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Mice deficient in TNF receptors are protected against dopaminergic neurotoxicity: Implications for Parkinson's disease

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ABSTRACT

The pathogenic mechanisms underlying idiopathic Parkinson's disease (PD) remain enigmatic. Recent findings suggest that inflammatory processes are associated with several neurodegenerative disorders, including PD. Enhanced expression of the proinflammatory cytokine, tumor necrosis factor (TNF)-α, has been found in association with glial cells in the substantia nigra of patients with PD. To determine the potential role for TNF-α in PD, we examined the effects of the 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), a dopaminergic neurotoxin that mimics some of the key features associated with PD, using transgenic mice lacking TNF receptors. Administration of MPTP to wild-type (+/+) mice resulted in a time-dependent expression of TNF-α in striatum, which preceded the loss of dopaminergic markers and reactive gliosis. In contrast, transgenic mice carrying homozygous mutant alleles for both the TNF receptors (TNFR-DKO), but not the individual receptors, were completely protected against the dopaminergic neurotoxicity of MPTP. The data indicate that the proinflammatory cytokine TNF-α is an obligatory component of dopaminergic neurodegeneration. Moreover, because TNF-α is synthesized predominantly by microglia and astrocytes, our findings implicate the participation of glial cells in MPTP-induced neurotoxicity. Similar mechanisms may underlie the etiopathogenesis of PD.

Key words: brain • neurodegeneration • neuroprotection • MPTP

The neuropathological basis of Parkinson’s disease (PD) involves a progressive degeneration of midbrain dopaminergic neurons and their terminals in the striatum (1). The etiology of PD remains unknown; however, several mechanisms have been proposed to serve as the initiator of the neuronal damage and the subsequent cell death that characterize this disorder. These include mitochondrial dysfunction (2–4), oxidative damage (5–7), excitotoxicity, and α-synuclein deposition (8). In addition to these specific mechanisms, inflammatory processes that involve a host of cytokines have been shown to be associated with ongoing neuronal degeneration seen in several neurodegenerative diseases, including PD (9, 10). Proinflammatory cytokines are known to play a role in mitochondrial

impairment and oxidative stress; therefore, an inflammatory response may serve as an integral feature of the mechanistic underpinnings related to the pathogenesis of PD.

Astrocytes and microglia are thought to play a major role in brain inflammatory responses (11, 12). Both of these cell types exhibit a reactive phenotype in association with neurodegenerative diseases (13–19) as well as in response to neurotoxic insults (20–26). Recent evidence indicates that brain injury is associated with enhanced expression of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-4, IL-6, transforming growth factor (TGF)-α, and TGF-β1 (27–30). Indeed, an elevation in these proinflammatory cytokines has been linked to neurodegenerative disorders such as PD (14, 31), Alzheimer's disease (32, 33), multiple sclerosis (34), and stroke (35), conditions also associated with activation of microglia and astroglia. Thus, ample evidence exists to implicate cytokines as participants in the pathological processes underlying both neuronal and glial responses associated with PD and other degenerative diseases of the central nervous system (CNS).

Of the cytokines associated with PD, TNF-α appears to be the most strongly implicated. TNF-α is known to induce generation of reactive oxygen intermediates associated with necrotic cell death (36), and it also induces changes in mitochondrial ultrastructure and function (37, 38). Both of these damage mechanisms have been implicated in PD. Moreover, significant increases in the expression of TNF-α and its receptors have been reported in the caudate and putamen of postmortem brain samples from patients with PD (14, 39, 40). More recently, a polymorphism of the TNF gene in patients with PD has been associated with the early onset of the disease, suggesting a toxic role for TNF (41). To determine whether TNF-α may be involved in the pathogenesis of PD, we examined the effects of the dopaminergic neurotoxin, MPTP, in mice lacking the receptors for TNF.

MATERIALS AND METHODS

Chemicals and reagents

MPTP-HCl was obtained from Aldrich (Milwaukee, WI). Solutions of 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), calculated as free-base, were prepared fresh in isotonic saline. Purified bovine GFAP was purchased from American Research Products (Belmont, MA). Mouse anti-GFAP monoclonal antibody (clone G-A-5) was obtained from Chemicon International (Temecula, CA). Rabbit anti-cow GFAP polyclonal antibody was obtained from Dako Corporation (Carpenteria, CA). Mouse anti-rat TH monoclonal antibody and rabbit anti-rat TH polyclonal antibody were procured from Calbiochem-Novabiochem (San Diego, CA). Alkaline phosphatase conjugated anti-mouse IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Horseradish peroxidase conjugated anti-rabbit IgG and ECL immunoblotting substrate were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Horseradish peroxidase conjugated anti-mouse IgG and the fluorogenic peroxidase substrate, Quantablu, were purchased from Pierce (Rockford, IL). Alkaline phosphatase substrate, *p*-nitrophenyl phosphate, was obtained from Bio-Rad (Hercules, CA). Anti-rabbit IgG fluorescent kit, anti-mouse IgG fluorescent kit, Vectashield,

and antigen unmasking solution were obtained from Vector Laboratories (Burlingame, CA). AMV reverse transcriptase and *Taq* DNA polymerase were procured from Promega (Madison, WI). Nitrocellulose membranes were purchased from Schleicher & Schuell (Keene, NH). All other chemicals and reagents were of analytical grade and were purchased from Sigma (St. Louis, MO)

Animals

All animal experiments were carried out in accordance with CDC and NRC guidelines for Care and Use of Laboratory Animals. Male mice aged 4–6 months (28–35 g) were used in all experiments. Mice carrying homozygous mutant alleles for TNF receptors (TNFR1, TNFR2, or TNFR1/TNFR2) were maintained on a C57BL/6J background. The TNFR knockout mice and wild-type C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) 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. Mice were administered a single dose of MPTP (12.5 mg/kg, s.c.) or vehicle (saline) alone. Animals were killed at various time intervals (1–48 h postdosing) by cervical dislocation and decapitated, and brains were rapidly removed and placed on a cold plate. The striatum from both hemispheres was dissected free hand. Striatal tissues from the left hemisphere were used for extraction of RNA or estimation of dopamine, and those from the right hemisphere were used for analysis of specific proteins. For histopathological assessment, brains were post-perfusion fixed in 4% paraformaldehyde before cutting sagittal sections of the whole brain or coronal sections at the level of striatum.

RNA isolation, cDNA synthesis, and polymer chain reaction (PCR) amplification

Total RNA from striatum (pooled from two animals) was isolated by acid guanidinium thiocyanate:phenol:chloroform extraction method (42). DNase treated RNA (1 µg) was subjected to first-strand synthesis at 42°C for 60 min in a 20-µl reaction mixture containing 15 U of avian myeloblastosis virus (AMV) reverse transcriptase and 0.5 µg oligo $(dT)_{15}$ primer. At the end of the incubation period, the samples were maintained at 94°C for 5 min and then placed on ice to inactivate the enzyme. The cDNA (2–5 µl) was amplified by 27–40 PCR cycles in a 50-µl reaction containing 1.25 U of *Taq* polymerase and 10–50 pmol of template-specific primers for TNF-α, GFAP, and β-actin. The primer sequences were as follows: TNF-α, 5'-ATG AGC ACA GAA AGC ATG ATC-3' and 5'-CAG AGC AAT GAC TCC AAA GTA-3' (704 bp; ref 43); GFAP, 5'-CGA AGC TAA CGA CTA TCG CC-3' and 5'-TCA CAT CAC CAC GTC CTT GT-3' (462 bp); β-actin, 5'-AGG CAT TGT GAT GGA CTC CG-3' and 5'-AGT GAT GAC CTG GCC GTC AG-3' (300 bp). The thermal cycle profile was 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C, followed by a final 7-min elongation cycle. A portion (15 µl) of the PCR mixture was subjected to electrophoresis on a 2% agarose gel containing ethidium bromide. Gels were visualized in a multi-image light cabinet (Alpha Innotech, San Leandro, CA) and documented, and the band intensities were analyzed using image analysis software (Scion, Frederick, MD). The experiment was performed with two sets (n=2 in each set) of striatal samples, and each set was analyzed by at least tow independent reverse transcriptase (RT)-PCR reactions.

Tissue preparation for total and specific protein analysis

Tissues were weighed, homogenized in a cell disrupter (Kontes, Vineland, NJ) with 10 volumes of hot (90–95°C) 1% sodium dodecyl sulfate (SDS), and stored at −75°C until use. Total protein concentration in the SDS homogenate was determined by bicinchoninic acid (BCA) method (44) using bovine serum albumin as the standard.

GFAP ELISA

GFAP was assayed in accordance with a previously described procedure (45, 46). In brief, a rabbit polyclonal antibody to GFAP was coated on the wells of Immulon-2 microtiter plates (Dynatech Laboratories, Chantilly, VA). The SDS homogenates and standards were diluted in phosphate-buffered saline (PBS) (pH 7.4) containing 0.5% Triton-X 100 solution (PBS-T). After blocking nonspecific binding with 5% nonfat dry milk in PBS, 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, Menlo Park, CA). The amount of GFAP in the samples was calculated as micrograms GFAP per milligrams total protein.

TH ELISA

TH protein levels were assessed by fluorescence-based ELISA developed in this laboratory (J.P. O'Callaghan, unpublished results). A mouse monoclonal antibody to tyrosine hydroxylase was coated onto Immulon-4 microtiter plates (Dynatech Laboratories) and incubated at 37°C for 1 h. Following appropriate washes with PBS and blocking of nonspecific binding with 5% nonfat dry milk in PBS, aliquots of SDS homogenates of samples and standards were added to the wells and incubated further for 1 h. A rabbit polyclonal antibody was added to capture and sandwich the TH protein, which was then detected using a peroxidase-labeled antibody directed against rabbit IgG. Peroxidase activity was detected using a fluorogenic substrate, Quantablu (Pierce), that has excitation/emission maxima of 325/420 nm. The amount of TH in the samples was calculated as micrograms TH per milligrams total protein.

HPLC determination of dopamine and its metabolites

Dopamine and its metabolites were quantified by high-performance liquid chromatography (HPLC) with electrochemical detection (HPLC-EC; Waters, Milford, MA). Tissues were homogenized in 300 µl of ice-cold 0.2 M perchloric acid, containing 1 µM dihydroxybenzylamine (DHBA) as internal standard, and were centrifuged at 10,000*g* for 10 min at 4°C. The supernatant was filtered through a 0.2-µm membrane, and an aliquot (10 µl)

was injected from a temperature-controlled (4°C) automatic sample injector (Waters 717plus Autosampler) connected to a Waters 515 HPLC pump. Catecholamines were separated on a C18 reverse-phase column (LC-18 RP; Waters SYMMETRY, 25 cm × 4.6 mm; 5 μm), electrochemically detected (Waters 464 Pulsed Electrochemical Detector; range 10 nA, potential +0.7 V), and analyzed using Millennium^{32} Software. The mobile phase (pH 3.0) for isocratic separation of dopamine consisted of dibasic sodium phosphate (75 mM), octane sulfonic acid (1.7 mM), acetonitrile (10% v/v), and EDTA (25 μm). Flow rate was maintained at 1 ml/min. Dopamine, dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) standards (0.5–25 pmoles) were prepared in 0.2 M perchloric acid containing dihydroxybenzylamine (DHBA). Recovery of each analyte was adjusted with the internal standard and quantified from a standard curve. The levels of dopamine and its metabolites are expressed as micrograms per gram wet tissue.

Immunoblot analysis of GFAP and TH

In addition to analysis by ELISA, changes in the expression of TH and GFAP were analyzed from immunoblots of these proteins. A linear range for the protein load in the immunoblot analysis of these proteins has previously been established in our laboratory (47). Aliquots of brain homogenates (1–3 μg) were diluted in sample buffer, boiled, and loaded on 10% sodium dodecyl sulfate-polyacrylamide gels (48). Proteins then were electrophoretically resolved and transferred to 0.1-μm nitrocellulose membranes (49). Following transfer, we performed immunoblot analysis for TH and GFAP. All steps were carried out at room temperature. In brief, membranes were blocked for 1 h in 5% nonfat 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) or GFAP (rabbit polyclonal, 1:500) for 2 h. Following incubation with primary antibodies, we washed blots with PBS-T (1 × 15 min; 2 × 5 min) and incubated them with anti-rabbit IgG-HRP conjugate (1:2500) for 1 h. Membranes were washed (1 × 15 min; 4 × 5 min) in PBS-T, and signals detected using a chemiluminescent substrate (Amersham ECL, Piscataway, NJ) were captured on an X-ray film (Fuji Medical Systems, Stamford, CT), typically by exposure from 10 s to 3 min depending on the signal intensity.

Immunohistochemistry

For histological evaluation, animals (saline or MPTP treated +/+ and TNFR-DKO mice) were perfused transcardially with 50 ml of saline followed by freshly prepared and cooled 4% phosphate-buffered paraformaldehyde. Brains were postfixed for 24 h in 4% paraformaldehyde and bisected into the two hemispheres. The right hemisphere was processed in increasing grades of sucrose (10–30%), and frozen sections (coronal, 25 μm) at the level of striatum were cut on a cryomicrotome (Leica CM3000, Leica Microsystems, Deerfield, IL) and mounted on Superfrost plus slides (Fisher Scientific, Pittsburgh, PA). The left hemisphere was embedded in paraffin, and sagittal sections of 8 μm were cut on a microtome. Paraffin-embedded sagittal sections were deparaffinized, rehydrated, and processed for antigen retrieval by using an antigen-unmasking solution (Vector Laboratories). Two sets of coronal and sagittal sections were independently stained in each analysis. Immunohistochemistry for TH (rabbit polyclonal, 1:1000) and GFAP (rabbit polyclonal,

1:5000) were performed 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 and then permeabilized in a solution containing 1.8% L-lysine, 4% normal horse serum, and 0.2% Triton-X-100 in PBS. Primary antibody diluted in a solution containing PBS and 4% normal horse serum was then added to the sections and incubated overnight at 4°C. Following overnight incubation, sections were washed in PBS (3 × 5min) and the corresponding FITC-conjugated secondary antibody (1:500) was added and incubated for 2 h at room temperature. Sections were then washed (3 × 5min) with PBS and mounted in an antifade solution (Vectashield; Vector Laboratories). Epifluorescence was visualized in a photomicroscope (Olympus AX70) under blue light (for FITC; excitation 460–490 nm; emission filter 515 nm; wide band pass), and images were captured in TIFF format using a Sony 3CCD color video camera (DXC9000, Sony Electronics, New York, NY) and Simple32 software (Compix Imaging Systems, Cranberry Township, PA).

Statistical analysis

All analyses were performed using SigmaStat (version 2.03) statistical software. The test of significance was performed using 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 ± SE.

RESULTS

Time course of TNF-α and GFAP mRNA expression following administration of MPTP

Systemic administration of MPTP to wild-type +/+ mice resulted in a time-dependent increase in the mRNA expression of TNF-α and GFAP (Fig. 1). TNF-α mRNA was detectable as early as 3 h (Fig. 1A) following MPTP in the striatum but was undetectable in saline treated controls (Fig. 1A) or in a non-target region, hippocampus (data not shown). Southern blot analysis of the PCR amplimer, using a digoxigenin (DIG)-labeled human cDNA probe to TNF-α, confirmed the expression of TNF-α mRNA in the striatum (Fig. 1B). TNF-α mRNA expression preceded the expression of GFAP mRNA (12 h, Fig. 1C and 1D) and the loss of the dopaminergic markers, dopamine, and TH. These findings suggest that processes associated with enhanced expression of TNF-α precede neurodegeneration-induced astrogliosis.

MPTP-mediated loss of striatal dopamine is abolished in mice lacking TNF receptors

Systemic administration of MPTP to wild-type +/+, *TNFR1 –/–*, or *TNFR2 –/–* mice resulted in a significant loss of striatal dopamine by 70% ($P<0.001$), 63% ($P<0.001$), and 41% ($P<0.05$), respectively (Fig. 2A). However, the TNFR-DKO mice were protected against the dopamine-depleting effects of MPTP (Fig. 2A). Similarly, marked decreases in levels of DOPAC and HVA were observed (data not shown) in wild-type mice and *TNFR1 –/–* but not

among TNFR-DKO mice, findings suggestive of a role for TNF-α in MPTP-induced dopaminergic neurotoxicity.

MPTP-mediated loss of striatal TH is abolished in mice lacking TNF receptors

Concurrent with the loss of striatal dopamine, MPTP also decreased striatal TH levels that were measured by a fluorescence-based ELISA. Administration of MPTP to wild-type +/+, *TNFR –/–*, or *TNFR2 –/–* mice resulted in 54% ($P<0.001$), 50% ($P<0.001$), and 47% ($P<0.01$) decreases in striatal TH levels, respectively, 48 h postdosing (Fig. 2B). However, TNFR-DKO mice were completely protected against MPTP-induced changes in the levels of TH (Fig. 2B). When striatal TH levels were measured at 6, 12, and 48 h following MPTP, significant loss of TH was observed only at time points 12 h and beyond, thereby confirming that TNF-α expression preceded the loss of TH (data not shown). Immunoblot analysis of TH revealed similar differences between +/+ and TNFR-DKO mice in agreement with the results from TH ELISA (data not shown). The normal physiological levels of TH were not altered in either the single or double TNF-receptor-deficient mice, suggesting that these genetic manipulations did not have a regulatory effect on the expression of TH.

Double knockout of TNF receptors abrogates MPTP-mediated up-regulation of striatal GFAP

GFAP was assayed as an index of glial response to neuronal injury. Administration of MPTP to wildtype +/+ mice or to mice carrying a mutant allele for either of the TNF receptors resulted in upregulation of GFAP gene expression, leading to accumulation of GFAP protein. Striatal GFAP mRNA levels increased by 1.7-2.7 fold in each of these groups ($P<0.05$, Fig. 2C). By 48 h post-dosing, significant increases in striatal GFAP protein (fourfold over respective controls, $P<0.001$, Fig. 2D) was also observed in these groups of mice. However, in mice lacking both the receptors for TNF (TNFR-DKO), no significant upregulation of GFAP expression was seen (Figs. 2C&D). The normal physiological levels of GFAP were not altered in either the single or double TNF receptor deficient mice, suggesting that these genetic manipulations did not have a regulatory effect on the expression of GFAP. Together, these results reconfirm the involvement of TNF in the neurotoxicity of MPTP.

Mice deficient in TNF receptors are protected against MPTP-mediated loss of striatal TH immunoreactivity and associated reactive gliosis

MPTP treatment resulted in loss of TH immunoreactivity, and it induced astrogliosis in wildtype +/+ mice. A marked loss of TH immunoreactivity was observed following degeneration of dopaminergic terminals in the striatum (Fig. 3A, left panels). Loss of TH immunoreactivity was associated with a concomitant astrogliosis as reflected by an increase in GFAP immunostaining (Fig. 3B, left panels). In TNFR-DKO mice, immunostaining for TH (Fig. 3A, right panels) and GFAP (Fig. 3B, right panels) further confirmed the neuroprotection afforded by the TNFR double receptor knockouts. Immunohistochemical evaluation of TH and GFAP substantiated the changes that were observed by ELISA for TH and GFAP, respectively.

DISCUSSION

We have demonstrated that enhanced expression of TNF-α is associated with the earliest stages of damage in the MPTP model of dopaminergic neurotoxicity. Moreover, using mice lacking receptors for TNF, we showed complete protection against MPTP-induced neurotoxicity. The early onset of TNF-α expression after MPTP and the neuroprotective effect afforded to dopaminergic neurons by TNF receptor deficiency implicate this proinflammatory cytokine as a potential upstream effector in the neurodegenerative processes underlying PD.

The roles of cytokines in various forms of neurodegenerative disorders are extensively documented. Proinflammatory cytokines such as IL-6 and TNF-α are known to be associated with the neuropathological effects underlying neurological disorders such as Alzheimer's disease (33), PD (39), multiple sclerosis (50, 51), and traumatic brain injury (52, 53). These cytokines also are expressed following experimentally induced neurodegeneration. For example, dopaminergic selective neurotoxins such as 6-hydroxydopamine and MPTP, agents commonly used to create animal models of PD, are able to induce expression of TNF-α in the nigrostriatal pathway (54). Moreover, enhanced expression of TNF-α has been shown to lower tyrosine hydroxylase immunoreactivity in the caudate-putamen (55). Consistent with these observations, we found that MPTP was able to induce the expression of striatal TNF-α mRNA within 3 h of s.c. administration with a peak increase in expression at 12 h after MPTP. This time course of TNF-α mRNA expression preceded the loss of striatal dopamine and TH, indices of damage to dopaminergic nerve terminals affected by MPTP (56). The effects on TNF-α mRNA also preceded the induction of GFAP mRNA and protein, indices of astrogliosis caused by MPTP-induced damage to dopaminergic nerve terminals (56). Together, these data suggest that enhanced expression of TNF-α is an early feature of the sequence of events associated with degeneration of the nigrostriatal dopaminergic pathway.

The known association of TNF-α expression with neuropathology and our demonstration of its early induction in the MPTP model of dopaminergic neurotoxicity are suggestive of a participatory role for this cytokine in damage to the nigrostriatal dopaminergic pathway. We explored this possibility by using mice deficient in TNF receptors. Our results demonstrate that the effects of MPTP on multiple indices of damage to dopaminergic nerve terminals can be completely blocked in the TNFR-DKO mice, but not in the individual receptor-deficient mice. These results strongly implicate TNF-α and its receptors as mediators of damage to dopaminergic nerve terminals caused by MPTP. Moreover, these data suggest that neuroprotection requires the absence or deficiency of both TNF receptor subtypes. Earlier studies suggested that TNFR1 was the essential subtype for TNF-induced modulation of gene expression and cytotoxicity (57). Nevertheless, TNFR2 alone is capable of signaling cell death (58). Although each TNF receptor was thought to mediate distinct cellular responses, more recent studies provided evidence for receptor cooperation in mediating the cytocidal effect of TNF-α (59). Thus, inhibition or deficiency of either of the receptors alone may not prevent detrimental aspects of TNF-α-mediated events. Our observations are in concordance with these findings.

Our results stand in contrast to the results of an earlier study, in which lack of TNF receptors aggravated hippocampal excitotoxic injury (60). This disparity in the role of TNF-α in neurotoxicity may be explained by taking into consideration the region-specific effects of this cytokine. Because TNF-α is predominantly expressed by microglia (61–63) and astrocytes (63–65) in the CNS, differences in the intrinsic characteristics among these glial cell types and in the glial-neuronal interactions in specific brain regions may contribute to the altered responses to toxic insults. For example, a previous study demonstrated reduction of striatal excitotoxic injury by a TNF-α inhibitor, whereas exacerbation of the injury occurred in the hippocampus (66).

Activation of microglia and the induction of astrogliosis can be modulated by cytokines, and these cellular responses to brain injury often have been linked to inflammatory changes in the CNS (12, 67, 68). Our results demonstrate that induction of TNF-α mRNA precedes the induction of GFAP mRNA after MPTP. This time-effect relationship is consistent with the time course for microglial activation after MPTP (69). Indeed, activated microglia are known to express proinflammatory cytokines, including TNF-α. Because the results of the experiments with the TNF-receptor-deficient mice are suggestive of an obligatory role of TNF-α in MPTP-induced neurotoxicity, TNF-α producing microglia appear to be the most likely cellular effectors in the damage cascade. Consistent with this notion is the fact microglia are capable of producing damaging reactive nitrogen and oxygen intermediates (70).

Oxidative stress has been implicated in a variety of neurological diseases states, including PD (71, 72). Mitochondrial dysfunction also is known to be involved in several neurodegenerative disorders, including PD (73), as well as in neurotoxin-mediated neurodegeneration (74–79). Oxidative stress and mitochondrial dysfunction are the major factors that have been implicated in the dopaminergic neurotoxicity of MPTP. The early induction of striatal TNF-α observed in this study that precedes the generation of reactive oxygen species (ROS) following MPTP (76) is suggestive of the involvement of TNF-α in inducing oxidative stress. Indeed, ROS are generated through the signaling cascade mediated by TNF-α (80). Furthermore, mitochondria have been shown to participate in TNF-induced cell damage by generating ROS (81). Thus, MPTP-induced oxidative stress and mitochondrial dysfunction may be mediated by signaling cascades triggered by TNF-α. Previous demonstrations of the presence of TNF receptors on nigrostriatal dopaminergic neurons (14), the increased nuclear translocation of NF-κB in dopaminergic neurons (82), and the existence of a cytokine/CD23-dependent activation pathway of iNOS and proinflammatory cytokines in glial cells (83) in PD strengthen the proposition that TNF-α may be involved in the oxidant injury to the dopaminergic neurons in MPTP neurotoxicity as well as in PD.

In summary, our results implicate TNF-α as an obligatory component of dopaminergic neurotoxicity caused by MPTP, and they suggest that microglia may serve as the cellular effectors of TNF-α-mediated neurodegeneration. The broad implication of these findings is that pharmacological modulation of the TNF receptors or intermediates in its signaling

cascade may provide novel and, perhaps, a mechanistically based approach for the treatment of PD.

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Fig. 1

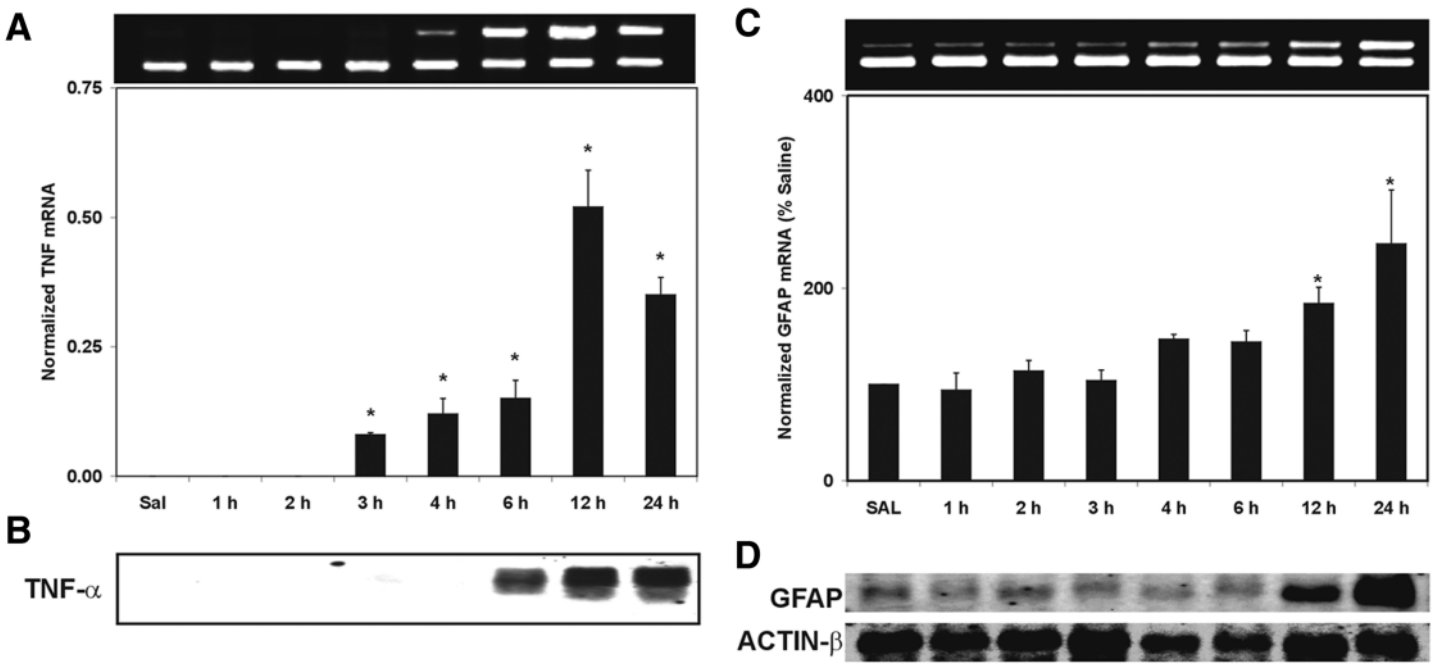

Figure 1. 1-Methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP)-mediated expression of striatal tumor necrosis factor (TNF)-α mRNA precedes the induction of GFAP mRNA. Wild-type +/+ mice were administered either saline or MPTP (12.5 mg/kg, s.c.) and killed at various time intervals (1–24 h). **A)** TNF-α mRNA expression was analyzed by reverse transcriptase-polymer chain reaction (RT-PCR). **B)** Southern blot analysis of the PCR amplimer with a digoxigenin-labeled human cDNA probe to TNF-α and detection with CDP-Star confirmed the specificity and size of the amplified product. **C)** GFAP mRNA expression was analyzed by RT-PCR. **D)** Northern blot analysis with a digoxigenin-labeled mouse cDNA probe to GFAP and detection with CDP-Star confirmed the expression of GFAP mRNA.Values are mean ±SE (n = 4 per time point). *Significantly different from saline-treated controls (P<0.01). Newman-Keuls pair-wise comparisons were used for post hoc statistical analysis.

Fig. 2

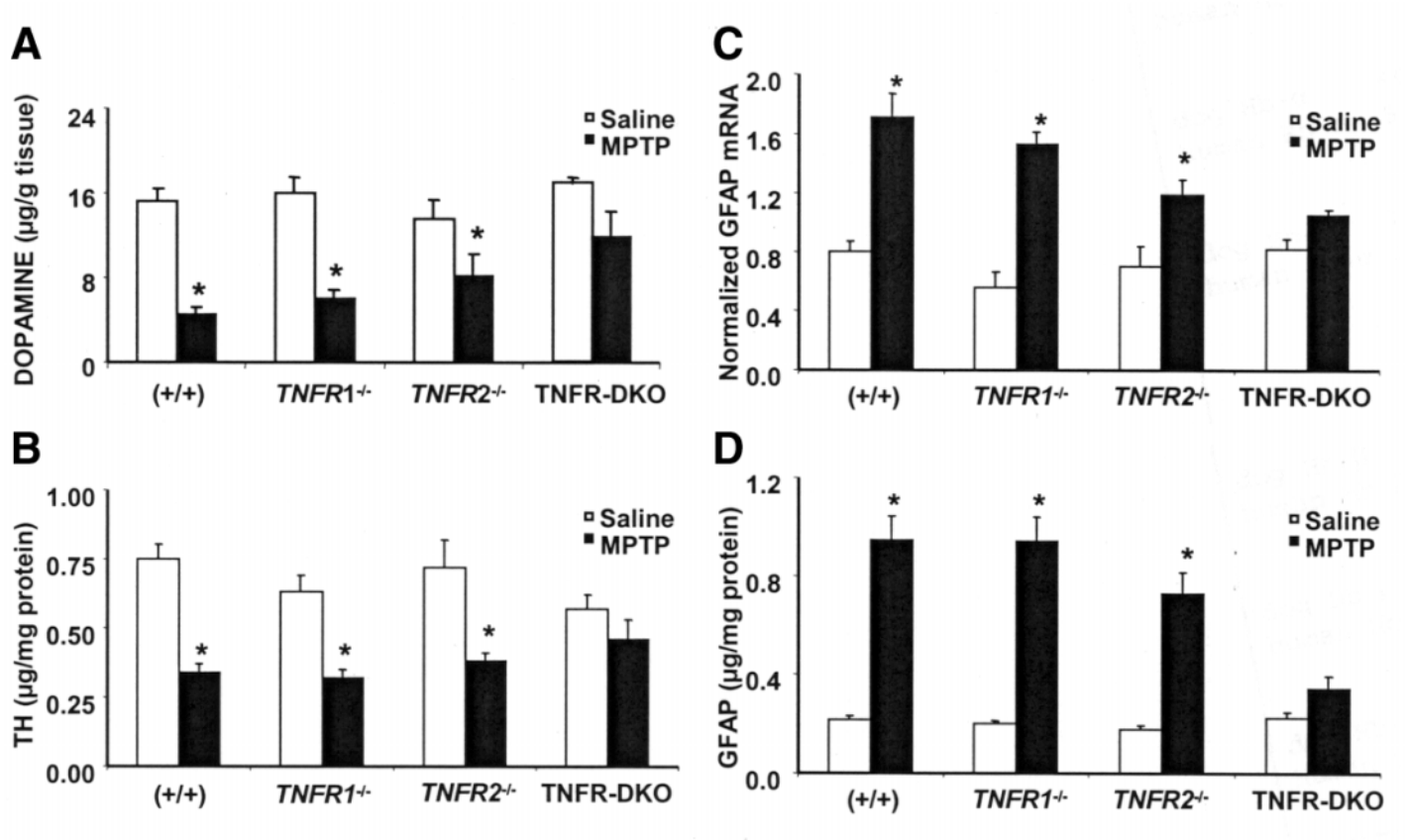

Figure 2. MPTP-mediated loss of striatal dopaminergic markers and induction of astrogliosis are abolished in TNFR-DKO mice. Wild-type +/+, *TNFR1–/–*, *TNFR2–/–*, and TNFR-DKO mice were administered either saline or MPTP (12.5 mg/kg, s.c.) and killed at 12 h or 48 h after treatment. **A)** Striatal levels of dopamine were quantified by high-performance liquid chromatography with electrochemical detection, 12 h postdosing (n = 5 per group). **B)** Striatal TH protein levels 48 h postdosing was quantified by ELISA (n = 8–10 per group). **C)** Striatal GFAP mRNA expression was analyzed by RT-PCR, 48 h posttreatment with MPTP (n = 4 per group). **D)** Striatal GFAP protein levels 48 h posttreatment were quantified by ELISA (n = 8-10 per group). Values are mean ±SE. *Significantly different from respective saline-treated controls (P<0.01). Newman-Keuls pair-wise comparisons were used for post hoc statistical analysis.

Fig. 3

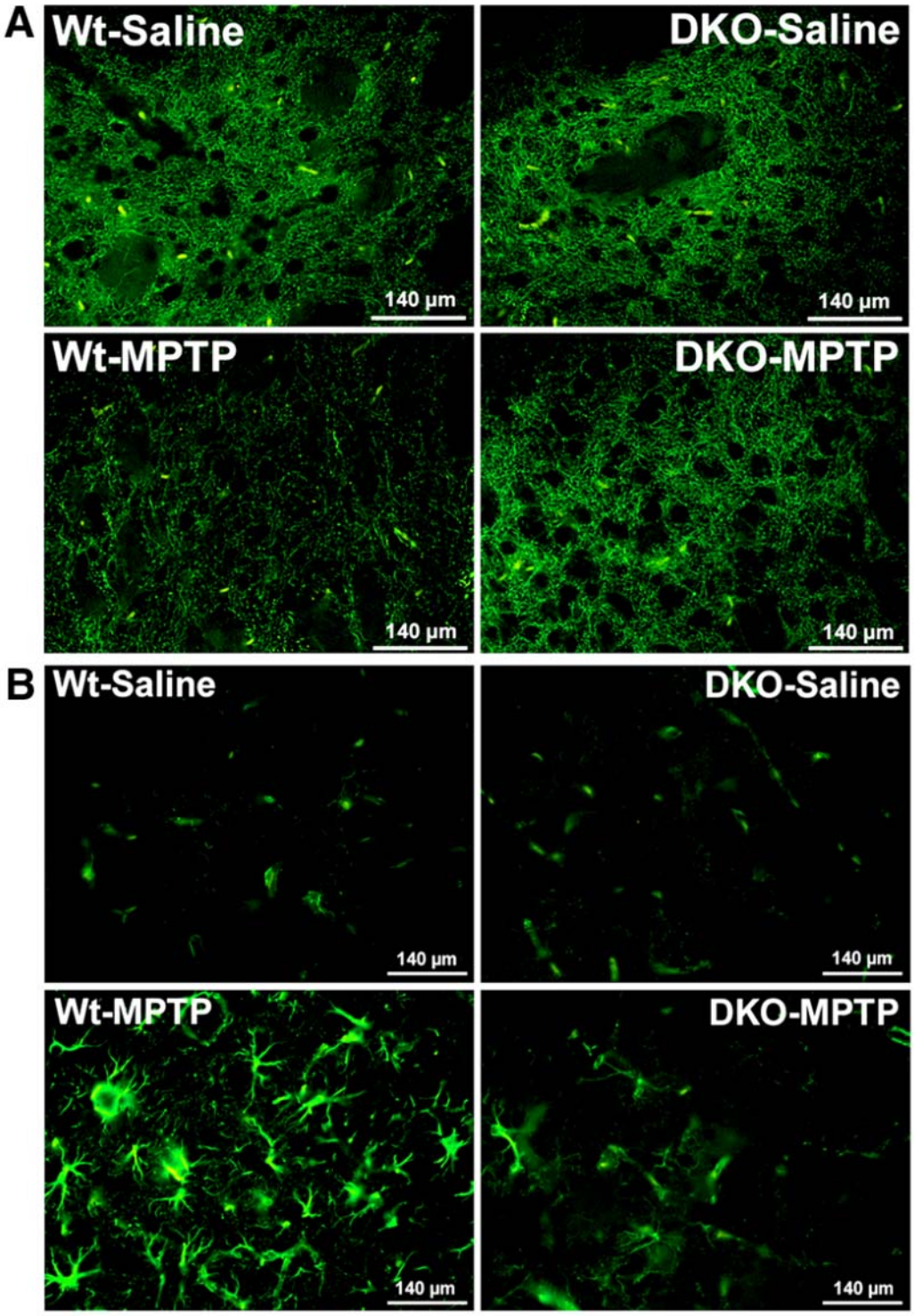

Figure 3. MPTP-mediated loss of TH immunoreactivity and increase in GFAP immunoreactivity are abolished in TNFR-DKO mice. Wild-type +/+, *TNFR1–/–*, *TNFR2 –/–*, and TNFR-DKO mice were administered either saline or MPTP (12.5 mg/kg, s.c.) and killed 48 h later. Two sets of coronal or sagittal serial sections were independently stained in each analysis for TH (polyclonal, 1:1000) and GFAP (polyclonal, 1:5000). **A)** TH immunoreactivity in striatum showing decreased staining in MPTP-treated wild-type +/+ mice and complete protection against MPTP neurotoxicity in TNFR-DKO mice. **B)** Concomitant increase in GFAP immunoreactivity (reactive astrogliosis) seen in the same field in wild-type +/+ mice is abolished in TNFR-DKO mice. Scale bar, 140 µm.