-T and the signals detected with a chemiluminescent substrate, Amersham ECL (Piscataway, NJ, USA), were captured on X-ray film (Fuji Medical Systems, Stamford, CT, USA), typically by exposure for 10 s–3 min depending on the signal intensity.

Histology

For histological evaluation of microglia, animals (saline or MPTP-treated wild-type mice; *n*=3 per group) were perfused transcardially with 100 mL of saline followed by 150 mL of fresh 4% phosphate-buffered paraformaldehyde. Brains then were post-fixed for 24 h in 4% paraformaldehyde and frozen sections (35 µm) were cut on a cryostat and stored as free-floating sections. Microglial staining was performed using 10 µg/mL of biotinylated GSA-IB₄ in the presence of 0.1 mM CaCl₂ and 0.1 mM MgCl₂. The sections were incubated with GSA-IB₄ overnight at 4°C. After washes in PBS (3×5 min), secondary amplification was performed using the ABC kit according to the manufacturer's recommendations. After washes in PBS (3×5 min), the sections were incubated with DAB substrate (0.5 mg/mL) for 5 min. The sections were then rinsed in PBS, mounted on Colorfrost + slides (Fisher Scientific, Pittsburgh, PA, USA), air-dried overnight, dehydrated through a series of graded alcohol, and cover-slipped with Permount.

For histological analysis of neuronal damage, another set of animals (saline or MPTP-treated wild-type or TNFR-DKO mice; *n*=4 per group) were perfused as above. After post-fix in 4% paraformaldehyde, the sections were embedded in paraffin. Sagittal 8 µm-thick sections were cut on a microtome and serial sections were mounted on Superfrost plus slides (Fisher Scientific). The paraffin embedded sagittal sections were deparaffinized, rehydrated and processed for antigen retrieval using an antigen unmasking solution (Vector Laboratories). Serial sections, in quadruplicate, were stained with hematoxylin and eosin (H&E) for anatomical/histopathological evaluation. Similarly, corresponding sections, from each group were stained with Fluoro-Jade B solution, a marker for localizing degenerating neurons (41). Sections were successively rinsed in 80% alcohol containing 1% sodium hydroxide and 70% alcohol for 2 min, respectively. After washes in distilled water (3×2 min), the sections were incubated in a 0.06% solution of potassium permanganate for 10 min and

washed in distilled water again as above. The sections were then incubated for 20 min in 0.001% Fluoro-Jade B prepared in 0.1% glacial acetic acid. After washes in distilled water the sections were air-dried for 5–10 min prior to mounting in an anti-fade solution, Vectashield (Vector Laboratories). The sections were examined under a fluorescence microscope (Olympus AX70) with a filter cube for FITC (excitation 460–490 nm; emission filter 515 nm; wide band pass). Digital (.tif) images were captured using Sony 3CCD color video camera (DXC9000, Sony Electronics Inc., NJ, USA) and Simple32 software (Compix Inc. Imaging Systems, Cranberry Township, PA, USA) and documented.

Immunohistochemistry for TH (mouse monoclonal, 1:1000), nitrotyrosine (rabbit polyclonal, 1:1000) or microtubule associated protein (MAP)-2 (mouse monoclonal, 1:1000), another marker for neuronal damage, were performed similarly on corresponding serial sections. In brief, sections were treated with 10% methanol/hydrogen peroxide solution for 15 min to quench endogenous peroxidase. The sections were washed (3×5 min) in PBS, then permeabilized in a solution containing 1.8% L-lysine, 4% normal horse serum and 0.2% Triton-X-100 in PBS. Primary antibody (1:1000) diluted in a solution containing PBS and 4% normal horse serum was then added to the sections and incubated overnight at 4°C. After overnight incubation, sections were washed in PBS (3×5 min) and incubated for 2 h at room temperature in FITC-conjugated anti-mouse IgG (1:500). Sections were then washed (3×5 min) with PBS and mounted in Vectashield. Epifluorescence was visualized in a photomicroscope (Olympus AX70) under blue light (for FITC; excitation 460–490 nm; emission filter 515 nm; wide band pass). Digital (.tif) images were captured using Sony 3CCD color video camera (DXC9000, Sony Electronics Inc., NJ, USA) and Simple32 software (Compix Inc. Imaging Systems, Cranberry Township, PA, USA) and documented.

Pathology scoring

Slides were scored for severity of pathological profiles after Fluoro-Jade B staining using a semiquantitative 4-point rating scale: 0 = no pathology; 1 = mild pathology; 2 = moderate pathology; 3 = severe pathology. Pathology scores were determined by low magnification microscopy (10×) of the entire hippocampus. Evaluation of pathology was performed by one author (SAB) and was verified by an independent observer who was blind with respect to treatment groups (KS).

Statistical analysis

Statistical analyses of data from biochemical assays were performed using SigmaStat (version 3.0) statistical software (Systat Software, Inc., Point Richmond, CA, USA). The test of significance was performed using one way ANOVA (ANOVA) followed by Student-Newman-Keuls (SNK) test. Values were considered statistically significant at 5% level of significance ($P < 0.05$). Graphical representations are mean \pm SE.

Statistical analysis of pathology quantification was performed using JMP (SAS Institute, Cary, NC, USA). Data were analyzed using a one-way nonparametric Wilcoxon/Kruskal-Wallis ANOVA, and differences in means were considered significant when $\text{Prob} > \chi^2$ values were less than 0.05.

RESULTS

MPTP causes a loss of striatal TH protein that is attenuated in TNFR-DKO mice

Administration of MPTP to wild-type C57BL/6J mice causes striatal dopaminergic neurodegeneration and

an attendant astrogliosis, as determined by loss of striatal dopamine and TH, and an increase in striatal GFAP (16, 17, 27, 42). Similarly, B6;129S mice administered MPTP exhibited an identical loss of striatal TH. This latter experiment was performed to confirm that no significant differences existed between the two strains, because the TNFR-DKO mice were originally on a B6;129S background prior to being outbred and maintained on a C57BL/6J background, as described in Materials and Methods. In TNFR-DKO mice, however, the loss of striatal TH protein was significantly attenuated compared with the TH protein decreases seen in wild-type mice (Fig. 1). Within 12 h of MPTP treatment, striatal TH protein levels decreased by 40–55% in wild-type C57BL/6J and B.129S mice, as determined by ELISA ($P < 0.05$; Fig. 1A). By 48 h post-dosing, the loss of TH was nearly 60% ($P < 0.01$; Fig. 1A). In contrast, the maximal decrement of striatal TH protein in TNFR-DKO mice was only 17%, indicating significant attenuation of TH loss (Fig. 1A). Since no significant differences were observed between the two strains, all subsequent experiments were performed using the C57BL/6J strain as wild-type controls. The choice of this strain was appropriate since the TNF receptor knockouts were maintained on this background for >10 generations. Immunoblot analysis of TH revealed similar differences between wild-type and TNFR-DKO mice, in agreement with the results from TH ELISA (Fig. 1B). The changes in striatal TH were verified by immunohistochemical analysis using coronal brain sections from wild-type and TNFR-DKO mice treated with saline or MPTP (Fig. 1C). These data, while confirming and extending the time course of our previous observation (16), also suggest that microglia-derived TNF- α may play a role in the neurotoxic effects of MPTP.

MPTP-induced striatal dopaminergic neurodegeneration elicits microglial activation

While activated microglia are implicated as etiological factors in several neurodegenerative diseases, their role in dopaminergic neurodegeneration has not been examined in great detail. Therefore, we evaluated microglial activation after administration of MPTP. MPTP-induced microglial activation was confirmed by staining with isolectin-B₄ (Fig. 2). Corresponding brain sections from saline and 24 h MPTP-treated wild-type mice stained for isolectin B₄ revealed microglial activation in the striatum after MPTP treatment (Fig. 2). Isolectin B₄ staining appeared to be confined to striatum; other brain areas were not affected (data not shown), consistent with the known targets of MPTP using our dosing regimen (17, 27). If microglial activation is engendered by MPTP-induced damage to dopaminergic nerve terminals, then neuroprotection with dopamine reuptake inhibitors should block the activation of microglia. Pretreatment with a selective dopamine reuptake inhibitor, nomifensine, abolished the striatal microglial activation elicited by MPTP, as revealed by isolectin-B₄ staining (Fig. 2).

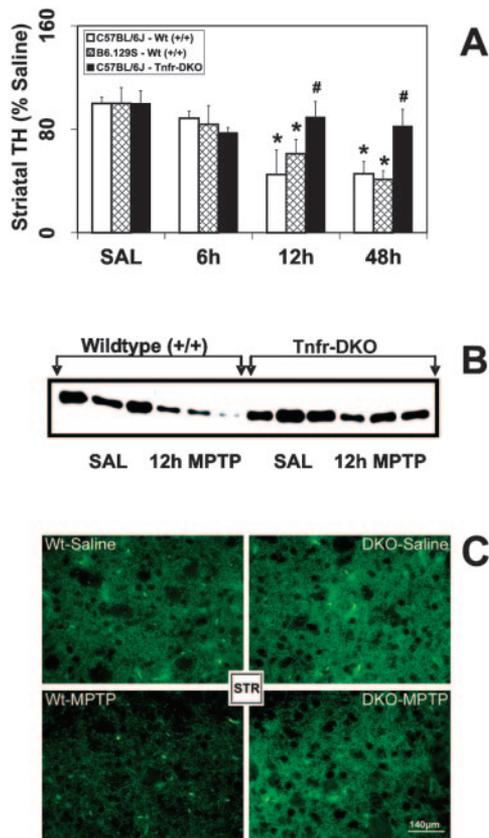


Figure 1. Loss of striatal TH protein due to MPTP is attenuated in TNFR-DKO mice. Wild-type C57BL/6J (□), wild-type B6.129S (⊠), and TNFR-DKO (■) mice were administered either saline or MPTP (12.5 mg/kg, s.c.) and killed at various intervals of time (6–48 h). *A*) Loss of striatal TH protein seen in wild-type C57BL/6J or B6.129S mice is attenuated in mice lacking both TNF receptors. Striatal TH protein levels were quantified by a fluorescence-based ELISA. Graphical representations are mean \pm SE ($n=5$ animals per time point) and TH levels are expressed as $\mu\text{g}/\text{mg}$ total protein. *Significantly different from saline-treated controls ($P<0.01$). #Significantly different from corresponding controls in wild-type group ($P<0.05$). Newman Keuls pairwise comparisons were used for post hoc statistical analysis. *B*) Immunoblot analysis of striatal TH protein. Representative samples (in triplicate) from saline and MPTP (12 h)-treated groups were used for immunoblot analysis. *C*) Wild-type and TNFR-DKO mice ($n=4$ per group) were administered either saline or MPTP (12.5 mg/kg, s.c.), killed 48 h after injection and processed for histopathological analysis. Four sets of serial coronal sections from each treatment group were independently stained in each analysis. Immunohistochemical analysis of striatal (STR) TH was performed to confirm the loss of striatal TH determined by ELISA and immunoblots. MPTP treatment caused extensive loss of striatal TH protein. The loss of striatal TH caused by MPTP was significantly attenuated in TNFR-DKO mice. Representative photomicrographs from each experimental group are depicted here. Scale bar = 140 μm .

MPTP treatment causes enhanced expression of microglial markers and microglia-derived cytokines and chemokines in the striatum

In addition to histological verification of MPTP-induced striatal microglial activation by analysis of isolectin B4 staining, we observed enhanced expression of

several markers known to be associated with activated microglia in the striatum of wild-type mice. This included increased striatal mRNA expression of cytokines and chemokines typically expressed by microglia, such as, TNF- α , MCP-1, and IL-1 α (Fig. 3A–C) and of the microglial marker F4/80 (Fig. 3D). The expression of these microglia-associated mRNAs was quantified by TaqMan[®] real-time PCR analysis. Within 2–4 h of dosing with MPTP, striatal TNF- α , MCP-1, and IL-1 α mRNA levels increased by 2.2, 4.3- and 3.7-fold ($P<0.05$), respectively (Fig. 3A–C). The mRNA levels increased in a time dependent manner and by 12–24 h after MPTP, the levels of these mRNAs increased by nearly 8- to 20-fold ($P<0.01$; Fig. 3A–C). A small increase (2.5-fold; $P<0.05$) in the mRNA expression of the microglial cell surface marker, F4/80, was observed by 12–24 h after MPTP (Fig. 3D). The time course of striatal F4/80 mRNA expression is consistent with histological evidence for microglial activation reported for a single subcutaneous dose of MPTP (43).

In the hippocampus, a nontarget region for the neurotoxic effects of MPTP, no up-regulation of TNF- α , IL-1 α , or F4/80 mRNAs was observed (Fig. 3E, G, H), consistent with a lack of damage and, therefore, of microglial activation in this structure. Nevertheless, a rapid and large increase in MCP-1 mRNA was observed in hippocampus that did not persist beyond 4 h post-dosing (Fig. 3F). The time course of the increase in MCP-1 mRNA in hippocampus did not coincide with the apparent time course for microglial activation seen with all other microglial markers analyzed in striatum, including MCP-1. Although an early activation of hippocampal microglia cannot be ruled out based on the hippocampal MCP-1 mRNA response, the significance of this increase was not further evaluated in the present study.

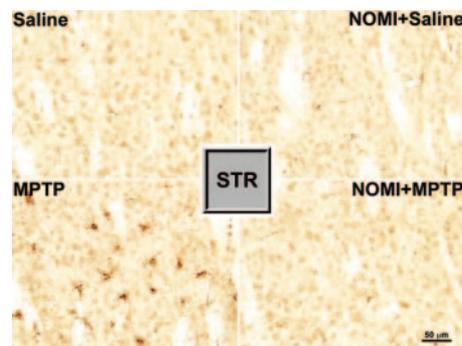


Figure 2. Striatal dopaminergic neurotoxicity due to MPTP is associated with microglial activation. Wild-type mice were administered either saline or MPTP (12.5 mg/kg, s.c.) and 24 h later were processed for immunohistochemical analysis of microglial activation using isolectin B₄. Immunostaining for activated microglia in brain sections from saline or nomifensine + saline-treated animals reveals no staining for striatal (STR) microglia in these control sections. Similar staining in MPTP-treated animals reveals intense staining (dark brown) of activated microglia in the striatum. Nomifensine pretreatment abolishes microglial activation associated with MPTP neurotoxicity, thus confirming pharmacological neuroprotection by nomifensine. Scale bars: A–D) 50 μm .

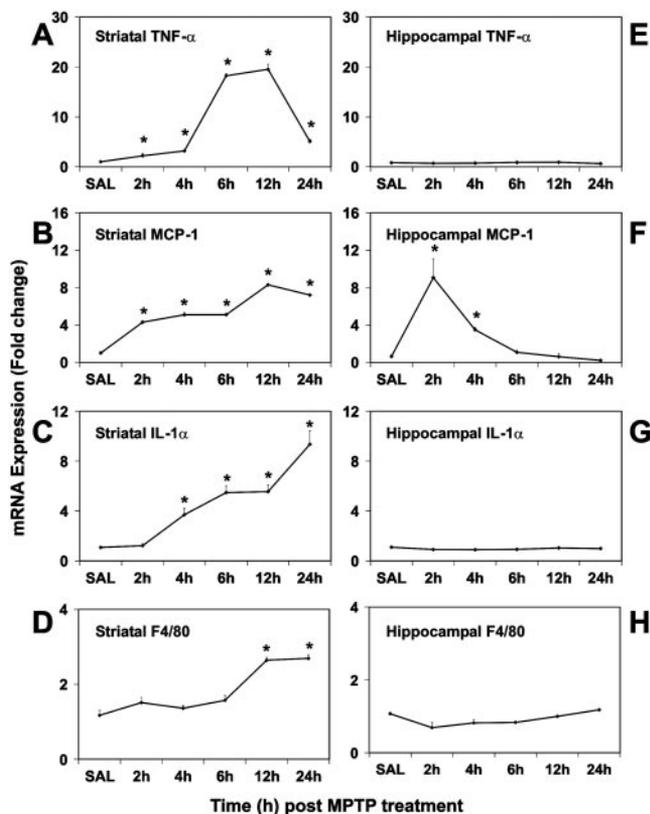


Figure 3. Striatal dopaminergic neurotoxicity due to MPTP is associated with up-regulation of microglial markers. Wild-type mice were administered saline or MPTP (12.5 mg/kg, s.c.) and killed at various intervals of time (2–24 h). Striatal mRNA expression of microglial factors, A) TNF- α , B) MCP-1, C) IL-1 α , and D) F4/80 were measured by TaqMan[®] real-time PCR analysis. E–H) The mRNA expressions of these factors in the hippocampus were similarly analyzed. Graphical representations are mean \pm SE ($n=6$ animals per time point; from 2 independent experiments) and are expressed as fold change over saline-treated controls. *Significantly different from respective saline-treated controls ($P<0.05$ – 0.01). Newman Keuls pairwise comparisons were used for post hoc statistical analysis.

Nomifensine pretreatment blocks MPTP-induced expression of microglial markers and microglia-derived cytokines and chemokines

It was possible that the enhanced expression of microglial markers and microglia-associated cytokines and chemokines seen in striatum in response to MPTP treatment was unrelated to nerve terminal damage and the subsequent activation of microglia. Therefore, as we did to confirm the origins of isolectin-B₄ staining (Fig. 2), we administered nomifensine prior to MPTP to block its neurotoxic effects. This pretreatment completely abolished the induction of TNF- α , MCP-1, IL-1 α , and F4/80 mRNAs in the striatum of wild-type mice (Fig. 4A–D), indicating that the enhanced expression of these microglia-associated factors occurs in response to striatal dopaminergic nerve terminal damage.

MPTP-induced up-regulation of TNF receptors is region-selective: Increased levels are observed in striatum but not in hippocampus

Of the microglia-associated cytokines and chemokines affected by MPTP treatment, TNF- α appears to exhibit the greatest response. A predicted consequence of this elaboration of TNF- α would be modulation of its receptors, TNFR1 and TNFR2. Analysis of the mRNA expression of these receptors by real-time PCR analysis indicated that TNFR1 and TNFR2 mRNAs were selectively up-regulated only in the striatum and not in the hippocampus of wild-type mice (Fig. 5A). Striatal TNFR1 mRNA expression increased by 2.7-fold ($P<0.05$) within 6 h of MPTP administration and continued to increase in a time-dependent manner. By 24 h after MPTP, an 8-fold increase ($P<0.001$) in TNFR1 mRNA was observed in the striatum. Striatal TNFR2 mRNA, however, did not significantly increase until 12 h after MPTP (2-fold, $P<0.05$). Neither TNFR1 nor TNFR2 mRNAs were induced by MPTP in the hippocampus (Fig. 5A). These effects are consistent with MPTP-induced expression of microglia-associated TNF- α in striatum but not in hippocampus.

Lack of nuclear factor- κ B (NF κ B) activation after MPTP treatment

Since MPTP treatment caused a rapid increase in the expression of TNF- α and its receptors, we examined whether MPTP also caused an induction of NF κ B, the downstream transcription factor associated with TNF

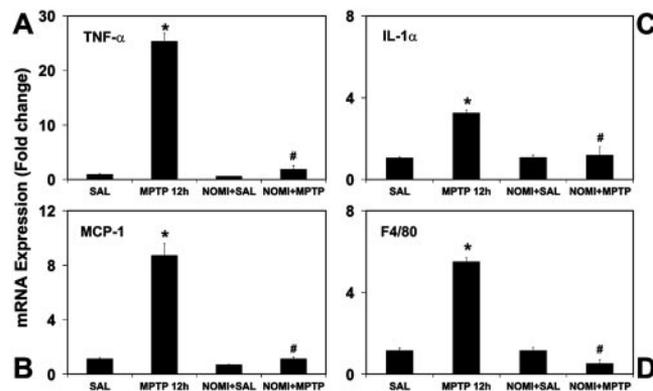


Figure 4. Striatal microglial activation due to MPTP is blocked by pharmacological modulation with nomifensine. Wild-type mice were pretreated with nomifensine (25 mg/kg, s.c.) 30 min prior to administration of either saline or MPTP (12.5 mg/kg, s.c.) and killed 12 h later. A–C) The striatal mRNA expression of microglia-derived factors, TNF- α , MCP-1, IL-1 α , and D) the microglial marker, F4/80, were measured by TaqMan[®] real-time PCR analysis. Graphical representations are mean \pm SE ($n=6$ animals per time point; from 2 independent experiments) and are expressed as fold change over saline-treated controls. *Significantly different from respective saline-treated controls ($P<0.05$ – 0.01). #Significantly different from MPTP-treated group ($P<0.001$). Newman Keuls pairwise comparisons were used for post hoc statistical analysis.

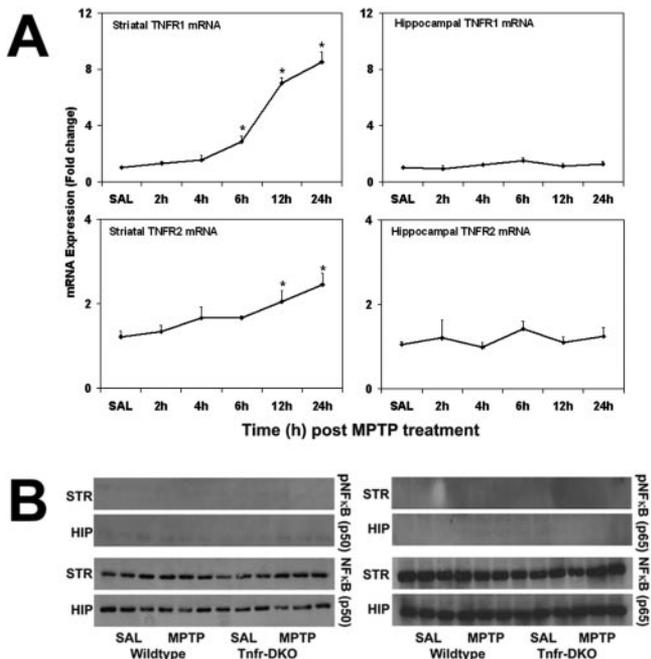


Figure 5. TNF receptors are selectively expressed in the striatum after MPTP treatment. Wild-type mice were administered either saline or MPTP (12.5 mg/kg, s.c.) and killed at various intervals of time (2–24 h). *A*) Striatal and hippocampal TNFR1 and TNFR2 mRNA expression was measured by TaqMan[®] real-time PCR analysis. Graphical representations are mean \pm SE ($n=6$ animals per time point; from 2 independent experiments) and are expressed as fold change over saline-treated controls. *Significantly different from respective saline-treated controls ($P<0.05$ – 0.01). Newman Keuls pairwise comparisons were used for post hoc statistical analysis. *B*) Immunoblot analysis of phosphorylated and unphosphorylated forms of NF κ B (p50 and p65) in striatum and hippocampus of wild-type or TNFR-DKO mice treated with MPTP. Representative samples (in triplicate) from saline and MPTP (48 h) -treated groups were obtained after sacrifice of mice by focused microwave irradiation to preserve steady-state protein phosphorylation.

signaling. Interestingly, MPTP treatment failed to induce NF κ B (levels or phosphorylation) in either the striatum or hippocampus of wild-type and TNFR-DKO mice (Fig. 5*B*). These findings suggest that NF κ B-independent mechanisms may be responsible for striatal dopaminergic nerve terminal damage caused by MPTP.

MPTP-induced microglial activation in the striatum is attenuated in TNFR-DKO mice

To further characterize the involvement of microglia in MPTP neurotoxicity and to evaluate the region-specific role exhibited by the proinflammatory cytokine, TNF- α , we examined the effects of MPTP in mice lacking TNF receptors. Specifically, we determined whether variations in microglial activation between wild-type and TNFR-DKO mice could underlie the observed regional differences in response to MPTP. To achieve this, we analyzed the mRNA expression of microglia-associated genes TNF- α , MCP-1, IL-1 α and

F4/80 mRNAs in these two groups of mice (Fig. 6*A–D*). By 12 h of MPTP administration, a 4- to 20-fold increase ($P<0.05$; Fig. 6*A–D*) in the levels of these mRNAs was observed in the striatum of wild-type mice, which was significantly attenuated in TNFR-DKO mice (Fig. 6*A–D*), findings consistent with decreased microglial activation. Since microglial activation has been associated with increased neurotoxicity, the above observation appeared to be consistent with striatal neuroprotection observed in these mice (16). Microglial activation, however, was not observed in the hippocampus of wild-type or TNFR-DKO mice (Fig. 6*E–H*), findings consistent with lack of microglial activation and of MPTP-induced damage in this structure.

Neuronal injury in the hippocampus of MPTP-treated TNFR-DKO mice

Histopathological analysis of the brains based on H&E staining revealed no significant differences between

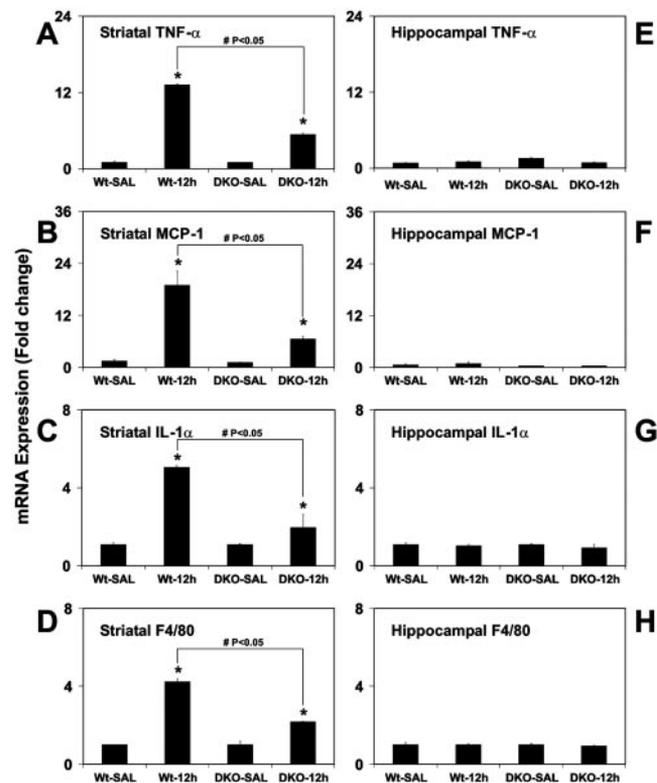


Figure 6. Striatal microglial activation due to MPTP is attenuated in mice lacking TNF receptors. Wild-type and TNFR-DKO mice were administered either saline or MPTP (12.5 mg/kg, s.c.) and killed 12 h later. *A–C*) The striatal mRNA expression of microglia-derived factors, TNF- α , MCP-1, IL-1 α , and *D*) the microglial marker, F4/80 were measured by TaqMan[®] real-time PCR analysis. *E–H*) The mRNA expressions of these factors were similarly measured in the hippocampus. Graphical representations are mean \pm SE ($n=6$ animals per time point; from 2 independent experiments) and are expressed as fold change over saline-treated controls. *Significantly different from respective saline-treated controls ($P<0.01$). #Significantly different from MPTP-treated group ($P<0.05$). Newman Keuls pairwise comparisons were used for post hoc statistical analysis.

wild-type and TNFR-DKO mice with respect to overall cytoarchitecture and apparent cellular density (Fig. 7A–C). A limitation of H&E staining, however, is that significant cellular loss must occur before pathological changes are observed (e.g., see ref 44). This drawback can be overcome by employing more sensitive indicators of neuronal damage, such as Fluoro-Jade B staining (44). Brains of wild-type mice administered MPTP did not exhibit any significant neuronal perikaryal damage based on Fluoro-Jade B staining (Fig. 7F–H). This was expected given the fact that the neurotoxicity of MPTP in our dosing model is predominantly restricted to damage of dopaminergic nerve terminals that do not stain with Fluoro-Jade B. Surprisingly, however, deficiency of TNF receptors rendered the hippocampus vulnerable to neuronal damage by MPTP, as evidenced by Fluoro-Jade B staining of degenerating neurons (Fig. 7F–H) and follow-up immunostaining of MAP-2 to

detect loss of dendritic processes (Fig. 7D, E). Fluoro-Jade B staining revealed neuronal degeneration in the CA3 area (Fig. 7G) and dentate gyrus (Fig. 7H) of the hippocampus in TNFR-DKO mice (quantitation of these data are presented in Fig. 7I). Similarly, MAP-2 immunostaining revealed significant loss of immunoreactivity in the hippocampus (CA3 region shown here) of TNFR-DKO mice (Fig. 7C). Thus, neuronal damage due to MPTP was seen in the hippocampus of TNFR-DKO mice in the absence of an associated microglial activation or astrocytic activation (data not shown). Fluoro-Jade B staining or MAP-2 immunostaining did not reveal any neuronal loss in the striatum of TNFR-DKO mice (data not shown). These data suggest that TNF- α plays region-specific roles in the CNS.

Basal levels of microglial markers and microglia-derived factors are higher in hippocampus than striatum

To determine the possible basis of the regional difference in the neurotoxic effects of MPTP seen above, we analyzed the expression of various microglia-associated factors in the striatum and hippocampus of wild-type mice. The mRNA expression of the microglial markers F4/80 and MAC-1 were significantly higher (2- and 5-fold, respectively; $P < 0.05$) in the hippocampus, suggesting that the number of microglia and/or their activation state varied across brain regions (Fig. 8A). The mRNA expression of cytokines and chemokines released from microglia, such as TNF- α , MCP-1, and IL- α , exhibited a similar pattern. The basal levels of mRNA expression of all these microglial factors were 3-fold higher in the hippocampus than in the striatum (Fig. 8B). Such regional differences in the microglial number and/or content may determine the role microglia take in influencing the fate of the neurons in that particular region.

Oxidative damage may underlie MPTP-induced hippocampal neuronal injury in TNFR-DKO mice

To determine the possible cause for increased vulnerability of hippocampal neurons in TNFR-DKO mice to MPTP, we examined the role of oxidative stress in mediating the neurotoxic outcome. As basal levels of microglia are higher in the hippocampus (as evaluated above) it seemed plausible that this region may be more vulnerable to oxidative damage, because microglia are known to be initiators as well as mediators of oxidative stress. Immunohistochemical analysis using anti-nitrotyrosine antibody revealed that MPTP treatment increased peroxynitrite-mediated oxidative injury selectively in the hippocampus of TNFR-DKO mice (Fig. 9). Extensive nitrotyrosine staining was observed in the CA1, CA3 (shown here; Fig. 9) and dentate gyrus regions of the hippocampus of MPTP-treated TNFR-DKO mice. These observations are consistent with the increased Fluoro-Jade B staining and decreased MAP-2 immunoreactivity (indicating neuronal degeneration)

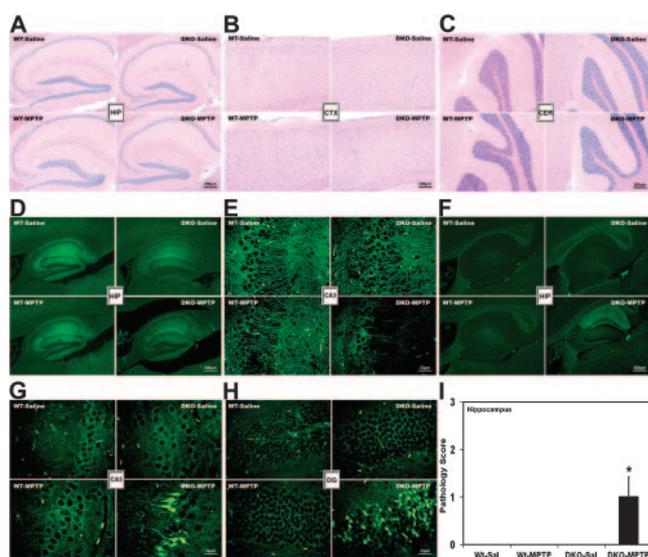


Figure 7. Lack of TNF receptors renders the hippocampus susceptible to MPTP-induced neurodegeneration: detection of increased Fluoro-Jade B staining and decreased MAP-2 immunostaining. Wild-type and TNFR-DKO mice ($n=4$ per group) were administered either saline or MPTP (12.5 mg/kg, s.c.), killed 48 h after injection and processed for histopathological analysis. Four sets of serial sagittal sections from each treatment group were independently stained in each analysis. A–C) H&E staining revealed no developmental anomalies in TNFR-DKO mice. (D, E) MAP-2 immunostaining revealed axonal and neurite loss in the hippocampus of TNFR-DKO mice, treated with MPTP. Extensive loss of MAP-2 immunoreactivity was observed in the CA3 region of the hippocampus. No loss of MAP-2 immunoreactivity was seen in wild-type mice treated with MPTP. F–H) Fluoro-Jade B staining reveals extensive neuronal degeneration in the CA3 and dentate gyrus (DG) regions of the hippocampus of TNFR-DKO mice after MPTP treatment. No neurodegeneration was detected in the hippocampus of wild-type mice. Representative photomicrographs from each experimental group are depicted here. I) Quantitation of MPTP-induced degeneration, as detected by Fluoro-Jade B staining, revealed significant damage in the hippocampus of TNFR-DKO mice. HIP, hippocampus; CTX, cortex; CER, cerebellum; CA3, cornu ammonis 3 field of hippocampus; DG, dentate gyrus. Scale bar: A–C) 200 μ m; D, F) 500 μ m; E, G, H) 33 μ m.

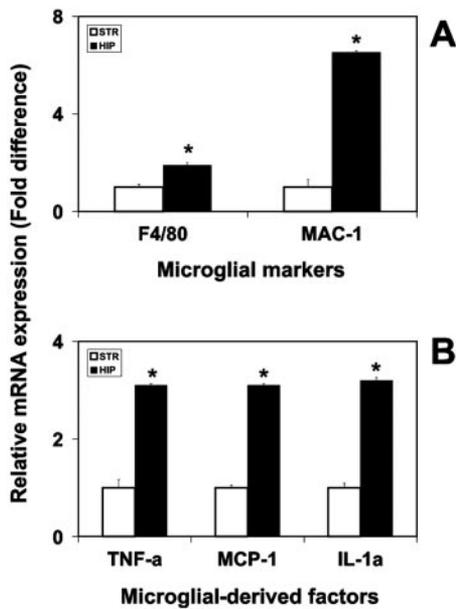


Figure 8. Regional differences in number and expression of microglial markers and microglia-derived factor. Wild-type mice were administered saline and killed 12 h later. *A*) mRNA expression of the microglial markers, F4/80 and MAC-1 in striatum (□) and hippocampus (■) reveals increased basal levels of expression in hippocampus. *B*) mRNA expression of the microglia-derived factors, TNF- α , MCP-1, and IL-1 α in striatum (□) and hippocampus (■) reveal a similar increase in basal levels of expression in hippocampus. The mRNA expression of these markers was measured by TaqMan[®] real-time PCR analysis. Graphical representations are mean \pm SE ($n=3$ animals) and are expressed as fold change over corresponding levels in striatum. *Significantly different from corresponding level in striatum ($P<0.05$ – 0.01). Newman Keuls pairwise comparisons were used for post hoc statistical analysis.

seen in the hippocampus of TNFR-DKO mice treated with MPTP.

DISCUSSION

Microglial activation and the elaboration of microglia-associated cytokines and chemokines have been linked to various forms of neurological and neurodegenerative disorders (2, 5, 18). Key among the cytokines associated with neurodegenerative processes is TNF- α , a proinflammatory cytokine that we previously implicated in the neurodegenerative effects of the dopaminergic neurotoxicant, MPTP. Here we have provided morphological and biochemical evidence for microglial activation in response to the dopaminergic nerve terminal damage caused by MPTP. We expanded the repertoire of microglial cytokines and chemokines associated with MPTP exposure beyond TNF- α to include MCP-1, and IL-1 α . Because TNFR-DKO mice were protected against MPTP-induced neurotoxicity, TNF- α and other microglia-derived cytokines and chemokines may play a role in dopaminergic nerve terminal degeneration caused by this toxicant. Nevertheless, our unex-

pected observation that the hippocampus of TNFR-DKO mice was rendered vulnerable to MPTP-induced neurotoxicity is indicative of a dual role of TNF- α in the brain: promoter of neurodegeneration in the striatum and protector against neurodegeneration in the hippocampus. A schematic illustration of the region-specific role for microglia-derived TNF- α in brain injury/neurotoxicity is represented in Fig. 10.

The low-dose MPTP regimen used in this study was established in our laboratory to cause selective degeneration of striatal dopaminergic nerve terminals and to elicit a glial response without affecting dopaminergic cell bodies in the substantia nigra (16, 17, 27, 42). While we have extensively documented that the astroglial response to MPTP in our model is linked to dopaminergic nerve terminal damage and restricted to the striatum (e.g., see ref 17), until now, we had no evidence for microglial activation in response to MPTP using our regimen. As with the astroglial response to MPTP (17, 27), we can now link the activation of microglia to dopaminergic nerve terminal damage. This view is based on the fact that protection of dopaminergic nerve terminals against MPTP in the striatum with a selective dopamine reuptake inhibitor, nomifensine, blocked microglial activation, the expression of microglia-associated cytokines and chemokines,

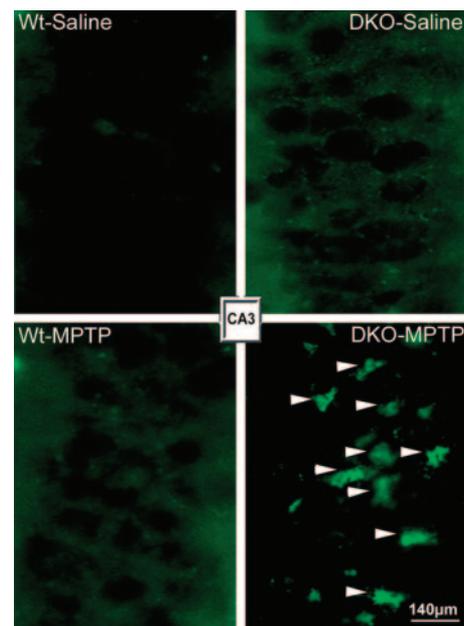


Figure 9. Lack of TNF receptors renders the hippocampus susceptible to MPTP-mediated oxidative injury: detection of increased nitrotyrosine staining. Wild-type and TNFR-DKO mice ($n=4$ per group) were administered either saline or MPTP (12.5 mg/kg, s.c.), killed 48 h later and processed for histopathological analysis. Four sets of serial sagittal sections from each treatment group were independently stained in each analysis. Immunohistochemical analysis of nitrotyrosine in saline or MPTP-treated wild-type mice or in saline-treated TNFR-DKO mice did not reveal any staining in the hippocampus. However, in MPTP-treated TNFR-DKO mice extensive nitrotyrosine staining was observed in the CA3 region. Representative photomicrographs from each experimental group are depicted. Scale bar = 140 μ m.

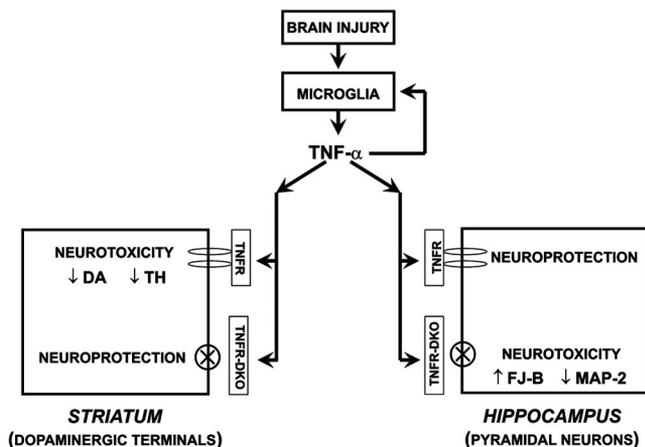


Figure 10. Schematic diagram depicting a dual role for TNF- α in brain. In the striatum, MPTP treatment results in activation of microglia and elaboration of microglia-derived proinflammatory cytokines, such as TNF- α . TNF- α triggers degeneration of the dopaminergic nerve terminals by signaling through its receptors localized on the dopaminergic neurons. Deficiency of both the TNF receptors results in attenuation of microglial activation and as a consequence the neurotoxicity. However, the lack of TNF receptors rendered the hippocampus vulnerable to MPTP-induced neuronal degeneration. Thus TNF- α plays a dual role in brain: neurotoxic in the striatum and neuroprotective in the hippocampus.

and the expression of a microglial selective cell-surface marker. The time course for these microglial-associated responses in striatum precedes the time course for astroglial activation in our model (17), findings consistent with a microglial response to neural injury that precedes astroglial activation in a variety of other neural damage models (45–47).

While extremely high doses of MPTP, or co-treatments that potentiate its toxicity, have been shown to cause moderate damage to targets other than the nigrostriatal dopaminergic pathway (48–52), we find damage to be restricted to the neostriatum with our dosing regimen (e.g., see ref 17). Thus, we did not expect to find evidence of a microglial reaction in hippocampus after MPTP. The lack of an effect of MPTP on TNF- α , IL-1 α , F4/80, TNFR1 and TNFR2 mRNA expression in the hippocampus is consistent with this expectation. The early and transient rise in hippocampal MCP-1 mRNA, a chemokine known to be associated with hippocampal microglial activation due to chemically induced neurodegeneration (53), could be viewed as evidence for an early microglial reaction to MPTP-induced damage to this structure. Although we cannot speculate as to the significance of the rise in hippocampal MCP-1 mRNA due to MPTP, it is unlikely to be due to microglial activation. This argument is supported by the fact that lectin staining did not reveal morphological evidence for microglial activation in hippocampus after MPTP and the time course for the increase and decline of MCP-1 mRNA was earlier than the time course for morphological evidence for microglial activation seen in striatum. Moreover, microglial activation usually is accompanied by astrogliosis, which

was not observed in hippocampus with our MPTP dosing regimen (e.g., see ref 17), nor was there evidence for neuronal degeneration in wild type mice as assessed by Fluoro-Jade staining or MAP-2 immunohistochemistry.

In TNFR-DKO mice we observed markedly attenuated MPTP-induced striatal dopaminergic neurotoxicity (16; also see Fig. 1). These data were suggestive of a detrimental role of microglia, as a source for TNF- α , in the observed neurotoxic effects of MPTP. However, there are contrasting reports on the involvement of TNF receptors in dopaminergic neurotoxicity, many of which are modeled on multiple dose regimens of MPTP (54, 55). Rousset et al (54) failed to observe neuroprotection of nigral dopaminergic neurons in TNF receptor deficient mice after an acute dosing regimen of MPTP. Leng et al. (55) using a chronic MPTP regimen also failed to observe neuroprotection. Nevertheless, an earlier study from the same group (56) shows that deficiency of the *Tnfa* gene decreases the loss of dopaminergic markers in the striatum but not in the nigra. Dose and dosing regimens of MPTP seem likely to have affected the outcome of examinations of the role of TNF- α in nigral, striatal or nigrostriatal damage caused by MPTP.

The extensive evidence obtained in the present study that document a striatal-localized elaboration of microglia-derived factors, in addition to TNF- α , is consistent with a role for microglial activation in association with MPTP-induced neurotoxicity. The diminished expression of microglial markers in the striatum of TNFR-DKO mice after MPTP argues for a contributory role for microglia in the observed dopaminergic neurotoxicity. These arguments notwithstanding, the role of microglia in the neurotoxicity of MPTP remains quite complex. For example, minocycline, a tetracycline derivative with anti-inflammatory properties distinct from its antimicrobial effects, has been shown to be neuroprotective against dopaminergic neurotoxicity caused by MPTP (57, 58). In contrast, other investigators have demonstrated that minocycline treatment worsened or exacerbated MPTP-induced injury (59). We recently observed (60) that minocycline did not afford neuroprotection against the neurotoxic effects of MPTP and methamphetamine in striatum, despite suppression of several markers of microglial activation in striatum. We attribute these results to the failure of minocycline to completely suppress TNF- α expression and signaling. Thus, in aggregate, we think the data suggest that microglia, in general, and TNF- α , in particular, are crucial players in striatal dopaminergic neurotoxicity caused by MPTP. Nevertheless, we note that microglia cannot serve as the primary target for initiation of neurotoxicity, given that pharmacological protection of dopaminergic nerve terminals with nomifensine blocks microglial activation and the production of microglia-associated factors. In addition, we cannot rule out the remote possibility that the apparent adverse effects of TNF- α and, potentially, of other proinflammatory cyto-

kines and chemokines, may not emanate from a microglial source after administration of MPTP.

The data obtained in the striatum of TNFR-DKO mice treated with MPTP demonstrated a neurotoxic role for TNF- α . Thus, it was quite unexpected to find evidence of neuronal damage in hippocampus of TNFR-DKO mice treated with MPTP, observations based on two independent measures of neuronal damage, Fluoro-Jade B staining and MAP-2 immunohistochemistry. Equally notable was the fact that microglial and astroglial activation, typical responses to neural injury, were not observed in hippocampus of TNFR-DKO mice after MPTP. With respect to TNF- α these observations are indicative of a neuroprotective role of this cytokine in hippocampus but a neurodegenerative role in the striatum. More generally, these observations suggest that activation of microglia (and perhaps astroglia as well) in hippocampus is neuroprotective, while microglial activation in striatum facilitates neurodegeneration. These observations are not without precedent. Hippocampal injury has been shown to be exacerbated in TNF-deficient mice (29) and inhibition of TNF- α in the striatum and hippocampus has been shown to be neurotoxic and neuroprotective to these structures, respectively (61). None of these observations rule out the possibility that physiological levels of hippocampal TNF- α can be neurotrophic, whereas specific pathological conditions resulting in an increase in hippocampal TNF- α could play a role in neurodegenerative responses in this structure, as observed for striatum after MPTP.

The regional differences in the apparent role of TNF- α in MPTP-induced neurotoxicity are indicative of a dual nature for TNF signaling in the brain. One of the implications of these findings is that the vulnerability/susceptibility of various brain regions or neuronal cell types could be attributed to differences in number and function of microglia across brain regions, including the repertoire of cytokines and chemokines elicited from activated microglia and the signaling pathways activated by microglial-derived cytokines or chemokines, such as TNF- α . Our analysis of the mRNA expression of select microglial markers revealed marked differences between striatum and hippocampus. Specifically, the microglial content in hippocampus was found to be significantly greater than that of striatum, as determined by the basal levels of F4/80 and MAC-1 mRNA. Similarly, the basal levels of TNF- α , MCP-1, and IL-1 α mRNA expression were significantly higher in the hippocampus than the striatum. Our findings are in accordance with earlier studies that reported differences in regional microglial content (62, 63) and are consistent with the regional differences we observed in the levels of the astroglial marker GFAP (64). Such variations in their distribution and content raise the possibility that regional differences exist in the way that microglia interact/cross-talk with their immediate neuro-astroglial microenvironment, as well as in the way they respond to external stimuli. Although basal levels of TNF- α are not necessarily indicative of a neuroprotective vs. a neurotoxic role of this cytokine, both of which have been reported (65–67), differences in the levels and activation state of the down-stream effectors of this

mediator may serve to confer differences in its functional roles. In this regard, earlier studies have indicated that TNF- α -mediated induction of nuclear factor kappa B (NF- κ B) is associated with neuronal survival (68–70). On the other hand, the inability of TNF- α to induce NF- κ B has been shown to be associated with increased neurotoxicity (71). Thus, one possible reason for the differential role exhibited by TNF- α could be attributed to the differences in the expression of NF- κ B among various brain regions. Indeed, such differences in the expression of NF- κ B have been known to occur in the CNS (61, 72). After our MPTP dosing regimen in wild-type and TNFR-DKO mice, we were unable to detect activation of NF- κ B in the striatum despite the observed increases in TNF- α . This observation is consistent with previous findings for MPTP (73) and suggests that lack of NF- κ B activation may result in increased neurotoxicity (71); thereby confirming that TNF- α may elicit a neurotoxic response in the striatum. In contrast, activation of NF- κ B by TNF- α in the hippocampus has been shown to be neuroprotective after neurotoxic insult (74). Thus, regional differences in the expression of TNF- α and in the signaling pathways that it activates, including downstream effectors such as NF- κ B, may determine the neurotoxic or neurotrophic outcome in that particular brain region.

Oxidative stress due to production of reactive oxygen species such as superoxide and hydroxyl radicals, or due to reactive nitrogen species such as peroxynitrite, has been implicated in a variety of neurological diseases states. These include PD (75, 76) as well as experimental models of dopaminergic neurotoxicity (77–79). In the present study, we have demonstrated that increased nitrotyrosine formation, an index of peroxynitrite-mediated oxidative injury, is associated with the degeneration of hippocampal neurons of TNFR-DKO mice to MPTP. Thus, consistent with above observations that TNF- α plays a neurotrophic role in the hippocampus, the lack of TNF signaling due to deficiency of its receptors rendered the hippocampus more susceptible to oxidative injury.

Taken together, our results are suggestive of a region-specific and dual role for microglia-derived TNF- α in the brain. One implication of these observations is that anti-TNF therapy directed against certain auto-immune and inflammatory disorders may have unexpected and negative consequences on the nervous system. Moreover, there is no assurance that such concerns should be limited to effects on nervous tissue. Finally, from a drug discovery and therapeutic intervention point of view, our findings suggest the need for comprehensive screening of potential drug candidates across all brain regions and to demonstrate caution in extrapolating the results of preliminary/preclinical anti-TNF studies into clinical practice. EJ

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