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Exacerbation of Dopaminergic Terminal Damage in a Mouse Model of Parkinson's Disease by the G-Protein-Coupled Receptor Protease-Activated Receptor 1 [S]

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ABSTRACT

Protease-activated receptor 1 (PAR1) is a G-protein-coupled receptor activated by serine proteases and expressed in astrocytes, microglia, and specific neuronal populations. We examined the effects of genetic deletion and pharmacologic blockade of PAR1 in the mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease, a neurodegenerative disease characterized by nigrostriatal dopamine damage and gliosis. After MPTP injection, PAR1−/− mice showed significantly higher residual levels of dopamine, dopamine transporter, and tyrosine hydroxylase and diminished microgliosis compared with wild-type mice. Comparable levels of dopaminergic neuroprotection from MPTP-induced toxicity were obtained by infusion of the PAR1 antagonist, BMS-200261 into the right lateral cerebral ventricle. MPTP administration caused changes in the brain protease system, including increased levels of mRNA for two PAR1 activators, matrix metalloprotease-1 and Factor Xa, suggesting a mechanism by which MPTP administration could lead to overactivation of PAR1. We also report that PAR1 is expressed in human substantia nigra pars compacta glia as well as tyrosine hydroxylase-positive neurons. Together, these data suggest that PAR1 might be a target for therapeutic intervention in Parkinson's disease.

Parkinson's disease is a neurodegenerative disease characterized by bradykinesia, tremor, loss of dopaminergic neurons in the *substantia nigra pars compacta*, the appearance of Lewy bodies, and a reduction in dopaminergic projection terminals to the striatum (Olanow and Tatton, 1999). Although some Parkinson's cases have been linked to genetic mutations, the majority are idiopathic (Olanow and Tatton, 1999). Whereas current therapy focuses on restoration of dopamine, no existing treatments alter progression of the disease.

Although the causes of Parkinson's disease are not understood, considerable evidence suggests a role for gliosis in the etiology and progression of the disease. Microglia, the major immune cell in the CNS, are found at highest density in the ventral midbrain (Lawson et al., 1990; Kim et al., 2000). Post mortem brain tissue from patients with Parkinson's disease show elevated levels of reactive microglia compared with age-matched control subjects (McGeer et al., 1988; Vila et al., 2001; Ouchi et al., 2005). Ouchi et al. (2005) demonstrated that increased microglia correlates with duration of disease,

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ABBREVIATIONS: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PAR1, protease-activated receptor 1; HPLC, high-performance liquid chromatography; BMS-200261, *trans*-cinnamoyl-*p*-fluoro-Phe-*p*-guanidino-Phe-Leu-Arg-Arg-NH_2; MPP+, 1-methyl-4-phenylpyridinium; WIN 35,428, 2β-carbomethoxy-3β-(4-fluorophenyl)tropane; TBS, Tris-buffered saline; ANOVA, analysis of variance; DAT, dopamine transporter; TH, tyrosine hydroxylase; PCR, polymerase chain reaction; CNS, central nervous system; MMP1, matrix metalloprotease-1; GFAP, glial fibrillary acidic protein; WT, wild type; ERK, extracellular signal-regulated kinase.

and occurs concurrently with decreased levels of the dopamine transporter in Parkinson's disease patients. Furthermore, epidemiological studies indicate that events leading to compromise of the blood-brain barrier increase the risk of Parkinson's disease, whereas daily use of nonaspirin, nonsteroidal anti-inflammatory drugs lowers risk of development by 40% (Taylor et al., 1999; Chen et al., 2003; Mayeux, 2003). Human and nonhuman primates exposed to the dopaminergic neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) show damage to the nigrostriatal dopamine system and increased reactive microglia years after the exposure, indicating chronic inflammation (Langston et al., 1983; Langston et al., 1999; McGeer et al., 2003). Treatments that dampen inflammation are neuroprotective in the MPTP model of Parkinson's disease in mice (Rousselet et al., 2002; Wu et al., 2002; Sánchez-Pernaute et al., 2004), suggesting that dopaminergic projections to striatum may be uniquely vulnerable to inflammation.

Protease-activated receptor 1 (PAR1) is a G-protein-coupled receptor that is expressed in the mammalian brain (Weinstein et al., 1995; Niclou et al., 1998; Junge et al., 2004), with particularly high levels in glia and dopaminergic neurons (Weinstein et al., 1995; Hamill et al., 2005). Serine proteases cleave the extracellular N terminus of PAR1 at Arg41 to reveal a new N terminus that acts as a tethered ligand, initiating a complex signaling cascade (Macfarlane et al., 2001). PAR1 function has been reported in neurons, astrocytes, and microglia (Suo et al., 2002; Wang et al., 2002a; Junge et al., 2004; Hamill et al., 2005). Moreover, PAR1 activation plays multifaceted roles in a number of neuropathological situations (Gingrich and Traynelis, 2000; Macfarlane et al., 2001; Ruf, 2003; Wang and Reiser, 2003; Suo et al., 2004), including stimulation of astrocytic and microglial proliferation (Suo et al., 2002; Sorensen et al., 2003; Wang and Reiser, 2003; Nicole et al., 2005). PAR1 is expressed throughout the basal ganglia, including the *substantia nigra pars compacta* and striatum (Weinstein et al., 1995; Ishida et al., 2006). Direct injection of serine proteases into the nigrostriatal pathway is selectively toxic to dopaminergic neurons (Carreño-Muller et al., 2003; Choi et al., 2003a), although some of the damage may reflect protease receptors other than PAR1 (Choi et al., 2003b). Thrombin also can induce delayed injury to corticostriatal cultures, with striatal damage dependent on PAR1 activation (Fujimoto et al., 2006). There also seems to be an up-regulation of PAR1 in post mortem samples from human patients diagnosed with Parkinson's disease (Ishida et al., 2006). Here we examine whether PAR1 activation plays a role in degeneration of dopaminergic terminal projections to striatum in the mouse MPTP model of parkinsonism. In this study, we used a moderate MPTP dosing paradigm that leads to damage of dopaminergic terminals (Tillerson et al., 2002) in an effort to model early events in Parkinson's disease. Our data suggest that removal or blockade of PAR1 can blunt the neurotoxic effects of MPTP on dopaminergic nerve terminals, raising the idea that PAR1 may be a plausible therapeutic target for Parkinson's disease.

Materials and Methods

MPTP Model of Parkinson's Disease. Male PAR1+/− mice (>95% C57BL/6; provided by Dr. Shaun Coughlin, University of California, San Francisco, CA) (Connolly et al., 1996) were bred with female C57BL/6 wild-type mice (The Jackson Laboratory, Bar Harbor, ME). Heterozygous littermates were bred to generate homozygous null mutants that were 97.5% C57BL/6. These mice were backcrossed again with wild-type C57 Bl/6 mice (The Jackson Laboratory), and the homozygous littermate wild-type or PAR1−/− used to establish the colony (>98.8% C57BL/6). PAR1−/− mice used in this study have been backcrossed > 7 generations with C57/Bl6 mice. Animals used for these studies were within five generations of the initial homozygous breeding pairs. All mice were maintained on a standard 12-h light/dark cycle and given ad libitum access to food and water. All procedures were approved by the Emory University Institutional Animal Care and Use Committee.

Mice (aged 90–120 days) were injected subcutaneously twice with 10 mg/kg MPTP or saline between the shoulder blades (at 7:00 AM and 7:00 PM) (Tillerson et al., 2002). This level of MPTP produces sustained damage of dopaminergic terminal projections in striatum without overt loss of dopaminergic cells, mimicking the early stages of parkinsonian degeneration. Mice were sacrificed by decapitation at 2 or 7 days, and a 1-mm section of the striatum (+1 mm to bregma) was dissected for both the left and right hemispheres. The left hemisphere was used in HPLC analysis, whereas the right hemisphere was used for immunoblot analysis. Otherwise, mice were overdosed on sodium pentobarbital and transcardially perfused with phosphate-buffered saline; the brains were drop-fixed in 4% paraformaldehyde for 1 week, cyroprotected in 20% sucrose, and cut into 40 μM coronal sections. Each experiment group contained at least three mice.

PAR1 Antagonist BMS-200261 Treatment. For studies involving pharmacologic blockade of PAR1, 3-day, 1 μl/h Alzet Minipumps were used for drug delivery. Empty pumps with flow moderators were weighed, filled with 6 mM BMS-200261 (Bernatowicz et al., 1996) or 10 mM HEPES-buffered saline, and then reweighed. Flow moderators were connected to a catheter, which was connected to an infusion cannula. Pumps were stored overnight at 37°C in sterile saline (0.9% NaCl) to prime drug release. The following day, 90- to 120-day-old mice were anesthetized using 5% isoflurane, placed in a small animal stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), and maintained with 2% isoflurane. The scalp was shaved, cleaned, and opened with a scalpel. Using hemostats, a subcutaneous pocket was created between the shoulder blades for the minipump. A small burr hole was drilled 1 mm lateral and 0.2 mm caudal from bregma (Hof et al., 2000). The cannula was lowered into the right ventricle (depth, 2.5 mm ventral) and affixed with dental cement (Hof et al., 2000), and the opening was sutured shut. Placement was verified in a subset of animals by injection of 0.5 μl of Evan's blue. Mice recovered for 12 h before treatment with MPTP. Because the minipump was placed between the shoulder blades, MPTP could not be administered as previously and was instead injected subcutaneously into the flank. The same dosing regimen (2 × 10 mg/kg) was used for the minipump-implanted animals. Mice were sacrificed by decapitation after 2 days.

High Performance Liquid Chromatography. HPLC was performed as described previously (Richardson and Miller, 2004). In brief, left striata were sonicated in 0.1 M perchloric acid with 347 μM sodium bisulfite/134 μM EDTA and centrifuged at 16,000*g* for 20 min (4°C). The supernatant was removed, centrifuged at 16,000*g*, and analyzed for levels of dopamine, 3,4-dihydroxyphenylacetic acid, and homovanillic acid by HPLC (column, MD 150, 3.2 mm × 8 cm, eight-channel coulometric electrode array; femtomole sensitivity; model 5600; ESA Inc., Chelmsford, MA). Quantification was made by reference to calibration curves. To measure conversion of MPTP to MPP+, animals were injected intraperitoneally with 20 mg/kg MPTP or phosphate-buffered saline (Giovanni et al., 1991). After 90-min survival, mice were sacrificed by decapitation and both striata were isolated, weighed, rapidly frozen on dry ice, and maintained at −80°C until HPLC analysis.

Immunoblot Analysis for Markers of Dopaminergic Neurons and for Gliosis. Samples were homogenized in 320 mM sucrose and 5 mM HEPES containing protease-inhibitor cocktail (1 μM aprotinin, 1 μM leupeptin, and 1 μM pepstatin A), and centrifuged at 2000*g* for 5 min. The supernatant was removed and centrifuged at 30,000*g* for 30 min. The pellets were resuspended in the homogenization buffer, protein concentrations were measured, and samples were subjected to polyacrylamide gel electrophoresis (10%). Samples were electrophoretically transferred to a polyvinylidene difluoride membrane and blocked with 7.5% nonfat milk in Tris-buffered saline composed of 135 mM NaCl, 2.5 mM KCl, 50 mM Tris, and 0.1% Tween 20, pH 7.4. Membranes were incubated in a monoclonal antibody to the dopamine transporter (rat anti-DAT antibody; Chemicon, Temecula, CA) in Tris-buffered saline with 2% nonfat milk diluted 1:5000. Antibody binding was detected using a goat anti-rat horseradish peroxidase secondary antibody (MP Biomedicals, Irvine, CA) and enhanced chemiluminescence (Pierce, Rockford, IL). The chemiluminescent signal was captured with a FluoroImager (Alpha Innotech, San Leandro, CA), stored as a digital image, and analyzed. Membranes were then stripped for 15 min at 25°C (stripping buffer; Pierce, Rockford, IL), and reprobed consecutively with a polyclonal rabbit tyrosine hydroxylase antibody (1:375; Chemicon), a polyclonal rabbit GFAP antibody (1:5000; Sigma, St. Louis, MO), a polyclonal rabbit GLUT5 antibody (1:5000; Chemicon), and monoclonal mouse α-tubulin antibody (1:5000; Sigma). Rabbit primary antibodies were visualized with goat anti-rabbit secondary diluted 1:5000 (Bio-Rad Laboratories, Hercules, CA). Mouse primary antibody was visualized with goat anti-mouse secondary diluted 1:10,000 (Bio-Rad Laboratories). For all, membranes were incubated in primary antibody for 8 to 12 h at 4°C and in secondary antibody for 1 h at 25°C. All samples were controlled for protein loading through normalization to the signal for tubulin.

Immunohistochemistry. Human tissue was obtained by Emory University Hospital Autopsy Service from patients who died from non-neurologic causes and whose brains were free of evidence of neurodegenerative disease. Tissue was obtained from four patients: two men (age 70 and 40) and two women (age 74 and 57). Tissue was fixed in 4% paraformaldehyde, paraffin-embedded, and cut into 8-μm sections. Paraffin was removed and sections were rinsed/incubated in 3% hydrogen peroxide to quench endogenous peroxidases. To remove neuromelanin, sections were incubated in 2.5% potassium permanganate (30 min) and 5% oxalate solution (5 min). Sections were rinsed and microwaved in citrate buffer (10 mM citrate monohydrate; 10 min). After cooling, sections were blocked in normal goat serum and incubated in the monoclonal PAR1 antibody (1:200; WEDE15) and/or polyclonal rabbit tyrosine hydroxylase antibody (1:1000; Chemicon) for 48 h at 4°C. PAR1 signal was visualized with tyramide-signal amplification with Alexa Fluor 546-conjugated tyramide (Invitrogen, Carlsbad, CA). Tyrosine-hydroxylase signal was visualized with Alexa Fluor 488-conjugated secondary antibody. Primary antibody was excluded for negative controls.

Mouse brain sections were incubated in 3% hydrogen peroxide (10 min) and blocked in 10% NGS, and incubated overnight at 4°C in antibody against tyrosine hydroxylase (1:1000; Chemicon), dopamine transporter (1:1000; Chemicon), or anti-GFAP (1:5000; Sigma). Sections were then incubated in biotin-conjugated goat anti-mouse antibody (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and signal detected using the avidin-biotin complex method of staining (which contains avidin complexed to peroxidase; Vector Labs, Burlingame, CA) and visualized with 3,3-diaminobenzidine tetrachloride (Sigma). For GFAP, staining was visualized using a Cy3-conjugated secondary antibody (1:200 goat-anti rabbit). Staining for MAC-1 was similar except that slices were incubated in primary antibody (1:500; Serotec, Raleigh, NC) for 48 h at 4°C before secondary incubation (biotin-conjugated goat anti-rat; Jackson ImmunoResearch Laboratories). For IB4 microglia staining, slices were incubated for 2 h with Cy3-conjugated IB4-lectin in a solution composed of 22 mM sucrose, 10 mM glucose, 150 mM NaCl, 10 mM HEPES, 3 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. Blood-brain barrier permeability staining was performed as described previously (Schmidt-Kastner et al., 1993). Sections were then incubated in biotin-conjugated goat secondary antibody (1:200; Jackson ImmunoResearch) and signal detected using the avidin-biotin complex method (Vector Laboratories, Burlingame, CA), and visualized with 3,3-diaminobenzidine tetrachloride (Sigma). Median eminence was examined as a positive control as this region normally has no blood-brain barrier.

Synaptosomal Dopamine and MPP+ Uptake, and [^{3}H]WIN 35,428 Binding. Dopamine uptake studies were performed as described previously (Elwan et al., 2006). In brief, crude synaptosomes were prepared from striatal tissue and incubated in assay buffer composed of 4 mM Tris, 6.25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.6 mM ascorbate, 5.5 mM glucose, and 0.01 mM pargyline, pH 7.4, containing a saturating concentration of dopamine (1 μM) and trace amount of [^{3}H]dopamine (20 nM). Likewise, MPP+ uptake was performed with a single concentration of MPP+ (1 μM) and trace amount of [^{3}H]MPP+ (20 nM). Uptake proceeded for 3 min at 37°C and was terminated by adding ice-cold buffer, followed by vacuum filtration over GF/B filter paper. Filters were washed twice, air-dried, and placed in scintillation vials containing 8 ml of ScintSafe Econo (Fisher Scientific, Pittsburgh, PA) for scintillation counting. Uptake rates were calculated from total uptake minus nonspecific uptake, with nonspecific uptake defined by the inclusion of 10 μM nomifensine for both dopamine and MPP+ studies. After determination of synaptosomal protein concentration (Bradford, 1976), uptake rates were calculated and expressed as picomoles per minute per milligram of protein.

Determination of [^{3}H]WIN 35,428 binding to the dopamine transporter was performed as described previously (Coffey and Reith, 1994) with modifications to reduce the total volume to 200 μl in 96-well microtiter plates (Elwan et al., 2006). In brief, binding studies with crude synaptosomes were conducted with a single concentration (10 nM) of [^{3}H]WIN 35,428 in 25 mM sodium phosphate buffer for 1 h at 4°C. Incubations were terminated by vacuum filtration onto GF/B filter plates; radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined by the inclusion of 10 μM nomifensine and specific binding was calculated as the total binding minus nonspecific binding. All experiments were performed at room temperature (23°C).

Extraction of mRNA and Real-Time Quantitative Reverse Transcriptase PCR. Extraction of mRNA was performed as described by the manufacturer (QIAGEN, Valencia, CA). RNA was eluted into 50 μl of RNase-free water, and concentrations measured using a spectrophotometer (Eppendorf Biophotometer; wavelength, 260/280 nm; Eppendorf North America, New York, NY). cDNA was generated using 1 μg of RNA, 10 μl of 10$\times$ random primers, and MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA). The solutions were brought to 50 μl with RNase-free water and incubated at 25°C (10 min) and at 37°C (2 h). Samples were stored at $-$20°C.

Real-time PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Bedford, MA). Reactions were performed in a total volume of 25 μl using SyBr Green Master Mix (Applied Biosystems); 2 μl of cDNA template synthesized as described above per sample was used, with 10 μM forward and reverse primers; the final concentration of template was 40 ng/reaction. Both the target and 18S amplifications were performed in duplicate. Thermal cycling conditions included 2 min at 50°C, 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 1 min at the annealing temperature. Primers for MAC-1(CD11b) were as follows: forward primer, 5′-tgtgccgagtcgctgaagtttgat-3′; reverse primer, 5′-ctaccacggtgccccctacgat-3′. Primers for protease, protease inhibitors, and protease-activated receptors were obtained from Superarray Bioscience Corporation (Frederick, MD) and used as specified. To normalize the amount of total mRNA present in each reaction, levels of the 18S ribosomal subunit were monitored in parallel samples.

Results were expressed as relative levels of mRNA, referred to as control samples chosen to represent 1× gene expression. The amount in the treated sample, normalized to reference (18S) and relative to the control sample, was defined by the C_t method (Livak and Schmittgen, 2001). Primer sets yielded a single PCR product as monitored using melting curves in each reaction well.

Ventral Midbrain Cultures and Treatment. Ventral midbrain was dissected from embryonic day 14.5 C57BL/6 mice and pooled in Dulbecco's phosphate-buffered saline without calcium or magnesium. Tissue was washed twice and dissociated using the Papain Dissociation System from Worthington Biochemicals (Freehold, NJ). In brief, tissue was incubated for 1 h at 37°C in Earle's balanced salt solution containing 20 U/ml papain, 1 mM L-cysteine, 0.5 mM EDTA, and 100 U/ml DNase, and then triturated through a fire-polished Pasteur pipette followed by a 1.5-inch 22G needle. The tissue suspension was centrifuged at 300*g* for 5 min, cells were taken up in 2 ml of protease inactivating solution (Earle's balanced salt solution containing 10% ovomucoid protease inhibitor and 100 U/ml DNase), layered onto 4 ml of ovomucoid protease inhibitor, and centrifuged at 70*g* for 7 min. Cells were taken up in 2 ml of plating medium (Invitrogen Neurobasal medium supplemented with B27 supplement, 2% FBS, 30 mM glucose, 0.5 mM glutamine, and 25 μg/ml gentamicin), counted using a hemacytometer, diluted to 600,000 cells/ml, plated in a 50-μl drop onto the center of a 12-mm coverslip coated with poly-D-lysine (10 μg/ml) and laminin (10 μg/ml), and placed in a 37°C incubator (5% CO_2; 100% relative humidity). Four hours later, wells (24-well plates) were topped up to a 1-ml final volume using plating medium. After 24 h in culture, plating medium was replaced with Neurobasal medium supplemented with B27 supplement, 30 mM glucose, 0.5 mM glutamine, 25 μg/ml gentamicin, and 5 ng/ml glial cell line-derived neurotrophic factor. Cells were used after 8 to 12 days in culture. Under these conditions, neurons (approximately 7% of which are tyrosine hydroxylase-positive) are the predominant cell type on top of a monolayer of astrocytes. Cultured mouse neurons were treated for 15 min at 37°C with vehicle or a maximally effective concentration (30 μM) of the PAR1-selective modified peptide agonist TFLLR-NH_2 (TFLLR; chemical structure, Thr-Phe-Leu-Leu-Arg-NH_2) (Hollenberg et al., 1997; Nicole et al., 2005). Neurons were then fixed in 4% paraformaldehyde at room temperature for 10 min and washed in phosphate-buffered saline three times, 0.1% Triton-X, and blocked with 3% bovine serum albumin in Tris-buffered saline (TBS) for 20 min. Neurons were incubated overnight at 4°C in monoclonal mouse anti-tyrosine hydroxylase (1:1000; Chemicon) and monoclonal rabbit antibody recognizing extracellular signal-regulated kinase that was phosphorylated at both threonine-202 and tyrosine-204 (phospho-ERK, 1:250; Cell Signaling Technology, Danvers, MA). After washing in TBS, neurons were incubated at room temperature in goat anti-mouse Alexa Fluor 488 (1:500; Invitrogen) and goat anti-rabbit antibody conjugated to Texas red (1:200, 4 h; Invitrogen) for 2 h. After rinsing three times with TBS, neurons were mounted with Vectashield medium (Vector Laboratories, Burlingame, CA) and viewed with Olympus IX71 microscope.

Statistics. Statistical evaluation was performed using unpaired *t* test, one-factor ANOVA with Newman-Keuls post hoc test, or two-factor ANOVA with Bonferroni post hoc test where appropriate. The number of observations are given in the figure legends or text; all F values and degrees of freedom for two-factor ANOVAs are provided in Supplementary Table 1. $p < 0.05$ was considered to be significant. Data are given as mean ± S.E.M.

Results

PAR1−/− Mice Showed Reduced MPTP-Induced Damage of Nigrostriatal Dopamine Pathway. We used the mouse MPTP model of Parkinson's disease to determine whether PAR1 activation contributes to damage of the dopaminergic system. Reasoning that findings obtained using a model of early disease might identify clinically useful intervention strategies, we chose an MPTP dosing regimen that causes significant dopaminergic terminal destruction with little cell loss, thus mimicking the dying-back phenomena thought to occur in early Parkinson's disease (Aubin et al., 1998; Saporito et al., 1999; Schmidt and Ferger, 2001; Tillerson et al., 2002; Rommelfanger et al., 2004). For all parameters, MPTP induced a significant change compared with saline-injected control mice (Supplementary Table 1).

PAR1−/− mice showed considerably less striatal damage compared with age-matched wild-type control mice 2 days after MPTP injection, as determined by immunohistochemistry (Fig. 1a) and protein levels of dopamine transporter and tyrosine hydroxylase (Fig. 1b), key markers of striatal integrity. Wild-type mice treated with MPTP lost 56 ± 4% of the dopamine transporter and 42 ± 4% of tyrosine hydroxylase compared with vehicle-treated control mice. By contrast, the PAR1−/− mice treated with MPTP lost only 22 ± 8% (Fig. 1b; $p < 0.01$; two-factor ANOVA) of the dopamine transporter and 8 ± 6% (Fig. 1b; $p < 0.01$; two-factor ANOVA) of the tyrosine hydroxylase compared with vehicle-treated control mice. These immunoblot results were supported by immunohistochemical analysis within the mouse striatum. Wild-type mice treated with MPTP showed a large reduction in dopamine transporter and tyrosine hydroxylase, as indicated by noticeably lighter staining in MPTP-treated wild-type mice compared with PAR1−/− mice (Fig. 1a). MPTP caused uniform decreases in dopamine transporter and tyrosine hydroxylase protein expression across wild-type striatum, whereas PAR1−/− mice showed staining evenly throughout the striatum that was similar to saline-injected control mice.

To confirm that the reduction in damage to dopaminergic markers did not reflect simply a delay in damage in PAR1−/− mice, we analyzed mice 1 week and 1 month after MPTP treatment. As shown in Fig. 1c, PAR1−/− mice showed significantly higher levels of dopamine transporter and tyrosine hydroxylase than vehicle-treated controls 7 days after MPTP treatment ($p < 0.01$ for both; two-factor ANOVA). This result was supported by immunohistochemistry, which revealed attenuated loss of dopamine transporter and tyrosine hydroxylase throughout the striatum in PAR1−/− mice after MPTP treatment (data not shown; $n = 3$). Likewise, we found that after 30 days, the PAR1−/− mice still maintained higher levels of striatal dopamine transporter (DAT) and tyrosine hydroxylase (TH) than their wild-type counterparts, documenting the persistence of the terminal damage and suggesting that long-term protective effects accompany the removal of PAR1 (Fig. 1d; $p < 0.1$ for both; two-factor ANOVA).

PAR1−/− Mice Showed Reduced MPTP-Induced Dopamine Loss. To evaluate the functional consequence of reduced expression of dopamine transporter and enzymes for dopamine synthesis, we measured striatal dopamine levels as well as the levels of metabolites 3,4-dihydroxy-phenylacetic acid, and homovanillic acid. Again, the PAR1−/− mice had significantly higher residual levels of dopamine than their wild-type counterparts after MPTP exposure. After 2 days, wild-type mice lost 54 ± 5% of striatal dopamine, whereas PAR1−/− mice lost 35 ± 4% (Fig. 2a; $p < 0.01$; two-factor ANOVA). Similar results were seen for the dopamine metabolites (data not shown). The higher levels of do-

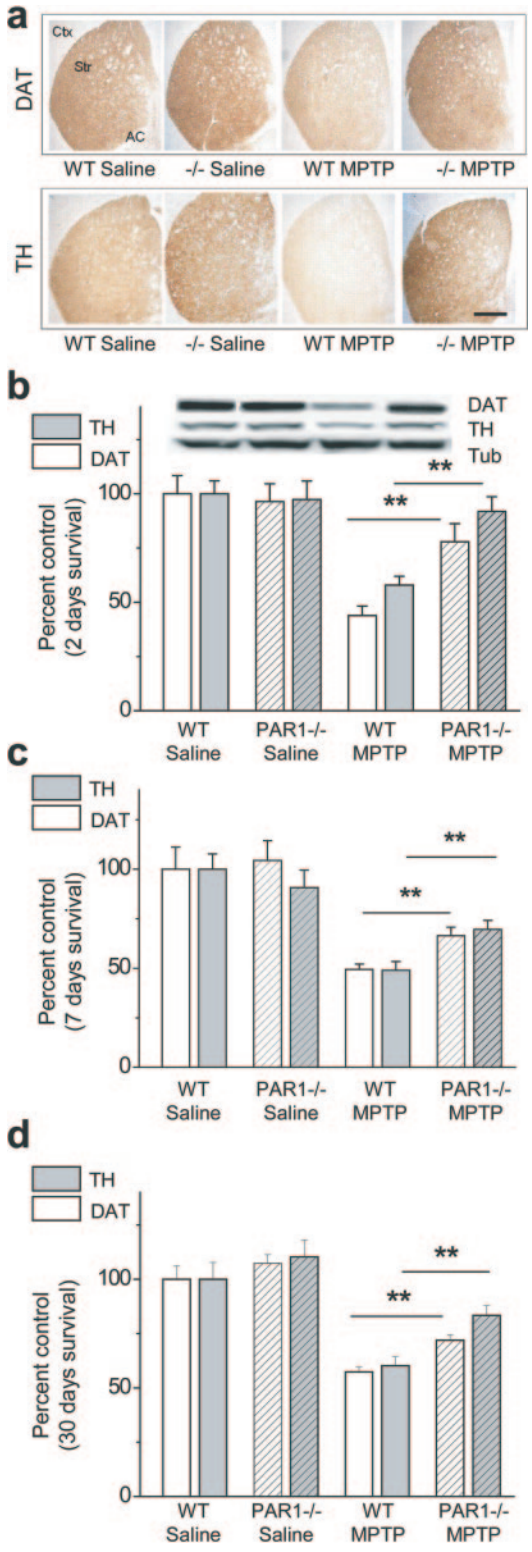

Fig. 1. PAR1−/− mice are structurally protected against MPTP-induced damage. a, immunohistochemistry for DAT and TH show that MPTP treatment reduced staining in wild-type mice, whereas PAR1−/− mice resemble saline controls after 2 days' survival. Staining was visualized with diaminobenzadine (Ctx, cortex; Str, striatum; AC, anterior commissure). Scale bar represents 500 μm. b–d, the quantification of DAT and TH immunoblots is shown. Wild-type (WT) mice expressed significantly less DAT and TH (**, $p < 0.01$; two-factor ANOVA; $n = 4–8$) after MPTP-treatment than their PAR1−/− counterparts after 2 (b), 7 (c), and 30 (d) days' survival. Inset, representative immunoblot showing DAT and TH protein levels compared with tubulin levels (Tub).

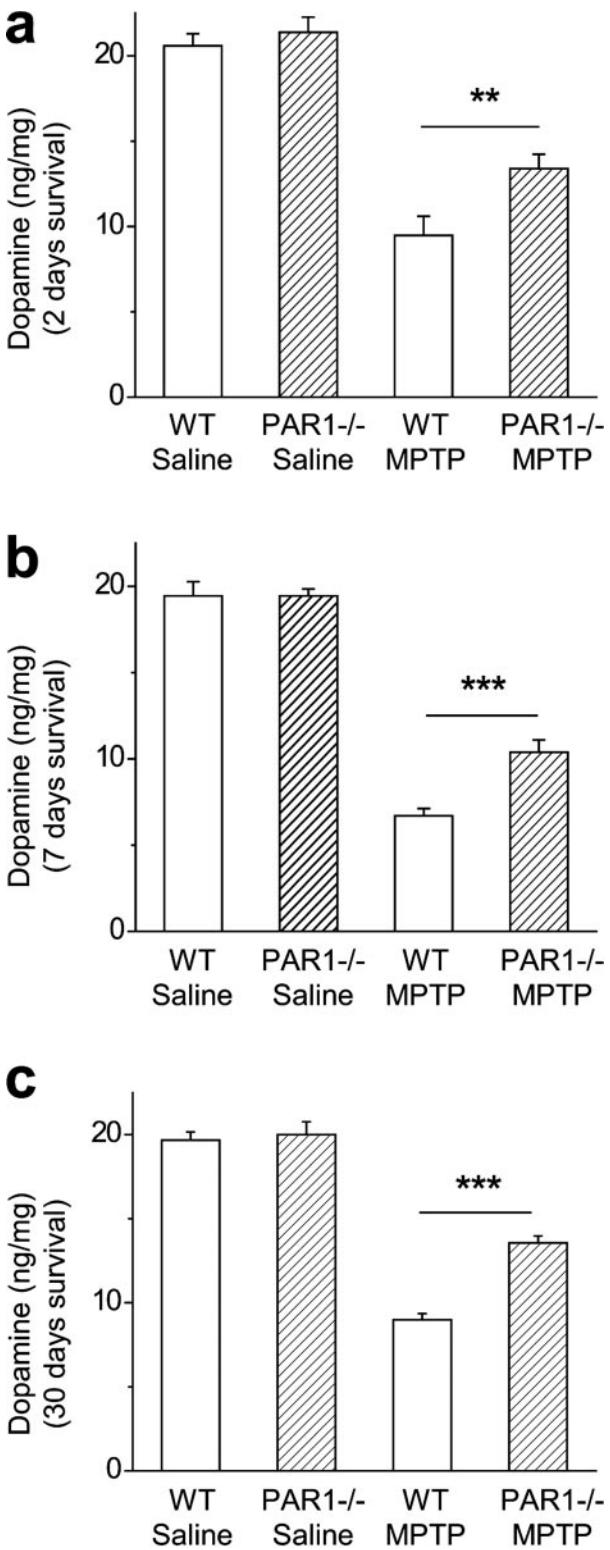

Fig. 2. PAR1−/− mice are functionally protected against MPTP-induced damage. a, PAR1−/− mice have significantly more striatal dopamine after MPTP than wild-type mice (2-day survival, 9.5 ± 1.0 ng/mg of tissue in wild-type mice versus 13.4 ± 0.8 ng/mg tissue PAR1−/− mice; **, $p < 0.01$; two-factor ANOVA; $n = 7–8$). b–c, higher levels of dopamine are observed in PAR1−/− mice at 7 days survival (6.7 ± 0.4 ng/mg of tissue in wild-type mice versus 10.4 ± 0.7 ng/mg of tissue in PAR1−/− mice; ***, $p < 0.001$; two-factor ANOVA; $n = 4–8$) and after 30 days' survival (9.0 ± 0.4 ng/mg of tissue in wild-type mice versus 13.6 ± 0.4 ng/mg of tissue in PAR1−/− mice; ***, $p < 0.001$; two-factor ANOVA).

pamine indicate that not only are the dopaminergic terminals more structurally intact (as indicated by immunoblot data), but that the neurons also maintain metabolic activity close to normal levels. Dopamine from wild-type and PAR1−/− mice was also measured after a 1-week survival. PAR1−/− mice continued to show higher levels of dopamine than the wild-type mice. After MPTP treatment, wild-type mice lost 66 ± 2% of their dopamine; PAR1−/− mice lost 47 ± 4% (Fig. 2b; $p < 0.001$; two-factor ANOVA). The protective effect observed in the PAR1−/− mice continued through 30-day survival. At this time point, PAR1−/− mice showed a decrease of 31 ± 2% of their striatal dopamine, whereas wild-type mice had a decrease of 55 ± 2% (Fig. 2c; $p < 0.001$; two-factor ANOVA).

PAR1 Antagonist Reduced MPTP-Induced Damage of Nigrostriatal Dopamine Pathway. To ensure that the protection seen in PAR1−/− mice was specific to PAR1 and not due to epigenetic or compensatory changes in the knockout animal, we examined the role of PAR1 activation in MPTP-induced damage in wild-type mice by pharmacologically blocking the receptor with the selective PAR1 antagonist BMS-200261 (Bernatowicz et al., 1996; Kawabata et al., 1999). BMS-200261 potently binds to PAR1 (K_D, 20 nM), preventing cleavage at Arg41 from activating the receptor (Bernatowicz et al., 1996). One micromolar BMS-200261 is sufficient to fully block PAR1-induced Ca^{2+} signaling in astrocytes (Nicole et al., 2005). Antagonist was administered intracerebroventricularly via a brain cannula connected to an osmotic minipump. Mice received 1 μl/h of 6 mM PAR1 antagonist or vehicle throughout the MPTP treatment and survival periods (approximately 6.2 mg/kg over 24 h). If we assume a mouse brain volume of ~1 ml with 20% extracellular volume, this would provide a maximum concentration of 30 μM; assuming peptide degradation and removal reduce the concentration 10-fold, there should still be sufficient antagonist (3 μM) to block PAR1. Five sets of mice were treated to evaluate the effect of antagonist on the response to MPTP. We treated one set of mice with vehicle-loaded minipumps and saline injection and a second set of mice with antagonist-loaded minipumps and saline injection. These data sets showed no difference in dopamine transporter and tyrosine hydroxylase protein levels and therefore were combined. A third set of mice was treated with vehicle-loaded minipumps and MPTP injection, whereas a fourth set of mice received no minipump and was injected subcutaneously with MPTP. The third and fourth sets of mice showed similar loss of biochemical markers and as such were combined. The fifth set of mice received antagonist-loaded minipump and subcutaneous MPTP injection. The results of this experiment showed that the mice treated with antagonist had significantly less damage after MPTP treatment than those that were not treated with antagonist. Mice that did not receive antagonist lost 40 ± 6% of dopamine transporter and 37 ± 7% of tyrosine hydroxylase, compared with antagonist-treated mice, which lost 2 ± 5% of dopamine transporter and 19 ± 5% of tyrosine hydroxylase (Fig. 3a; $p < 0.01$ and $p < 0.05$, respectively; one-factor ANOVA).

Pharmacologic blockade of the PAR1 receptor with BMS-200261 also inhibited MPTP-induced dopamine loss. Mice that received saline infusion over 3 days lost 46 ± 3% of their striatal dopamine, which was similar to wild-type mice that did not receive minipump implantation (54 ± 5%). In contrast, mice that were treated with PAR1 antagonist lost only 25 ± 3% of their dopamine, which was significantly different from vehicle-treated control mice (Fig. 3b; $p < 0.01$; one-factor ANOVA). Together, these data support our interpretation that the effects of PAR1 activation exacerbate MPTP-induced terminal damage.

PAR1−/− Mice Showed Attenuated Microglial Responses after MPTP Treatment. Microgliosis and astrogliosis have been shown to play a role in the damage seen in the MPTP model of Parkinson's disease (O'Callaghan et al., 1990; Kurkowska-Jastrzebska et al., 1999; McGeer et al., 2003). We measured several markers of gliosis in wild-type and PAR1−/− mice to determine whether PAR1 expression altered glial response to MPTP treatment. MPTP treatment

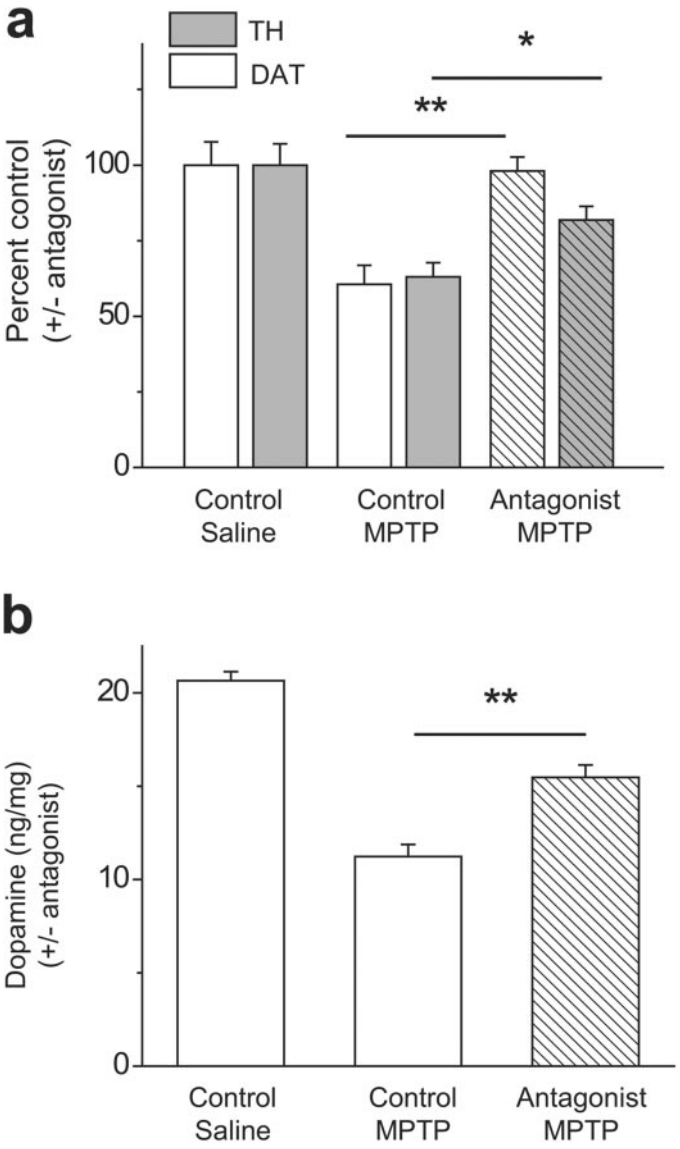

Fig. 3. Wild-type mice receiving a PAR1 antagonist are structurally and functionally protected against MPTP-induced damage. a, wild-type mice implanted with a minipump loaded with the PAR1 antagonist BMS-200261 show increased levels of DAT (**, $p < 0.01$; one-factor ANOVA; $n = 6–8$) and TH (*, $p < 0.05$; one-factor ANOVA; $n = 6–8$) after MPTP treatment compared with wild-type mice not treated with PAR1 antagonist. b, PAR1 antagonist-treated mice have significantly more striatal dopamine after MPTP administration than control mice (11.2 ± 0.7 ng/mg in control mice and 15.5 ± 0.7 ng/mg in mice receiving BMS-200261; **, $p < 0.01$; one-factor ANOVA; $n = 2–6$).

increased the extent of microglial activation in striatum of wild-type mice (Fig. 4a), with cells assuming an activated phenotype with hypertrophic cell bodies and shorter processes (Fig. 4a, arrows) (Kurkowska-Jastrzebska et al., 1999). By contrast, the microglial response was close to baseline in the PAR1−/− mice after MPTP injection (Fig. 4a). In PAR1−/− mice, most of the microglia retained small cell bodies and ramified processes (Fig. 4a). To determine effects of MPTP on microglia, we used reverse transcriptase-polymerase chain reaction (PCR) to quantify in each treatment group the level of MAC-1(CD11b) mRNA, a microglial marker. Saline-treated wild-type and PAR1−/− mice had similar levels, indicating no baseline genotypic differences (100 ± 23% in wild-type mice versus 116 ± 23% in PAR1−/− mice). Wild-type mice showed an increase of MAC-1(CD11b) mRNA after MPTP injection (343 ± 77%). MPTP had no significant effect on MAC-1(CD11b) mRNA in PAR1−/− mice compared with wild-type mice (120 ± 15%; $p < 0.05$; two-factor ANOVA). To quantify this microglial response on the protein level, we used immunoblot analysis of glucose transporter-5, another marker for microglia (Payne et al., 1997). We found no significant difference in glucose transporter-5 levels between the saline-treated wild-type and PAR1−/− animals (Fig. 4b). However, wild-type mice treated with MPTP showed 162 ± 21% of glucose transporter-5, whereas PAR1−/− mice showed no significant increase after MPTP (Fig. 4b; $p < 0.01$; two-factor ANOVA). To test whether the effect of PAR1 on microglial activation was persistent, we repeated these studies after 1 week of survival. Similar to that observed in mice by other studies (Francis et al., 1995; Czlonkowska et al., 1996; Kohutnicka et al., 1998; Liberatore et al., 1999), the microglial response had abated in wild-type mice by 7 days after injection, with sporadic phenotypically activated microglia visible. However, no activated microglia were observed in any of the slices examined from PAR1−/− mice (data not shown). These data indicate that the reduced presence of activated microglia at 2 days did not reflect a delay in microglial activation.

We studied the response of astrocytes to MPTP treatment by evaluating the astrocyte marker GFAP 2 or 7 days after MPTP injections. Although there was an up-regulation of GFAP in both wild-type and PAR1−/− mice after MPTP, we found no difference between wild-type and PAR1−/− mice at 2 or 7 days after injection by either immunoblot or immunohistochemistry (Fig. 4, c and d; 2 days: wild-type mice, 151 ± 18%; PAR1−/− mice, 143 ± 14%; $p > 0.05$; 7 days: wild-type, 212 ± 29%; PAR1−/−, 191 ± 15%; $p > 0.05$; two-factor ANOVA).

As a control, we examined levels of activated and nonactivated astrocytes and microglia before MPTP injection to determine whether PAR1−/− animals showed altered glial cell expression. PAR1−/− animals showed no difference of the astrocyte marker GFAP compared with wild-type controls

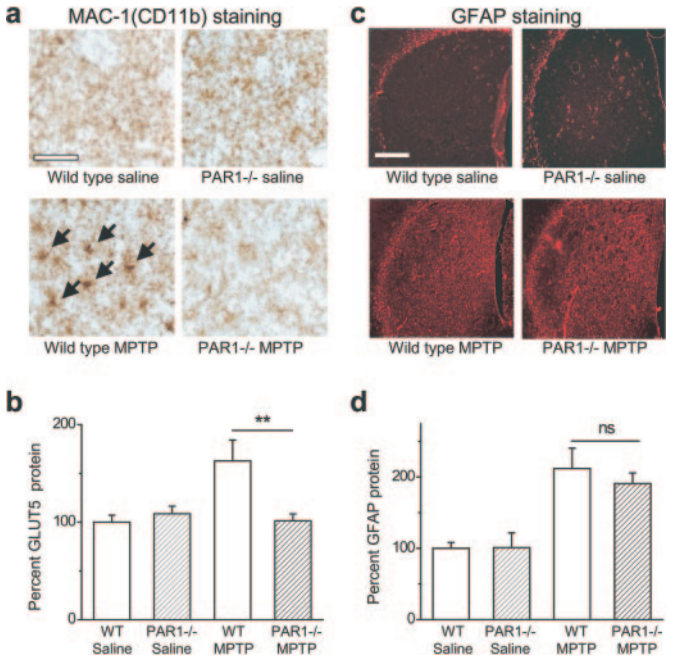

Fig. 4. PAR1−/− mice have dampened microglial responses but normal astrocyte responses to MPTP. a, staining for MAC-1(CD11b) shows that wild-type mice have more reactive striatal microglia after MPTP than PAR1−/− mice ($n = 3$); scale bar, 200 μm. b, immunoblot shows higher levels of GLUT5 protein, a microglial marker, in wild-type (WT) mice than in PAR1−/− mice after MPTP treatment (**, $p < 0.01$; two-factor ANOVA; $n = 6–8$). c, GFAP staining shows no differences in astrocytic response in WT and PAR1−/− mice 7 days after MPTP treatment; scale bar, 500 μm. d, immunoblot analysis of GFAP shows no significant difference between genotypes ($p > 0.05$; two-factor ANOVA; $n = 6–8$).

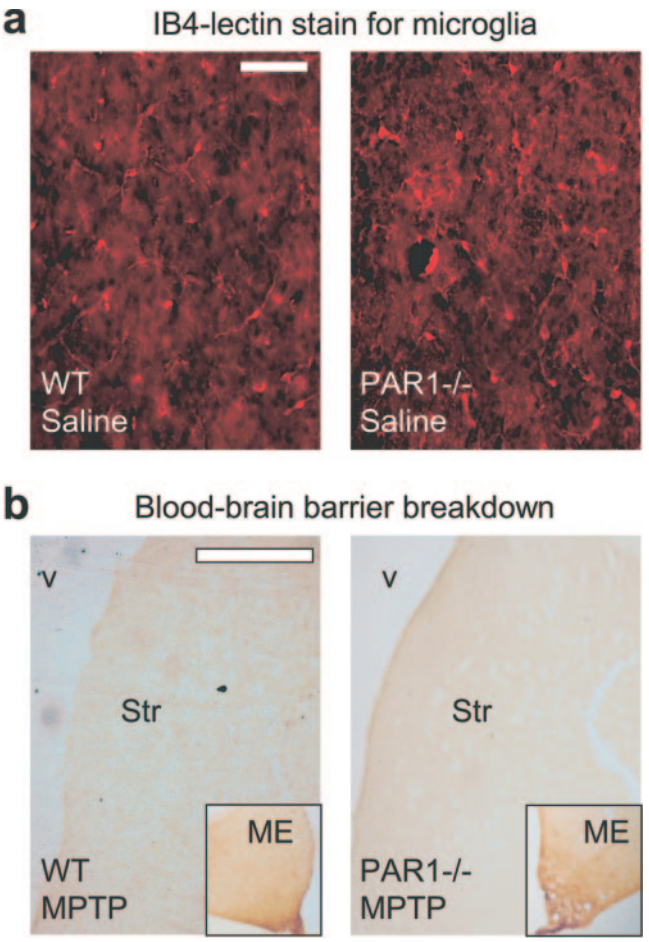

Fig. 5. Baseline microglial populations and post-MPTP blood-brain barrier breakdown. a, there were no differences between wild-type (WT) and PAR1−/− IB4-lectin stain, which labels all microglia regardless of activation state; scale bar 100 μm. b, after MPTP treatment, there was no detectable opening of the barrier in striatum (Str), as determined by IgG staining; scale bar, 500 μm. Inset, median eminence (ME) serves as a positive control, because blood-brain barrier is absent in this region.

(Fig. 4, c and d). Likewise, we found no difference in IB4 staining, a marker for both activated and nonactivated microglia, between wild-type and PAR1−/− mice before MPTP injection (Fig. 5a). These data suggest that the attenuated microglial response in PAR1−/− mice reflected reduced activation by MPTP-dependent processes.

MPTP Increased Two Known Activators of PAR1. To evaluate the effects of MPTP on upstream factors controlling PAR1 activation, we measured the effects of MPTP on the mRNA levels of several central nervous system proteases. Some of these proteases directly activate PAR1, whereas others indirectly lead to PAR1 activation through activation of zymogen precursors of PAR1 activators (Liu et al., 1991; Vu et al., 1991; Blackhart et al., 1994; Kuliopulos et al., 1999; Altrogge and Monard, 2000; Blanc-Brude et al., 2001; Macfarlane et al., 2001; Jiang et al., 2004; Shi et al., 2004; Boire et al., 2005). MPTP caused no significant changes in mRNA levels determined by real-time PCR for plasminogen or its activator, tissue plasminogen activator. Likewise, we found no difference in prothrombin, factor VII, neurotrypsin, or protein C mRNA levels (Table 1). However, we did find that MPTP administration significantly increased MMP1 in both wild-type and PAR1−/− mice (Table 1; treatment $p < 0.01$; two-factor ANOVA). Likewise, MPTP induced an increase in factor X mRNA in both genotypes (Table 1; treatment $p < 0.05$; two-factor ANOVA). Thus, MPTP may stimulate PAR1 activation through increased production of direct and indirect activating proteases. We also evaluated whether MPTP might cause changes in PAR1 signaling by increasing receptor expression. However, we did not find an MPTP-induced change in mRNA for PAR1 or any of the other protease-activated receptors (Table 1; $p > 0.05$; two-factor ANOVA). Increased PAR1 signaling could also be mediated by a decrease in the expression of the protease inhibitors, which may act to dampen tonic PAR signaling through inhibition of serine protease activators. We found no significant changes in the mRNA levels for protease-nexin 1, neuroserpin, or tissue inhibitor of matrix metalloprotease-1 (Table 1; $p > 0.05$; two-factor ANOVA). However, there was a significant decrease in the expression of plasminogen-activator inhibitor-1 in both genotypes (Table 1; $p < 0.05$; two-factor ANOVA). This decrease indicates that there may be enhanced PAR1 signaling via the tPA-plasmin pathway. Plasminogen is present in central nervous system (CNS) tissue and, when converted to plasmin, is capable of activating PAR1 (Junge et al., 2003). These data suggest that MPTP favors PAR1 activation through both increased transcription of a subset of PAR1 activators and decreased transcription of PAR1 inhibitors. These data also indicate that the mechanism of the protection observed in PAR1−/− mice was not due to altered response to MPTP in regard to these changes in gene expression, because there was no significant effect of genotype (Table 1; $p > 0.05$; two-factor ANOVA).

Deletion of PAR1 Did Not Alter Dopamine Transport, MPP+ Levels, or Blood-Brain Barrier Breakdown. Four control studies were performed to test whether the protection of nigrostriatal system observed in PAR1−/− mice reflected developmental differences that modified the response to MPTP. First, we found no significant difference in the expression of dopamine transporter in wild-type mice versus PAR1−/− mice, as measured using the radiolabeled ligand [^{3}H]WIN 35,428 (Table 2; wild-type, 984 ± 39 fmol/mg protein; PAR1−/−, 1014 ± 36 fmol/mg protein; $p > 0.05$; t test) (Coffey and Reith, 1994; Elwan et al., 2006). Second, we tested whether the ability of the dopamine transporter to move dopamine or MPP+ into cells was different between wild-type and PAR1−/− animals. Dopamine uptake in picomoles per minute per milligram of protein was 309 ± 9 for wild-type mice and 306 ± 17 for PAR1−/− mice (Table 2; n = 3–4; $p > 0.05$; t test). Likewise, uptake of MPP+ was indistinguishable between wild-type versus PAR1−/− mice (Table 2; 198 ± 13 versus 195 ± 18 pmol/min/mg of protein), respectively; $p > 0.05$; n = 3–4; t test). Third, we measured the ability of wild-type and PAR1−/− mice to convert MPTP to the active toxin MPP+. We found no difference in the level of the active neurotoxin MPP+, indicating that the protection in PAR1−/− mice was not due to decreased toxin exposure. Levels of MPP+ were 3.5 ± 0.3 ng/mg of tissue for wild-type and 3.2 ± 0.2 ng/mg of tissue for PAR1−/− mice (Table 2; n = 3–4; $p > 0.05$; t test). Fourth, we examined whether PAR1−/− mice had abnormalities in the permeability of the blood-brain barrier after MPTP injection, because PAR1 is expressed in endothelial cells and may influence maintenance of the blood-brain barrier (Riewald et al., 2002). We stained for the presence of serum-borne IgG (molecular mass, ~150 kDa) into the brain parenchyma after MPTP injection and 2-day survival, which would indicate compromised barrier function at this point. Consistent with many other stud-

TABLE 1

MPTP increases the mRNA levels of PAR1 activators

Real-time reverse transcriptase-PCR measurements of mRNA relative to 18S mRNA are expressed as a percentage of saline-injected controls. PAR1 signal was only found in wild-type mice. For all, n = 4. There was no significant difference between genotypes.

	Wild Type	PAR1−/−
Protease Receptors		
PAR1	114 ± 16	N.D.
PAR2	107 ± 39	70 ± 18
PAR3	95 ± 8	89 ± 12
PAR4	84 ± 20	124 ± 15
Proteases		
Prothrombin	124 ± 36	107 ± 18.
Plasminogen	138 ± 58	93 ± 21
Tissue plasminogen activator	119 ± 20	82 ± 8
Matrix metalloprotease 1	201 ± 15*	248 ± 18*
Factor X	283 ± 65**	345 ± 55**
Factor VII	94 ± 19	74 ± 5
Neurotrypsin	99 ± 13	84 ± 24
Protein C	76 ± 12	104 ± 22
Protease inhibitors		
Neuroserpin	118 ± 25	105 ± 16
Protease nexin 1	128 ± 23	124 ± 20
Plasminogen activator inhibitor 1	55 ± 13*	52 ± 10*
Tissue inhibitor of matrix metalloprotease-1	90 ± 47	93 ± 21

* Treatment $P < 0.05$; ** Treatment $P < 0.01$, two-factor ANOVA.
N.D., not determined.

TABLE 2

Wild-type and PAR1−/− mice show baseline similarities in the dopaminergic system

All studies were performed in triplicate with at least three animals per group. No significant differences were found between the wild-type and PAR1−/− values (t test).

	Wild Type	PAR1−/−
[^{3}H]WIN 35,428 binding (fmol/mg protein)	984 ± 39	1014 ± 36
[^{3}H]Dopamine uptake (pmol/min/mg protein)	309 ± 9	306 ± 17
[^{3}H]MPP + uptake (pmol/min/mg protein)	198 ± 13	195 ± 18
MPP+ levels (ng/mg of striatal tissue)	3.5 ± 0.3	3.2 ± 0.2

ies showing that MPTP does not alter blood-brain barrier breakdown (Adams et al., 1989; O'Callaghan et al., 1990; Kurkowska-Jastrzebska et al., 1999), we were unable to detect IgG in the striata of either wild-type or PAR1−/− mice after MPTP injection, suggesting that the protection observed in PAR1−/− mice was not due to differences in blood-brain barrier permeability (Fig. 5b). Together, these data suggest that the protection seen in PAR1−/− mice did not reflect production or uptake of neurotoxic MPTP metabolites or altered permeability of the blood-brain barrier in PAR1−/− animals.

Expression of PAR1 in Human and Mouse Dopaminergic Neurons. Previous work has shown that PAR1 mRNA expression is highest in rat dopaminergic neurons (Weinstein et al., 1995; Ishida et al., 2006). Additional studies have colocalized PAR1 protein with astrocytic markers in the CNS of rats as well as humans (Weinstein et al., 1995; Niclou et al., 1998; Junge et al., 2004; Hamill et al., 2005; Ishida et al., 2006). We used antibodies against PAR1 and tyrosine hydroxylase, a reliable marker of dopaminergic neurons, to examine whether PAR1 protein was present in the dopaminergic neurons of normal human substantia nigra pars compacta. Previous studies have found PAR1 protein expression in substantia nigra pars compacta astrocytes but not neurons (Ishida et al., 2006). Immunohistochemical analysis of four cases from normal patients all showed clear colocalization of immunoreactivity for tyrosine hydroxylase and PAR1 in a subset of substantia nigra pars compacta cells; other tyrosine hydroxylase-positive cells lacked PAR1 immunoreactivity, suggesting PAR1 expression varies among dopaminergic neurons (Fig. 6a). On average, approximately 71% of tyrosine hydroxylase-positive neurons also expressed PAR1. A subset of small cells that expressed PAR1 but lacked tyrosine hydroxylase were likely to be glial cells (Junge et al., 2003; Hamill et al., 2005). These data provide evidence that PAR1 is expressed not only in astrocytes but also in dopaminergic neurons of the substantia nigra.

We also tested for functional expression of PAR1 on cultured mouse dopaminergic neurons. It has been previously shown that activation of PAR1 causes signaling via the mitogen-activated protein kinase pathway and phosphorylation of ERK (Macfarlane et al., 2001; Hollenberg, 2002; Wang et al., 2002b; Sorensen et al., 2003; Nicole et al., 2005). After a 15-min exposure to either vehicle or 30 μM TFLLR, coverslips containing neuronal cultures were fixed and stained for phospho-ERK and tyrosine hydroxylase (Nicole et al., 2005). The peptide agonist, TFLLR is known to be specific for PAR1 over other protease-activated receptors and was administered at a high dose (Hollenberg et al., 1997). Throughout the mixed culture of both dopaminergic and GABAergic neurons, astrocytes, and microglia, we saw a strong increase in phospho-ERK expression (Fig. 6b). In particular, we observed a clear colocalization of phospho-ERK and tyrosine hydroxylase in cultures treated with PAR1 activators, suggesting that there is functional expression of PAR1 in mouse dopaminergic neurons. Of 24 tyrosine-hydroxylase positive neurons observed on three coverslips, 22 showed increased phospho-ERK staining.

Discussion

In this study, we show that MPTP caused an up-regulation of PAR1 activators and that genetic deletion or pharmacologic blockade of PAR1 protected mice from MPTP-induced damage as well as MPTP-triggered microglia activation. These data demonstrate that PAR1 activation can contribute to dopaminergic terminal damage in this model of early Parkinson's disease. Furthermore, we show that PAR1 was expressed in the dopaminergic neurons of human and mouse, suggesting that the results obtained in this animal model could have relevance for humans. Because protease-activated receptors such as PAR1 and PAR4 are known to influence microglial activation (Suo et al., 2002; Nicole et al., 2005), these findings raise the idea that PAR1 activation may be an important molecular link between inflammation and damage to dopaminergic neurons in Parkinson's disease.

Activation of PAR1 and Parkinson's Disease. The results of this study suggest that the proteases that activate PAR1 may influence the pathophysiology of Parkinson's disease, and focus attention on how potential activators or inhibitors of PAR1 might affect the disease progression. Previous studies that have examined PAR1 activation in the context of stroke and ischemia have provided a great deal of information about PAR1 signaling and neuroprotection as well as neuronal death. One view emerging is that PAR1 activation can have both harmful and helpful effects in ischemia that involve different pathways and different levels of

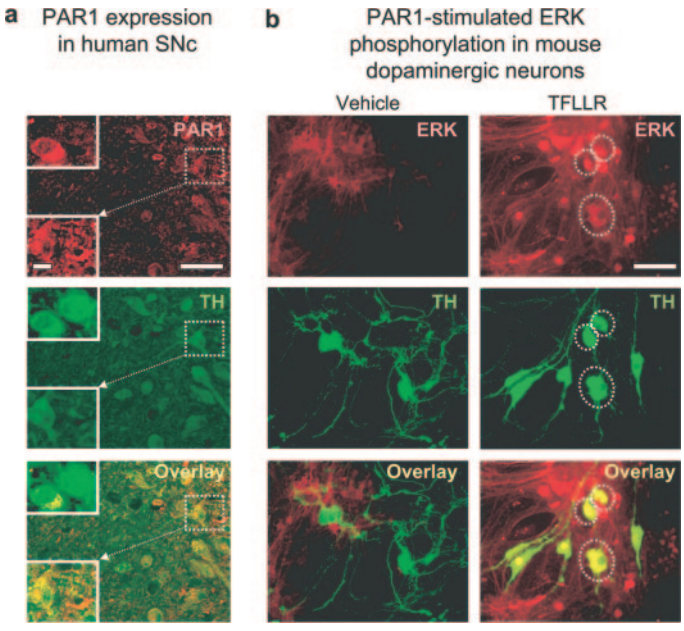

Fig. 6. PAR1 protein colocalizes with tyrosine hydroxylase in normal human substantia nigra pars compacta and is functionally expressed in mouse dopaminergic neurons. a, A representative photomicrograph of PAR1 (top) and tyrosine hydroxylase (TH; middle) staining and their overlay (bottom) is shown. The main panels show 20× magnifications (scale bar, 100 μm); insets show 40× magnifications (scale bar, 10 μm). Most neurons that stain for TH also show PAR1 expression. There are some TH-positive cells that are negative for PAR1 (cell on right in upper insets; from a different field of view) and some PAR1 positive cells that do not stain for TH (cell on right in lower inset). Data are representative of four different experiments. b, representative images showing that TFLLR (right) but not vehicle (left) causes increased phospho-ERK expression in tyrosine hydroxylase-expressing neurons. Top, phospho-ERK staining; middle, TH staining; bottom, overlay. Cells coexpressing PAR1 and TH are circled with dotted lines.

PAR1 activation (Wang and Reiser, 2003). Ishida et al. (2006) suggest a similar paradigm may occur in models of Parkinson's disease, the neurotoxicity of thrombin being mitigated by PAR1-activation of astrocytes. Cannon et al. (2005, 2006) have presented evidence that thrombin preconditioning can reduce behavioral deficits after 6-hydroxydopamine lesions, but not 6-hydroxydopamine-induced dopamine depletion. These data support a potential neuroprotective effect of low levels of PAR1 activation, which may engage neuroprotective intracellular pathways or down-regulate surface expression of PAR1. However, the pathophysiology of Parkinson's disease and MPTP-induced damage is complex, with hypothesized roles for both neurons and glia (Beal, 2003; Wu et al., 2003) in addition to a range of molecular mechanisms, including oxidative stress, inflammation, and mitochondrial dysfunction. Our findings (that PAR1 removal or block can protect dopaminergic nerve terminal damage) support the idea that PAR1 can have multiple effects, some of which exacerbate nerve terminal damage in the MPTP model of Parkinson's disease. We expect the harmful PAR1-linked mechanisms that contribute to terminal damage after the modest MPTP dosing paradigm used here will also be engaged in models of parkinsonism employing higher MPTP doses that lead to substantial neuronal loss.

MPTP-induced degeneration of the dopaminergic system has long been known not to cause direct detectable breakdown of the blood-brain barrier (Adams et al., 1989; O'Callaghan et al., 1990; Kurkowska-Jastrzebska et al., 1999; Nicole et al., 2005). Our data are consistent with this view and support the idea that breakdown of the blood-brain barrier is unlikely to influence the effects of PAR1 in the acute MPTP model of Parkinson's disease. However, environmental factors such as head trauma and infection may increase the risk of developing Parkinson's disease (Taylor et al., 1999; Mayeux, 2003). It is noteworthy that both head trauma and infection should temporarily increase permeability of the blood-brain barrier long before onset of Parkinson's disease, allowing blood-derived serine proteases access to brain tissue. Our data further raise the possibility that activation of PAR1 in brain parenchyma could stimulate microglial activation, perhaps increasing inflammatory processes that may favor initial nerve terminal damage and thereby elevating the risk of Parkinson's disease.

Ishida et al. (2006) have suggested that Parkinson's disease is associated with an up-regulation of PAR1 and an increase in potential expression of PAR1 activators. In general agreement with these data, we have found that administration of MPTP alters the brain serine protease system in favor of PAR1 activation, although we did not detect increased PAR1 expression. Rather, we find up-regulation of several PAR1 activators, and propose these changes exacerbate harmful actions of MPTP via their activation of PAR1. MMP1, which has been shown to have a role in substantia nigral disorders, can activate PAR1 directly (Gardner and Ghorpade, 2003; Lorenzl et al., 2004; Shi et al., 2004; Boire et al., 2005). Factor Xa can activate PAR1 and can convert prothrombin to thrombin, which is a potent PAR1 activator (Jones and Geczy, 1990; Yamada and Nagai, 1996; Shikamoto and Morita, 1999). Factor X is activated by factor VIIa and factor IXa, which may also be present in the CNS, although this has not been studied extensively. However, macrophages produce and release a factor VII-like protein that activates factor X (Shands, 1983, 1985; Tsao et al., 1984). Several reports suggest that MMP1 and factor X can be made by microglia and astrocytes (Nakagawa et al., 1994; Yamada and Nagai, 1996; Shikamoto and Morita, 1999; Ghorpade et al., 2001; Lorenzl et al., 2002, 2004; Gardner and Ghorpade, 2003; Kunapuli et al., 2004; Hamill et al., 2005; McCready et al., 2005). In addition, there is strong evidence that prothrombin, the precursor to thrombin, is expressed throughout the CNS (Soifer et al., 1994; Weinstein et al., 1995). Ishida et al. (2006) report an increase in prothrombin-expressing astrocytes in substantia nigra from Parkinson's patients. With increased levels of factor X, we would predict increased activation of thrombin and consequent PAR1 signaling. Likewise, we found a decrease in plasminogen activator inhibitor-1 expression. This could enhance conversion of plasminogen to plasmin in the central nervous system, which has been shown to play a role in learning and memory, synaptic plasticity, and neuronal damage. Furthermore, plasmin is also known to cleave and activate PAR1 (Sharon et al., 2002; Pang et al., 2004; Nagai et al., 2005). Together, these changes indicate that the MPTP-induced toxicity causes a shift in the protease system of the brain toward PAR1 activation.

Microglia and Parkinson's Disease. The data presented here are consistent with the idea that microglial activation is detrimental to neuronal survival in the MPTP model of Parkinson's disease. Although it is not clear whether the reduced microglial response in PAR1−/− mice reflects a reduction in neuronal damage and consequent inflammatory response, we note that astrogliosis is unaffected by the absence of PAR1 in this model. The role of microglia in the pathogenesis of Parkinson's disease has been an area of active investigation. Several groups have reported that activation of microglia with substances such as lipopolysaccharide or thrombin, a potent PAR1 agonist, can lead to dopaminergic neuronal death that is due in part to release of cytokines (Adams et al., 1989; Gayle et al., 2002; Carreño-Muller et al., 2003; Choi et al., 2003ab; Gao et al., 2003; Qin et al., 2004; Lee da et al., 2005). Likewise, others have found that pharmacologic inhibition of microglia, for example with minocycline, protected against neurotoxins like MPTP and thrombin, although the nonmicroglial-specific effects of such drugs may have other actions (Du et al., 2001; Wu et al., 2002; Yang et al., 2003; Diguet et al., 2004; Choi et al., 2005). The ability of PAR1 stimulation to activate microglia (Suo et al., 2002), coupled with the findings presented in this study, further support a role for inflammation as a mediator of dopaminergic terminal damage in the parkinsonian brain. Clearly more work is needed to both elucidate the nature by which microglia promote projection terminal degeneration as well as clarify the role of PAR1 and other protease receptors on microglia. Although our experiments with acute intracerebroventricular administration of the PAR1 antagonist BMS-200261 support our working hypothesis, the lack of brain penetration of the modified peptide limits experimental design. If brain penetrable PAR1 antagonists were available, a number of important questions could be addressed about the role of PAR1 in chronic neuroinflammation.

Despite advances in our understanding of Parkinson's disease, therapy is still limited to the treatment of symptoms; no medication to date slows the progression of the disease in patients. Recent studies using transgenic mice have begun to

identify key molecular components in the progression of Parkinson's disease. Mice lacking pro-inflammatory signaling components are partially protected from MPTP-induced damage (Rousselet et al., 2002; Wu et al., 2003; Ferger et al., 2004; Hébert et al., 2005). The results presented here provide further evidence that serine proteases and their receptors may have a role in dopamine neuron toxicity as it relates to Parkinson's disease. These data offer a reasonable link between events that disrupt the blood-brain barrier and the future risk of development of Parkinson's disease (Mayeux, 2003). Moreover, these data place PAR1 among only a handful of therapeutically tractable drug targets (Chen et al., 2001; Xu et al., 2005) that can slow or halt MPTP-induced damage (Chen et al., 2001; Rousselet et al., 2002; Hébert et al., 2005). Indeed, blockade of PAR1 may represent a novel therapeutic approach to slow disease progression in a Parkinson's patient or decrease disease incidence in at-risk patient populations.

Acknowledgments

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