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Erythropoietin is Neuroprotective in a Transgenic Mouse Model of Multiple System Atrophy

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Abstract

Multiple system atrophy is a rapidly progressive neurodegenerative disorder with a markedly reduced life expectancy. Failure of symptomatic treatment raises an urgent need for disease-modifying strategies. We have investigated the neuroprotective potential of erythropoietin in (proteolipid protein)-α-synuclein transgenic mice exposed to 3-nitropropionic acid featuring multiple system atrophy-like pathology including oligodendroglial α-synuclein inclusions and selective neuronal degeneration.

Mice were treated with erythropoietin starting before (early erythropoietin) and after (late erythropoietin) intoxication with 3-nitropropionic acid. Nonintoxicated animals receiving erythropoietin and intoxicated animals treated with saline served as control groups. Behavioral tests included pole test, open field activity, and motor behavior scale. Immunohistochemistry for tyrosine hydroxylase and dopamine and cyclic adenosine monophosphate-regulated phosphoprotein (DARPP-32) was analyzed stereologically.

Animals receiving erythropoietin before and after 3-nitropropionic acid intoxication scored significantly lower on the motor behavior scale and they performed better in the pole test than controls with no significant difference between early and late erythropoietin administration. Similarly, rearing scores were worse in 3-nitropropionic acid-treated animals with no difference between the erythropoietin subgroups. Immunohistochemistry revealed significant attenuation of 3-nitropropionic acid-induced loss of tyrosine hydroxylase and DARPP-32 positive neurons in substantia nigra pars compacta and striatum, respectively, in both erythropoietin-treated groups without significant group difference in the substantia nigra. However, at striatal level, a significant difference between early and late erythropoietin administration was observed.

In the combined (proteolipid protein)-α-synuclein 3-nitropropionic acid multiple system atrophy mouse model, erythropoietin appears to rescue dopaminergic and striatal gabaergic projection neurons. This effect is associated with improved motor function. Further studies are warranted to develop erythropoietin as a potential interventional therapy in multiple system atrophy.

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Multiple system atrophy (MSA) is an atypical parkinsonian disorder clinically characterized by dysautonomia, parkinsonism, cerebellar symptoms, and pyramidal signs in any combination.[1] Histopathological hallmarks are α-synuclein-positive glial cytoplasmic inclusions (GCIs) associated with a distinctive neuronal multisystem degeneration involving striatonigral, olivopontocerebellar, and central autonomic pathways.[2] Because of loss of striatal projection neurons, parkinsonism in MSA is usually unresponsive to dopaminergic (DA) therapy. Disease is rapidly progressive, and life expectancy markedly reduced with a median survival of 7 to 9 years.[3,4] Lack of symptomatic treatment combined with ongoing neurodegeneration raise an urgent need for disease-modifying strategies.

Both toxin-induced and transgenic MSA animal models are available to screen novel interventions.[5] The combination of both genetic and toxic factors by exposing transgenic α-synuclein mice with GCI-like inclusions[6] to mitochondrial stress [using 3-nitroproprionic acid (3-NP)] induces selective neurodegeneration in striatonigral and olivopontocerebellar pathways and results in a distinct motor phenotype.[7] In this test bed, we have shown neuroprotective effects for minocycline[8] and rasagiline.[9]

Circulating erythropoietin (EPO) is a large, highly glycosylated, and negatively charged molecule. Therefore, EPO was thought not to be able to cross the blood–brain barrier.[10-12] However, more recent studies provided evidence that peripherally administered EPO indeed passes the blood–brain barrier in a dose-dependent fashion in several species including humans.[13-17] After brain injury, increased cerebral concentrations of EPO were observed.[17] EPO is mostly known for its effects in stimulating erythropoiesis. However, EPO protein and EPO receptors (EPO-R) are also expressed in the human brain throughout development.[18,19] In response to hypoxia/ischemia, EPO expression is increased,[20] and recombinant EPO protects against hypoxia/ischemia insults in rodent models.[21-24] Exposure of rat embryonic mesencephalic DA precursor cells to higher environmental oxygen levels (18–20%) in vitro elicits a decrease in the number of tyrosine hydroxylase (TH) immunoreactive neurons when compared with cells grown at lower oxygen levels (1–2%).[25] Addition of EPO to the culture medium of cells grown in the higher oxygen culture conditions results in a significant increase in the differentiation of precursor cells to TH-positive (TH+) neurons, an effect that is blocked by the addition of EPO-neutralizing antibodies.[25] Genc et al.[26] have shown in vivo that supranigral delivery of EPO provides neuroprotection against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MTPT) insult in mice, which was associated with an upregulation of nitric oxide and glutathione peroxidase[27] in the nigrostriatal pathway. Treatment of mesencephalic grafts with EPO enhances survival rate of TH+ cells by more than 100% corresponding with better behavioral recovery.[28] Neuroprotective effects of EPO have also been observed in models of epilepsy,[29] multiple sclerosis,[30] and amytrophic lateral sclerosis.[31] A clinical trial of EPO in patients with Friedreich's ataxia showed a partial reversal of reduced frataxin levels and improved motor outcome.[32,33] As opposed to that, a recently published double-blind,

placebo-controlled, randomized, multicenter stroke trial failed to exert a beneficial effect of EPO.[34] Whereas in MSA, EPO is known to be related to anemia and orthostatic hypotension,[35,36] nothing is known about its neuroprotective potential in this atypical parkinsonian disorder. Long-term treatment with EPO may be limited due to the necessity of safety monitoring with regular blood cell counts and parameters of iron metabolism and of repeated bloodletting. Unfortunately, asialoEPO, which is a natural nonerythropoietic EPO metabolite, did not exert any beneficial effects in the R6/2 line of Huntington's disease (HD) mice that share striatal pathology with MSA[37] and was therefore not used in this study.

Materials and Methods

Animals

A total of 40 homozygous transgenic mice with targeted overexpression of human α-synuclein in oligodendrocytes driven by the proteolipid protein (PLP) promoter [(PLP)-α-synuclein mice][6] were used. All animals were 6 months or older. Further, mice were housed under standard conditions with a 12-hour light/dark cycle and free access to food and water. All efforts were made to minimize the number of animals used and their suffering. In particular, all attempts were made to minimize discomfort from invasive procedures (e.g., intraperitoneal injections) to avoid a significant impact of pain on experimental results. The following in vivo protocols were approved by the Federal Ministry of Science and Research of Austria.

Animals were randomized into four experimental groups. Thirty mice were chronically intoxicated with 3-NP according to the following low-dose paradigm[7]: 4 × 10 mg/kg, 4 × 20 mg/kg, 4 × 40 mg/kg, 3 × 50 mg/kg—which results in a total dosage of 430 mg/kg 3-NP—were dissolved in saline and injected intraperitoneally (i.p.) every 12 hours during the intoxication period. Intoxicated animals were treated with saline, early EPO, and late EPO with treatment starting before (saline, early EPO) or after (late EPO) intoxication (each: n = 10). Nonintoxicated animals receiving EPO from the beginning served as control group (n = 10).

Commercially available EPO (Erypo; Janssen-Cilag, Vienna, Austria) was delivered i.p. three times per week during the whole study period of 4 weeks (i.e., 12 injections) in the early EPO group. The late EPO group was treated only following the last 3-NP administration for five injections.

Because of the necessity of long-term EPO treatment, when applied in neurodegenerative diseases a human dose of 100 IU/kg was chosen to be the long-term maintenance dose in chronic renal failure, which is considered safe in humans.[38] The corresponding mouse dose (1,250 IU/kg) was calculated according to published Food and Drug Administration (FDA) Guidelines (http://www.fda.gov/cder/guidance/5541fnl.htm).

Behavioral Testing

The following behavioral tests were performed blinded to the treatment status according to a standardized protocol: motor behavior scale (MBS), pole test, and spontaneous locomotor activity at the time points shown in the flowchart (Fig. 1).

The MBS is used to score 3-NP-induced motor disability in mice[39] and assesses general locomotor activity, hind limb clasping, hind limb dystonia, truncal dystonia, and postural challenge response. Higher scores indicate higher disability; the maximum total score is 10.

The pole test consists of a wooden vertical pole with rough surface, 1-cm wide and 50-cm high. After habituation, the animal is placed head up at the top of the pole, and both time for turning downwards (T_{turn}) as well as total time for climbing down (T_{total}) were taken in three trials. The best performance of all three trials was kept for statistical analysis.[7]

Open field activity was tested with the Flex Field Activity System (San Diego Instruments, CA), which allows monitoring and real-time counting of horizontal and vertical locomotor activity by 544 photobeam channels. Mice were tested for a 15-min period during the dark cycle.

The general observations (standardized MBS) were performed at the beginning, during the intoxication period, and on the day before scarification. The remaining tests (pole test and open field activity) were performed at the beginning of the study and on day 27 (day before sacrifice).

Immunohistochemistry

Animals were sacrificed at day 29 under at least three-fold thiopental (i.e., 120–150 mg/kg) overdose and perfused with 10 mL of 0.9% phosphate buffered saline (PBS) followed by 50 mL of ice-cold 4% paraformaldehyde (PFA) dissolved in PBS (pH = 7.4). After overnight postfixation in PFA and immersion in 25% sucrose (in PBS) until they sank, the brains were frozen in isopentane. Serial coronal sections were cut on a freezing microtome.

Free-floating sections (40 μm) were stained with antibodies for TH (Sigma) and dopamine and cyclic adenosine monophosphate (cAMP)-regulated phosphoprotein (DARPP-32; kindly provided by Dr. Hugh Hemmings, Jr.; The New York Hospital-Cornell Medical Centre).[6]

Image and Data Analysis

Nikon E-800 microscope and computerized analysis system (*Stereo Investigator software*; MicroBrightField Europe e.K., Magdeburg, Germany) were used for image analysis.

The number of TH+ (substantia nigra) and DARPP-32 positive cells (striatum) were assessed using the stereology module of the *Stereo Investigator software*. To obtain unbiased estimates of cell numbers, five coronal sections containing comparable regions of the substantia nigra pars compacta as well as seven coronal sections containing comparable striatal structures were assessed using the optical dissector method. Structures of interest (striatum and substantia nigra) were outlined manually according to the *Paxinos and Franklin Mouse Brain Atlas* (1997, Academic Press, San Diego). Analysis was performed blinded to the treatment group. The quality of quantitative estimates was assessed using the Scheaffer coefficient of error (CE).

Statistical analysis was performed using *SPSS 15.0* (SPSS). All data are expressed as mean ± standard deviation. Groups were subdivided into two strata comprising intoxicated and

nonintoxicated animals. Within the intoxication group, group differences in behavioral and histology data were analyzed using a one-way ANOVA followed by Bonferroni post hoc correction. In cases of missing normality of distribution, a nonparametric Mann–Whitney *U* test was performed. Differences between the two strata were analyzed using a one-way ANOVA. Correlations between histopathology and behavioral variables were obtained using Spearman's rho. The significance level was set at $P < 0.05$; all tests were two-sided.

Results

Survival

During the 3-NP intoxication period, four mice died in the 3-NP (treated with saline only) group, three mice in the early EPO group, and five mice in the late EPO group (no significant difference; 3-NP vs. early EPO, $P = 0.648$; 3-NP vs. late EPO, $P = 0.661$; early EPO vs. late EPO, $P = 0.374$). In the EPO control group (nonintoxicated animals receiving EPO from the beginning) all animals survived.

MBS

At the baseline assessment, no significant differences between the three intoxicated groups were observed (Fig. 2). During the intoxication period, early EPO-treated animals (0.00 ± 0.00) as well as late EPO-treated animals (0.00 ± 0.00) failed to score significantly lower than 3-NP-treated animals (0.67 ± 0.82, $P = 0.759$; Fig. 2).

At the end of the study, animals receiving early EPO (0.14 ± 0.38) performed significantly better than that of 3-NP animals (5.33 ± 1.03, $P = 0.001$). Animals receiving late EPO treatment (1.00 ± 1.22) also showed lower MBS scores than that of 3-NP animals (5.33 ± 1.03, $P = 0.004$). There was no significant difference between early and late EPO administration. Overall, EPO- and saline-treated 3-NP animals (2.11 ± 2.52) scored significantly higher than nonintoxicated animals (0.00 ± 0.00, $P = 0.016$; Fig. 2).

Pole Test

Pole test performance was good in all groups; no animal was unable to perform the test. At baseline, all animals showed homogenous pole test performance without any significant difference between groups. At the end of the study, 3-NP animals (5.83 ± 0.41) were significantly slower in turning downwards than either EPO-treated group (early EPO, 3.42 ± 1.72, $P = 0.006$; late EPO, 2.60 ± 0.55, $P = 0.001$). Total time to descend was also significantly longer within the 3-NP group (12.33 ± 0.52) compared with the EPO-treated groups (early EPO, 7.57 ± 2.23, $P < 0.001$; late EPO, 5.60 ± 0.89, $P < 0.001$). No differences were observed between both the EPO-treated groups (Fig. 3).

Open Field Activity

Comparison of nonintoxicated with intoxicated animals resulted in a significant difference in number of rearings ($P = 0.048$) but failed to differ significantly in central ($P = 0.643$), peripheral ($P = 0.052$), and total ($P = 0.076$) activity counts.

In the intoxicated group, no significant differences between different treatments were observed for number of rearings ($P = 0.692$) as well as central ($P = 0.169$), peripheral ($P = 0.887$), and total counts ($P = 0.854$) (Table 1).

Histology

All 3-NP-treated animals ($3.96 \times 10^3 \pm 1.17 \times 10^3$) showed a significant loss of TH+ neurons in the substantia nigra pars compacta compared with nonintoxicated controls ($6.69 \times 10^3 \pm 0.64 \times 10^3$, P 0.001; Fig. 4).

Both EPO treatment groups had significantly higher TH+ cell numbers (early EPO, $4.87 \times 10^3 \pm 0.69 \times 10^3$, $P < 0.001$; late EPO, $4.35 \times 10^3 \pm 0.64 \times 10^3$, $P = 0.007$) than the 3-NP group ($2.72 \times 10^3 \pm 0.79 \times 10^3$). There was a numerical difference between the EPO groups favoring early administration; however, no significant difference was observed (Figs. 4 and 5).

In the striatum, intoxicated animals ($2.97 \times 10^5 \pm 0.68 \times 10^5$) did not show significant loss of dopamine and cAMP-regulated phosphoprotein positive (DARPP-32+) neurons compared with nonintoxicated animals ($3.48 \times 10^5 \pm 0.67 \times 10^5$, $P = 0.062$). The 3-NP group ($2.17 \times 10^5 \pm 0.30 \times 10^5$) showed significant lower numbers of DARPP-32+ cells than animals receiving early ($3.61 \times 10^5 \pm 0.29 \times 10^5$, P <0.001) or late ($3.03 \times 10^5 \pm 0.23 \times 105$, P <0.001) EPO treatment. Additionally, a significant difference between both EPO treatment groups was observed ($P = 0.007$; Figs. 4 and 5).

Regarding the quality of quantitative estimates, a CE of 0.1741 ± 0.0515 was obtained within the substantia nigra. At striatal level, the CE was 0.0599 ± 0.0092.

In the nonintoxicated group, pole test performance correlated significantly with cell counts [T_{turn} with striatal DARPP-32+ cell number ($r = 0.750$, $P = 0.012$), T_{total} with nigral TH+ cell number ($r = 0.675$, $P = 0.032$)]. Intoxicated animals, EPO treated as well as saline treated, showed an inverse correlation between striatal DARPP-32+ cell number and pole test's T_{total} ($r = -0.478$, $P = 0.045$). Additionally, in these three groups (early EPO, late EPO, and 3-NP) nigral TH+ cell number and pole test performance correlated inversely [nigral TH + cells with T_{total} ($r = -0.563$, $P = 0.019$), nigral TH+ cells with T_{turn} ($r = -0.535$, $P = 0.027$)]. In the intoxicated group, an inverse correlation between MBS score and stereologically estimated cell number at striatal as well as nigral level was observed [striatal DARPP-32+ cell number with MBS ($r = -0.839$, $P < 0.001$); nigral TH+ cell number with MBS ($r = -0.712$, $P = 0.001$)].

Locomotor activity failed to correlate with nigral and striatal cell counts.

Discussion

This study shows that EPO reduces both behavioral impairment and striatonigral pathology in an established transgenic MSA mouse model.[7]

The MBS used to assess the overall disease severity in MSA mice comprises different aspects related to clinical symptoms of human MSA. Reduced locomotor speed, impaired

postural stability, dystonic postures of limbs, and especially truncal dystonia are characteristic for MSA.[40] Hind limb clasping in rodents is characteristic for striatal lesions in models of HD and MSA.[39] EPO had a significant effect on 3-NP toxicity; animals receiving EPO from the beginning did not show elevated MBS scores. Animals receiving EPO post-intoxication, although significantly worse than early EPO animals, scored significantly lower than 3-NP controls. The pole test animals receiving EPO performed significantly better than 3-NP controls with no difference between early and late EPO administration. In contrast to other studies, where animals were unable to perform the test and a default value of 120 seconds was taken into account,[9,39] in the present experiments no animal took longer than 15 seconds to complete the test thus eliminating the bias introduced by nonperformers. Regarding spontaneous locomotor activity, animals receiving 3-NP (EPO treated as well as saline treated) showed a significant lower number of rearings compared with nonintoxicated animals. Similar behavior was observed in previous studies as impaired rearing is part of the transgenic MSA mouse model's phenotype and is exacerbated by intoxication with 3-NP.[6,7] Unfortunately, we were not able to observe significant differences between treatment groups.

The observed improvement of motor deficits may (partly) reflect peripheral effects on erythropoiesis and oxygen transport. Indeed, asialoEPO, a nonhematogenic variant of EPO, failed to provide neuroprotection in a transgenic model of HD which shares striatal pathology with MSA.[37] EPO-R is also expressed in the cardiovascular system and skeletal muscle. It may promote vascularization via vascular endothelial growth factor.[41] In a rat model, EPO was shown to induce changes in muscle fiber type[42] and promote functional and histological recovery of traumatized skeletal muscle.[43] However, no direct effects on skeletal fiber thickness and vascularization were observed in humans.[44] The effect of EPO, however, in our study was not limited to motor improvement.

Stereological analysis of TH+ neurons in substantia nigra pars compacta and DARPP-32 neurons in the striatum demonstrated reduced cell loss in the EPO-treated animal groups. The oligodendroglial α-synucleinopathy in the (PLP)-α-synuclein mice increases vulnerability to 3-NP-induced oxidative stress.[7] 3-NP is a mitochondrial toxin which inhibits succinate dehydrogenase activity throughout the brain, but nevertheless causes selective neurodegeneration in the striatum, or in presence of GCIs, in striatum, substantia nigra pars compacta, cerebellum, and pontine nuclei. The exact mechanisms underlying this specificity are not yet understood. In HD models, it has been shown that 3-NP induces selective DNA fragmentation and increased bax/bcl-2 ratio in the centre of severe striatal lesions.[45] Furthermore, bcl-2 overexpression has been shown to prevent 3-NP-induced cell death.[46]

The possible central mechanisms through which EPO may have provided neuroprotection in this study include upregulation of antiapoptotic signals (i.e., Bcl-2 and Bcl-XL), inhibition of caspases, inhibition of glutamate release, and upregulation of enzymes that scavenge oxygen radicals.[47,48] On the other hand, MSA is thought to be a primary oligodendrogliopathy with secondary neurodegeneration.[49] EPO and EPO-R are expressed in human astrocytes and play an important role in oligodendrocyte maturation.[50] Astrocyte EPO expression is reduced by proinflammatory cytokines;[51] exogenous EPO protects oligodendrocytes in vitro from cytotoxicity induced by inflammatory stimuli.[52] In a model

of spinal cord trauma, EPO has been shown to counteract secondary oligodendrocyte death enhancing functional recovery.[53] These glioprotective effects might have contributed to neuroprotection observed in this study. Therefore, discrepancy between the above-mentioned study on asialoEPO in HD mice[37] and this study might be a result of not only peripheral impacts but also oligodendroglial protection due to modulatory effects of EPO.

It is important to take into account that both EPO groups—the “early” group administering EPO ahead of 3-NP intoxication as well as the “late” group which started treatment 2 days after the end of the intoxication period—provided rescue of the DA phenotype. This is important, since the diagnosis of MSA is clinical and usually made 2 to 3 years after disease onset when patients may already be disabled. Thus, this study provides evidence that EPO might become a prospective disease-modifying drug candidate.

To sum up, striatonigral degeneration is the key neuropathological substrate of parkinsonism in MSA. Our finding of EPO-derived effects within the striatonigral pathway suggest that further studies to explore the underlying mechanisms and to define the preclinical rationale of EPO-based therapies in MSA are highly warranted.

Some limitations have to be acknowledged. The model used in this study combines 3-NP-induced oxidative stress and transgenic overexpression of synuclein to induce MSA-like pathology. However, the relative contribution of genetic and environmental factors in the pathogenesis of MSA remains unknown at present and therefore the EPO effects observed in the mouse model may not be reproducible in humans. In addition, we have not investigated whether EPO interferes with synuclein aggregation itself as it has been demonstrated for rifampicin. Further, we cannot exclude nonspecific effects of EPO on motor behavior and neuronal integrity due to lack of a non-EPO nonintoxicated control group.

In summary, we have shown that EPO improves motor deficits and rescues TH+ as well as DARPP32 neurons in the combined toxin/transgenic model of MSA. Further studies are warranted to develop EPO as a potential interventional therapy in MSA.

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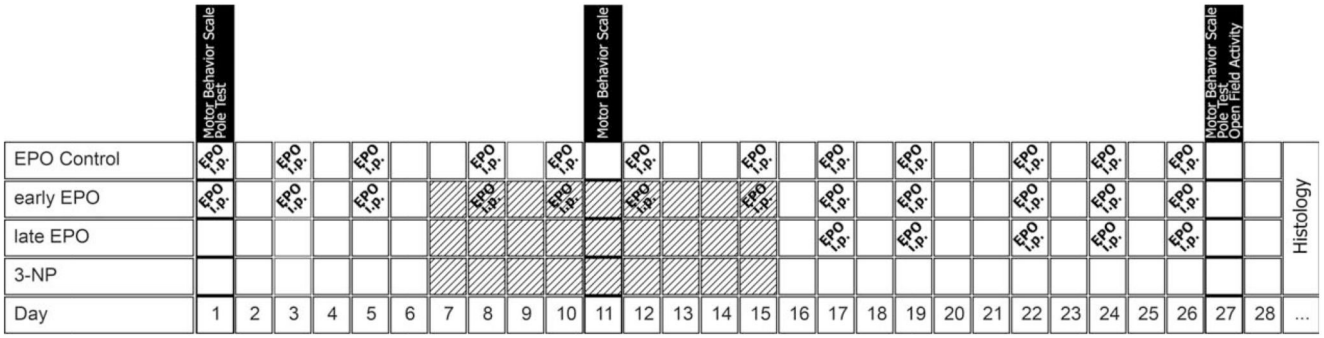

FIG. 1.

Flowchart of the study. Diagonally shaded boxes represent injection of 3-NP intraperitoneally every 12 hours; dosage according to low-dose intoxication paradigm.

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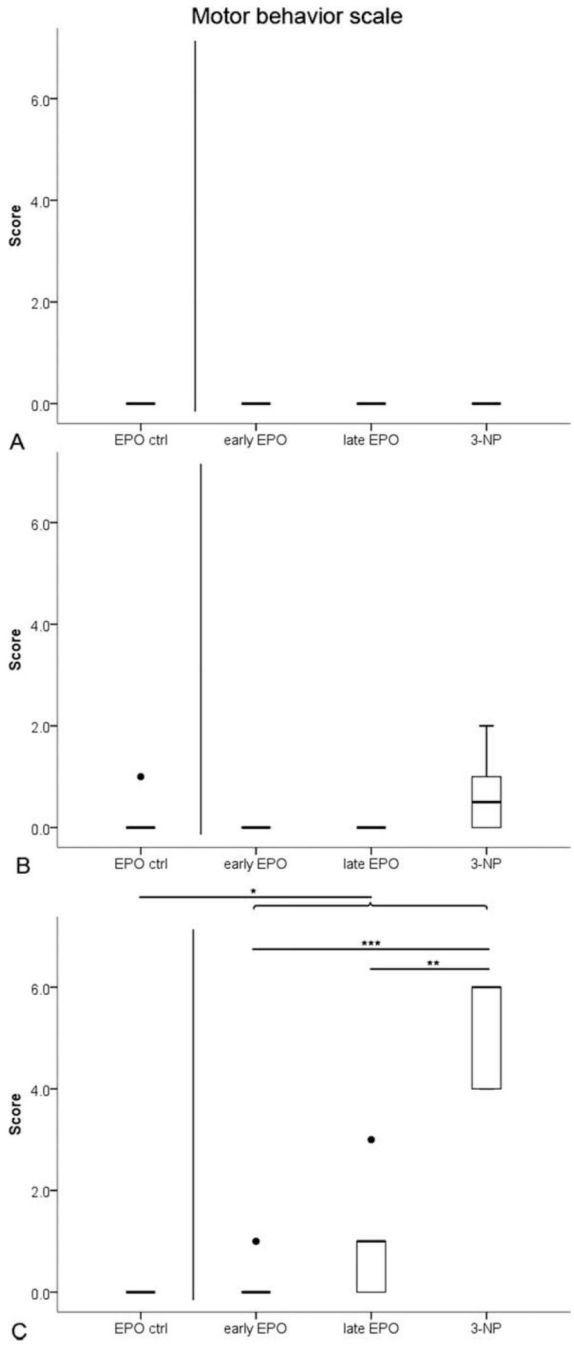

FIG. 2.

Motor behavior scale. Motor scores at baseline assessment (**A**), during the intoxication period (**B**) and at the end of the study (**C**). Dark gray vertical line indicates segmentation into two strata. The box shows interquartile range (from lower to upper quartile, Q25 and Q75 respectively), a horizontal line within the box represents median. Whiskers extend box to minimum and maximum value. Discordant values are emphasized by symbols. Curly brackets indicate group composition. Statistical significance figured as **P* < 0.05, ***P* < 0.01, and ****P* ≤0.001.

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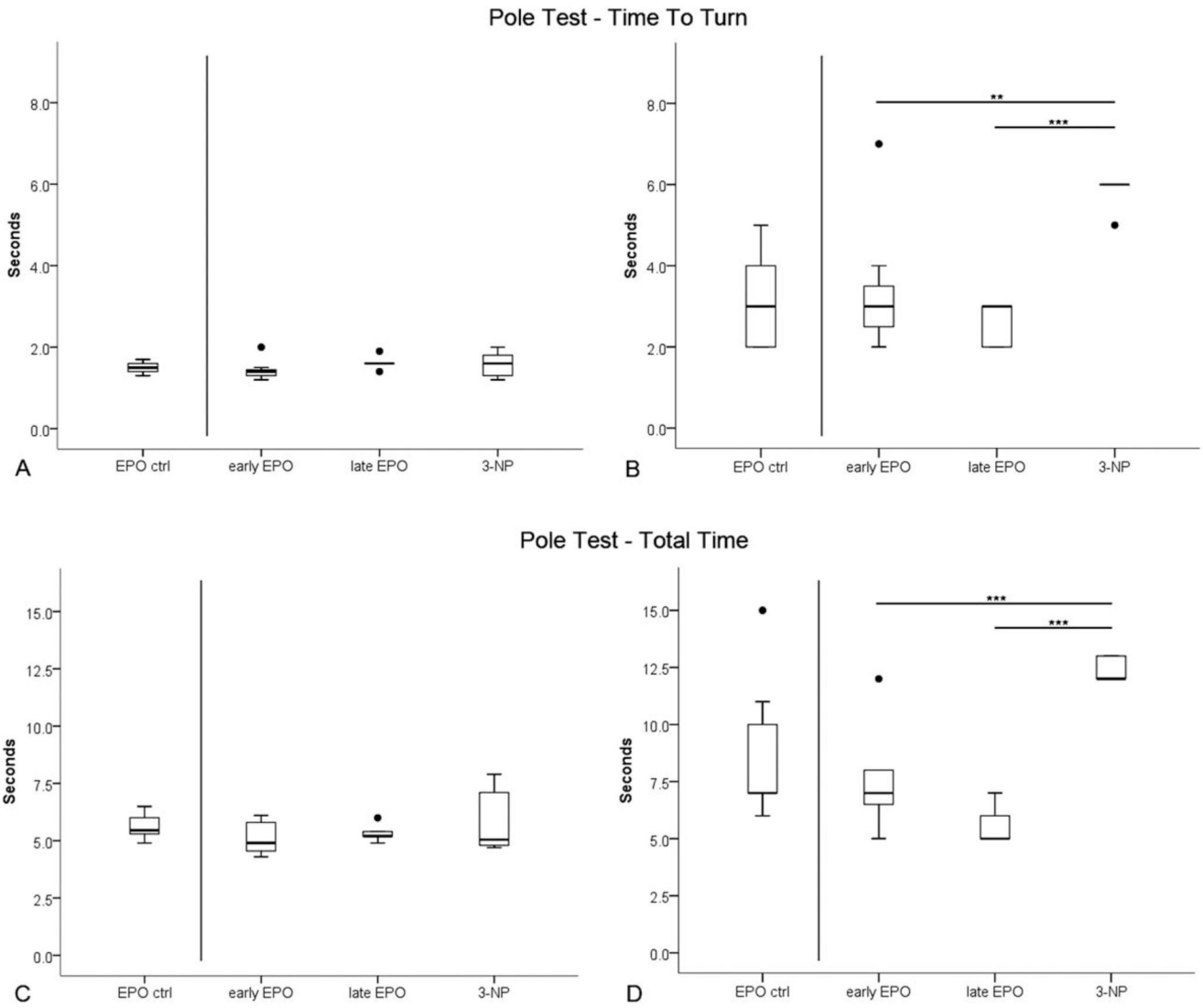

FIG. 3.

Pole test. Time until animals turned downwards (**A** and **B**) and total time to descend the pole (**C** and **D**). Left-sided box plots (A and C) represent baseline behavior. Dark gray vertical line indicates segmentation into two strata. The box shows interquartile range (from lower to upper quartile, Q25 and Q75, respectively), and a horizontal line within the box represents median. Whiskers extend box to minimum and maximum value. Discordant values are emphasized by symbols. Statistical significance after post hoc testing figured as $*P < 0.05$, $**P < 0.01$, and $***P \leq 0.001$

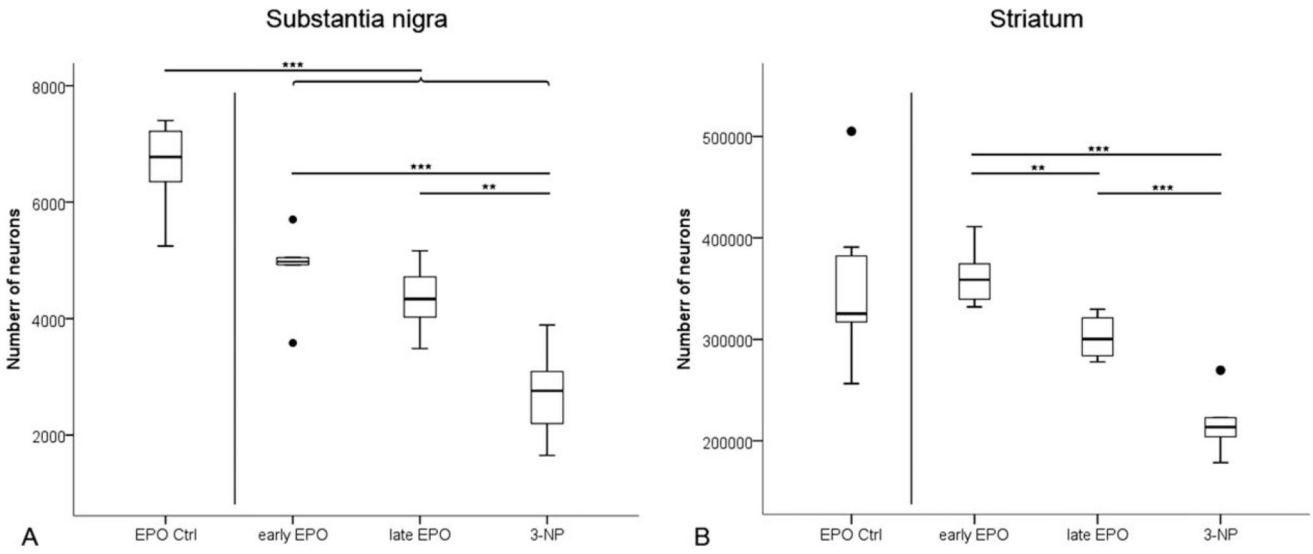

FIG. 4.

Cell counts. Cell counts in substantia nigra and striatum. Dark gray vertical line indicates segmentation into two strata. The box shows interquartile range (from lower to upper quartile, Q25 and Q75, respectively), a horizontal line within the box represents median. Whiskers extend box to minimum and maximum value. Discordant values are emphasized by symbols. Curly brackets indicate group composition. Statistical significance after post hoc testing figured as $*P < 0.05$, $**P < 0.01$, and $***P \leq 0.001$.

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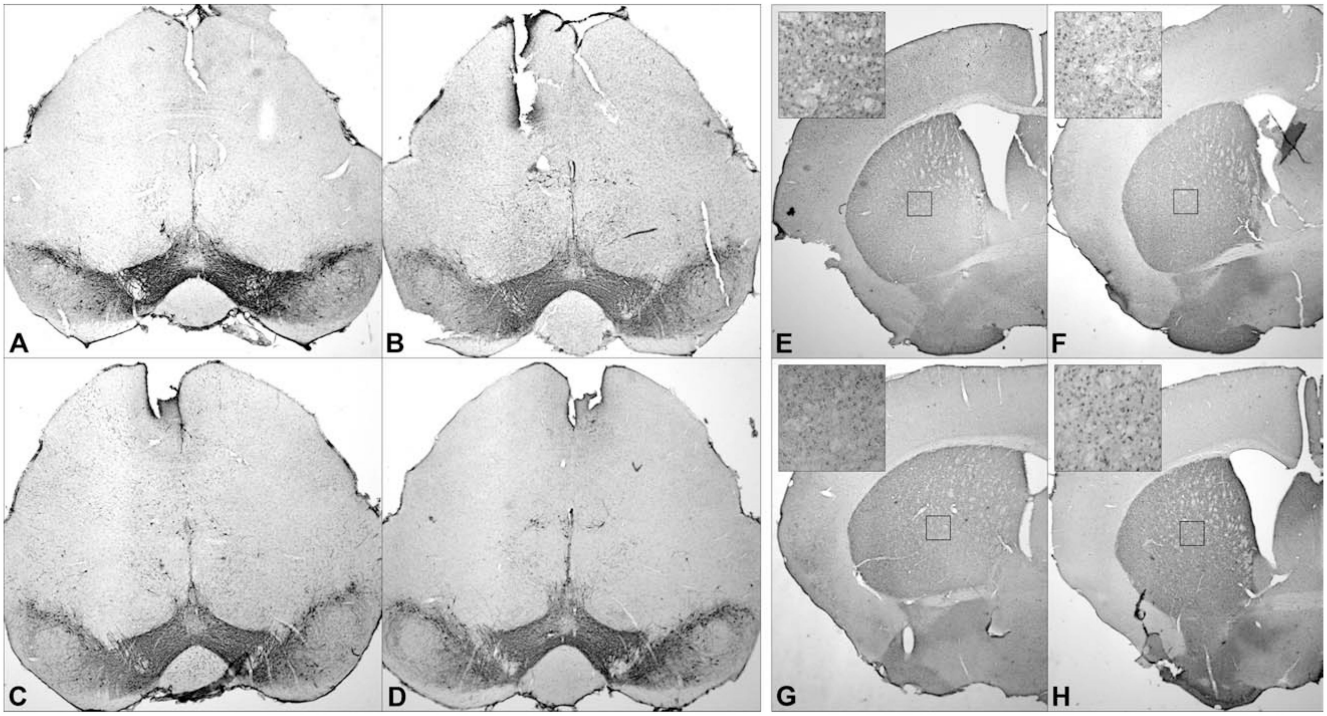

FIG. 5.

Histology. TH staining of substantia nigra (**A–D**) and DARPP-32 staining of striatum (**E–H**). A and E showing EPO control; B and F, early EPO; C and G, late EPO; and D and H, indicating 3-NP controls.

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TABLE 1

Results of spontaneous open field activity

	Rearing	Central	Peripheral	Total
EPO control	158.60 ± 31.75	598.50 ± 319.03	3944.90 ± 829.56	4702.00 ± 1077.00
Early EPO	137.71 ± 24.20	137.71 ± 213.09	3086.85 ± 583.45	3647.14 ± 798.80
Late EPO	116.80 ± 39.67	701.00 ± 172.41	3068.60 ± 915.16	3886.40 ± 1072.29
3-NP	122.50 ± 58.20	564.67 ± 314.41	3346.67 ± 1509.23	4047.17 ± 1754.91

Data shown as mean ± standard deviation. Statistical significance is not shown.

EPO, erythropoietin; 3-NP, 3-nitropropionic acid.