Supporting Information

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SI Materials and Methods

Human Samples. Human paraffin-embedded sections (7 μm) were obtained from the Parkinson Brain Bank at Columbia University and processed as described previously (1) before incubation with an antibody against Oct3 (1:100, Alpha Diagnostics International). Clinical and neuropathological information about these samples has been described (2). As a negative control, Oct3 antibody was preabsorbed with excess Oct3 peptide (10-fold, 50 μg/mL; Alpha Diagnostics) overnight at 4 °C, centrifuged to remove the complexes, and the supernatant was used to immunostain the adjacent sections. Immunostaining was visualized using 3,3′-diaminobenzidine with cobalt/nickel enhancement.

MPTP and Methamphetamine Treatments. Ten to 12-week old *Oct3*$^{-/-}$ mice and their wild-type littermates, and C57BL/6 mice infused s.c. with either vehicle or the Oct3 inhibitor, decynium 22 (D22, 1,1′-diethyl-2,2′-cyanine iodide, Sigma-Aldrich), were randomly assigned to receive either 4 i.p. injections of MPTP (18 mg/kg) or saline at 2-h intervals. For the D22 studies, 1 day before the MPTP injections, Alzet miniosmotic pumps (delivery rate of 1 μL/hr) containing varying doses of D22 (0, 0.1, 1, or 2 μmol/kg/d) or vehicle (propylene glycol plus 0.375% to 7.5% DMSO) were s.c. implanted below the shoulder blades. For the methamphetamine study, mice were injected with the following regimens: (*i*) Five milligrams/kg i.p., either with a single injection or 4 injections every 2 hours. (*ii*) Thirty milligrams/kg s.c. either with a single injection or with 2 injections 4 hours apart. Subcutaneous injection was used for this high dose of methamphetamine to reduce mortality in animals (3). In all studies, mice were killed 7 days after the last injection and perfused transcardially with 4% paraformaldehyde. Brains were removed and postfixed overnight in the same perfusion fixative, cryoprotected in successive 15% and 30% sucrose phosphate buffer for 2 days then frozen at −80 °C for immunohistochemical studies.

Immunostaining and Colocalization. Coronal sections (30 μm) from MPTP-treated mice were incubated in MOM mouse IgG blocking reagent (Vector Laboratories) before incubation with polyclonal anti-Oct3 (1:100, Alpha Diagnostic International) and monoclonal antibodies against tyrosine hydroxylase (TH; 1,000; Calbiochem), glial fibrillary acidic protein (GFAP, 1:1,000, Chemicon) or microtubule-associated protein (MAP-2, 1:200, Chemicon), followed by the corresponding secondary antibodies Alexa Fluor 594 and 488 (Invitrogen). Images were scanned at 1-μm intervals throughout the whole section and analyzed using confocal microscopy (FV500; Olympus).

Real Time Quantitative RT-PCR Analysis of *Oct3* Expression in LCM Samples. RNA from laser-captured cells was isolated using an RNeasy Micro isolation kit (Qiagen) according to the company's instruction. Oligonucleotide primers to amplify a segment of mouse Oct3 (accession no. NM_011395) were designed using Primer Express 1.5 (Applied Biosystems): Fwd 5′-AGCCAGCCCGACTACTATTGGT-3′ and Rev 5′-TGAGCTCTGAGCTGGTATTAGT-3′; likewise, a portion of mouse β-actin (accession no. NM_007393) was amplified as an internal standard using Fwd 5′-ACCCTGTGCTGCTCACCGA-3′ and Rev 5′-CTGGATGGCTACGTACATGGCT-3′ primers. Reactions were conducted and analyzed on a Rotor-Gene 3000 real time light cycler from Corbett Robotics. BioRad's iScript One-Step RT-PCR kit with SYBR Green was used for real time RT-PCR analysis, and expression levels are reported as a ratio to mouse β-actin.

Stereological Nigral Cell Count and Striatal Optical Density. Brains from saline and MPTP-treated mice were sectioned (30 μm) and processed for stereological cell counts using the optical fractionator method as described (1). Striatal optical densities of TH and DAT immunoreactivity were also quantitated as described (1).

Measurements of MPTP Metabolism. To assess whether D22 treatment or Oct3 ablation interferes with the conversion of MPTP into MPP$^+$, wild-type C57BL/6 mice infused with D22 (2.0 μmol/kg/d) or vehicle as well as *Oct3*$^{-/-}$ and *Oct3*$^{+/+}$ mice were injected with 4 i.p. injections of MPTP (18 mg/kg) at 2-h intervals. All mice were killed 90 min after the fourth injection. Striatal tissue levels of MPP$^+$ were measured using HPLC as previously described (1).

Laser Capture Microdissection (LCM). Brains were snap frozen in liquid nitrogen, sectioned at 8 μm, and directly collected onto PEN membrane slides. All solutions were made in RNase-free water and RNase-Secure (Ambion) was added to the antibody solutions to remove RNases. Briefly, after blocking for 1 hour, sections were incubated with primary antibodies for 1 hour at 37 °C, followed by biotinylated secondary antibody for 30 min. Immunoreactivity was visualized using DAB. Sections were counterstained with cresyl-violet. At least 800–1,000 cells of each type ($\approx$1- to 1.5 $\times$ 10^6 μm^2) were captured using UV Laser Microbeam technology (PALM, Carl Zeiss). Only cells with immunoreactivity with no nearby Nissl positive cells were captured to avoid contamination with other cell types.

In Vivo Microdialysis. *Oct3*$^{-/-}$ mice and their *Oct3*$^{+/+}$ littermates were anesthetized with ketamine/xylazine (65/6 mg/kg i.p.) and placed in a stereotaxic frame (model SAS-4100, ASI Instruments). A hole was drilled using a fine trephine drill above the right striatum at the following coordinates, relative to bregma: anterior-posterior = +0.5 mm, lateral = −2.0 mm, dorsal-ventral = −1.5 mm (from surface of brain). A locking intracerebral guide and stylet (Bioanalytical Systems, Inc.) was implanted through this hole and held tight by a layer of acrylic dental cement and the animals were returned to their cages and allowed to recover for 2 days. The microdialysis probe (2-mm membrane, Bioanalytical Systems, Inc.) was inserted into the intracerebral guide and connected to a low torque-dual channel swivel (Instech Laboratories, Inc.) which was connected to a syringe pump perfusing with artificial cerebrospinal fluid at 2 μL/min. After at least a 2-h equilibration period, the dialysates were collected every 30 min. Control samples were collected for an hour before a single injection of MPTP (30 mg/kg, i.p.) or methamphetamine (5 mg/kg i.p. or 30 mg/kg s.c.) to determine a baseline and for an additional 4 hours after the injection. The 2 baseline fractions were pooled and presented as zero time point before the injection. Histological examination subsequent to the experiments was performed to verify the placement of the probe in each animal. Samples from the same animals were measured for the contents of MPP$^+$, DA, and its metabolites. Levels of these molecules were calculated on the basis of the standard curves, probe efficiency ($\approx$8%), flow rate, and duration of sample collection. Of note, since the in vitro probe recovery calculation is not an accurate estimate of in vivo recovery (4–6),

these values may not reflect the real in vivo concentrations in the brain.

Measurements of Striatal DA and MPP^+ Levels. A 4-channel CoulArray (ESA Inc.) equipped with a highly sensitive amperometric microbore cell (model 5041, ESA Inc.) was used to analyze the content of DA and its metabolites with the cell potential set at +220 mV. Briefly, 20 μL samples were injected manually into a sample injector (with 20 μL sample loop) and eluted on a narrowbore (ID: 2 mm) reverse-phase C18 column (MD-150, ESA, Inc.) using MD-TM (ESA, Inc.) mobile phase. Another 20 μL of the same samples were used for MPP^+ measurement using a UV detector (model no. 526, ESA Inc.) at 280 nm λ. Samples were injected manually and separated by a narrowbore column (ID: 2.1 mm, Altima HP C18, Alltech Associates, Inc.) using mobile phases consisting of 80.5% 50 mM KH_2PO_4 and 9.5% acetonitrile, pH 3.2. The flow rate was set at 0.2 mL/minute for all measurements by using a solvent delivery pump (Model 585, ESA Inc.).

Cell Cultures. ***Primary astrocyte cultures.*** Postnatal astrocyte cultures from regular C57BL, $Oct^{+/+}$ or $Oct^{-/-}$ (generated from $Oct^{+/+}$ or $Oct^{-/-}$ breeders, respectively) pups were prepared as described previously (7). This method yields more than 95% GFAP positive cells. Because the expression of Oct3, as verified by immunocytochemistry and RT-PCR (data not shown), was detectable in cortical astrocytes at this developmental stage, we used cortical tissues to increase the yield for our experiments.

EM4 cells with stable expression of OCT3. Modified human embryonic kidney (HEK) 293 cells, stably transfected with macrophage scavenger to increase their adherence to tissue culture plastic (8) (referred to as EM4 cells), were used to generate stable cells overexpressing rat Oct3. To generate stable Oct3 cells, pIREShygro3 (Clontech) was cut with Mlu1 and Kas1 and rat Oct3 (provided by Dr. Bruno Giros) was inserted using standard molecular biology methods. The correct orientation of the Oct3 insert was confirmed by both restriction enzymes and sequencing. Transfection was performed using lipofectamine 2000 (Gibco/BRL). Stably transfected cells were selected and maintained in DMEM supplemented with 10% FBS and hygromycin (250 μg/mL). The stable expression of Oct3 was assessed on the basis of the efficiency of MPP^+ transport, immunoreactivity, and transcript level. With the latter analysis, real time RT-PCR was used. Oligonucleotide primers to amplify a segment of rat Oct3 (accession no. NM_019230) were designed using Primer Express 1.5 (Applied Biosystems): Fwd 5′-TTAGACCTTGTGAGGACTCCC-3′ and Rev 5′-CAGAAGGATCAAGAGAGCTCC-3′ primers. Likewise, a portion of human β-actin (accession no. NM_001101) was amplified as an internal standard using Fwd 5′-CCCAAGGCCAACCGCGAGAAGATGA-3′ and Rev 5′-GTCCCGGCCAGCCAGGTCCAGA-3′ primers. Expression levels are reported as a ratio to human β-actin within each cell line examined.

Transport Assays. Cells were grown in 24-well plates for all transport studies.

(*i*) EM4 Cells Overexpressing Oct3 or Empty Vector. ***Uptake dose–response studies.*** EM4 cells were washed twice and then preincubated for 20 min at 37 °C in Krebs Ringer Hepes (KRH) buffer (125 mM NaCl, 25 mM hepes, 5.6 mM glucose, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, pH 7.4) (9), before being incubated with 10 nM [^{3}H]-MPP^+ and increasing concentrations of unlabeled MPP^+ (1, 5, 10, 25, 50, and 100 μM) for 1 min. To stop the reaction, cells were rinsed with ice-cold buffer and the proteins were solubilized with 0.2 N NaOH and 1% SDS. Radioactivity was measured using a liquid scintillation counter and protein concentration was measured using the BCA assay.

Uptake time-course studies. EM4 cells were incubated with 10 nM [^{3}H]-MPP^+ plus 5 μM MPP^+ at 37 °C in KRP buffer as described above for various periods of time (0.5, 1, 2.5, 5, 10, and 15 min).

Efflux dose–response studies. EM4 cells stably transfected with Oct3 were preloaded with MPP^+ by being incubated with 10 nM [^{3}H]-MPP^+ and increasing concentrations of unlabeled MPP^+ (1, 5, 25, 50, and 100 μM) for 1 min as described above. Cells were then washed twice with ice-cold assay buffer with or without 5 μM D22 before being incubated at 37 °C with prewarmed (37 °C) buffer in the presence or absence of 5 μM D22. The radioactivity released from cells into the buffer over a 1-min period was counted using a scintillation counter.

Efflux time-course studies. EM4 cells with stable expression of Oct3 were preloaded for 1 min with 10 nM [^{3}H]-MPP^+ (plus 5 μM MPP^+) at 37 °C and then washed with ice-cold KRH buffer. Cells were then incubated at 37 °C and the radioactivity released from cells into the buffer over various lengths of time (0, 15, 30, 60, 150, 300 seconds) and the fraction of radioactivity remaining in cells were counted using a scintillation counter.

(*ii*) Primary Astrocytes. ***Uptake dose–response studies.*** Primary astrocytes cultured from postnatal C57BL/6, $Oct3^{+/+}$ or $Oct3^{-/-}$ pups were washed twice and then preincubated for 20 min at 37 °C in KRH buffer in the presence or absence of 5 μM D22 before being incubated with 10 nM [^{3}H]-MPP^+ and increasing concentrations of unlabeled MPP^+ (50, 100, 200, 400, 800, 1,600 μM) for 30 min. To stop the reaction, cells were rinsed with ice-cold buffer and the proteins were solubilized with 0.2 N NaOH and 1% SDS. Radioactivity was measured using a liquid scintillation counter. Note: due to the stickiness of these lysed samples, it was difficult to measure protein concentrations of these cells reliably; therefore, the transport activities of astrocytes are expressed as rate per total cells per well in a 24-well plate, rather than per-protein quantity.

Uptake time-course studies. Astrocytes were incubated with 10 nM [^{3}H]-MPP^+ plus 100 μM MPP^+ at 37 °C in KRP buffer as described above for various periods of time (2.5, 5, 10, 20, 30, 60, and 120 min).

Efflux time-course studies. Astrocytes were preloaded for 30 min with 10 nM [^{3}H]MPP^+ (plus 100 μM MPP^+) at 37 °C and then washed with ice-cold KRH buffer. Cells were then incubated at 37 °C and the radioactivity released from cells into the buffer over various lengths of time (10, 20, 30, 60, and 120 min) and the fraction of radioactivity remaining in cells was counted using a scintillation counter.

Cell viability assessment. N27 and primary astrocytes were plated in 96-well plates (8,000 cells/well) 1 day before experiments. N27 ($1RB_3AN_{27}$, were kindly provided by Anumantha G. Kanthasamy, Iowa State University) is an immortalized dopaminergic neuronal cell line created from rat fetal mesencephalic cells (10, 11). These N27 cells express dopamine transporter and functional tyrosine hydroxylase. One day after plating, cells were treated with 50, 100, 200, and 400 μM of MPTP, MPP^+, or PQ for 24 h. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as previously described (12).

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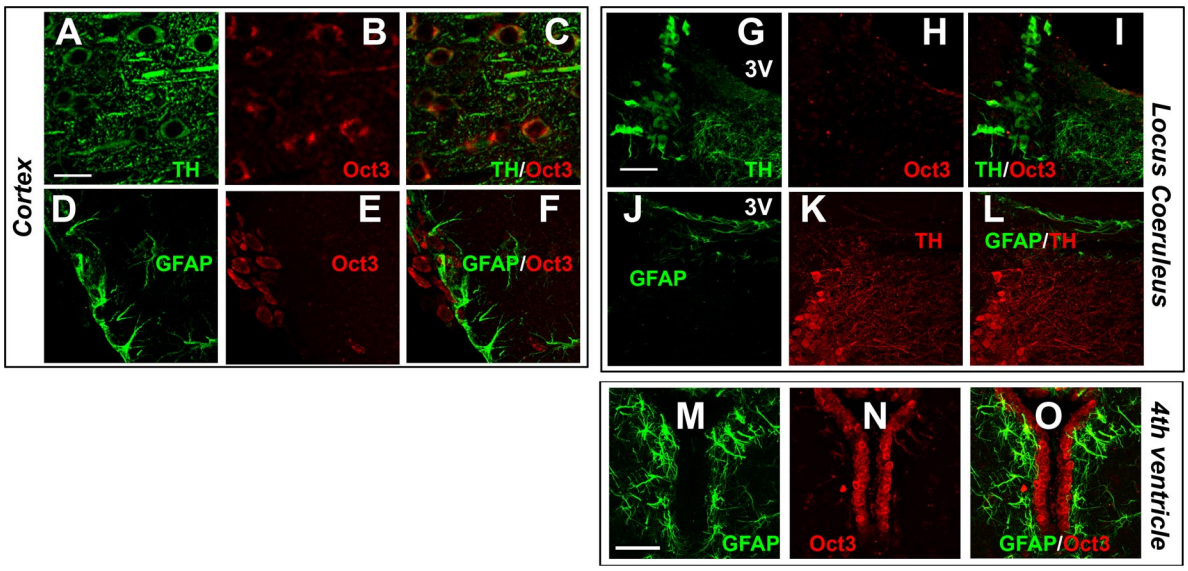

Fig. S1. Expression of Oct3 in the mouse brain. Coronal brain sections were immunolabeled with antibodies against Oct3, GFAP, TH, and MAP2. Oct3 was detected in cortical (*A–C*) but not in adrenergic neurons in the locus coeruleus (*G–I*), despite its high expression level in cells that line the nearby third ventricle (3V). The latter observation is common in all ventricles as Oct3 was found to be expressed abundantly in ependymal cells and, to a lesser extent, astrocytes that radiate from those regions as shown in the fourth ventricle (*M–O*). Oct3 was not detectable in cortical (*D–F*) nor locus coeruleus (data not shown) astrocytes. In the locus coeruleus, GFAP immunoreactivity was not detectable but it was highly abundant in the adjacent ventricle (*J–L*). [Scale bars: 20 μm (*A–F*), 40 μm (*M–O*), and 50 μm (*G–L*)].

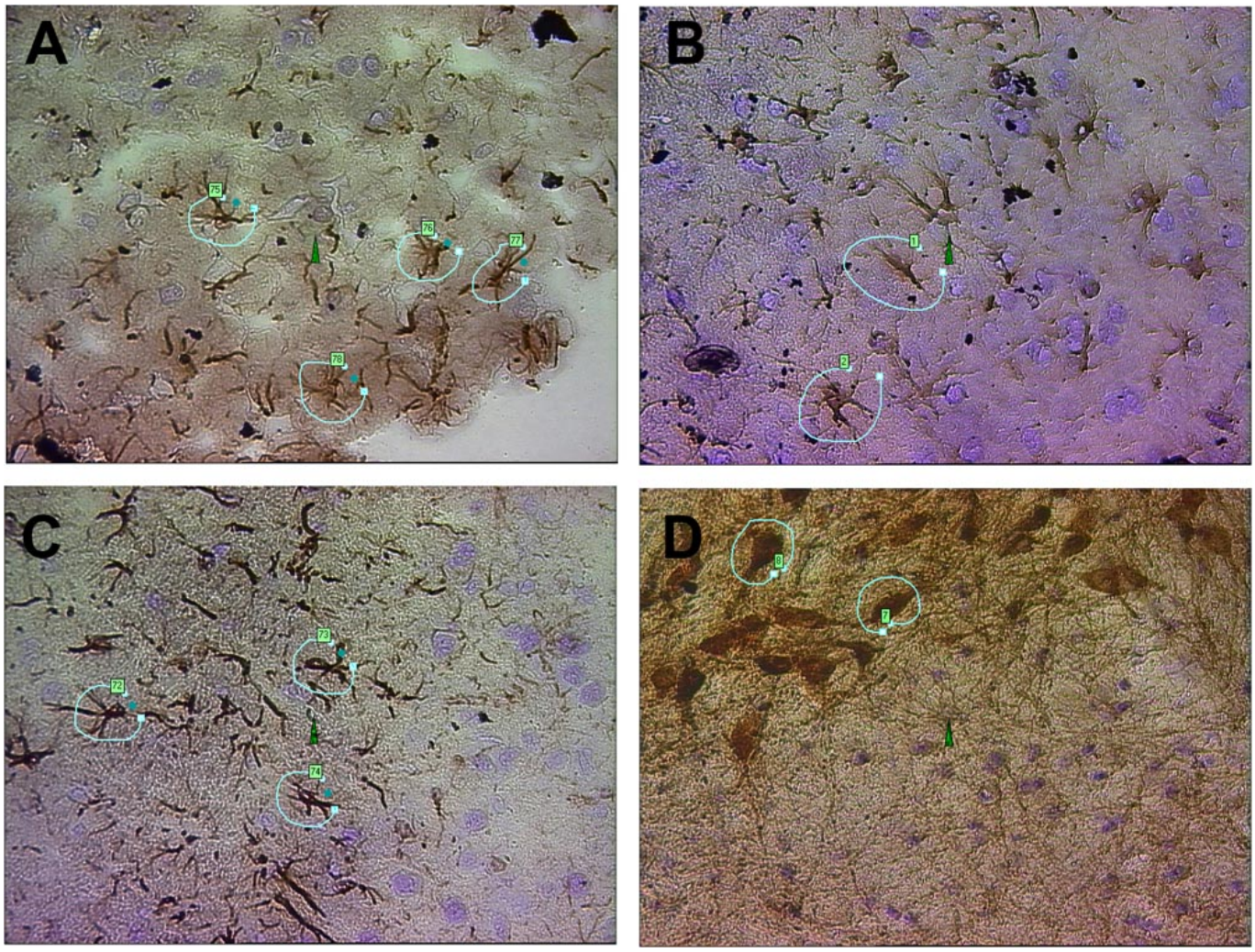

Fig. S2. Laser capture microdissection. Coronal sections (8 μm) of snap-frozen brain from MPTP-treated mice were immunolabeled with either GFAP (*A*–*C*) or TH (*D*). Sections were counterstained with Cresyl Violet. Individual immunopositive cells were captured. Special care was taken to avoid Nissl positive and immunonegative cells. (*A*) nigral astrocytes; (*B*) striatal astrocytes; (*C*) hippocampal astrocytes; (*D*) nigral dopaminergic neurons.

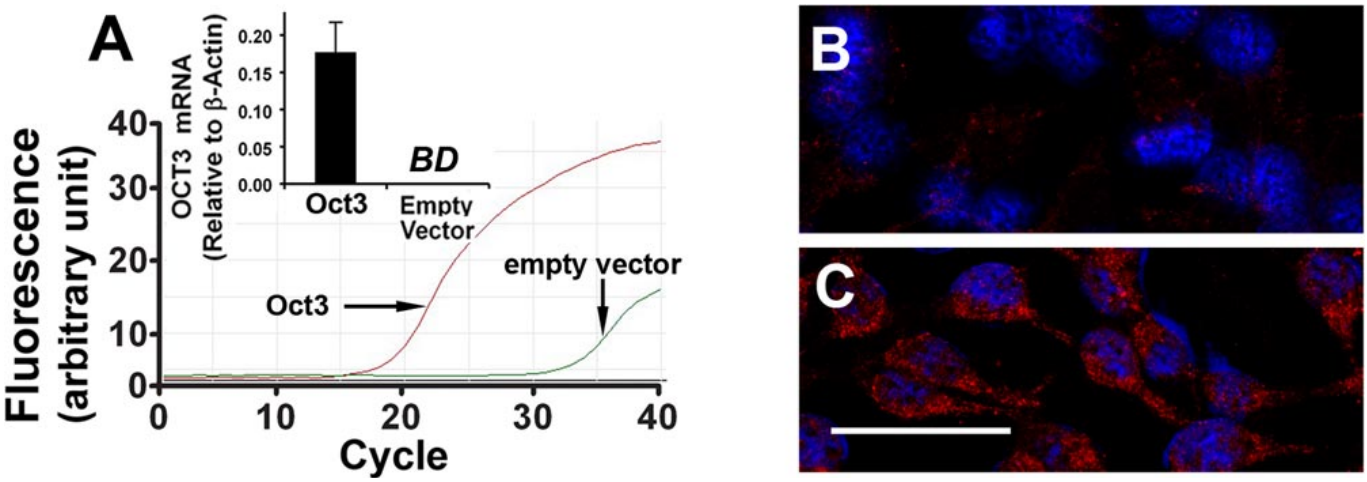

Fig. S3. EM4 cells stably overexpressing Oct3. Stable overexpression of rat Oct3 (*A* and *C*) or empty vector (*A* and *B*) was confirmed by real time quantitative RT-PCR (*A*) and immunocytochemistry (*B* and *C*). BD, below detection.

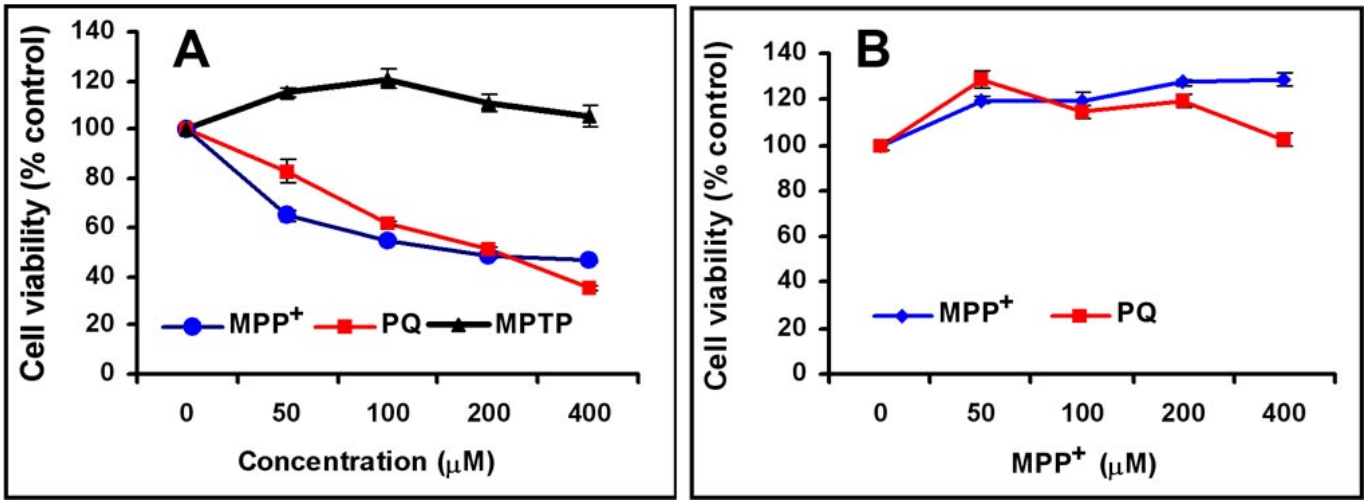

Fig. S4. Resistance of astrocytes to MPP^+-induced toxicity. Cells were grown in 96-well plates for 24 h before being exposed to different concentrations of indicated compounds for 24 h. Cell viability was assessed using MTT assay and expressed as percentage of untreated control. In N27 cells (*A*), which are immortalized dopaminergic neurons created from rat fetal mesencephalic cells, MPP^+ or paraquat (PQ) induced significant loss of cell viability. Consistent with the lack of MAO-B in midbrain dopaminergic neurons, N27 cells are resistant to the MPTP toxicity, suggesting the loss of cell viability induced by MPP^+ and PQ in these cells was not simply a nonspecific effect. Astrocytes (*B*), however, were resistant to toxicity induced by these toxic compounds. $n = 6$/time point performed in 3 separate experiments.

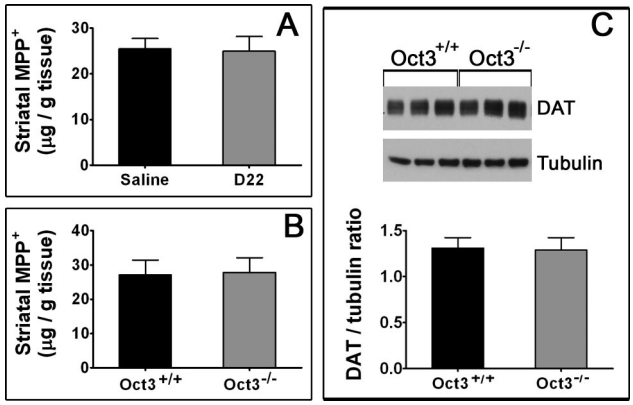

Fig. S5. MPP^+ and DAT abundance in striatal tissues. C57BL/6 mice infused with either vehicle or D22 (*A*) and Oct3 mice (*B*) were injected with MPTP (4 × 18 mg/kg, 2 h apart). Striatal MPP^+ levels were measured 90 min after the last injection using HPLC. Data represent mean ± SEM of $n = 5$/group. DAT levels (*C*) in striatal tissues of $Oct3^{-/-}$ and $Oct3^{+/+}$ littermates were assessed using immunoblotting (rat anti-DAT antibody, Chemicon) and quantified as the ratio over tubulin, $n = 3$/group.

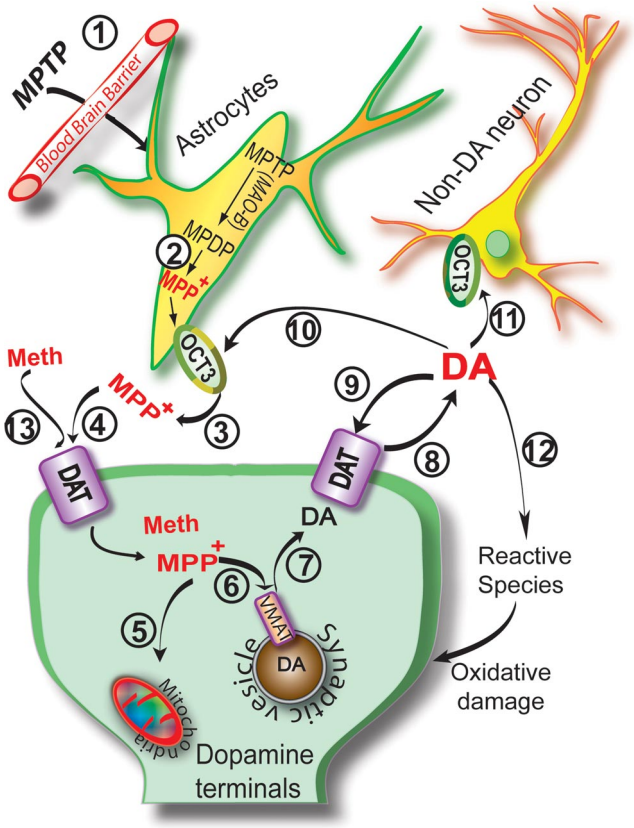

Fig. S6. Proposed role of Oct3 in modulating dopaminergic neurotoxicity induced by organic cations. MPTP is a lipophillic prototoxin that rapidly crosses the blood–brain barrier (1). In astrocytes, MPTP is metabolized by monoamine oxidase-B (MAO-B) and subsequently converted to the active toxic cation MPP^+ (2). From astrocytes, MPP^+ is extruded into the extracellular space through Oct3 (3). Since MPP^+ has a high affinity for the dopamine transporter (DAT), it is taken up by the neighboring dopaminergic neurons and terminals (4). Within these neurons, MPP^+ inhibits mitochondrial function (5). MPP^+ also enters (6) and induces effluxes (7) of synaptic DA through the vesicular monoamine transporter-2 (VMAT2) into the cytosol and subsequently, the extracellular compartment (8). Some of the extracellular DA is taken back up into dopaminergic terminals through DAT (9), some is taken into astrocytes (10) or the neighboring GABAergic cells (11) through Oct3, and some is converted extracellullary into dopamine-related reactive species (12), which can induce oxidative damage. Methamphetamine is also a substrate for Oct3 but it enters dopaminergic cells largely through DAT (13) and induces extracellular effluxes of dopamine. Because Oct3 is bidirectional, it can modulate neurodegeneration by releasing or removing toxic cations from the extracellular compartment.

Table S1. Oct3 expression in mouse brain

Brain regions	Astrocytes	Neurons
Substantia nigra	++	+ (in GABA-like but not dopaminergic neurons)
Striatum	+	++
Cortex	BD	+
Cerebellum	BD	+++ (in granule but not purkinje cells)
Hippocampus	BD	+++
Locus coeruleus	BD	BD
Ventricles	++	+++

Variable intensity of Oct3 immunofluorescent signal (denoted with the plus sign) was detected in different brain regions. BD, below detection.