Supporting Information

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SI Materials and Methods

Culture Studies. CC and VM areas were removed separately from embryonic day 15 Sprague-Dawley rats. Then CC areas from ICR WT and *Glp1r*$^{-/-}$ mice, dissociated by mild trypsination, and equal numbers of cells were seeded onto 96-well plates in plating media [DMEM-12 media containing 2% B27 supplement (Invitrogen); 10% heat-inactivated FBS; for CCs, 0.5 mM L-glutamine and 25 μM L-glutamate; and for VMs, 1 mM L-glutamine] at a density of $\approx 6 \times 10^4$ cells/well. From the third day in vitro (DIV), cultures were maintained in feeding media [Neurobasal medium containing 2% B-27 supplement (Invitrogen) and 0.5 mM L-glutamine] in a 5% CO_2/21% O_2 atmosphere at 37 °C. Human SH-SY5Y neuroblastoma cells (American Type Culture Collection) were grown to 80% confluence [1:1 Eagle's Minimal Essential Medium and Ham's F12 medium supplemented with 10% heat-inactivated FBS and 100 U/mL of penicillin/streptomycin (Invitrogen), at 37 °C, 5% CO_2, and 95% air].

RT-PCR. Total RNA was extracted from CC and VM cells at DIV 10, or from SH-SY5Y cells, using TRIzol reagent (Invitrogen). Cells from 10 wells were pooled and used for RNA extraction, in which the quality and quantity of RNA were assessed by spectrophotometry at 260 and 280 nM. Before RT-PCR, 1 μg of RNA was first treated with DNase I (Ambion) to degrade genomic DNA; subsequently, 50 ng of treated RNA was used for each one-step RT-PCR (QIAGEN OneStep RT-PCR Kit). The following specific primers for CC and VM cells were used: rat GLP-1R, forward: 5′ AGTAGTGTGCTCCAAGGGCAT 3′, reverse: 5′ AAGAAAGTGCGTACCCCACCG 3′ (expected PCR product, 190 bp); rat GAPDH, forward: 5′ GACCTGCAGAGCTCCAATCAAC 3′, reverse: 5′ CACGACCCTCAGTACCAAAGGG 3′ (expected PCR product, 214 bp). RNA extracted from CHO-GLP-1R cells (CHO cells permanently transfected with rat GLP-1R) was used as a positive control. RT-PCR conditions for both GLP-1R and GAPDH were 50 °C for 30 min; 95 °C for 15 min; 35 cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s; and then 72 °C for 10 min.

The following primers for human SH-SY5Y cells were used: human GLP-1R, forward: 5′ TCAAGGTCAACGGCTTATTAG 3′; reverse: 5′ TAACGTGTCCCTAGATGAACC 3′ (expected PCR product, 480 bp). Secondary primers (a second pair of human GLP-1R primers) were forward: 5′ TTCTGCAACCGGACC 3′; reverse: 5′ CAAGTGCTCAAGCCG 3′ (expected product size, 1.1 kb).

Treatments. To establish the functionality of GLP-1Rs, time-dependent levels of cAMP were quantified (EIA kit; Assay Designs) in DIV10 cell cultures with GLP-1 (10 nM). CC DIV11 cells were treated with feed media using B27 without antioxidants (B27-AO; Invitrogen). On DIV 13, cells were treated with Ex-4, GLP-1, or vehicle with or without the GLP-1R antagonist Ex-9–39, and then placed in a hypoxic incubator (37 °C, 1% O_2, 5% CO_2; Thermo Forma) for 3 h. Normoxic control cells (21%O_2, 5% CO_2, 37 °C) with similar treatments also were incubated. Subsequently, all cells were placed under normoxic conditions, and after 48 h, cell viability was assessed using MTS (Promega) or LDH (Sigma) assays. This study was repeated using cells from *Glp1r*$^{-/-}$ mice.

VM DIV10 cells were pretreated with feeding media containing 100 nM GLP-1 or Ex-4, or vehicle, for 3 h at 37 °C. Half of the dells were then treated with 6-OHDA (30 μM in O_2-free PBS) for 90 min, to induce dopaminergic neuron injury. Then the cells were washed, incubated for 24 h, and fixed in 4% PFA for TH immunostaining. Immunostaining was performed with a mouse anti-TH monoclonal antibody (1:500; Chemicon) and counterstained with DAPI nuclear dye (Invitrogen). TH-immunostained cultures were imaged with a Nikon Eclipse TE2000-E inverted microscope using a Diagnostic Instruments Spot RT slider camera and Molecular Devices MetaMorph v6.2 software. In brief, the microscope stage was programmed to move to the center of each well of a 96-well plate for manual focusing. Once focused, the stage moved to 4 locations per well and acquired images using a UV filter (DAPI) and an FITC filter (TH). Exposure times were kept constant for each filter. Immunoreactive pixel densities (TH) or cell counts (DAPI) were determined using the integrated morphometry feature of Metamorph (n = 5 wells/per treatment group).

Parallel studies were undertaken in SH-SY5Y cells. In specific studies, biochemical markers of cell death (caspase-3, Bax, and Bcl-2) were assessed by Western blot analysis, as described previously (1–3). In others, inhibitors of PI3K (LY294002; 10 μM), MAPK (PD98059; 20 μM), and PKA (H89; 10 μM) were used (4).

Animal Studies. Rodents were maintained under temperature- and light-controlled conditions (20–23 °C, 12-h light/dark cycle) with continuous access to food and water.

Stroke. Adult male Sprague-Dawley rats were anesthetized (chloral hydrate 0.4 g/kg i.p.) and, to ensure no impact on the subsequent area of infarction, Ex-4 (1 μM $\times$ 20 μL; 83 ng) or vehicle (PBS) was administrated into the left lateral ventricle 15 min before right-sided 60-min MCAo, induced with a 10-O suture (5). The coordinates for intraventricular administration were 0.8 mm posterior to the bregma, 1.5 mm lateral to the midline, and 3.7 mm below the dura surface. Core body temperature was maintained at 37 °C during and after surgery. Cortical blood flow was measured using a laser Doppler flowmeter (PF-5010, Periflux system; Perimed). The animal was placed in a stereotaxtic frame, and a burr hole was created in the right frontoparietal region. A Perimed blood flow probe (probe 411, 0.45 mm diameter, PeriFlux system) was placed in the cortex stereotaxically (3.5–4.0 mm posterior, 3.5–4.0 mm lateral to the bregma, and 1.0 mm below the dura). Blood pressure measurements and blood gas analyses were performed as described previously (5).

Locomotor Measures. The animal was placed in an Accuscan activity monitor for 30 min at 48 h after MCAo. Motor activity was determined using infrared beam sensors.

TTC Staining. Animals were killed 48 h after MCAo. Brains were removed, sliced into 2.0-mm-thick sections, incubated in 2% wt/vol TTC, fixed in 4% wt/vol PFA, and then digitally scanned (5). The area of infarction was analyzed by observers blinded to treatment group. In parallel studies, adult male ICR WT (n = 13) and *Glp1r*$^{-/-}$ (n = 15) mice were anesthetized (chloral hydrate 0.4 g/kg i.p.), and Ex-4 (1 μM $\times$ 5 μL) or vehicle (saline) was administered into the left lateral ventricle 15 min before right-sided transient MCAo. Ligation was performed with a 10–0 suture for 90 min and then released. The coordinates for intraventricular administration were 0 mm anterior-posterior to the bregma, 0.9 mm lateral to the midline, and 2.0 mm below the dura surface. Core body temperature was maintained at 37 °C during and after surgery. Animals were killed 48 h after ligation. Brains were removed, sliced into 1-mm-thick sections, incubated

in 2% wt/vol TTC, fixed in 4% wt/vol PFA, and then digitally scanned. The area of infarction was analyzed by observers blinded to treatment group. A volume of infarction in each animal was obtained from the sum of infarction areas in all 1-mm brain slices examined.

PD (MPTP) Model. Adult male C57BL/6 mice (22–24 g) were implanted with an osmotic minipump (Alzet) containing either PBS or Ex-4 (20 nM, 0.25 μL/h over 7 days) attached to a cannula for lateral ventricle administration. After 2 h, MPTP (20 mg/kg in 0.1 mL of PBS given i.p. at 2-h intervals in 4 doses; Sigma M-0896) was administered (1). Control mice received PBS. At designated times thereafter, motor function was evaluated. In some studies, the brain was removed and the striatum was dissected and stored at -80 °C for monoamine measurements. In others, after perfusion with 4% PFA, the brain was removed, fixed (4% PFA, 12 h, 4 °C), and then stored in 30% sucrose for TH immunohistochemistry.

Monoamine/Immunoblot Analyses. The striatum was prepared and extracted (1), and concentrations (ng/mg tissue weight) of DA and metabolites DOPAC, HVA, and 5-hydroxyindolacetic acid [5-HIAA]) were measured by HPLC with electrochemical detection. TH immunoblotting (1) was done using a TH (phospho S40) antibody (AbCam).

Immunohistochemistry. Sections (50 μm) throughout the SN were processed for immunohistochemistry using TH antibody (Sigma T-1299, diluted 1:1000). TH immunoreactivity was visualized using a monoclonal anti-TH antibody (1), and quantification of TH-immunopositive cells was undertaken by image analysis. Sections were dried and mounted on coverslips. Cells were counted using a computer-assisted stereologic toolbox. Counts were done blinded to drug treatment and performed at 100-fold magnification.

Behavioral Assessment. A "mean score of behavior" was provided by a gross neurologic screen that included vibrissae placing, reaching reflex, righting reflex, and turning and gait. In the vibrissae placing test, mice were grasped by the tail and held so that their vibrissae, but not skin, brushed the edge of a table, to assess their reaction to the table edge. In the reaching reflex test, mice were grasped by the tail and slowly lowered toward a table surface to determine whether they reached out their paws. The righting reflex test assessed the mice's ability to regain their posture after being placed on their back. The turning and gait test assessed their ability move across a flat surface.

Rotarod. Mice were placed on a stationary rod and trained to remain on it as it rotated at 5 rpm. The mean time spent on the rotating rod (3 consecutive trials) to an established maximal time of 180 s (1) was assessed at day 7 post-MPTP.

Pole Test. Mice were placed head up on a 50 × 1 cm high gauze-taped pole below a cork ball (diameter 1.5 cm) at its top. The times taken to reach the floor (T-Total) and to turn around completely (T-Turn) were determined, to an established maximal time of 60 s. The test was performed under dim red light (1 lux) on day 4 after MPTP, and the best performance of 5 trials was recorded (6, 7).

Beam Walk. The time taken for mice placed at one end of a brightly lit beam (50 × 0.9 cm and 50 cm above the floor) to enter a dark shelter at the other end was measured. The number of slips and falls were recorded. Mice were habituated to the test over 4 days and were assessed at day 4 after MPTP (mean of 6 trials) (6).

Open Field. Mice were assessed in an open-field apparatus (San Diego Instruments), in which activity was measured using infrared beams. Mice were tested for 10 min on 4 consecutive days, starting 1 h after the final MPTP dose. The mean and maximal velocity (V_{mean} and V_{max}), distance traveled, and number of rearings were measured.

Statistics. Throughout, Dunnett's *t*-test and 1-way ANOVA were used for statistical comparisons, with a significance level of $P \leq .05$. In the event of multiple comparisons, Bonferroni correction was performed. Data are presented as mean ± SEM.

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7. Matsuura K, Kabuto H, Makino H, Ogawa N (1997) Pole test is a useful method for evaluating the mouse movement disorder caused by striatal dopamine depletion. *J Neurosci Methods* 73:45–48.

GLP-1: H A E G T F T S D V S S Y L E G Q A A K E F I A W L V K G R

Exendin-4: H G E G T F T S D L S K Q M E E E A V R L F I E W L K N G G P S S G A P P P S

Exendin (9-39): E G T F T S D L S K Q M E E E A V R L F I E W L K N G G P S S G A P P P S

Fig. S1. Amino acid sequence of GLP-1, the long-acting agonist Ex-4, and the antagonist Ex-9–39.

PNAS PNAS PNAS PNAS

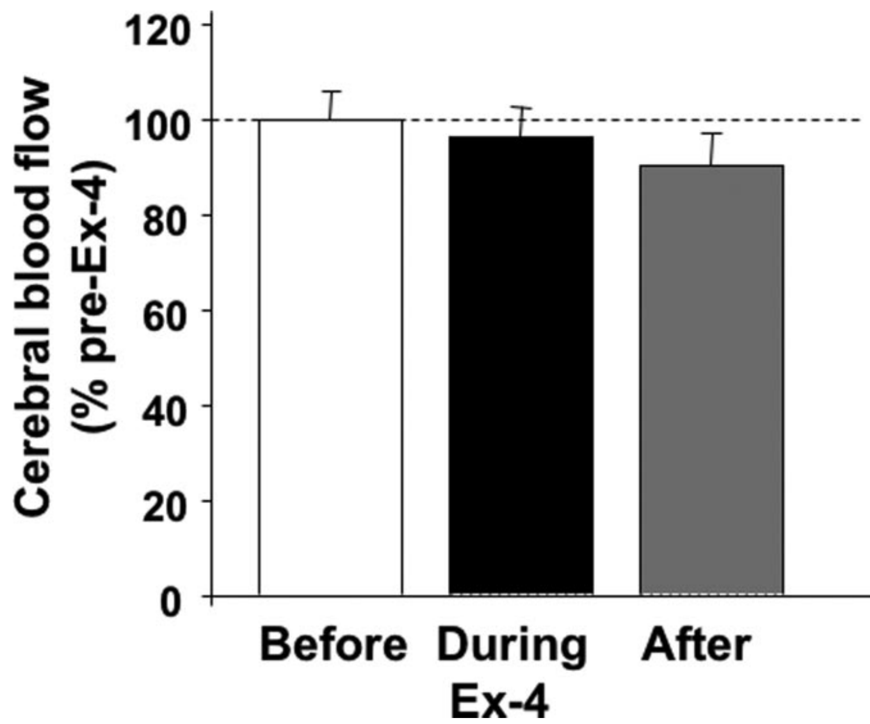

Fig. S2. Cortical cerebral blood flow was unaffected by Ex-4 (1 μM x 20 μL lateral ventricle: 83 ng), as measured by laser Doppler flowmetry.

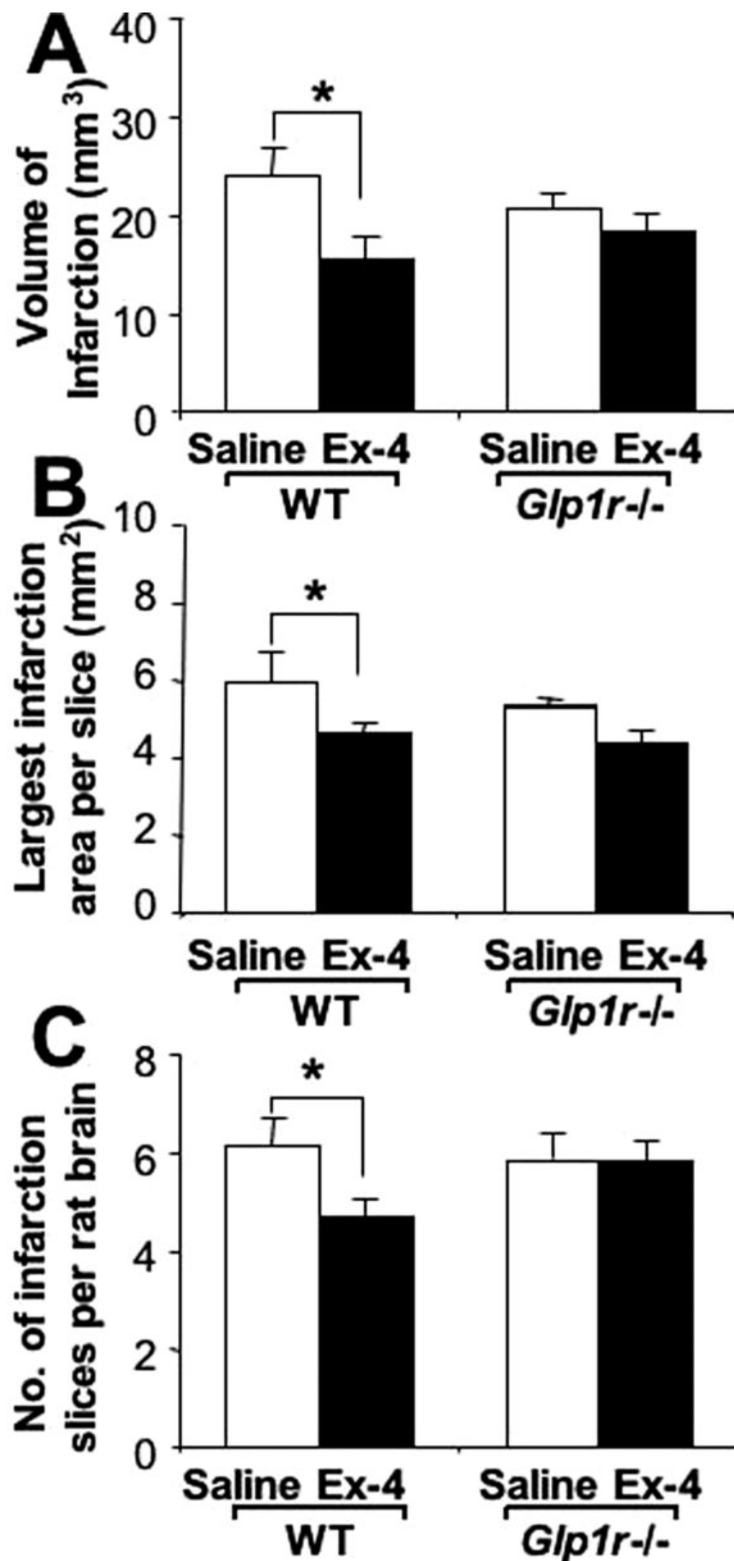

Fig. S3. Ex-4 markedly reduced cortical infarction induced by transient MCAo in WT but not *Glp1r*$^{-/-}$ mice. (*A*) After administration of Ex-4 (1 μM $\times$ 5 μL; 21 ng) or vehicle (saline) into the left (L) lateral ventricle, the right (R) middle cerebral artery was ligated for 90 min. At 48 h after ischemia/reperfusion, the mice were killed, and their brains were sliced into 1-mm sections, and stained with TTC. The volume of infarction = [sum of the infarction area in all brain slices (mm^2)] $\times$ [slice thickness (1 mm)]. (*B*) The area of the largest infarction in a slice from each mouse brain. (*C*) The number of infarcted slices from each mouse brain. WT control (saline), n = 6; WT Ex-4, n = 7; *Glp1r*$^{-/-}$ control (saline), n = 8; *Glp1r*$^{-/-}$ Ex-4, n = 7. *$P \leq .05$, 1-way ANOVA and Student *t*-test.

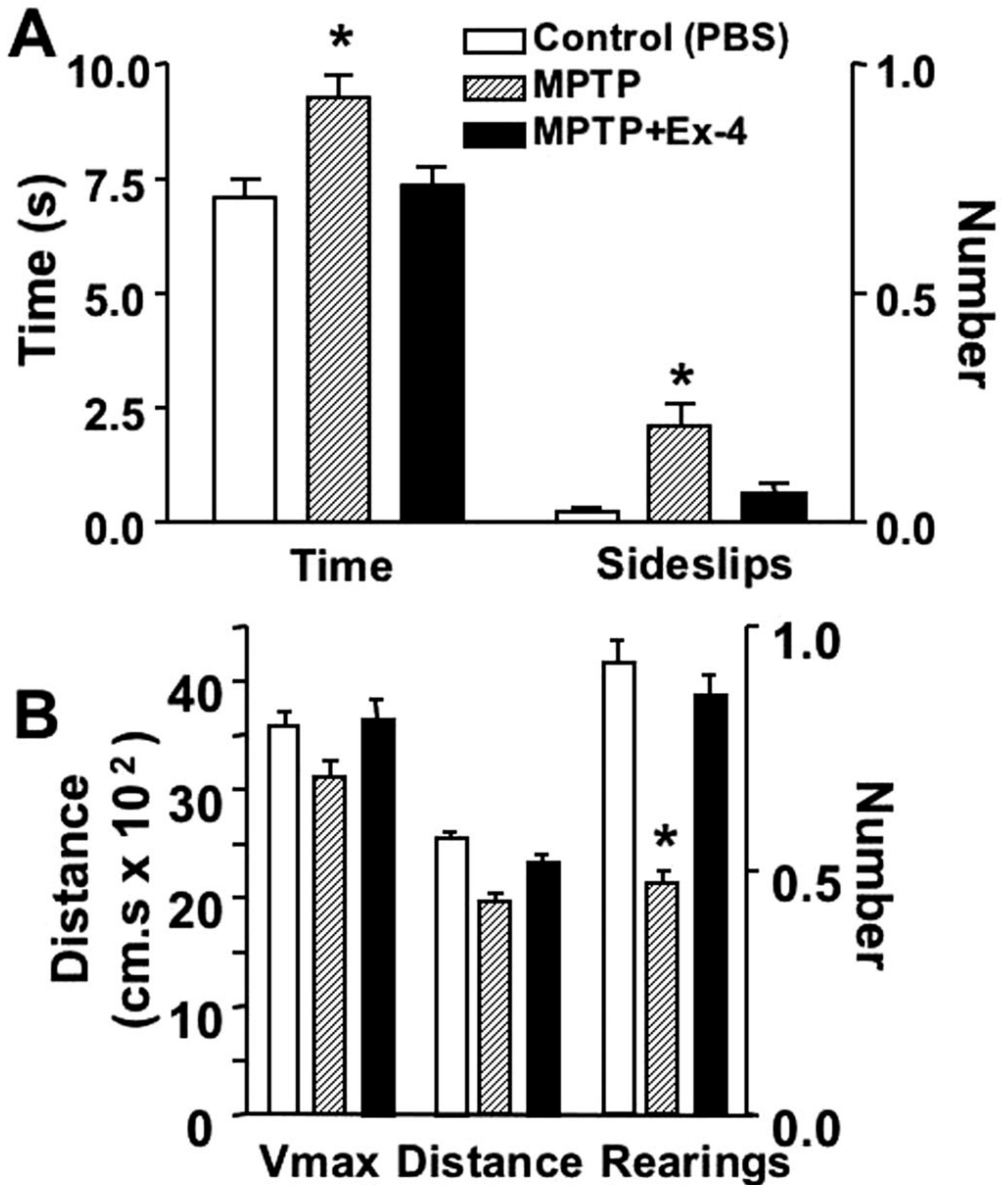

Fig. S4. Ex-4 treatment protected mice against MPTP-induced deficits in motor functions, as assessed by beam traverse (*A*) and open-field activity (*B*). (*A*) The time taken for mice placed on a brightly lit beam to enter a dark shelter at the end of the beam was measured, and slips and falls were recorded (mean of 6 trials). (*B*) Activity of mice was monitored in an open-field apparatus (San Diego Instruments). V_{max}, maximum velocity; distance, distance traveled; rearings, number of rearings. For both paradigms, mice were habituated to the test over 4 days and were assessed at day 4 after MPTP. $^*P < .05$ versus controls, Dunnett's *t* test, $n = 10$/group.

Table S1. Arterial blood gas, blood pressure, and brain temperature in rats was unaffected by Ex-4 treatment

	Vehicle (n = 8)		Ex-4 (n = 6)	
	Before, mean ± SEM	After, mean ± SEM*	Before, mean ± SEM	After, mean ± SEM*
pH	7.39 ± 0.02	7.41 ± 0.02	7.40 ± 0.01	7.38 ± 0.03
$PaCO_2$, mm Hg	44.75 ± 0.75	41.0 ± 1.6	42.5 ± 1.0	41.0 ± 2.4
PaO_2, mm Hg	86.5 ± 1.8	87.7 ± 2.6	95.5 ± 1.8	88.0 ± 3.4
Blood pressure, mm Hg	113.3 ± 5.5	112.5 ± 8.6	98.7 ± 6.8	102.0 ± 7.1
Brain temperature (° C)		37.0 ± 0.1		36.8 ± 0.1
Body temperature (° C)		36.8 ± 0.1		36.8 ± 0.1

*Between 20 and 30 min after L ventricle Ex-4; $P > .05$ vs. before (Student's t test).