A single intramuscular injection of rAAV-mediated mutant erythropoietin protects against MPTP-induced parkinsonism

A. Dhanushkodi[†,‡], **E. O. Akano**[†,‡], **E. E. Roguski**[¶], **Y. Xue**[†,‡], **S. K. Rao**[†,‡], **S. G. Matta**[§], **T. S. Rex**[¶], and **M. P. McDonald**[†,‡,*]

[†]Department of Neurology, University of Tennessee Health Science Center, Memphis, TN, USA

[‡]Department of Anatomy & Neurobiology, University of Tennessee Health Science Center, Memphis, TN, USA

[§]Department of Pharmacology, University of Tennessee Health Science Center, Memphis, TN, USA

[¶]Department of Ophthalmology, University of Tennessee Health Science Center, Memphis, TN, USA

Abstract

Erythropoietin (Epo) is neuroprotective in a number of preparations, but can lead to unacceptably high and even lethal hematocrit levels. Recent reports show that modified Epo variants confer neuroprotection in models of glaucoma and retinal degeneration without raising hematocrit. In this study, neuroprotective effects of two Epo variants (EpoR76E and EpoS71E) were assessed in a model of Parkinson's disease. The constructs were packaged in recombinant adeno-associated viral (rAAV) vectors and injected intramuscularly. After 3 weeks, mice received five daily injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and were killed 5 weeks later. The MPTP-lesioned mice pretreated with rAAV.eGFP (negative control) exhibited a 7- to 9-Hz tremor and slower latencies to move on a grid test (akinesia). Both of these symptomatic features were absent in mice pretreated with either modified Epo construct. The rAAV.eGFP-treated mice lesioned with MPTP exhibited a 41% reduction in tyrosine hydroxylase (TH)-positive neurons in the substantia nigra. The rAAV.EpoS71E construct did not protect nigral neurons, but neuronal loss in mice pretreated with rAAV.EpoR76E was only half that of rAAV.eGFP controls. Although dopamine levels were normal in all groups, 3,4-dihydroxyphenylacetic acid (DOPAC) was significantly reduced only in MPTP-lesioned mice pre-treated with rAAV.eGFP, indicating reduced dopamine turnover. Analysis of TH-positive fibers in the striatum showed normalized density in MPTP-lesioned mice pretreated with rAAV.EpoS71E, suggesting that enhanced sprouting induced by EpoS71E may have been responsible for normal behavior and dopaminergic tone in these mice. These results show that systemically administered rAAV-generated non-erythropoietic Epo may protect against MPTP-induced parkinsonism by a combination of neuroprotection and enhanced axonal sprouting.

*Corresponding author: M. P. McDonald, PhD, Department of Neurology, UTHSC, 855 Monroe Ave., 415 Link Bldg., Memphis, TN 38163, USA. mike@tennessee.edu.

Keywords

Akinesia; dopamine; erythropoietin; gene therapy; MPTP; neuroprotection; parkinsonism; striatum; substantia nigra; tremor

---

Parkinson's disease is characterized neuropathologically by gradual degeneration of dopaminergic neurons in the substantia nigra and behaviorally by bradykinesia, akinesia, tremor, postural instability and impaired executive function (Blanchet *et al*. 2000; Monchi *et al*. 2007; Mondon *et al*. 2007). There is no cure for Parkinson's disease, and current interventions are typically directed toward diminishing behavioral symptoms and enhancing quality of life. The most commonly used treatments at the moment are only partially and transiently effective, or are only effective in a minority of patients (Bronstein *et al*. 2011; Poulopoulos & Waters 2010; Wood 2010). Importantly, they do not prevent the progressive destruction of nigrostriatal neurons.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been used extensively to model Parkinson's disease in species as diverse as monkeys, mice and worms (Braungart *et al*. 2004; Pope-Coleman & Schneider 1998; Pope-Coleman *et al*. 2000; Schneider & DiStefano 1994; Schneider & Yuwiler 1989; Schneider *et al*. 1998). Injected systemically, it destroys nigrostriatal dopaminergic neurons rapidly and reliably. The MPTP readily enters the brain and is converted to the toxic metabolite 1-methyl-4-phenylpyridinium (MPP+) by monoamine oxidase (MAO; Cicchetti *et al*. 2009). MPP+ is then taken up by the dopamine transporter where it depletes vesicular dopamine and inhibits mitochondrial respiration, ultimately resulting in neuronal death. In monkeys, this produces a profound akinesia and tremor; however, in mice, Parkinson-like behavioral consequences are typically minimal and transient, even at large doses (Hallman *et al*. 1984; Heikkila & Sonsalla 1992; Sedelis *et al*. 2001; Willis & Donnan 1987).

Erythropoietin (Epo) is a glycoprotein responsible for generating red blood cells. The volume of blood occupied by red blood cells, or hematocrit, is tightly controlled by an Epo-mediated feedback loop. It has long been known that Epo is neuroprotective (Bernaudin *et al*. 1999; Sinor & Greenberg 2000); however, long-term administration raises hematocrit to unacceptably high levels and may be fatal. Intracranial administration is an alternative but requires surgery. Genc *et al*. (2001) showed that Epo can protect against MPTP-induced neurotoxicity when injected in the central nervous system (CNS). They injected native Epo directly into the substantia nigra of mice either 24 h before or immediately after MPTP administration, and found that in both cases Epo prevented the hypokinesia associated with subchronic MPTP. Similarly, Puskovic *et al*. (2006) showed that HSV-mediated native Epo injected intrastriatally a week before MPTP injections prevented dopaminergic loss. These studies show that the neuroprotective properties of Epo are effective against MPTP-induced neurotoxicity. Indeed, Wu *et al*. (2010) showed region-specific upregulation of Epo receptors in the substantia nigra following five daily injections of 30 mg/kg MPTP. We have recently developed some novel Epo variants designed to retain their neuroprotective properties without raising hematocrit. One of them has shown effectiveness in mouse models of retinal degeneration and glaucoma (Sullivan *et al*. 2011). The experiments

described herein were designed to determine whether the mutant Epo variants would protect against MPTP-induced neurotoxicity. We chose two Epo variants that have demonstrated neuroprotective properties without raising hematocrit. We hypothesized that the novel Epo variants would be neuroprotective in the MPTP model.

Material and methods

Subjects

Subjects were 94 male wild-type C57BL/6J mice (Stock #000664; Jackson Laboratories, Bar Harbor, ME, USA), 9 months old at the start of testing. Mice were housed five per cage in tub cages under standard conditions in a vivarium approved by the Association for Accreditation of Laboratory Animal Care International (AALAC), except for the 5 days during and 5 days following the MPTP injection regimen as described below. Mice had free access to food and water for the duration of the study and were maintained on a 12:12-h light/dark cycle, with lights on at 0600 h. All experiments were conducted during the light cycle and were approved by the Institutional Animal Care and Use Committee.

Generation and administration of rAAV vectors

EpoR76E and EpoS71E were produced by site-directed mutagenesis of rhesus Epo (ARIAD Pharmaceuticals, Cambridge, MA, USA) using the Quickchange multi-site kit according to the manufacturer's protocol (Stratagene, La Jolla, CA, USA). Two nucleotides were altered to result in a conversion of arginine to glutamate at position 76 for EpoR76E and a serine to glutamate at position 71 for EpoS71E. The sequences were subcloned into an AAV2 backbone plasmid (gift of Dr James M. Wilson, University of Pennsylvania). The recombinant adeno-associated viral (rAAV) vectors were produced by the University of Iowa Vector Core using the triple transfection protocol. The rAAV was isolated and purified from the cells by double cesium chloride gradient centrifugation and dialyzed in HEPES-buffered saline (pH 7.8), and the viral titer was determined using the LightCycler 480 real-time quantitative polymerase chain reaction machine (Roche Applied Science, Branford, CT, USA) and primers specific to the polyA tail. The AAV2/5 serotype has been used safely in rodents, monkeys and humans, and is currently used in three clinical trials with Parkinson's patients with no adverse effects (Christine *et al*. 2006; Gasmi *et al*. 2007; Herzog *et al*. 2007; Kordower & Olanow 2008; Kordower *et al*. 2006; Starr *et al*. 2007). Pilot data showed that it takes 3 weeks to reach maximal transcriptional activity after transduction with AAV2/5 *in vivo*. The vector was diluted to the appropriate concentration, and mice were given a single injection in the gastrocnemius muscle with 10 μl (1.0×10^{11} genome copies) of AAV2/5.CMV.EpoR76E, AAV2/5.CMV.EpoR71E or AAV2/5.CMV.eGFP (negative control; Rex *et al*. 2004; Sullivan & Rex 2011; Sullivan *et al*. 2011, 2012). Experiments with additional mice showed that this dose of EpoR76E does not affect levels of endogenous Epo or Epo mRNA (data not shown). Confirmation of injection of the vectors was reported previously, in a study performed in parallel with this study and using the same vector, lot and dilution (Sullivan *et al*. 2011). In that study, the presence of enhanced green fluorescent protein (eGFP) was confirmed by examination of leg muscle tissue, and the presence of Epo variants in serum was 67 ± 89 mU/ml in treated mice and undetected in rAAV.eGFP controls.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine

The MPTP HCl (#M0896, lot #48K1861; Sigma, St. Louis, MO, USA) was dissolved in physiological (0.9%) saline at a concentration of 1.8 mg/ml free base (2.11 mg/ml salt). Starting 3 weeks after injection of the rAAV constructs, mice were given one injection of MPTP saline per day for 5 consecutive days. Injections were given subcutaneously at a volume of 10 ml/kg to achieve a dose of 18 mg/kg. A 30 mg/kg dose was used initially, the standard dose with the subchronic regimen. However, this dose proved fatal to a large number of mice so it was reduced to 18 mg/kg. Our pilot data showed that 18 mg/kg given once daily reduces striatal dopamine 37.2% in MPTP-treated vs. saline-treated mice ($F_{1,11}$ = 7.9, $P = 0.0167$), when killed on the fifth day following the last MPTP injection (saline 92.39 ± 6.31 ng/mg protein, $n = 8$; MPTP 57.99 ± 11.85, $n = 5$). Dopamine turnover [3,4-dihydroxyphenylacetic acid (DOPAC)/dopamine] was increased by MPTP in these mice (0.080 ± 0.003 vs. 0.062 ± 0.005; $P = 0.0254$).

While receiving MPTP injections, mice were housed in disposable cages under a fume hood to protect personnel from aeration of the toxic MPP+ metabolite excreted in the urine and feces. The cages remained under the hood for 5 days after the last injection, when the mice were transferred to clean tub cages and returned to the vivarium. The MPTP and associated hazardous waste were handled and administered in strict accordance with the safety guidelines outlined by Przedborski *et al.* (2001), and all procedures were approved and monitored by the Institutional Biosafety Committee. All behavioral procedures were conducted after the MPTP injections and the 5-day post-MPTP quarantine period.

Locomotor activity

Locomotor activity was assessed in 60-min sessions in commercially available activity monitors (MED Associates, Georgia, VT, USA) as previously described (Harrison *et al.* 2008, 2009, 2010; McDonald *et al.* 1998, 2001; Siesser *et al.* 2005, 2006). The activity monitors measured 27×27 cm, with 16 infrared beams equally spaced in the *x* and *y* axes of the horizontal plane, 1 cm from the floor of the monitor. An additional vector of 16 photobeams was situated 4 cm above the floor to track rearing. Mice were placed in the activity monitors for a 30-min session before receiving MPTP, and matched on activity levels for assignment to MPTP or saline groups, with the constraint that every subject within a cage was required to receive either MPTP or saline for biosafety reasons. Following MPTP, mice were given an additional 60-min session in the activity monitors.

Sensorimotor function

A number of tasks were conducted to measure balance, coordination and movement, including rotorod, horizontal beam, rope climb, block test, inverted screen, vertical pole, grid test and sticker test, as previously described (Dhanushkodi & McDonald 2011; Liu *et al.* 1997; Norflus *et al.* 1998; Sango *et al.* 1995, 1996). Balance and coordination were assessed using a Rotamex-5 rotorod (Columbus Instruments, Columbus, OH, USA). After a single practice trial, mice were trained for three trials per day for 3 consecutive days to balance on a rotating rod 3 cm in diameter. The rotation speed increased from 0 to 80 r.p.m., incrementing 0.8 r.p.m. every 3 seconds. If a mouse fell within 15 seconds it was given a second opportunity. In some cases, mice would grasp the rod and rotate around with it.

When this occurred, the time at which the first rotation occurred was noted and latency to fall or to the first rotation was the measure of interest. The horizontal beam task required the mouse to traverse a 0.64-cm diameter, 80-cm long beam elevated 40 cm above a blanket. Mice were motivated by a 25-W white light bulb at the starting platform, and reinforced with entry into a dark box on the other side of the beam. Mice were placed on the 5-cm^2 starting platform, and latency to initiate (all four paws on the beam), latency to traverse, and number of paw slips were recorded. The rope climb involved a similar avoidance of a 25-W white light bulb and escape into a dark box. Mice were placed on the 1.5-cm-diameter rope facing down to start the trial. Latency to turn around and latency to climb the 25 cm into the dark box were recorded. On the block test, the mouse was placed on a block, 10 cm^2 and 3 cm high. The latency to put its forepaws and then all four paws on the table were recorded. A second version was used in which the mouse was placed on the table with its forepaws on the block, and the latency to move the forepaws to the table was recorded. On the inverted screen task, mice were confined to a 15-cm^2 section of 1-cm^2 hardware cloth. The screen was waved in the air several times to induce a gripping response and then inverted. The duration to fall was recorded with a maximum of 60 seconds. On the vertical pole task, mice were placed on top of a 2.5-cm-diameter polystyrene ball situated atop a 40-cm-long pole covered in SELF-GRIP athletic tape (98% cotton & 2% latex; Dome Industries, Warwick, RI, USA). The pole was rooted in a sling and mounted in a cage containing clean bedding. The latency to come of the ball and place all four paws on the pole and the latency to climb down the pole and place all four paws on the bedding were recorded. On the grid task, the subject was placed on a screen of 1-cm^2 hardware cloth situated vertically. The latency to move all four paws was recorded. On the sticker test, a small round sticker was placed on the mouse's snout and the mouse was placed in a clean tub cage free to move about. The latency to the first attempt to remove the sticker was recorded, as well as the latency to remove the sticker.

Tremor

Tremor was assessed using a force-plate actometer (FPA; BASi, West Lafayette, IN, USA). The FPA consisted of a 44-cm^2 carbon fiber load plate situated on four force transducers, one at each corner, within a ventilated sound-attenuating cubicle illuminated by an 8-W fluorescent bulb. The position of the subject was tracked by the relative force impinging of each of the transducers, producing measures such as horizontal locomotor activity, stereotypy and bouts of low activity. With spatial resolution of 1 mm and temporal resolution of 1 millisecond, the FPA tracks variations in power to quantify tremors ranging from 0.1 to 24.6 Hz (Fowler *et al.* 2001, 2002; McKerchar *et al.* 2006). Mice were placed individually in the FPA enclosure for a period of 5 min and then removed to their home cages. The apparatus was cleaned with 15% ethanol after each session.

Histology and immunohistochemistry

Mice were killed 5 weeks following the last MPTP injection. The mice whose brains were used for immunohistochemistry were perfused transcardially under isoflurane anesthesia, first with ice-cold saline and then with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 30 min. Brains were removed and fixed overnight in the same fixative, cryo-protected and then 40-μm coronal cryostat sections were taken throughout the

extent of the substantia nigra and striatum for histological analysis. Free-floating sections were treated first with PBS containing 20% methanol and 3% hydrogen peroxide for 30 min and rinsed thoroughly in PBS. Sections were then incubated in 10% normal horse serum in PBS containing 0.1% Triton X-100 for 30 min and incubated overnight at 4°C in the primary antibody (dilution 1:200) targeting tyrosine hydroxylase (TH;#AB152; Millipore, Billerica, MA, USA). Following incubation, sections were rinsed three times in PBS, incubated for 1 h in a biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA), washed thrice, treated with avidin–biotin complex (Vectastain Elite ABC kit, Vector Labs) reagent for 1 h and visualized using diaminobenzidine. Sections were then mounted on slides and air-dried. Some sections were incubated in the anti-TH primary antibody and processed with a fluorescent donkey anti-rabbit secondary antibody (FITC, Vector Labs). Optical density of TH-positive fibers in the striatum was quantified from two to six grayscale sections per subject using the public domain software ImageJ v1.44n (http://rsb.info.nih.gov/ij). Fluorescent images of striatal TH-positive fibers were captured using a Zeiss 710 confocal microscope.

HPLC with electrochemical detection

The mice whose brains were used for high-performance liquid chromatography (HPLC) were killed by cervical dislocation without anesthesia, and trunk blood was collected for measurement of hematocrit. Brains were dissected rapidly and punches 1 mm in diameter were taken from the dorsal striatum of 2-mm thick coronal sections and flash-frozen in liquid nitrogen. The frozen striatal punches were suspended in 400 μl of artificial cerebrospinal fluid and 600 μl of 0.2 mM perchloric acid, homogenized for 30 seconds and then centrifuged at 9201 g for 15 min at 4°C. The supernatant was filtered through a nylon syringe filter (0.2 μm) and frozen at −80°C. Frozen samples (20 μl; 1:1 dilution with polished HPLC grade water) were automatically injected by an ESA 542 refrigerated autosampler (ESA, Chelmsford, MA, USA) onto a 150 ± 2 mm ODS C18 column connected to an ESA model 580 HPLC pump. The mobile phase, containing 80 mM sodium dihydrogen phosphate monohydrate, 2.0 mM 1-octanesulfonic acid sodium salt, 100 μl/l triethylamine, 5 nM ethylenediaminetetraacetic acid and 10% acetonitrile, pH 3.0, was perfused at 0.25 ml/min. Dopamine levels were determined using an ESA 5041 high-sensitivity analytical cell, an ESA 5020 guard cell and an ESA Coulochem II 5200A electrochemical detector at a potential of 220 mV with the current gain at 10 nA and the guard cell at +350 mV. Under these conditions, the limit of detection for dopamine is 100 fg per injection.

Stereological quantification of cells in SNc

For unbiased stereological quantification of TH-positive cells in four sections per mouse, every eighth section through the rostro-caudal extent of the substantia nigra pars compacta (SNc) was measured using the optical fractionator counting method and Stereo Investigator v9.0 software (MicroBrightField, Williston, VT, USA) in the Neuroscience Institute's Imaging Center at University of Tennessee Health Science Center (UTHSC; http://cns.utmem.edu/imaging-center). The contour of the SNc was first delineated using Stereo Investigator's anatomical mapping tool at low power. The TH-positive cells within the SNc were counted from 10 to 20 frames measuring 25×25 μm in each of the selected sections,

generated using Stereo Investigator's random sampling grid. The frames were selected using the systematic random sampling scheme, which provides an unbiased and efficient sampling technique. In every counting-frame location, the top of the section was identified, after which the plane of the focus was moved 4 μm deeper through the section (guard zone) to prevent counting inaccuracies due to uneven section surfaces. The resulting focal plane served as the first point of the counting process. All TH-positive cells that came into focus in the next 8-μm segment (dissector height) were counted if they were entirely within the counting frame or touching the upper or right side of the counting frame. On the basis of these parameters and counts, the total number of TH-positive cells per selected region was counted using the optical fractionator formula $N = 1/ssf \times 1/asf \times 1/hsf \times Q$, where ssf is the section sampling fraction, asf is the area sampling fraction, hsf is the height sampling fraction (dissector height divided by the section thickness after shrinkage) and Q denotes the total count of particles sampled for each region.

Statistical analysis

Statistical tests were conducted using JMP Pro 9.0.2 (SAS Institute, Cary, NC, USA). Most behavioral, histological and neurochemical data were analyzed using two-way analysis of variance (ANOVA) or repeated-measures ANOVA (RMANOVA), with lesion (saline or MPTP) and treatment (eGFP, EpoS71E or EpoR76E) as between-subjects variables. Follow-up comparisons were made using orthogonal paired *t*-tests. Degrees of freedom on repeated-measures analyses were corrected for variations in sphericity using Huyn–Feldt ε. To protect against spurious type I errors, follow-up analyses were conducted only after a significant omnibus effect, except for comparisons having specific *a priori* hypotheses. Assessment of hematocrit levels was made using one-way ANOVAs for lesion and treatment separately, excluding the native Epo control group because they did not receive MPTP. All statistical tests were two tailed with $a = 0.05$.

Results

Five weeks following the first MPTP injection, mice were killed and brains processed for immunohistochemistry or HPLC. Trunk blood was collected and hematocrit was measured in experimental mice, as well as a positive control group receiving native Epo (rAAV.Epo) and given five daily injections of saline. Most of the mice in this positive control group died, but those that survived had elevated hematocrit ($71.5 \pm 0.5\%$; Fig. 1) compared with the normal range for male mice of 44–48% as exemplified by the rAAV.eGFP negative control group, and compared with all other mice in the study ($F_{1,26} = 72.9$, $P < 0.0001$). There was no overall effect of MPTP on hematocrit ($F_{1,24} = 0.42$, $P = 0.525$). There was a significant effect of rAAV-mediated treatment on hematocrit ($F_{2,23} = 10.86$, $P = 0.0018$). Follow-up analysis showed that hematocrit levels were slightly but significantly elevated in mice pretreated with rAAV.EpoS71E compared with rAAV.eGFP controls [$t(23) = 3.9$, $P = 0.0008$], although nowhere near the native Epo levels. Hematocrit levels were in the normal range for rAAV.EpoR76E-treated mice and not different from those of rAAV.eGFP-treated controls [$t(23) = 0.3$, $P = 0.783$].

Although mice do not exhibit the robust behavioral deficits observed in monkeys after MPTP lesioning, only subtle behavioral changes are observed with the subchronic regimen using the standard 30 mg/kg dose. Similarly, with the 18 mg/kg dose we saw very little in the way of behavioral symptoms. In the activity monitors, there were no main or interaction effects of MPTP or either Epo treatment on distance traveled, beam breaks, time active or ambulatory episodes (F's < 2.8, P's > 0.104; data not shown). Velocity also did not differ by lesion or treatment conditions (F's < 1.1, P's > 0.437; data not shown). Similarly, performance on the rope climb, horizontal beam, vertical pole, rotorod and block test was unaffected by MPTP (F's < 1.4, P's > 0.246; data not shown). Initial analyses of force-plate and grid-test data did not show significant main or interaction effects (F's 2.5, P's 0.101). However, follow-up tests showed that MPTP induced a significant tremor in the 7- to 9-Hz range in mice pretreated with rAAV.eGFP [Fig. 2a; $t(29) = 2.3$, $P = 0.0268$]. MPTP did not affect tremor in either of the modified Epo groups (t's < 0.6, P's > 0.592). MPTP also induced a significant impairment on the grid task in rAAV.eGFP-treated mice (Fig. 2b). When first placed on the grid, mice normally adapt quickly and move all four paws within 3–4 seconds, as exemplified by saline control mice in all treatment groups. However, MPTP-lesioned mice took twice as long to start moving as regular mice when pretreated with rAAV.eGFP, indicative of akinesia [$t(28) = 3.2$, $P = 0.0037$]. When pretreated with either of the modified Epo constructs, MPTP did not significantly affect grid movement latencies (t's < 0.8, P's > 0.436).

Figure 3 shows that five daily injections of 18 mg/kg MPTP induced significant changes in the TH-positive cell count ($F_{1,12} = 48.3$, $P < 0.0001$). There was also a significant effect of Epo treatment ($F_{2,12} = 4.9$, $P = 0.0275$), indicating that not all groups were affected equally by the neurotoxin. The lesion × treatment interaction was not significant ($P = 0.170$). In terms of percentage control reduction from each group's own saline control, the percentage loss of TH-positive neurons was statistically significant in the rAAV.eGFP ($41.0 \pm 7.5\%$) and rAAV.S71E ($39.6 \pm 4.4\%$) groups (P's < 0.006), but not in mice pre-treated with rAAV.R76E ($20.4 \pm 8.8\%$; $P = 0.121$). Sections counterstained with cresyl violet (CV) showed that the loss of TH-positive neurons was not the result of metabolic compromise, as virtually all surviving large cell bodies in the SNc expressed TH (Fig. 3g–i). Across the six groups, the percentage of TH-neurons positive for CV ranged from 1.1 to 3.4%. The omnibus effect of treatment and the treatment × lesion interaction were both statistically significant (F's > 5.4, P's < 0.0452). Follow-up comparisons showed a significant difference only in mice pretreated with EpoR76E, with $3.1 \pm 1.3\%$ TH-/CV+ neurons in the SNc in MPTP-lesioned mice compared with $1.1 \pm 0.4\%$ in saline controls. Differences between MPTP and saline groups were not statistically different in EpoS71E- or eGFP-treated mice (P's > 0.419). The area of SNc used to count neurons did not differ across groups (F's < 1.4, P's > 0.295; data not shown).

There were no significant main or interaction effects of MPTP or Epo treatment on striatal dopamine (F's < 2.03, P's > 0.163). The effects of MPTP on striatal dopamine were also not significant in any of the treatment groups individually (Fig. 4a; t's < 1.92; P's > 0.174). This may reflect the effect of sprouting and reinnervation of dopaminergic neurons when mice are killed many weeks following MPTP lesions. The DOPAC also did not differ by

lesion or treatment group (F's < 1.7, P's > 0.200). However, analysis of treatment groups individually showed that DOPAC was significantly reduced in the rAAV.eGFP control group [Fig. 4b; $t(42) = 2.15$, $P = 0.0370$]. This loss in DOPAC reflects a decrease in dopamine turnover of 30.1% in the rAAV.eGFP-treated control mice. Although dopamine turnover is typically increased in MPTP-lesioned mice, it can be decreased when dopamine recovers more fully than DOPAC. In contrast, DOPAC did not change significantly (t's < 0.14, P's > 0.896) and turnover was unchanged in the rAAV.EpoS71E and rAAV.EpoR76E groups. To assess possible differences in DOPAC levels in saline-treated mice, we conducted follow-up statistics on these groups alone. There was no overall difference in DOPAC across the three saline-treated groups ($F_{2,24} = 0.68$, $P = 0.514$), and neither of the two Epo-treated groups differed significantly from eGFP control (t's < 1.2, P's > 0.254).

To investigate the reason for normal dopaminergic tone and lack of parkinsonism in rAAV.EpoS71E-treated mice despite a approximately 40% SNc lesion, we assessed TH-positive fibers in the striatum for possible differences in sprouting and regeneration. There was a significant lesion × treatment interaction on optical density ($F_{2,14} = 3.92$, $P = 0.0445$). Main effects of lesion and treatment were not significant (F's < 3.2, P's > 0.098), and none of the omnibus or follow-up comparisons was significant when only the dorsolateral quadrant was considered (F's < 2.1, P's > 0.115). Figure 5 shows that TH-positive optical density was significantly reduced (-23.7 ± 3.9%) in the rAAV.eGFP control mice lesioned with MPTP [$t(14) = 2.56$, $P = 0.0227$], consistent with the SNc lesion observed in Fig. 3. A smaller, non-significant loss of TH-positive fibers (-17.4 ± 5.6%) was observed in mice pretreated with rAAV.EpoR76E ($P = 0.125$). In contrast, optical density of TH-reactive fibers in MPTP-lesioned mice treated with rAAV.EpoS71E had normalized to that of their saline-treated counterparts (+12.1 ± 5.4%) by the time they were killed ($P = 0.249$).

Discussion

We show here for the first time that a peripherally-administered non-hematopoietic Epo can be neuroprotective in a model of CNS neurodegeneration. A relatively low dose of MPTP destroyed 41.0% of the dopaminergic neurons in the substantia nigra in control mice. When a single intramuscular injection of the EpoR76E vector was administered 3 weeks before the MPTP regimen, the lesion was less than half that size. The EpoS71E vector did not protect against MPTP-induced nigral cell loss, but increased optical density of TH-positive fibers 5 weeks following the lesion. Thus, by two different mechanisms, modified Epo constructs were able to maintain and/or restore good dopaminergic tone in MPTP-lesioned mice. The normal dopaminergic function was reflected in protection from MPTP-induced parkinsonism. Taken together, these results suggest that non-erythropoietic Epo delivered peripherally by viral vector may be a viable therapeutic approach for CNS neurodegenerative disorders.

It has long been known that Epo is neuroprotective, and that its neuroprotective properties are independent of its effects on erythropoiesis (Byts & Siren 2009). Rex *et al*. (2009) showed that direct delivery of Epo to the retina in 7-day-old mouse pups prevents retinal degeneration (*rd*) in mice homozygous for a mutant peripherin/rds allele (*rds*), which

induces complete blindness by 3 weeks of age. They followed up this study by showing that EpoR76E was protective in the same *rd* model when packaged in an rAAV2/5.CMV vector and delivered intramuscularly (Sullivan & Rex 2011). Systemic delivery of rAAV.EpoR76E also spared retinal ganglion cells from degeneration in a model of glaucoma (Sullivan *et al*. 2011). The EpoR76E variant was not fully protective in the MPTP model in this study. However, MPTP administration is not the slow, natural degenerative process that characterizes the glaucoma and *rds* models. Instead, it induces a much more rapid degeneration, recapitulating in 5 days the nigrostriatal damage that occurs over decades in Parkinson's disease. It is plausible that the EpoR76E construct may be fully protective in a model of slow CNS neurodegeneration. Despite only half the neuroprotection, EpoR76E-treated mice were fully protected from MPTP-induced tremor and akinesia. Consistent with this, dopamine and DOPAC levels in these mice were not significantly different from their saline-treated counterparts. This is not surprising given that large depletions of striatal dopamine are typically needed to observe parkinsonism in mice (Heikkila & Sonsalla 1992; Sedelis *et al*. 2001). Similarly, extensive nigrostriatal damage is thought to be needed in Parkinson's disease before symptoms are observable (Zigmond *et al*. 1990). The mechanism of Epo's neuroprotective effect is not fully understood but likely involves activation of the Epo receptor (Epo-r). Epo-r binding activates the Janus kinase 2 (Jak2) pathways to initiate expression of antiapoptotic genes (Noguchi *et al*. 2007; Sola *et al*. 2005). This induces phosphorylation of Akt, resulting in dissociation of Bcl-2-associated death promoter (BAD) from the Bcl-2/Bcl-X complex and activation of components of the reperfusion injury signaling kinase (RISK) pathway to increase mitochondrial membrane potential and reduce oxidative stress (Kobayashi *et al*. 2008; Miki *et al*. 2009; Smith & Yellon 2011; Weishaupt *et al*. 2004). Native Epo has also been shown to increase levels of anti-inflammatory interleukin-10 and attenuate inflammatory cytokines under pathological conditions (Chau *et al*. 2011; Juul *et al*. 2008; Lofrumento *et al*. 2011; Sajja *et al*. 2012; Zhang *et al*. 2012). MPTP induces a robust inflammatory response and oxidative stress secondary to inhibition of complex I of the mitochondrial respiratory chain (Karunakaran *et al*. 2007; Thomas *et al*. 2012). Indeed, Genc *et al*. (2001) showed that native Epo restores the reduction of glutathione peroxidase in the SNc induced by systemic MPTP. Thus, one or more of these mechanisms may have been involved in protection of half the striatal neurons from destruction by MPTP in the EpoR76E-treated mice.

In contrast to the partial neuroprotection observed in EpoR76E-treated mice, the MPTP lesion in the EpoS71E group was equivalent to that of the eGFP controls. However, like those in the EpoR76E group, mice pretreated with EpoS71E did not exhibit parkinsonism. The recovery of dopaminergic tone and associated function after MPTP lesion has been reported under some conditions, associated with generation of new TH-positive fibers on surviving neurons (Bezard & Gross 1998; Bezard *et al*. 2000a, Bohn *et al*. 1988; Domenger *et al*. 2012; Kang *et al*. 2007; Schneider & Yuwiler 1989). An examination of the time course of recovery in one study showed that sprouting of new TH-positive fibers starts almost immediately after MPTP lesion in mice, is time-dependent, and is accompanied by increased dopamine content in striatal punches (Mitsumoto *et al*. 1998). By 24 days post-lesion in that study, striatal dopamine had increased by 82% and TH-positive optical density more than doubled, with no change in the number of TH-positive cell bodies in the SNc. In

contrast, DOPAC had only recovered 10.5% in that same period. A similar dynamic was observed in MPTP-lesioned mice pretreated with the eGFP vector in this study. Striatal dopamine levels had nearly completely recovered in these mice 35 days following the last MPTP injection, but DOPAC was still significantly lower than in saline-treated mice. DOPAC is formed by the metabolism of dopamine by MAO intracellularly. When dopamine synthesis is compromised, MAO activity is reduced to maintain physiologic levels of dopamine, resulting in lower DOPAC. In contrast to this, DOPAC and dopamine were both normal in MPTP-lesioned mice pretreated with EpoS71E, despite a lesion size similar to that of mice in the eGFP group. Densitometric analysis showed a greater TH-positive optical density in the striatum of EpoS71E-pretreated mice, suggesting greater sprouting and reinnervation following MPTP lesion. It is well known that Epo enhances neuronal sprouting and regeneration in a number of central and peripheral lesion models, including MPTP, but not in non-pathogenic brain areas or conditions (Puskovic *et al*. 2006; Yin *et al*. 2010; Zhang *et al*. 2010). Optical density of striatal TH-positive fibers in MPTP-lesioned mice pretreated with EpoR76E did not appear to recover to the extent of those in the EpoS71E group, although the magnitude of difference between saline and MPTP groups was not as great as that in the eGFP controls. This may reflect the smaller lesion size, some moderately enhanced axonal sprouting or a combination of the two.

Although neuroprotective and adaptive mechanisms may explain maintenance and recovery of striatal dopamine in the three groups, the question remains as to why behavior was abnormal only in the rAAV.eGFP-treated group. Our pilot data show that 18 mg/kg MPTP induced a 37.2% loss of striatal dopamine on the fifth day following the last MPTP injection. The grid test was conducted 7 days following the last MPTP injection for all mice (the second day after removal from quarantine), and the FPA session on either the 18th or 19th day. Thus, it is likely that the rAAV.eGFP mice had substantial dopaminergic loss at the time the behavioral tests were conducted. However, MPTP induced a similar SNc lesion in EpoS71E-pretreated mice, but their behavior was normal. It is unlikely that enhanced sprouting or reinnervation of striatal TH-positive fibers would have occurred so quickly after the last MPTP injection. Additional studies are needed to address the time course of recovery, the possibility of other Epo-related changes following MPTP, and the possible involvement of other brain regions or neurotransmitter systems in the expression of the MPTP-induced deficits.

The primary goal of this line of inquiry was to develop an Epo variant that dissociates the erythropoietic effect of Epo from its neuroprotective effect. We show here that a third known effect of Epo, namely its ability to enhance axonal regeneration under pathological conditions, may be operational in the MPTP lesion model and may be independent of both neuroprotection and erythropoiesis. This may have important treatment implications. There is no cure for Parkinson's disease, and no known intervention that will prevent the neurodegeneration. The most common pharmacological interventions replace nigrostriatal dopamine, but the amelioration only lasts a few years and there is some concern that chronic treatment with dopaminergic agonists like L-3,4-dihydroxyphenylalanine (L-DOPA) may ultimately worsen symptoms. The most common surgical approach is deep-brain stimulation, which involves implantation of a stimulatory electrode in the subthalamic

nucleus to dampen the inhibitory tone on nigrostriatal dopaminergic neurons. Unfortunately, it is effective in less than one-third of the patients who undergo surgery. Both of these treatments result in greater stimulation of postsynaptic dopaminergic receptors in the striatum, either through mimicking dopamine or stimulating its endogenous release. However, they do nothing to actively prevent the ongoing neurodegeneration. In contrast, our data suggest that Epo can be administered to protect against further neurodegeneration, as well as to increase sprouting and reinnervation to provide dopamine replacement under conditions of substantial pre-existing nigrostriatal loss. This, together with the fact that only a single intramuscular injection is needed to confer these putative therapeutic benefits, suggests that further investigation of modified Epo variants as novel therapeutics for Parkinson's disease is warranted.

Acknowledgments

References

Bernaudin M, Marti HH, Roussel S, Divoux D, Nouvelot A, MacKenzie ET, Petit E. A potential role for erythropoietin in focal permanent cerebral ischemia in mice. J Cereb Blood Flow Metab. 1999; 19:643–651. [PubMed: 10366194]

Bezard E, Gross CE. Compensatory mechanisms in experimental and human parkinsonism: towards a dynamic approach. Prog Neurobiol. 1998; 55:93–116. [PubMed: 9618745]

Bezard E, Dovero S, Imbert C, Boraud T, Gross CE. Spontaneous long-term compensatory dopaminergic sprouting in MPTP-treated mice. Synapse. 2000a; 38:363–368. [PubMed: 11020240]

Bezard E, Jaber M, Gonon F, Boireau A, Bloch B, Gross CE. Adaptive changes in the nigrostriatal pathway in response to increased 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurodegeneration in the mouse. Eur J Neurosci. 2000b; 12:2892–2900. [PubMed: 10971632]

Blanchet S, Marie RM, Dauvillier F, Landeau B, Benali K, Eustache F, Chavoix C. Cognitive processes involved in delayed non-matching-to-sample performance in Parkinson's disease. Eur J Neurol. 2000; 7:473–483. [PubMed: 11054130]

Bohn MC, Marciano F, Cupit L, Gash DM. Recovery of dopaminergic fibers in striatum of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mouse is enhanced by grafts of adrenal medulla. Prog Brain Res. 1988; 78:535–542. [PubMed: 3266803]

Braungart E, Gerlach M, Riederer P, Baumeister R, Hoener MC. *Caenorhabditis elegans* MPP + model of Parkinson's disease for high-throughput drug screenings. Neurodegener Dis. 2004; 1:175–183. [PubMed: 16908987]

Bronstein JM, Tagliati M, Alterman RL, et al. Deep brain stimulation for Parkinson disease: an expert consensus and review of key issues. Arch Neurol. 2011; 68:165. [PubMed: 20937936]

Byts N, Siren AL. Erythropoietin: a multimodal neuroprotective agent. Exp Transl Stroke Med. 2009; 1:4. [PubMed: 20142991]

Chau M, Chen D, Wei L. Erythropoietin attenuates inflammatory factors and cell death in neonatal rats with intracerebral hemorrhage. Acta Neurochir Suppl. 2011; 111:299–305. [PubMed: 21725772]

Christine, C.; Starr, PA.; Larson, P.; Mah, R.; Eberling, J.; Jagust, WJ.; McGuire, D.; Aminoff, MJ. Aromatic L-amino acid decarboxylase gene transfer therapy for Parkinson's disease: initial results of an open-label, dose escalation, safety and tolerability study. Abstracts of the American Academy of Neurology Annual Meeting; San Diego, CA. 2006. p. S23.005

Cicchetti F, Drouin-Ouellet J, Gross RE. Environmental toxins and Parkinson's disease: what have we learned from pesticide-induced animal models? Trends Pharmacol Sci. 2009; 30:475–483. [PubMed: 19729209]

Dhanushkodi A, McDonald MP. Intracranial *V. cholerae* sialidase protects against excitotoxic neurodegeneration. PLoS One. 2011; 6:e29285. [PubMed: 22195039]

Domenger D, Dea D, Theroux L, Moquin L, Gratton A, Poirier J. The MPTP neurotoxic lesion model of Parkinson's disease activates the apolipoprotein E cascade in the mouse brain. Exp Neurol. 2012; 233:513–522. [PubMed: 22155743]

Fowler SC, Birkestrand BR, Chen R, Moss SJ, Vorontsova E, Wang G, Zarcone TJ. A force-plate actometer for quantitating rodent behaviors: illustrative data on locomotion, rotation, spatial patterning, stereotypies, and tremor. J Neurosci Methods. 2001; 107:107–124. [PubMed: 11389948]

Fowler SC, Zarcone TJ, Vorontsova E, Chen R. Motor and associative deficits in D2 dopamine receptor knockout mice. Int J Dev Neurosci. 2002; 20:309–321. [PubMed: 12175868]

Gasmi M, Herzog CD, Brandon EP, Cunningham JJ, Ramirez GA, Ketchum ET, Bartus RT. Striatal delivery of neurturin by CERE-120, an AAV2 vector for the treatment of dopaminergic neuron degeneration in Parkinson's disease. Mol Ther. 2007; 15:62–68. [PubMed: 17164776]

Genc S, Kuralay F, Genc K, Akhisaroglu M, Fadiloglu S, Yorukoglu K, Fadiloglu M, Gure A. Erythropoietin exerts neuroprotection in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated C57/BL mice via increasing nitric oxide production. Neurosci Lett. 2001; 298:139–141. [PubMed: 11163297]

Hallman H, Olson L, Jonsson G. Neurotoxicity of the meperidine analogue N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine on brain catecholamine neurons in the mouse. Eur J Pharmacol. 1984; 97:133–136. [PubMed: 6607840]

Harrison FE, Yu SS, Van Den Bossche KL, Li L, May JM, McDonald MP. Elevated oxidative stress and sensorimotor deficits but normal cognition in mice that cannot synthesize ascorbic acid. J Neurochem. 2008; 106:1198–1208. [PubMed: 18466336]

Harrison FE, Allard J, Bixler R, Usoh C, Li L, May JM, McDonald MP. Antioxidants and cognitive training interact to affect oxidative stress and memory in APP/PSEN1 mice. Nutr Neurosci. 2009; 12:203–218. [PubMed: 19761651]

Harrison FE, May JM, McDonald MP. Vitamin C deficiency increases basal exploratory activity but decreases scopolamine-induced activity in APP/PSEN1 transgenic mice. Pharmacol Biochem Behav. 2010; 94:543–552. [PubMed: 19941887]

Heikkila RE, Sonsalla PK. The MPTP-treated mouse as a model of parkinsonism: how good is it? Neurochem Int. 1992; 20:299S–303S. [PubMed: 1365446]

Herzog CD, Dass B, Holden JE, Stansell J 3rd, Gasmi M, Tuszynski MH, Bartus RT, Kordower JH. Striatal delivery of CERE-120, an AAV2 vector encoding human neurturin, enhances activity of the dopaminergic nigrostriatal system in aged monkeys. Mov Disord. 2007; 22:1124–1132. [PubMed: 17443702]

Juul SE, McPherson RJ, Bammler TK, Wilkerson J, Beyer RP, Farin FM. Recombinant erythropoietin is neuroprotective in a novel mouse oxidative injury model. Dev Neurosci. 2008; 30:231–242. [PubMed: 17960054]

Kang JM, Park HJ, Choi YG, Choe IH, Park JH, Kim YS, Lim S. Acupuncture inhibits microglial activation and inflammatory events in the MPTP-induced mouse model. Brain Res. 2007; 1131:211–219. [PubMed: 17173870]

Karunakaran S, Saeed U, Ramakrishnan S, Koumar RC, Ravindranath V. Constitutive expression and functional characterization of mitochondrial glutaredoxin (Grx2) in mouse and human brain. Brain Res. 2007; 1185:8–17. [PubMed: 17961515]

Kobayashi H, Miura T, Ishida H, Miki T, Tanno M, Yano T, Sato T, Hotta H, Shimamoto K. Limitation of infarct size by erythropoietin is associated with translocation of Akt to the mitochondria after reperfusion. Clin Exp Pharmacol Physiol. 2008; 35:812–819. [PubMed: 18346168]

Kordower JH, Olanow CW. Regulatable promoters and gene therapy for Parkinson's disease: is the only thing to fear, fear itself? Exp Neurol. 2008; 209:34–40. [PubMed: 17888424]

Kordower JH, Herzog CD, Dass B, Bakay RA, Stansell J 3rd, Gasmi M, Bartus RT. Delivery of neurturin by AAV2 (CERE-120)-mediated gene transfer provides structural and functional neuroprotection and neurorestoration in MPTP-treated monkeys. Ann Neurol. 2006; 60:706–715. [PubMed: 17192932]

Liu Y, Hoffmann A, Grinberg A, Westphal H, McDonald MP, Miller KM, Crawley JN, Sandhoff K, Suzuki K, Proia RL. Mouse model of GM2 activator deficiency manifests cerebellar pathology and motor impairment. Proc Natl Acad Sci U S A. 1997; 94:8138–8143. [PubMed: 9223328]

Lofrumento DD, Saponaro C, Cianciulli A, De Nuccio F, Mitolo V, Nicolardi G, Panaro MA. MPTP-induced neuroinflammation increases the expression of pro-inflammatory cytokines and their receptors in mouse brain. Neuroimmunomodulation. 2011; 18:79–88. [PubMed: 20938211]

McDonald MP, Wong R, Goldstein G, Weintraub B, Cheng SY, Crawley JN. Hyperactivity and learning deficits in transgenic mice bearing a human mutant thyroid hormone β1 receptor gene. Learn Mem. 1998; 5:289–301. [PubMed: 10454355]

McDonald MP, Miller KM, Li C, Deng C, Crawley JN. Motor deficits in fibroblast growth factor receptor-3 null mutant mice. Behav Pharmacol. 2001; 12:477–486. [PubMed: 11742142]

McKerchar TL, Zarcone TJ, Fowler SC. Use of a force-plate actometer for detecting and quantifying vertical leaping induced by amphetamine in BALB/cJ mice, but not in C57BL/6J, DBA/2J, 129X1/SvJ, C3H/HeJ, and CD-1 mice. J Neurosci Methods. 2006; 153:48–54. [PubMed: 16290200]

Miki T, Miura T, Hotta H, Tanno M, Yano T, Sato T, Terashima Y, Takada A, Ishikawa S, Shimamoto K. Endoplasmic reticulum stress in diabetic hearts abolishes erythropoietin-induced myocardial protection by impairment of phospho-glycogen synthase kinase-3beta-mediated suppression of mitochondrial permeability transition. Diabetes. 2009; 58:2863–2872. [PubMed: 19755525]

Mitsumoto Y, Watanabe A, Mori A, Koga N. Spontaneous regeneration of nigrostriatal dopaminergic neurons in MPTP-treated C57BL/6 mice. Biochem Biophys Res Commun. 1998; 248:660–663. [PubMed: 9703982]

Monchi O, Petrides M, Mejia-Constain B, Strafella AP. Cortical activity in Parkinson's disease during executive processing depends on striatal involvement. Brain. 2007; 130:233–244. [PubMed: 17121746]

Mondon K, Gochard A, Marque A, Armand A, Beauchamp D, Prunier C, Jacobi D, de Toffol B, Autret A, Camus V, Hommet C. Visual recognition memory differentiates dementia with Lewy bodies and Parkinson's disease dementia. J Neurol Neurosurg Psychiatry. 2007; 78:738–741. [PubMed: 17287240]

Noguchi CT, Asavaritikrai P, Teng R, Jia Y. Role of erythropoietin in the brain. Crit Rev Oncol Hematol. 2007; 64:159–171. [PubMed: 17482474]

Norflus F, Tifft CJ, McDonald MP, Goldstein G, Crawley JN, Hoffmann A, Sandhoff K, Suzuki K, Proia RL. Bone marrow transplantation prolongs life span and ameliorates neurologic manifestations in Sandhoff disease mice. J Clin Invest. 1998; 101:1881–1888. [PubMed: 9576752]

Pope-Coleman A, Schneider JS. Effects of chronic GM1 ganglioside treatment on cognitive and motor deficits in a slowly progressing model of Parkinsonism in non-human primates. Restor Neurol Neurosci. 1998; 12:255–266. [PubMed: 12671296]

Pope-Coleman A, Tinker JP, Schneider JS. Effects of GM1 ganglioside treatment on pre- and postsynaptic dopaminergic markers in the striatum of parkinsonian monkeys. Synapse. 2000; 36:120–128. [PubMed: 10767059]

Poulopoulos M, Waters C. Carbidopa/levodopa/entacapone: the evidence for its place in the treatment of Parkinson's disease. Core Evid. 2010; 5:1–10. [PubMed: 20694135]

Przedborski S, Jackson-Lewis V, Naini AB, Jakowec M, Petzinger G, Miller R, Akram M. The parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): a technical review of its utility and safety. J Neurochem. 2001; 76:1265–1274. [PubMed: 11238711]

Puskovic V, Wolfe D, Wechuck J, Krisky D, Collins J, Glorioso JC, Fink DJ, Mata M. HSV-mediated delivery of erythropoietin restores dopaminergic function in MPTP-treated mice. Mol Ther. 2006; 14:710–715. [PubMed: 16949343]

Rex TS, Allocca M, Domenici L, Surace EM, Maguire AM, Lyubarsky A, Cellerino A, Bennett J, Auricchio A. Systemic but not intraocular Epo gene transfer protects the retina from light-and genetic-induced degeneration. Mol Ther. 2004; 10:855–861. [PubMed: 15509503]

Rex TS, Wong Y, Kodali K, Merry S. Neuroprotection of photoreceptors by direct delivery of erythropoietin to the retina of the retinal degeneration slow mouse. Exp Eye Res. 2009; 89:735–740. [PubMed: 19591826]

Sajja VS, Tenn C, McLaws LJ, Vandevord PJ. A temporal evaluation of cytokines in rats after blast exposure. Biomed Sci Instrum. 2012; 48:374–379. [PubMed: 22846308]

Sango K, Yamanaka S, Hoffmann A, Okuda Y, Grinberg A, Westphal H, McDonald MP, Crawley JN, Sandhoff K, Suzuki K, Proia RL. Mouse models of Tay-Sachs and Sandhoff diseases differ in neurologic phenotype and ganglioside metabolism. Nat Genet. 1995; 11:170–176. [PubMed: 7550345]

Sango K, McDonald MP, Crawley JN, Mack ML, Tifft CJ, Skop E, Starr CM, Hoffmann A, Sandhoff K, Suzuki K, Proia RL. Mice lacking both subunits of lysosomal β-hexosaminidase display gangliosidosis and mucopolysaccharidosis. Nat Genet. 1996; 14:348–352. [PubMed: 8896570]

Schneider JS, DiStefano L. Oral administration of semisynthetic sphingolipids promotes recovery of striatal dopamine concentrations in a murine model of parkinsonism. Neurology. 1994; 44:748–750. [PubMed: 8164837]

Schneider JS, Yuwiler A. GM1 ganglioside treatment promotes recovery of striatal dopamine concentrations in the mouse model of MPTP-induced parkinsonism. Exp Neurol. 1989; 105:177–183. [PubMed: 2568945]

Schneider JS, Schroeder JA, Rothblat DS. Differential recovery of sensorimotor function in GM1 ganglioside-treated vs. spontaneously recovered MPTP-treated cats: partial striatal dopaminergic reinnervation vs. neurochemical compensation. Brain Res. 1998; 813:82–87. [PubMed: 9824674]

Sedelis M, Schwarting RK, Huston JP. Behavioral phenotyping of the MPTP mouse model of Parkinson's disease. Behav Brain Res. 2001; 125:109–125. [PubMed: 11682102]

Siesser WB, Cheng SY, McDonald MP. Hyperactivity, impaired learning on a vigilance task, and a differential response to methylphenidate in the TRβPV knock-in mouse. Psychopharmacology (Berl). 2005; 181:653–663. [PubMed: 15983791]

Siesser WB, Zhao J, Miller LR, Cheng SY, McDonald MP. Transgenic mice expressing a human mutant β1 thyroid receptor are hyperactive, impulsive, and inattentive. Genes Brain Behav. 2006; 5:282–297. [PubMed: 16594981]

Sinor AD, Greenberg DA. Erythropoietin protects cultured cortical neurons, but not astroglia, from hypoxia and AMPA toxicity. Neurosci Lett. 2000; 290:213–215. [PubMed: 10963901]

Smith CC, Yellon DM. Adipocytokines, cardiovascular pathophysiology and myocardial protection. Pharmacol Ther. 2011; 129:206–219. [PubMed: 20920528]

Sola A, Rogido M, Lee BH, Genetta T, Wen TC. Erythropoietin after focal cerebral ischemia activates the Janus kinase-signal transducer and activator of transcription signaling pathway and improves brain injury in postnatal day 7 rats. Pediatr Res. 2005; 57:481–487. [PubMed: 15718373]

Starr, PA.; Verhagen, L.; Larson, PS.; Bakay, R.; Taylor, R.; Cahn-Weiner, D.; Bartus, R.; Ostrem, JL.; Marks, WJJ. Intrastriatal gene transfer with AAV-neurturin for Parkinson's disease: results of a phase I trial. Abstracts of the American Association of Neurological Surgeons Annual Meeting; Washington, DC. 2007. p. Article ID 40735

Sullivan T, Rex TS. Systemic gene delivery protects the photoreceptors in the retinal degeneration slow mouse. Neurochem Res. 2011; 36:613–618. [PubMed: 20924671]

Sullivan TA, Geisert EE, Hines-Beard J, Rex TS. Systemic AAV-mediated gene therapy preserves retinal ganglion cells and visual function in DBA/2J glaucomatous mice. Hum Gene Ther. 2011; 22:1191–1200. [PubMed: 21542676]

Sullivan TA, Geisert EE, Templeton JP, Rex TS. Dose-dependent treatment of optic nerve crush by exogenous systemic mutant erythropoietin. Exp Eye Res. 2012; 96:36–41. [PubMed: 22306016]

Thomas B, Banerjee R, Starkova NN, Zhang SF, Calingasan NY, Yang L, Wille E, Lorenzo BJ, Ho DJ, Beal MF, Starkov A. Mitochondrial permeability transition pore component cyclophilin D distinguishes nigrostriatal dopaminergic death paradigms in the MPTP mouse model of Parkinson's disease. Antioxid Redox Signal. 2012; 16:855–868. [PubMed: 21529244]

Weishaupt JH, Rohde G, Polking E, Siren AL, Ehrenreich H, Bahr M. Effect of erythropoietin axotomy-induced apoptosis in rat retinal ganglion cells. Invest Ophthalmol Vis Sci. 2004; 45:1514–1522. [PubMed: 15111610]

Willis GL, Donnan GA. Histochemical, biochemical and behavioural consequences of MPTP treatment in C-57 black mice. Brain Res. 1987; 402:269–274. [PubMed: 3493826]

Wood LD. Clinical review and treatment of select adverse effects of dopamine receptor agonists in Parkinson's disease. Drugs Aging. 2010; 27:295–310. [PubMed: 20359261]

Wu Y, Sun X, Feng X, Liu R, Huang L, Shang Y, Liang Z, Chen J, Qiao X, Liu C, Sun S. Increased expression of erythropoietin receptor in 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine-induced Parkinsonian model. Physiol Res. 2010; 59:281–287. [PubMed: 19537929]

Yin ZS, Zhang H, Bo W, Gao W. Erythropoietin promotes functional recovery and enhances nerve regeneration after peripheral nerve injury in rats. AJNR Am J Neuroradiol. 2010; 31:509–515. [PubMed: 20037135]

Zhang Y, Xiong Y, Mahmood A, Meng Y, Liu Z, Qu C, Chopp M. Sprouting of corticospinal tract axons from the contralateral hemisphere into the denervated side of the spinal cord is associated with functional recovery in adult rat after traumatic brain injury and erythropoietin treatment. Brain Res. 2010; 1353:249–257. [PubMed: 20654589]

Zhang X, Li QY, Xiao BG. Anti-inflammatory effect of erythropoietin therapy on experimental autoimmune encephalomyelitis. Int J Neurosci. 2012; 122:255–262. [PubMed: 22176555]

Zigmond MJ, Abercrombie ED, Berger TW, Grace AA, Stricker EM. Compensations after lesions of central dopaminergic neurons: some clinical and basic implications. Trends Neurosci. 1990; 13:290–296. [PubMed: 1695406]
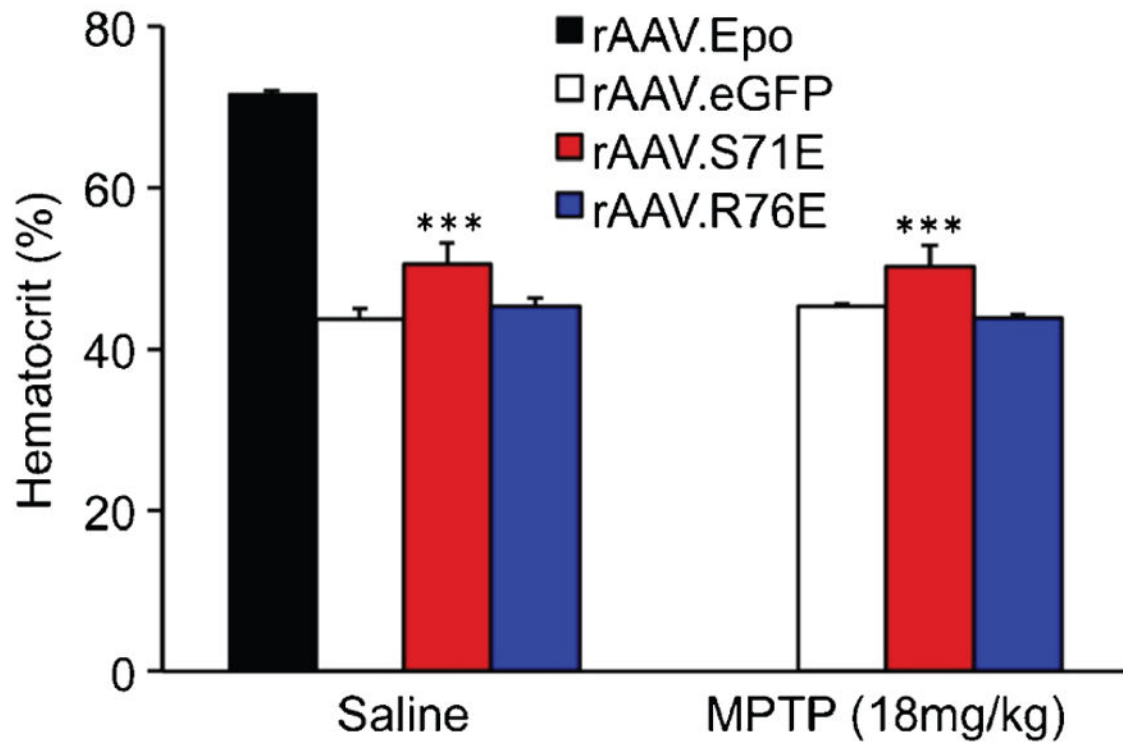
Figure 1. Modified Epo constructs are non-erythropoietic
Mice were injected intramuscularly with an rAAV vector harboring native Epo, eGFP control or modified variants EpoR76E or EpoS71E. Trunk blood was collected 3 weeks following the injection and hematocrit measured. Most of the mice injected with rAAV.Epo died; those that survived had significantly elevated hematocrit levels. Mice injected with rAAV.eGFP or rAAV.EpoR76E had normal hematocrit levels. The rAAV.EpoS71E group had slightly ($13.9 \pm 4.0\%$) elevated hematocrit that was statistically significant. ***$P < 0.001$ vs. rAAV.eGFP-treated groups (*t*-test).
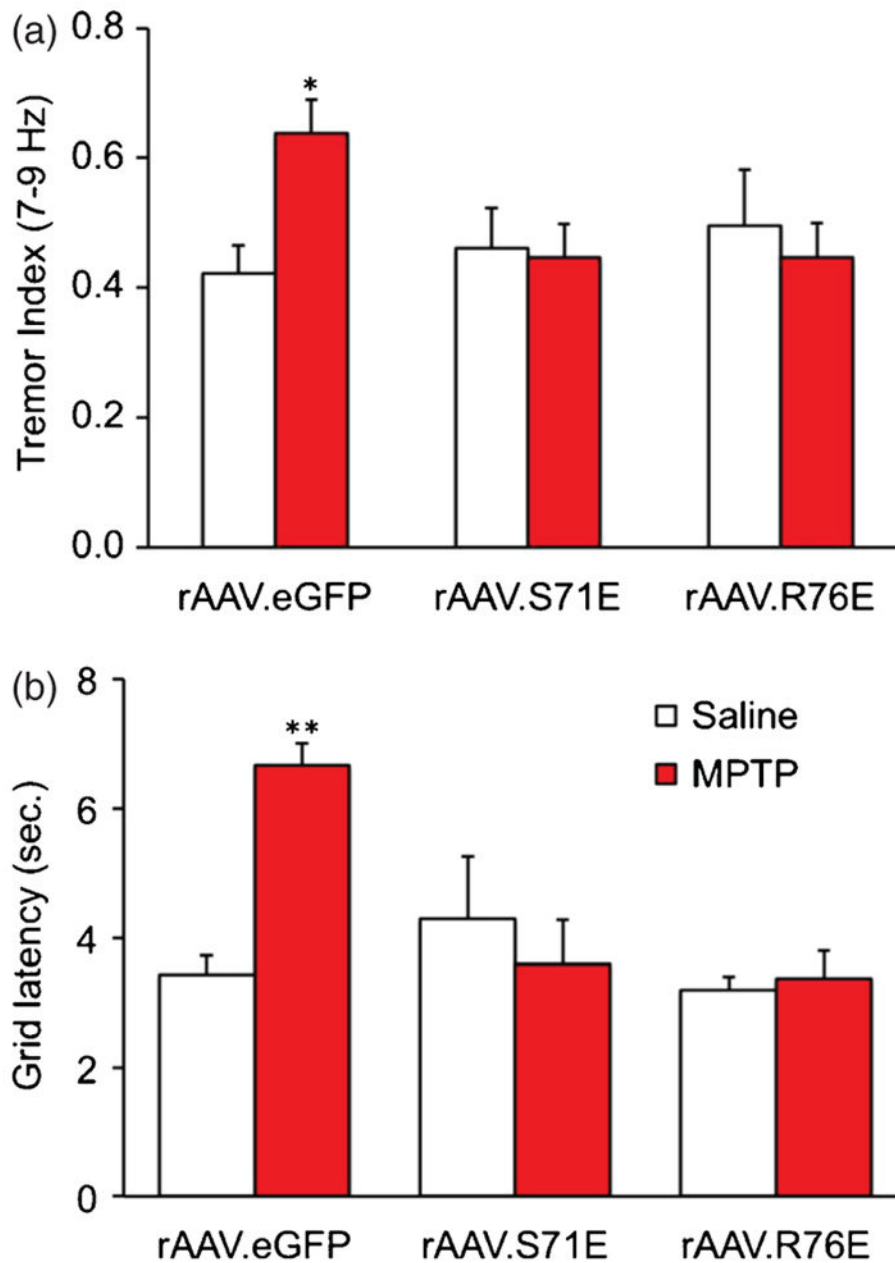
Figure 2. Modified Epo variants protect against MPTP-induced parkinsonism
(a) Mice were placed in a FPA and allowed to explore freely for 5 min. Minute variations in force were recorded on a millisecond time scale. MPTP induced a robust 7- to 9-Hz tremor in control mice pretreated with rAAV.eGFP. Mice injected with either of the modified Epo constructs did not exhibit MPTP-induced tremor. (b) Mice in the rAAV.eGFP control group and lesioned with MPTP were slower to move all four paws when placed on a horizontal grid. Mice pretreated with rAAV.EpoS71E or rAAV.EpoR76E did not exhibit this MPTP-induced akinesia. **$P < 0.01$, *$P < 0.05$ vs. rAAV.eGFP/saline-treated group (t-test).
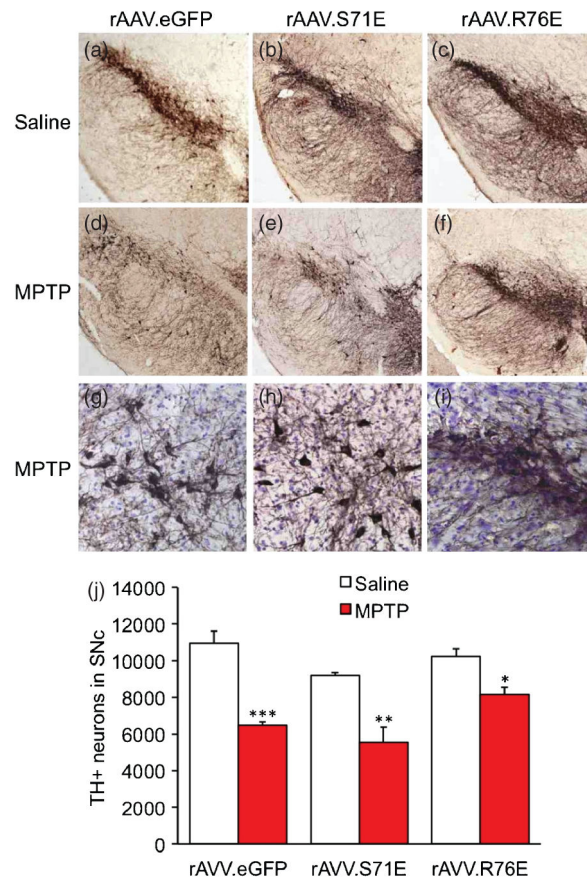
Figure 3. rAAV.EpoR76E protects against MPTP-induced destruction of neurons in the SNc
(a, d) MPTP destroyed approximately 40% of the TH-positive neurons in the SNc in control mice pretreated with rAAV.eGFP and induced a similar-sized lesion in the rAAV.EpoS71E group (b, e). (c, f) Mice pretreated with rAAV.EpoR76E were partially protected against the MPTP lesion, exhibiting a approximately 20% loss of TH-positive SNc cells. (g–i) Sections counterstained with cresyl violet showed that lesioned areas were largely devoid of large, Nissl-positive cell bodies. This confirms that the lesion represents neurons destroyed by MPTP and not a metabolic downregulation of TH synthesis. (j) Stereological quantification of the images presented in a–f. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs. respective saline-treated group (*t*-test).
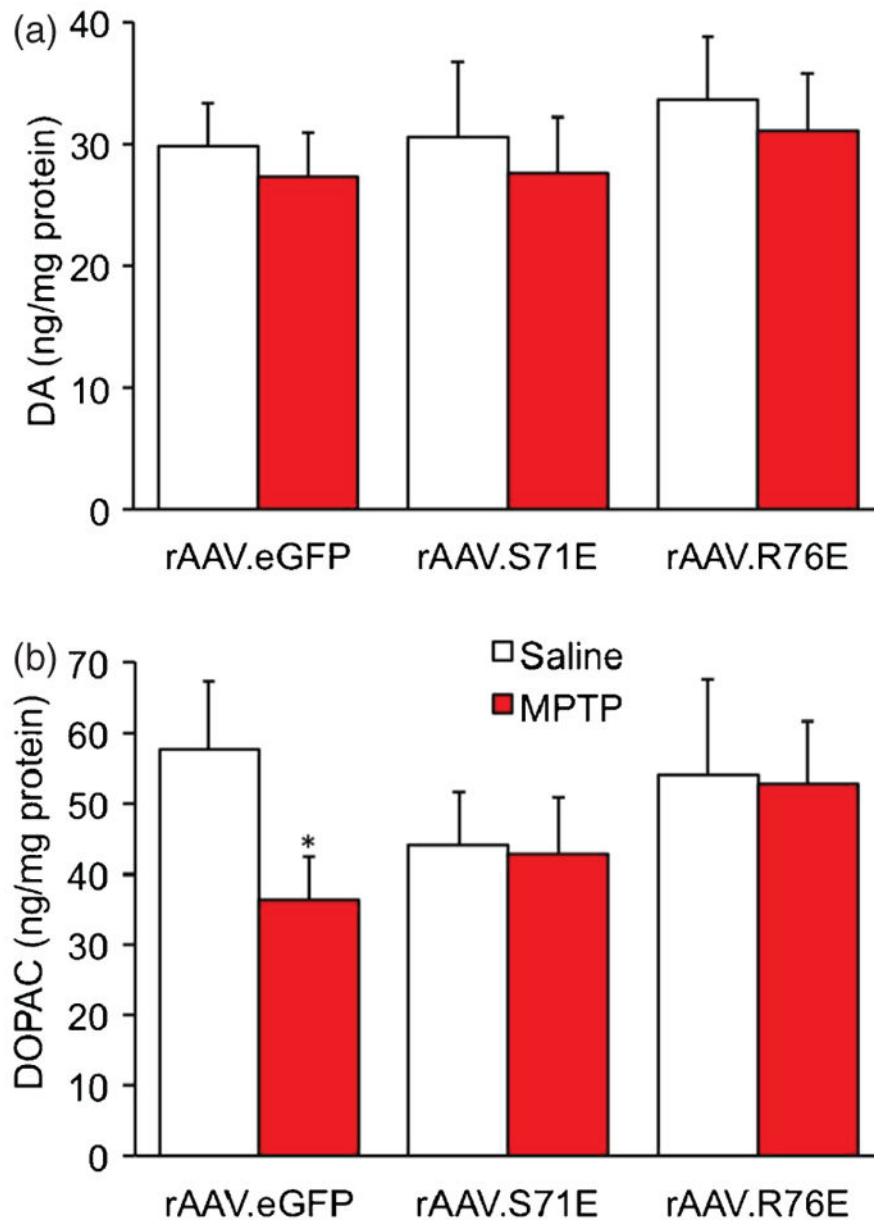
Figure 4. Modified Epo variants normalize dopamine turnover following MPTP lesion
(a) Intracellular striatal dopamine measured by HPLC 8 weeks following the last injection of saline or 18 mg/kg MPTP. There were no significant differences in dopamine across groups, possibly due in part to axon regeneration and sprouting of new terminals following MPTP lesion. (b) The dopamine metabolite DOPAC was significantly lower in MPTP-lesioned mice pretreated with rAAV.eGFP, compared with their saline-treated counterparts, suggesting reduced dopamine turnover. In contrast, mice pretreated with either of the modified Epo variants had normal DOPAC regardless of lesion status. $*P < 0.05$ vs. rAAV.eGFP/saline-treated group (*t*-test).
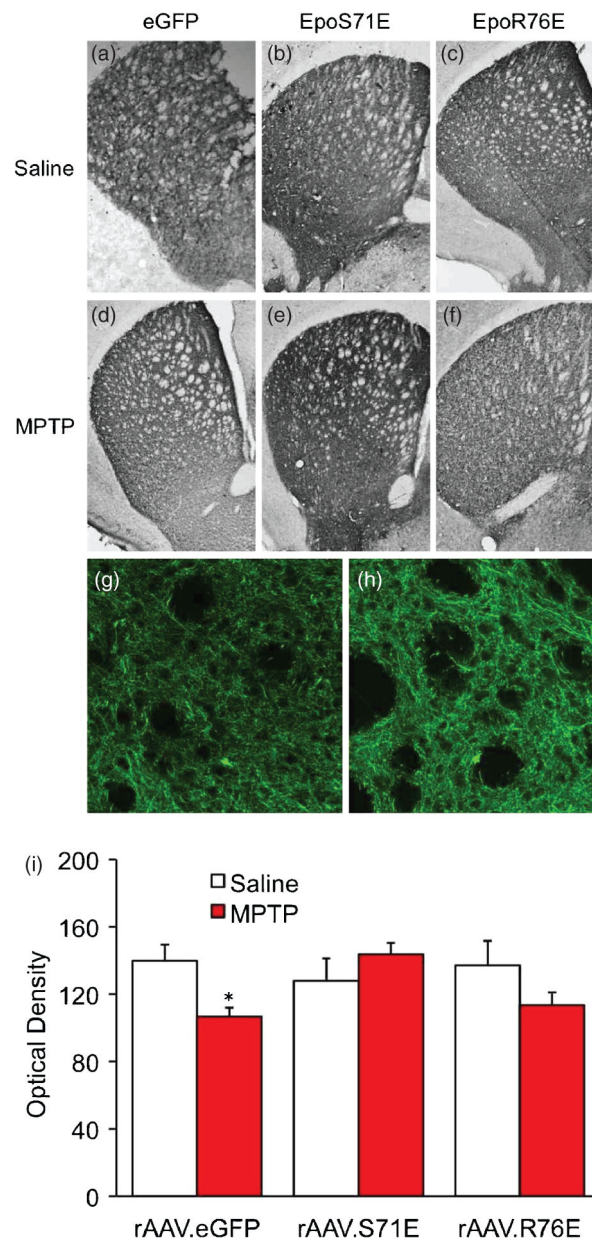
Figure 5. rAAV.EpoS71E pretreatment increases TH-positive optical density following MPTP lesion

(a–f) Density of TH-positive fibers was assessed in striatal sections 8 weeks following administration of MPTP or saline. MPTP-lesioned mice pretreated with rAAV.eGFP showed a characteristic loss of TH-positive fibers compared with saline-treated controls (a, d). Mice pretreated with rAAV.EpoR76E showed a similar magnitude of MPTP lesion (c, f). In contrast, mice in the rAAV.EpoS71E group exhibited no loss of TH-positive fibers after MPTP treatment, presumably due to increased axonal sprouting and regeneration (b, e). Confocal images of striatal sections show increased density of TH-positive fibers in MPTP-lesioned mice pretreated with either rAAV.eGFP (g) or rAAV.EpoS71E (h). (i)

Densitometric quantification of the images presented in a–f. *$P < 0.05$ vs. rAAV.eGFP/saline-treated group (*t*-test).