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The neuroprotective and neurorescue effects of carbamylated erythropoietin Fc fusion protein (CEPO-Fc) in a rat model of Parkinson's disease.

Judith Thomas Tayra [a], Masahiro Kameda [a,*], Takao Yasuhara [a], Takashi Agari [a], Tomohito Kadota [a], Feifei Wang [a], Yoichiro Kikuchi [a], Hanbai Liang [a], Aiko Shinko [a], Takaaki Wakamori [a], Brigitta Vcelar [b], Robert Weik [b], Isao Date [a] .

[a] Department of Neurological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama, 700-8558, Japan.

[b] Polymun Scientific GmbH, Vienna, Austria.

*Corresponding author Fax: +81 86 227 0191.

Email address: mrkameda@gmail.com (M. Kameda)

Abbreviations: BBB, blood brain barrier; CEPO, carbamylated erythropoietin; CEPO-Fc, carbamylated erythropoietin Fc fusion protein; CNS, central nervous system; DA, dopamine; EPO, erythropoietin; EPO-Fc, erythropoietin Fc fusion protein; PBS, phosphate buffered saline; PD, Parkinson's disease; SN, substantia nigra; TH, tyrosine hydroxylase; 6-OHDA, 6-hydroxydopamine.

Abstract

Parkinson's disease is characterized by progressive degeneration of dopaminergic neurons. Thus the development of therapeutic neuroprotection and neurorescue strategies to mitigate disease progression is important. In this study we evaluated the neuroprotective/rescue effects of erythropoietin Fc fusion protein (EPO-Fc) and carbamylated erythropoietin Fc fusion protein (CEPO-Fc) in a rat model of Parkinson's disease. Adult female Sprague-Dawley rats received intraperitoneal injection of EPO-Fc, CEPO-Fc or PBS. Behavioral evaluations consisted of rota-rod, cylinder and amphetamine-induced rotation tests. In the neuroprotection experiment, the CEPO-Fc group demonstrated significant improvement compared with the EPO-Fc group on the amphetamine-induced rotation test throughout the four-week follow-up period. Histologically, significantly more tyrosine hydroxylase (TH)-positive neurons were recognized in the substantia nigra (SN) pars compacta in the CEPO-Fc group than in the PBS and EPO-Fc groups. In the neurorescue experiment, rats receiving CEPO-Fc showed significantly better behavioural scores than those receiving PBS. The histological data concerning striatum also showed that the CEPO-Fc group had significantly better preservation of TH-positive fibers compared to the PBS and EPO-Fc groups. Importantly, there were no increases in hematocrit or hemoglobin levels in the CEPO-Fc group in either the neuroprotection or the neurorescue experiments. In conclusion, the newly developed CEPO-Fc might confer neuroprotective and neurorescue benefits in a rat model of Parkinson's disease without the side effects associated with polycythemia. CEPO-Fc might be a therapeutic tool for patients with Parkinson's disease.

Keywords: carbamylated erythropoietin, dopamine, neuroprotection, neurorescue, Parkinson’s disease.

1. Introduction

Parkinson's Disease (PD) is a neurodegenerative disease of the central nervous system (CNS) characterized by dopaminergic loss in the nigrostriatal system. The progressive dopaminergic loss leads to symptoms such as resting tremor, rigidity, akinesia and disturbances of postural reflex. The currently available modes of treatment suppress some of these symptoms but are unable to mitigate disease progression (Hornykiewicz et al., 1988; Fearnley et al., 1991; Sethi et al., 2002). Effectively modeling this neurodegenerative process is an important step toward developing neuroprotective and neurorescue therapeutic strategies.

Erythropoietin (EPO) is a pleiotropic cytokine originally identified for its role in erythropoiesis (Sasaki et al., 2003). Recent studies have revealed various other effects of EPO, however. Its neuroprotective effects were observed in animal models of stroke and traumatic brain injury (Cerami et al., 2001; Sakanaka et al., 1998; Wang et al., 2004; Lu et al., 2005; Mahmood et al., 2007). EPO has the ability to drive the production of neuronal progenitors from neural stem cells (Shingo et al., 2001). In addition, EPO also stimulates progenitor cell differentiation into neurons, and it is involved in the maturation of astrocytes and oligodendrocytes (Wang et al., 2004; Lee et al., 2004; Sugawa et al., 2002). These previous studies have motivated the search for additional roles of EPO, and a stroke clinical trial with EPO was recently conducted (Ehrenreich et al., 2009), yet some adverse effects have led to safety concerns regarding EPO. One of the concerns is that sustained high hematocrit causes endothelial damage and could increase susceptibility to vascular disease in mouse brains (Ongunshola et al., 2006). The fact that EPO increases hematocrit and hemoglobin levels thus

precludes any possible clinical applications of EPO. Consequently, the development of erythropoietin analogues that are predominantly neuroprotective and that lack hematopoietic activity should open new therapeutic possibilities in the treatment scenario of PD.

One new pharmaceutical composition that might meet these criteria is a fusion protein developed by Polymun Scientific that consists of the Fc domain and hinge region of human IgG1 and two recombinant human EPO molecules. This construct was chosen because the fusion protein contains the constant region of an immunoglobulin and is expected to have a prolonged half-life. This final product is called the EPO-Fc fusion protein (EPO-Fc). EPO-Fc is often carbamylated as one means of avoiding undesirable side effects. Carbamylation of a protein is a chemical modification characterized by non-enzymatic reaction of the cyanate ion with the free Epsilon NH2 group of lysines (Bar-or et al., 2011). Since this process alters protein conformation, the newly formed carbamylated erythropoietin Fc fusion protein (CEPO-Fc) does not bind the classic EPO receptor, which explains the absence of erythropoietic activity (Leist et al., 2004). Importantly, carbamylated erythropoietin (CEPO) can also cross the blood-brain barrier (BBB) in rats and humans, as EPO can (Brines et al., 2000; Ehrenreich et al., 2004; Verdonck et al., 2007; Siren et al., 2009; Bouzat et al., 2011).

Based on this background, we examined whether EPO-Fc and CEPO-Fc have neuroprotective and neurorescue effects in a rat model of PD in this study. Because PD is a chronic and progressive neurodegenerative process, strategies aimed at slowing its development may be important. Considering this, in the neuroprotection experiment we evaluated the ability of both EPO-Fc and CEPO-

Fc to mitigate dopaminergic neuronal loss induced by 6-OHDA unilateral right striatal lesion. In this experiment, rats received the first dose of the respective drug before 6-OHDA administration. In the neurorescue experiment, on the other hand, we evaluated if EPO-Fc and CEPO-Fc have potential therapeutic role in a rat model of Parkinson's disease. In this context, rats received the first dose of the respective drug two weeks after 6-OHDA lesion and were already diseased animals.

2. Results

2.1. Study 1: Neuroprotection experiment

2.1.1. Behavioural tests

2.1.1.1. Rota-rod test

On the rota-rod test, measuring the longest time that a rat was able to remain on a rota-rod device, both the EPO-Fc and CEPO-Fc groups demonstrated improvement throughout the follow-up period after 6-OHDA injection. In contrast, the PBS group tended to worsen over time (Fig. 2A).

2.1.1.2 Cylinder test

Scores on the cylinder test in the EPO-Fc group were significantly better than those in the PBS group at two and four weeks after the 6-OHDA lesion (Fig.2B. contralateral bias: 51±7.9%; 33±12.9% for EPO-Fc group; 82±4.8%; 74±7.5% for PBS group at two and four weeks after the lesion, respectively. ** $p<0.05$ CEPO-Fc, EPO-Fc vs PBS and $F=5.17$ at two weeks; #p <0.05 EPO-Fc vs PBS and $F=4.18$ at four weeks).

In the CEPO-Fc group, the scores ameliorated over time and were significantly better than those in the PBS group at one, two and three weeks after

the 6-OHDA lesion (contralateral bias: 56±6.3%; 54±8.9%; 41.5±9.2% for CEPO-Fc group one, two and three weeks after the lesion, respectively; 81±4.9%; 82±4.8%; 78±6%, for PBS group. *p<0.05 CEPO-Fc vs PBS at one (F=4.41) and three (F=4.58) weeks after the lesion; ** p<0.05 CEPO-Fc, EPO-Fc vs PBS and F=5.17 at two weeks after the lesion).

2.1.1.3. Amphetamine-induced rotation test

The number of amphetamine-induced rotations decreased significantly at four weeks after lesion in the EPO-Fc group (8.5±1.4turns/min) compared to that in the PBS group (13.9±1.4turns/min) (Fig. 2C. **p<0.05 EPO-Fc vs PBS).

Rats receiving CEPO-Fc had a significantly smaller number of amphetamine-induced rotations at both two and four weeks after 6-OHDA injection (5.7±0.4; 3.8±0.9, respectively) compared with those receiving PBS (9.9±0.1; 13.9±1.4, respectively) (Fig.2C. *p<0.05 CEPO-Fc vs EPO-Fc at two and four weeks; ***p<0.01 CEPO-Fc vs PBS at two and four weeks; F=7.89 and F=15.48 at two and four weeks, respectively).

Interestingly, the number of turns/min in the CEPO-Fc group was also significantly smaller than that observed in the EPO-Fc group at two and four weeks after 6-OHDA administration (9.1±1.3; 8.5±1.4 for EPO-Fc and 5.7±0.4; 3.8±0.9 for CEPO-Fc, respectively).

Moreover, in the PBS group, the number of amphetamine-induced rotations increased over time and was higher at four weeks than at two weeks after 6-OHDA administration (13.9±1.4; 9.9±0.1, respectively). Conversely, in both the EPO-Fc and CEPO-Fc groups, the number of rotations decreased during the follow-up and was smaller at four weeks than at two weeks after 6-OHDA

injection (8.5±1.4; 9.1±1.3 for EPO-Fc and 3.8±0.9; 5.7±0.4 for CEPO-Fc, respectively). This suggests that the PBS group worsened over time, while the CEPO-Fc and EPO-Fc groups, in contrast, improved their behavioural function during our experiment. It is important to note that the CEPO-Fc group showed significantly better scores than the EPO-Fc group at both two and four weeks after 6-OHDA administration.

2.1.2. Hematocrit and Hemoglobin

In the EPO-Fc group, hematocrit and hemoglobin levels raised precipitously, with peak values reached eight days after the first injection of EPO-Fc (EPO-Fc group: 47.6±1.7% and 51.3±2.5% at four and eight days after first injection of EPO-Fc). In contrast, hematocrit and hemoglobin levels in the CEPO-Fc and PBS groups showed no such increase, but rather remained stable within normal values. Interestingly, increments on both hematocrit and hemoglobin values in the EPO-Fc group were significantly greater than those in the CEPO-Fc and the PBS groups (Fig. 3A for hematocrit. *$p<0.01$ EPO-Fc vs PBS, CEPO-Fc and $F=12.81$ at four days after i.p.injection; **$p<0.0001$ EPO-Fc vs PBS, CEPO-Fc and $F=18.07$ at eight days after i.p. injection) (Fig. 3B for hemoglobin. *$p<0.01$ EPO-Fc vs PBS, CEPO-Fc. $F=12.92$ at four days after i.p. injection; **$p<0.0001$ EPO-Fc vs PBS, CEPO-Fc and $F=18.14$ at eight days after i.p. injection).

2.2.3. Immunohistochemistry

In the PBS group, severe destruction of tyrosine hydroxylase (TH) fibers (Fig. 4B) was observed. On the other hand, there was profound and significant preservation of TH-positive fibers in the EPO-Fc and the CEPO-Fc groups (Fig. 4C and D). Moreover, the preservation of TH-positive fibers in the EPO-Fc and

the CEPO-Fc groups was also significantly greater than that observed in the PBS group (Fig. 4E.EPO-Fc group: 65±4% relative to intact side; CEPO-Fc group: 71±2% relative to intact side; PBS group: 32±1% relative to intact side. ***$p<0.0001$ EPO-Fc vs PBS; ****$p<0.0001$ CEPO-Fc vs PBS and $F=76.74$). Similarly, significant preservation of dopaminergic TH-positive neurons was seen in the Substantia Nigra (SN) pars compacta and ventral tegmental area (VTA) in both the EPO-Fc (Fig. 5C) and the CEPO-Fc (Fig. 5D) groups compared to the PBS group (Fig. 5B.) (Fig.5E for SN: EPO-Fc group: 62±4% relative to intact side; CEPO-Fc group: 71±2% relative to intact side; PBS group: 46±6% relative to intact side. *$p<0.05$ EPO-Fc vs PBS; **$p<0.05$ CEPO-Fc vs EPO-Fc; ***$p<0.0001$ CEPO-Fc vs PBS and $F=14.11$) (Fig.5F for VTA. EPO-Fc group: 68±3% relative to intact side; CEPO-Fc group: 81±2% relative to intact side; PBS group: 31±2% relative to intact side.*$p<0.0001$EPO-Fc vs PBS; **$p<0.01$ CEPO-Fc vs EPO-Fc; ***$p<0.0001$CEPO-Fc vs PBS and $F=97.01$). Furthermore, there was more profound and significant preservation in the CEPO-Fc group even compared to the EPO-Fc group.

2.2. Study 2: Neurorescue experiment

2.2.1. Behavioural tests

2.2.1.1. Rota-rod test

Animals in the CEPO-Fc group earned significantly better scores on the rota-rod test than those in the PBS group did at two and three weeks after intraperitoneal administration of CEPO-Fc (CEPO-Fc group: 77±2.2%, 89±5.2% at two and three weeks after CEPO-Fc injection, respectively; PBS group: 53±1.5%, 52±5.3% at two, and three weeks after PBS injection, respectively) (Fig.

6A. $^{*}p<0.05$ CEPO-Fc vs PBS and $F=3.98$ at two weeks; $^{**}p<0.01$ CEPO-Fc vs PBS and $F=9.95$ at three weeks). It is worth noting that the beneficial effect in the CEPO-Fc group continued for at least three weeks after CEPO-Fc administration.

2.2.1.2. Cylinder test

After the CEPO-Fc injection, the scores for forelimb asymmetry in the CEPO-Fc group improved continually throughout the follow-up period (Fig.6B). The scores in the CEPO-Fc group continued to improve for at least four weeks after CEPO-Fc administration. Most importantly, the CEPO-Fc group earned its best scores at four weeks after administration of CEPO-Fc, with a statistically significant difference from the EPO-Fc group (24±6%, 46±9% for CEPO-Fc and EPO-Fc, respectively). We speculate that these progressive and persistent improvements in the scores of the CEPO-Fc group indicate a more prolonged beneficial effect of CEPO-Fc ($^{*}p<0.05$ CEPO-Fc vs EPO-Fc and $F=5.38$ at four weeks after i.p. injection).

2.2.1.3. Amphetamine-induced rotation test

The number of amphetamine-induced rotations in the CEPO-Fc and EPO-Fc groups decreased significantly at two weeks after the intraperitoneal administration of the respective drug (4.7±1.3; 4.1±1.2 and 8.1±2.6 turns/min for CEPO-Fc, EPO-Fc and PBS, respectively (Fig. 6C. $^{*}p<0.01$ CEPO-Fc, EPO-Fc vs PBS and $F=7.27$).

This improvement persisted at least four weeks after CEPO-Fc administration: the number of amphetamine-induced rotations at four weeks was smaller than that observed at two weeks after the intraperitoneal injection (3.3±1.5 and 4.7±1.3turns/min, respectively). The beneficial effect of CEPO-Fc was observed

even at four weeks after CEPO-Fc injection, although it was not statistically significant.

2.2.2. Hematocrit and Hemoglobin

In the neurorescue experiment, the levels of hematocrit and hemoglobin remained stable within normal values in both the CEPO-Fc and the PBS groups. In the EPO-Fc group, in contrast, these values were significantly higher, and the peak level occurred seven days after EPO-Fc injection (Fig. 3C and Fig 3D. hematocrit: 49±1.7%; hemoglobin: 16.9±0.5g/dl, respectively).

Moreover, in the EPO-Fc group, hematocrit and hemoglobin levels did not return to normal levels within twenty-eight days after EPO-Fc injection (hematocrit: 43.4±2%; haemoglobin: 14.7±0.75g/dl, respectively). These levels were significantly higher than those in the CEPO-Fc and the PBS groups (Fig. 3C for hematocrit. **$p<0.0001$ EPO-Fc vs PBS, CEPO-Fc and $F=26.95$ at seven days after i.p. injection; *$p<0.05$ EPO-Fc vs PBS, CEPO-Fc and $F=8.64$ at twenty-eight days after i.p. injection) (Fig. 3D **$p<0.0001$ EPO-Fc vs PBS, CEPO-Fc. $F=29.09$ at seven days after i.p. injection;*$p<0.05$ EPO-Fc vs PBS, CEPO-Fc and $F=8.48$ at twenty-eight days after i.p. injection).

2.2.3. Immunohistochemistry

In the neurorescue experiment, analysis of the striatum revealed significant preservation of TH-positive fibers both in the EPO-Fc (Fig. 7C) and the CEPO-Fc (Fig. 7D) groups compared with the PBS group (Fig. 7B). Moreover, TH-positive fibers in the CEPO-Fc group were significantly better preserved than those in the EPO-Fc group (Fig. 7E: EPO-Fc group: 47±4% relative to intact side; CEPO-Fc group: 59±5% relative to intact side; PBS group: 20±2% relative to intact side.

*p<0.05 EPO-Fc vs PBS, **p<0.01 CEPO-Fc vs EPO-Fc, ***p<0.0001 CEPO-Fc vs PBS and F=97.01).

In relation to these findings, there was also significant preservation of dopaminergic TH-positive neurons in the SN pars compacta in both the EPO-Fc and the CEPO-Fc groups (Fig. 8C and D). Importantly, the number of preserved dopaminergic TH-positive neurons in the EPO-Fc and the CEPO-Fc groups was significantly greater than that in the PBS group (Fig. 8E for SN: EPO-Fc group: 64±3% relative to intact side; CEPO-Fc group: 67±3% relative to intact side; PBS group: 45±2% relative to intact side. *p<0.05 EPO-Fc vs PBS, **p<0.05 CEPO-Fc vs PBS and F=7.84) (Fig.8F for VTA: *p<0.01 EPO-Fc vs PBS; **P<0.0001CEPO-Fc vs PBS. EPO-Fc group: 71±3% relative to intact side; CEPO-Fc group: 81±2% relative to intact side; PBS group: 35±2% relative to intact side and F=95.99).

3. Discussion

Because Parkinson's disease is a chronic and progressive neurodegenerative process, strategies aimed at slowing its development may be important. As a step toward such strategies, in this study we explored the neuroprotective and neurorescue effects of EPO-Fc and CEPO-Fc. Neuroprotection is preventive pretreatment before disease onset, while neurorescue is corrective treatment after onset (Yasuhara et al., 2006a).

First the proteins were tested for neuroprotective capacity. Animals in the CEPO-Fc group demonstrated statistically significant improvement compared with those in the EPO-Fc group on an amphetamine-induced rotation test throughout the follow-up period. These results are supported by the observation

that more TH-positive fibers were preserved in the CEPO-Fc group than in the PBS group. In addition, TH-positive neurons in the SN pars compacta were better preserved in the CEPO-Fc group than in the PBS or the EPO-Fc group.

Next the proteins were tested for neurorescue capacity. Animals in the CEPO-Fc group showed significantly better behavioural scores than those in the PBS group. TH-positive neurons in the SN pars compacta were better preserved in the CEPO-Fc group than in the PBS group. The histological data concerning the striatum also showed that TH-positive fibers were significantly better preserved in the CEPO-Fc group than in the PBS or EPO-Fc group.

The protective effects of EPO are mediated through a receptor complex consisting of the EPO-R and the common beta receptor (Brines et al., 2004, 2005). The hematopoietic effects, on the other hand, are due to EPO binding to the (EPO-R/EPO-R) receptor, a dimer composed of two EPO receptor units. Because the affinity of EPO to the tissue-protecting receptor is substantially lower than its affinity to (EPO-R/EPO-R), higher EPO concentrations are necessary to induce its tissue-protective effects in the central nervous system (CNS) (Cerami et al., 2010). Accordingly, some have reported a bell-shaped concentration response curve for the beneficial properties of EPO: although high levels are most conducive to neuroprotection and neurorescue, they can induce generation of reactive oxygen species (ROS), causing a loss of beneficial effects and even toxicity (Ehrenreich et al., 2006; Wu et al., 2010; Diaz et al., 2005; Erbayraktar et al., 2006). This is one of the limitations of the use of EPO in clinical settings.

During the neurorescue experiment in the EPO-Fc group, haemoglobin and hematocrit levels did not return to normal levels within four weeks after EPO-Fc injection. The haemoglobin and hematocrit values were also significantly higher in the EPO-Fc group than in the CEPO-Fc and PBS groups. These parkinsonian rats were exposed to persistent erythrocytosis induced by EPO-Fc. This increase in hematocrit and haemoglobin levels precludes any clinical application of EPO. The main risks of erythrocytosis include heart failure, myocardial infarction, seizures, peripheral thromboembolic events and pulmonary embolism (Piloto et al., 2009). These undesirable and life-threatening complications are of concern, especially among PD patients, who already have one chronic and neurodegenerative process.

In the CEPO-Fc group, on the other hand, we observed no increase in haemoglobin or hematocrit, even using the same multiple injection paradigm used in the EPO-Fc group. That is why the greatest advantage of the carbamylation of erythropoietin is the elimination of its erythropoietic activity while its neuroprotection and neurorescue properties remain intact (Hasselblat et al., 2006; Kennedy et al., 2005).

3.1. Advantages of Carbamylated Erythropoieitin Fc Fusion Protein (CEPO-Fc).

EPO is a hormone that is neuroprotective in models of neurodegenerative diseases (Zhang et al., 2006). In the CNS, however, therapeutic dosages of EPO are so high that they affect haematological parameters such as hematocrit and haemoglobin (Kikerby et al., 2008; Martin et al., 1991; Bath et al., 2004; Greisenegger et al., 2004).

In addition, EPO has a relatively short serum half life, and the design of a fusion protein like EPO but with a longer serum half life is an important medical goal (Schriebl et al., 2006). In order to enhance serum half life and improve EPO's pharmacokinetics, the fusion of EPO to the Fc region of an antibody is a promising step (Capon et al., 1989). Indeed, the *in-vivo* serum half life of EPO-Fc fusion protein is greater than that of naturally occurring EPO. Moreover, in order to promote the full neuroprotective and neurorescue effects of EPO-Fc without its side effects related to erythrocytosis, EPO-Fc fusion protein was also carbamylated.

Unlike EPO, CEPO does not bind the classical homodimeric receptor EPO-R. Its neuroprotective action appears to require the common beta receptor, which explains the absence of erythrocytosis as well as its retention of tissue-protective properties.

Recent studies have revealed that CEPO is even more favourable than previously thought, given its absence of erythropoietic activity (Nijboer et al., 2010). CEPO increases renal blood flow, promotes sodium excretion and reduces injury-induced increases in procoagulant activity and does not affect platelet production (Coleman et al., 2006). Furthermore, treatment with CEPO proved to be more effective than EPO in the reduction of inflammatory parameters in a brain death model (Nijboer et al., 2010).

Through chemical modification, such as the carbamylation of at least one primary amino group of the lysines and/or the N-terminal amino acid of EPO, the hematopoietic effect of this cytokine is considerably reduced while its neuronal protective effect remains substantially unaltered. This final product is the newly

developed CEPO-Fc, consisting of two molecules of CEPO fused to the Fc part of IgG1 (Schreibl et al., 2006).

Thus the selective stimulation of neuroprotective pathways by CEPO, which does not act on bone marrow, and the addition of a Fc fusion protein property, which increases half life, leads to CEPO-Fc. In the CEPO-Fc group in our study, there were no increases in hematocrit and haemoglobin levels, though the neuroprotective and neurorescue effects of EPO were maintained. Taken together, therefore, our data suggest that CEPO-Fc represents a new opportunity to exploit the full potential of EPO without unwanted side effects derived from polycythemia and with increased serum half life.

3.2. Mechanisms for neuroprotection/neurorescue

The mechanism underlying the neuroprotective/neurorescue effects of EPO and its analogue CEPO can be explored through findings of various kinds. Previous studies have suggested that EPO and CEPO reduce neuronal apoptosis (Zhou et al., 2011; Wei et al., 2006). In addition, there is an endogenous erythropoietin system in the brain, which may also protect neurons against oxidative stress. Ferrucci et al. (2007) observed a higher prevalence of PD in humans with low levels of EPO. Taken together, these anti-apoptotic and anti-oxidative effects may ameliorate the oxidative stress caused by the 6-OHDA neurotoxin in the nigro-striatal tract (Aluf et al., 2006).

In addition to exerting an anti-apoptotic effect, CEPO may induce Sonic hedgehog (SHH), a mitogen known to enhance neurogenesis and neuroprotection (Amankulor et al., 2009; Wu et al., 2010; Wang et al., 2007). It is possible that SHH is also related to neurotrophins, since BDNF up-regulation was

mutually linked to elevated levels of SHH (Hashimoto et al., 2008). In fact, SHH is responsible for mediating CEPO's effects on the proliferation and differentiation of neural progenitor cells, which explains its beneficial effects in the CNS (Ahn et al., 2005; Amankulor et al., 2009; Wu et al., 2010; Wang et al., 2007).

Bouzat et al. (2011) demonstrated that CEPO blocked the early development of cerebral edema by decreasing ERK-1/-2 phosphorylation. This decrease in phosphorylation is probably mediated by the CEPO-induced up-regulation of SHH (Bar-Or et al., 2011).

Besides its important effects on the SHH pathway, CEPO might exert its beneficial effects on nerve cells through its action on MHC-I (major histocompatibility complex type I) molecules. MHC-I molecules are involved in nerve plasticity and play an important role in nerve cell recovery by increasing outgrowth of axons in injured neurons (Oliveira et al., 2004; Fu et al., 2010; Huh et al., 2000). In this context, the immunomodulatory effects of CEPO induce stable improvement in neurite outgrowth, which is crucial in restoring the CNS after injury (Fu et al., 2010; Adembri et al., 2008; Schmidt et al., 2008). Thus CEPO not only has neuroprotective effects but also acts on immunomodulation.

In relation to its anti-inflammatory properties, CEPO attenuated the expression of inflammatory cytokines (Beck et al., 1986; Hirano et al., 1992). CEPO is also related to reduced expression of p-selectin. Increased levels of p-selectin have been observed in acute stroke patients and are associated with various pro-thrombotic conditions. Furthermore, CEPO was also associated with lower levels of TNF in a model of infectious peritonitis, possibly by decreasing

apoptosis and inflammation while improving the rate of healing and regeneration of tissue after damage (Erbayraktar et al., 2009; Cerami et al., 2010).

Consequently, CEPO not only acts as an anti-inflammatory pathway, but also elicits several other cytoprotective properties without causing the side effects associated with increased levels of hematocrit and haemoglobin.

4. Conclusion

We showed that CEPO-Fc, a newly developed carbamylated erythropoietin, may exert neuroprotective and neurorescue effects in a rat model of Parkinson's disease without the side effects associated with polycythemia. Consequently, this newly developed carbamylated erythropoietin Fc fusion protein is a potential therapeutic strategy for Parkinson's disease.

5. Experimental Procedures

5.1. Materials

In this study we evaluated the neuroprotective and neurorescue capacities of EPO-Fc and CEPO-Fc. EPO-Fc is an engineered erythropoietin fusion protein consisting of two human recombinant EPO molecules fused to the Fc domain of a human IgG1 antibody. Its hematopoietic effects are mediated by the binding of EPO to the (EPO-R/EPO-R) receptor, a dimer composed of two EPO receptor units. Its tissue-protective properties, on the other hand, are mediated by its binding to a heteroreceptor complex consisting of an EPO receptor monomer and the common beta receptor (Brines et al., 2004).

Since carbamylation of EPO prevents its binding to the (EPO-R/EPO-R) receptor, the hematopoietic activity of EPO-Fc is eliminated by carbamylation.

Considering that PD is a progressive and chronic neurodegenerative disease, CEPO-Fc may represent a promising alternative when long-term treatment is necessary and hematopoiesis is an unwanted side effect.

Based on previously published studies using EPO and CEPO (Liu et al.,2011; Leconte et al., 2011; Zhou et al., 2011), we performed preliminary experiments with 0.04, 0.4 and 4.0 mg/kg of body weight (BW). Finally we determined 0.4mg/kg per BW as the best dosage for i.p administration. EPO-Fc and CEPO-Fc used in experiments were diluted with PBS, accordingly to previous reports (Sturm et al., 2010). Consequently, we used PBS in control group.

5.2. Subjects

Our study is composed of two parts: neuroprotection experiments and neurorescue experiments. In the neuroprotection experiments, we evaluated the neuroprotective role of CEPO-Fc and EPO-Fc. Rats received their respective drug three times: one day before the 6-OHDA lesion (day -1), on the day of 6-OHDA lesion (day 0) and one day after the lesion (day 1) (Fig. 1A).

In order to evaluate the potential clinical application of EPO-Fc and CEPO-Fc, we also performed neurorescue experiments. In this context, the drugs were administered two weeks after the 6-OHDA lesion, to evaluate their potential in already diseased animals (Fig. 1B).

Adult female Sprague-Dawley rats (Charles River, Kanagawa, Japan) (n=36 for neuroprotection experiments, n=36 for neurorescue experiments and n=8 for non-lesioned control) weighing 200-250g at the beginning of the experiments were used according to the approved guidelines of the Institutional

Animal Care and Use Committee of Okayama University. They were housed two per cage in a temperature- and humidity-controlled room which was maintained on a 12-hour light/dark cycle with free access to food and water.

5.3. Experimental design

5.3.1. Study 1: neuroprotection

Rats (n=36) were divided into three groups: EPO-Fc (n=12), CEPO-Fc (n=13), PBS (n=11).Rats received their respective drug (0.4mg/kg per BW) or PBS intraperitoneally for three days. The first injection of the drug was administered one day prior to 6-OHDA injection. The second injection was administered immediately before the 6-OHDA lesion and the third injection one day after the 6-OHDA lesion.

Behavioural tests consisted of amphetamine-induced rotation, rota-rod and cylinder tests. All animals were evaluated weekly with the rota-rod and cylinder tests. Amphetamine-induced rotation tests were performed two and four weeks after the 6-OHDA lesion.

In order to evaluate hematocrit and haemoglobin parameters, blood samples were collected one day before administration of EPO-Fc, CEPO-Fc or PBS for control and at three days, one week and four weeks after the 6-OHDA lesion.

All rats were followed for four weeks after the 6-OHDA lesion and were then euthanized for histological analysis (Fig. 1A).

5.3.2. Study 2: neurorescue

Rats (n=36) were divided into three groups named EPO-Fc (n=12), CEPO-Fc (n=12) and PBS (n=12). Rats received their respective drug (0.4mg/kg per BW) or PBS intraperitoneally for three days.

Behavioural tests consisted of amphetamine-induced rotation, rota-rod and cylinder tests. All animals were evaluated weekly with the rota-rod and cylinder tests. Amphetamine-induced rotation tests were performed two, four and six weeks after 6-OHDA lesion.

The first dose of EPO-Fc, CEPO-Fc or PBS was administered intraperitoneally exactly 14 days after the 6-OHDA lesion and after the behavioural tests had confirmed that all animals were indeed Parkinson's disease models. The second and third doses were administrated 15 and 16 days after the 6-OHDA lesion, respectively.

For measurements of hematocrit and haemoglobin parameters, blood samples were collected on days 13, 17 and 21 after the 6-OHDA lesion.

All rats were followed for six weeks and were then euthanized for histological analyses (Fig. 1B).

5.4. Surgical Procedures

All rats were anesthetized with sodium pentobarbital (35mg/kg, i.p.) and placed in a stereotaxic apparatus (Narishige, Tokyo, Japan). A midline skin incision was made and a hole was then drilled in the skull. After this procedure, 6-OHDA (20μg, concentration: 5μg/μl, dissolved in 0.9% saline containing 0.2mg/ml ascorbic acid; Sigma-Aldrich, St. Louis, MO, USA) was injected into the right striatum with a 28-gauge Hamilton Syringe (1.0mm anterior, 3.0mm lateral to the bregma, and 5.0mm ventral to the surface of the brain with the tooth-bar set at -

3.0mm) (Paxinos and Watson, 1998). The injection rate was 1μl/min. After the injection, the syringe was left in for an additional 5 minutes before being retracted slowly (1mm/min).

5.5. Behavioral testing

Behavioral analysis consisted of the amphetamine-induced rotation test, the rota-rod test and the cylinder test. Each of these tests was performed at several time points (Fig. 1A and Fig. 1B).

5.5.1. Rota-rod Test

The rota-rod test was performed to evaluate the degree of akinesia and coordination of movements. Before 6-OHDA injection, all rats were pre-trained to stay on an accelerating rota-rod (Shinano Seisakusho, Tokyo, Japan) at a constant speed of 8rpm until they could remain on the rota-rod for 100sec. After three consecutive days of pre-training, we performed three trials, in which the rotational speed was gradually increased from 4 to 40rpm within 5min. The longest time that the rats remained on the rota-rod was recorded as the baseline. Thereafter, the data were presented as percentages of the longest time on the rota-rod of three trials relative to the baseline (Takahashi et al., 2008).

5.5.2. Cylinder Test

The cylinder test was used to assess the degree of forepaw asymmetry. Rats were placed in a transparent cylinder (diameter: 20cm, height: 30cm) for three minutes and the number of forepaw contacts to the cylinder wall were counted (Schallert et al., 2001).

5.5.3. Amphetamine-induced rotation Test

All rats received an injection of amphetamine (2.5 mg/kg, i.p., Dainippon Sumitomo Pharma, Osaka, Japan). Rotational behaviours were assessed for 90min with a video camera. The number of complete 360° body turns in the direction ipsilateral to the lesion was counted. The number of turns per minute was used for statistical analysis.

5.6. Blood sampling

Blood sampling was performed to evaluate both hematocrit and hemoglobin at several time points (Fig. 1A and Fig. 1B).

5.7. Fixation and Sectioning

Rats were deeply anesthetized with sodium pentobarbital (100mg/kg), perfused from the ascending aorta with 200ml of cold PBS followed by 200ml of 4% paraformaldehyde (PFA) in PBS. Brains were removed and post-fixed first in the same fixative for two days and then in 30% sucrose in phosphate buffer (PB) until completely submerged. Six series of coronal sections were cut at a thickness of 40μm with a freezing microtome and stored at -20°C.

5.8. Immunohistochemistry

Free-floating sections for tyrosine hydroxylase (TH) immunohistochemistry were blocked with 3% hydrogen peroxide in 70% methanol for 7min. Sections were washed three times for 5min each time in PBS. Sections were then incubated overnight at 4°C with rabbit anti-TH antibody (1:500; Chemicon, Temecula, CA, USA) with 10% normal horse serum. After several rinses in PBS, sections were incubated for 1h in biotinylated donkey anti-rabbit IgG (1:500; Jackson Immuno-Research Lab, West Grove, PA, USA), then for 30min in avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Subsequently, the

sections were treated with 3,4-diaminobenzidine (DAB; Vector) and hydroxygen peroxide, mounted on albumin-coated slides and embedded with cover glass.

5.9. Morphological analyses

The density of TH-positive fibers in the striata of animals was analyzed as described previously (Kadota et al., 2009). Five sections at 0.5 ± 1.0mm anterior to the bregma were selected for quantitative analysis (Paxinos and Watson, 1998). The two areas adjacent to the needle track on the lesioned side and the two symmetrical areas on the contralateral side were analyzed. The proportion of lesions on the intact side was evaluated in each section and the average was used for statistical analyses. The images were computer-processed into binary images using an appropriate threshold (Scion Image, Scion Corp., Frederick, MD, USA). The areas were then calculated and used for statistical analyses.

Accordingly to our previous publications (Yuan et al., 2008; Kikuchi et al., 2011), for counting the number of TH-positive neurons, every fifth 40μm-thick coronal tissue section through the substantia nigra pars compacta (SNc) was explored using 3 coronal sections respectively at 4.8 and 5.3 mm posterior to the bregma. A cell was considered when intact, round and with clear nucleus. The number of TH-positive cell bodies in the SNc was counted and the average was used for the statistical analysis.

5.10. Statistical analyses

All data are shown as average ± standard error. The data were evaluated statistically using analysis of variance one way (ANOVA) and subsequent post

hoc Bonferroni for behavioral tests or Mann-Whitney's U test for immunohistochemical investigations. Statistical significance was preset at $p<0.05$.

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Figure Legends

Fig. 1. Experimental design.

(A) Neuroprotection experiment: In this experiment 6-OHDA was injected into the striatum on day 0 (encircled by ellipse). Blood samples (dotted arrows) were collected one day prior to administration of drugs and at four days, eight days and twenty-nine days after the first i.p. administration. Behavioral tests (arrow heads) were performed weekly, with the exception of amphetamine-induced rotation, which was performed on days 14 and 28. After behavioral evaluation, all rats were euthanized (grey triangle) for immunohistochemical analysis.

(B) Neurorescue experiment: The striatum was lesioned with 6-OHDA on day 0 (encircled by ellipse). Blood samples (dotted arrows) were collected one day prior to administration of drugs and at three, seven and twenty-eight days after the first i.p. administration. Behavioral tests (arrow heads) were performed weekly, with the exception of amphetamine-induced rotation, which was performed on days 14, 28 and 42. After behavioral evaluation, all rats were euthanized (grey triangle) for immunohistochemical analysis.

Fig. 2. Behavioral tests in the neuroprotection experiment

(A) Rota-rod test. Dashed lines represent non-lesioned control group.

EPO-Fc and CEPO-Fc groups demonstrated improvement throughout the

follow-up period, as shown by the maximum times achieved on the rota-rod device. *p<0.05 CEPO-Fc, EPO-Fc, PBS vs non-lesioned control at one week after 6-OHDA lesion with F=4.44. **p<0.05 PBS vs non-lesioned control at four weeks after 6-OHDA lesion with F=3.61. ***p<0.01 PBS vs non-lesioned control at three weeks after 6-OHDA lesion with F=5.11.

(B) Cylinder test. The contralateral bias of rats in the CEPO-Fc group was significantly reduced at one, two, and three weeks after 6-OHDA lesion. In the EPO-Fc group, contralateral bias were significantly better than PBS at two and four weeks after 6-OHDA lesion.*p<0.05 CEPO-Fc vs PBS with F=4.41 and F=4.58 for one and three weeks after 6-OHDA lesion, respectively. **p<0.05 CEPO-Fc, EPO-Fc vs PBS with F=5.17 at two weeks after 6-OHDA lesion. #p<0.05 EPO-Fc vs PBS with F=4.18 at four weeks after 6-OHDA lesion.

(C) Amphetamine-induced rotation test. The number of amphetamine-induced rotations of rats in the EPO-Fc group was significantly reduced compared with that in the PBS group at four weeks after 6-OHDA lesion. **p<0.05 EPO-Fc vs PBS. In the CEPO-Fc group, a similar reduction was observed compared to the PBS group and the EPO-Fc group at both two and four weeks after 6-OHDA lesion. ***p<0.01 CEPO-Fc vs PBS and * p<0.05 CEPO-Fc vs EPO. Both EPO-Fc and CEPO-Fc rats improved over time; PBS rats, in contrast, worsened during follow-up. F=7.89 and F=15.48 at two and four weeks after 6-OHDA lesion, respectively.

Fig3. Hematocrit and hemoglobin measurements

(A) Hematocrit in neuroprotection experiment.

*$p<0.01$ EPO-Fc vs PBS, CEPO-Fc and $F=12.81$ at four days after i.p. injection; **$p<0.0001$ EPO-Fc vs PBS, CEPO-Fc and $F=18.07$ at eight days after i.p. injection.

(B) Hemoglobin in neuroprotection experiment.

*$p<0.01$ EPO-Fc vs PBS, CEPO- Fc and $F=12.92$ at four days after i.p. injection; **$p<0.0001$ EPO-Fc vs PBS, CEPO- Fc and $F=18.14$ at eight days after i.p. injection. In this experiment, rats received the first dose of the respective drug before 6-OHDA striatal lesion. In the EPO-Fc group, levels increased precipitously just four days after i.p. injection and peaked eight days after the i.p administration. In the CEPO-Fc and PBS groups, in contrast, measurements of both hematocrit and hemoglobin remained stable and within normal values.

(C) Hematocrit in neurorescue experiment.

**$p<0.0001$ EPO-Fc vs PBS, CEPO- Fc and $F=26.95$ at seven days after i.p. injection; *$p<0.05$ EPO-Fc vs PBS, CEPO-Fc and $F=8.64$ at twenty-eight days after i.p. injection.

(D) Hemoglobin in neurorescue experiment.

**$p<0.0001$ EPO-Fc vs PBS, CEPO-Fc and $F=29.09$ at seven days after i.p. injection; *$p<0.05$ EPO-Fc vs PBS, CEPO-Fc and $F=8.48$ at twenty-eight days after i.p. injection. In this experiment, rats received the first dose of the respective drug two weeks after 6-OHDA striatal lesion and were already diseased rats. In the EPO-Fc group, levels increased and peaked seven days after i.p. administration. In the EPO-Fc group, levels remained high and did not return to normal, even after twenty-eight days after i.p. injection. In the CEPO-Fc and PBS

groups, in contrast, measurements of both hematocrit and hemoglobin remained stable and within normal values.

Fig. 4. Tyrosine hydroxylase immunostaining (TH) in the striatum.

(A) Strong immunoreactivity in intact striatum.

(B) Severe destruction of TH-positive fibers in the PBS group.

(C) Some preservation of TH-positive fibers in the EPO-Fc group.

(D) Immunoreactivity is more clearly preserved in the CEPO-Fc group.

(E) Graphic. The EPO-Fc and CEPO-Fc groups demonstrated significantly better preservation than the PBS group. ***$p<0.0001$ EPO-Fc vs PBS; ****$p<0.0001$ CEPO-Fc vs PBS and $F=76.74$.

Fig. 5. Tyrosine hydroxylase immunostaining (TH) in the substantia nigra (SN) and VTA (ventral tegmental area).

(A) TH-positive cells on SN and VTA on intact side.

(B) Almost complete absence of TH-positive cells in the PBS group.

(C) Immunoreactivity is preserved in the EPO-Fc group.

(D) Better preservation of TH-positive cells in the CEPO-Fc group.

(E) Graphic. Substantia Nigra. *$p<0.05$ EPO-Fc vs PBS; **$p<0.05$ CEPO-Fc vs EPO-Fc; ***$p<0.0001$ CEPO-Fc vs PBS and $F=14.11$

(F) Graphic. Ventral Tegmental Area. *$p<0.0001$ EPO-Fc vs PBS; **$p<0.01$ CEPO-Fc vs EPO-Fc; ***$p<0.0001$ CEPO-Fc vs PBS and $F=97.01$.

TH-positive cells were significantly better preserved in the EPO-Fc and CEPO-Fc groups than in the PBS group. Moreover, there was more significant preservation in the CEPO-Fc group than in the EPO-Fc group.

Fig. 6. Behavioral tests in the neurorescue experiment

(A) Rota-rod test. Dashed lines represent non-lesioned control group.

The CEPO-Fc group preformed significantly better than the PBS group did at two and three weeks after i.p. administration of CEPO-Fc. *p<0.05 CEPO-Fc vs PBS and F=3.98 at two weeks after i.p. injection; **p<0.01 CEPO-Fc vs PBS and F=9.95 at three weeks after i.p. injection. #p<0.05 CEPO-Fc, EPO-Fc, PBS vs non-lesioned control at two weeks after striatal lesion(F=7.17) and one week after i.p injection of the respective drug (F=12.43). ##p<0.01 PBS vs non-lesioned control at one week after striatal lesion (F=4.05) and at two weeks after i.p. injection of the respective drug (F=5.24). ###p<0.01 EPO-Fc, PBS vs non-lesioned control at three(F=10.16) and four(F=21.57) weeks after i.p. injection of the respective drug.

(B) Cylinder test. In the CEPO-Fc group, contralateral bias decreased just one week after i.p. administration of CEPO-Fc. The CEPO-Fc group also performed better than the EPO-Fc and PBS groups did and were statistically significant at four weeks after i.p. injection of CEPO-Fc when compared to EPO-Fc. *p<0.05 CEPO-Fc vs EPO-Fc with F=5.38.

(C) Amphetamine-induced rotation test. In both the EPO-Fc and the CEPO-Fc group, the number of amphetamine-induced rotations decreased at two weeks after drug administration. *p<0.01 CEPO-Fc, EPO-Fc vs PBS. F=7.27.

Fig. 7. Tyrosine hydroxylase immunostaining (TH) in the striatum.

(A) Strong immunoreactivity found in intact striatum.

(B) Severe destruction of TH-positive fibers in the PBS group.

(C) Some preservation is seen in the EPO-Fc group.

(D) Immunoreactivity is more clearly preserved in the CEPO-Fc group.

(E) Graphic. Preservation of fibers was significantly better in both the EPO-Fc and the CEPO-Fc groups compared with the PBS group. Additionally, the CEPO-Fc group exhibited significantly better preservation than the EPO-Fc group did. *p<0.05 EPO-Fc vs PBS, **p<0.01 CEPO-Fc vs EPO-Fc, ***p<0.0001 CEPO-Fc vs PBS. F=97.01.

Fig. 8. Tyrosine hydroxylase immunostaining (TH) in the substantia nigra (SN) and VTA (ventral tegmental area).

(A)TH-positive cells on SN intact side.

(B) Almost complete absence of TH-positive cells in the PBS group.

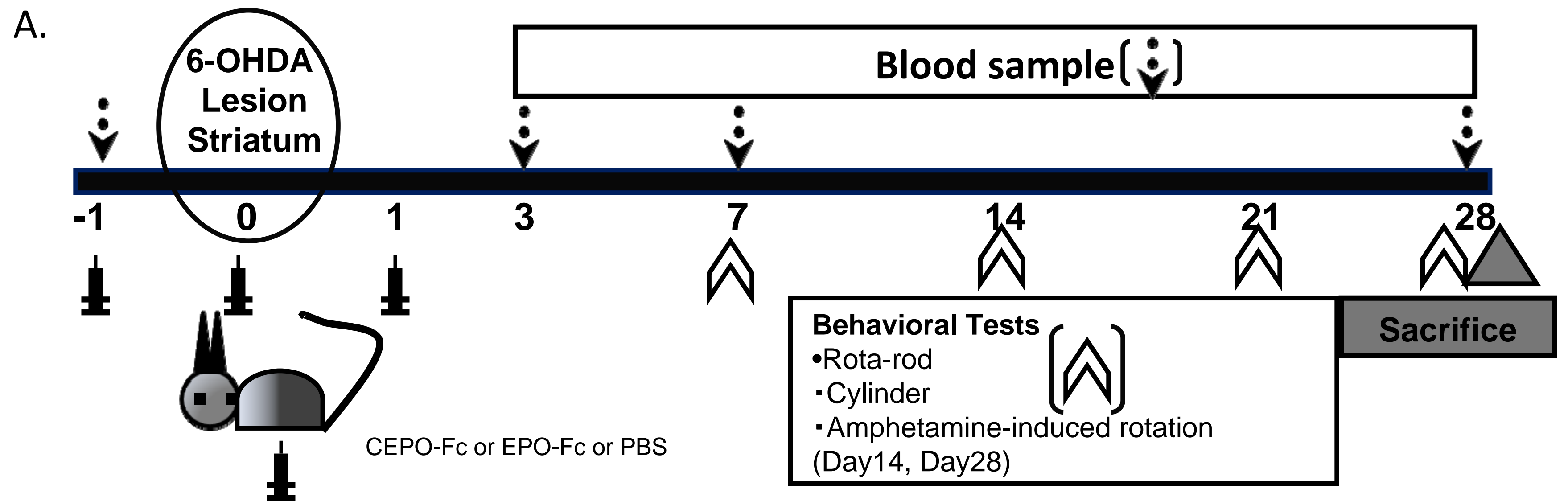
(C) Immunoreactivity is preserved in the EPO-Fc group.

(D) Better preservation of TH-positive cells in the CEPO-Fc group.

(E) Graphic. Substantia Nigra. *p<0.05 EPO-Fc vs PBS; **p<0.05 CEPO-Fc vs PBS and F= 7.84.

(F) Graphic. Ventral tegmental area. *p<0.01 EPO-Fc vs PBS; **p<0.0001 CEPO-Fc vs PBS and F=95.99.

TH-positive cells were significantly preserved in both the CEPO-Fc and EPO-Fc goups. Reversibly, there are severe loss of TH-positivel cells in PBS group.



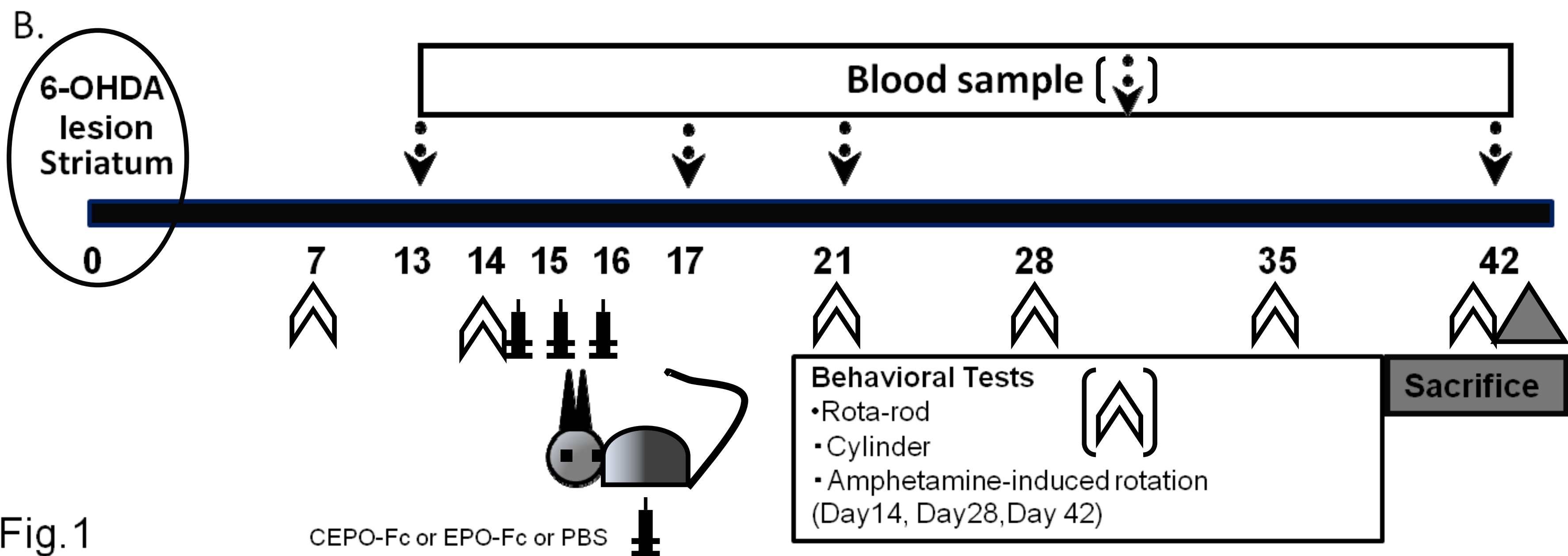


Fig. 1

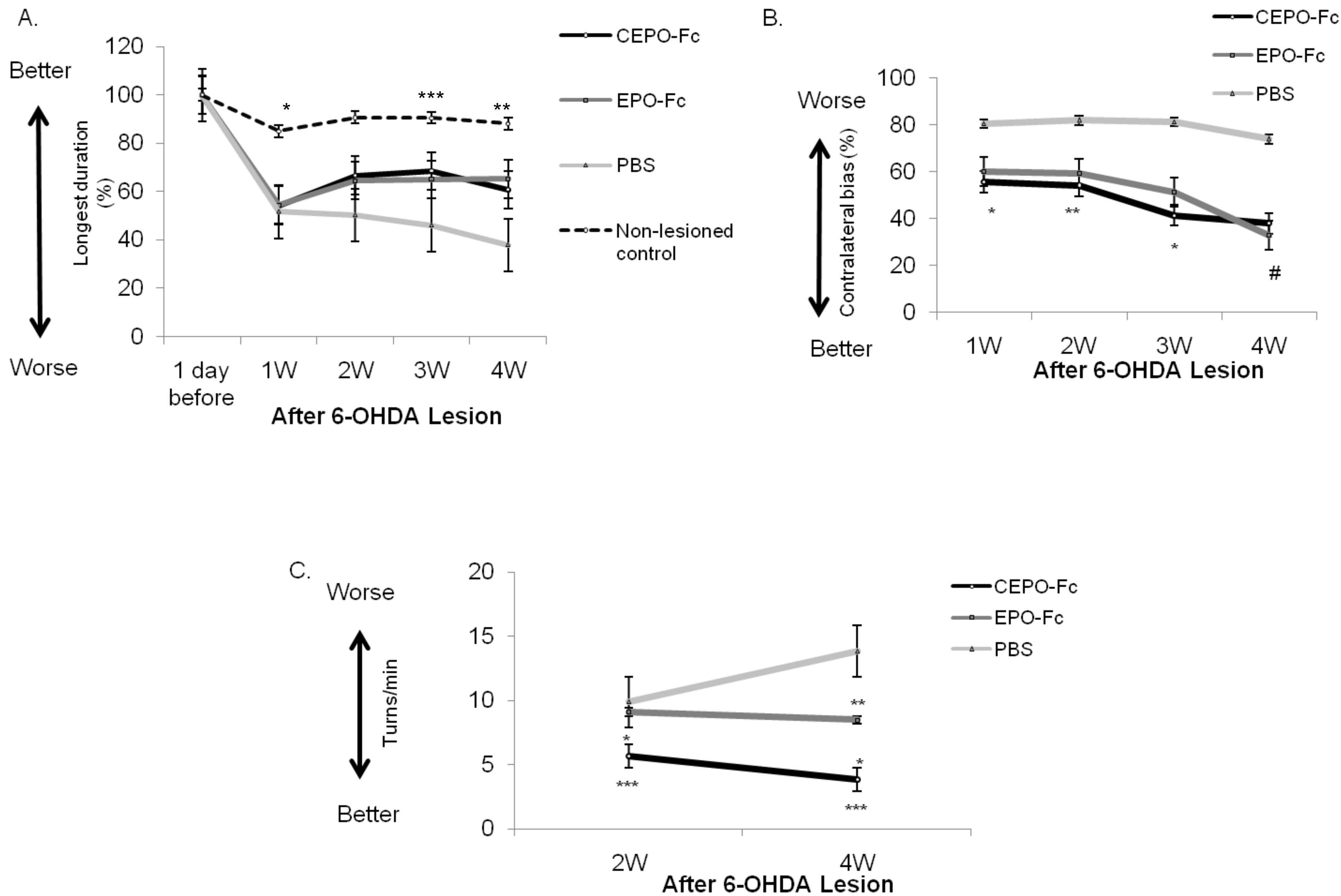


Fig.2

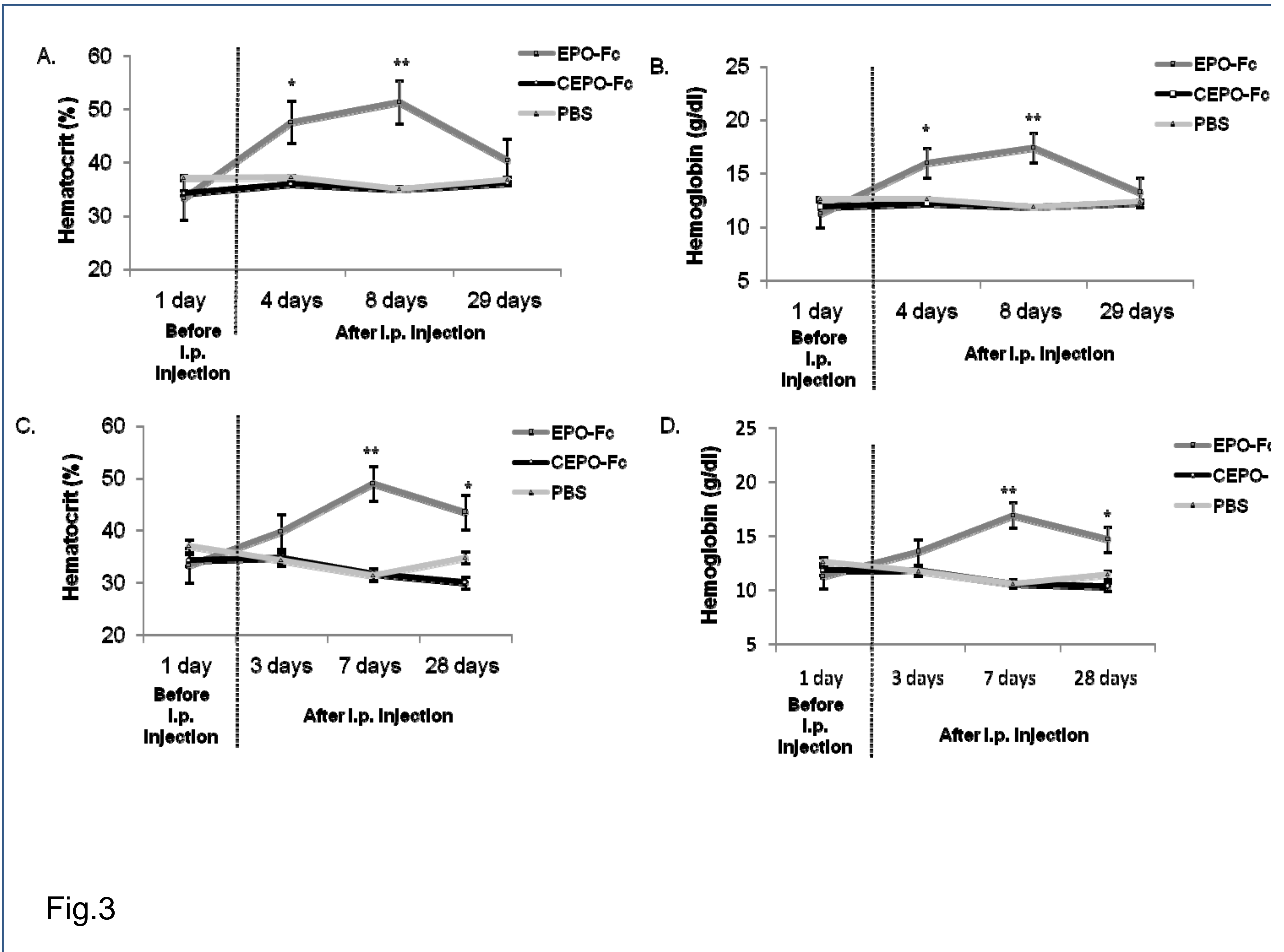
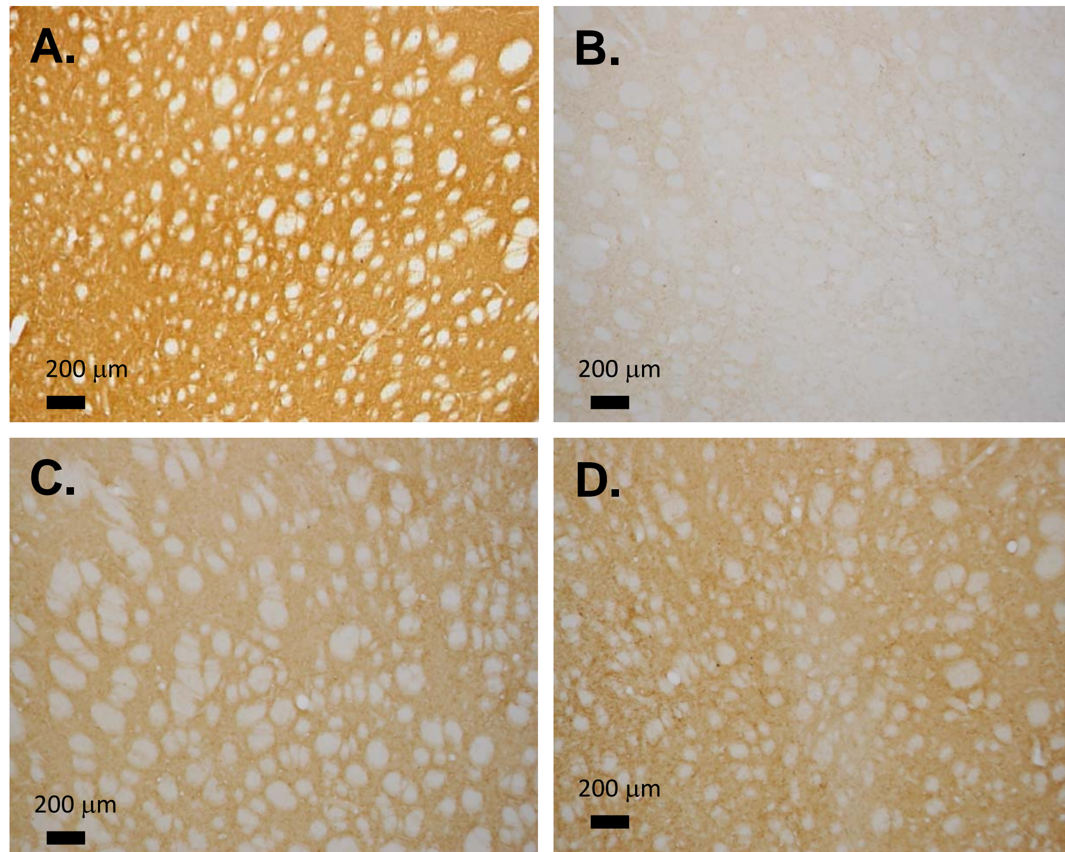


Fig.3



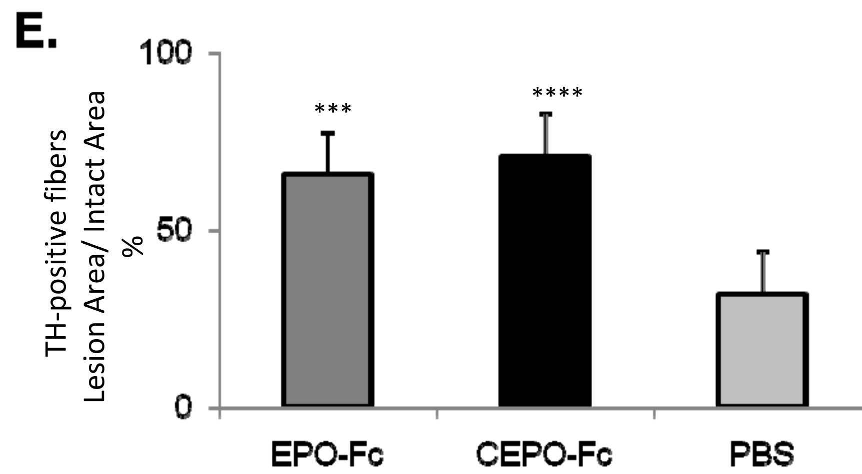
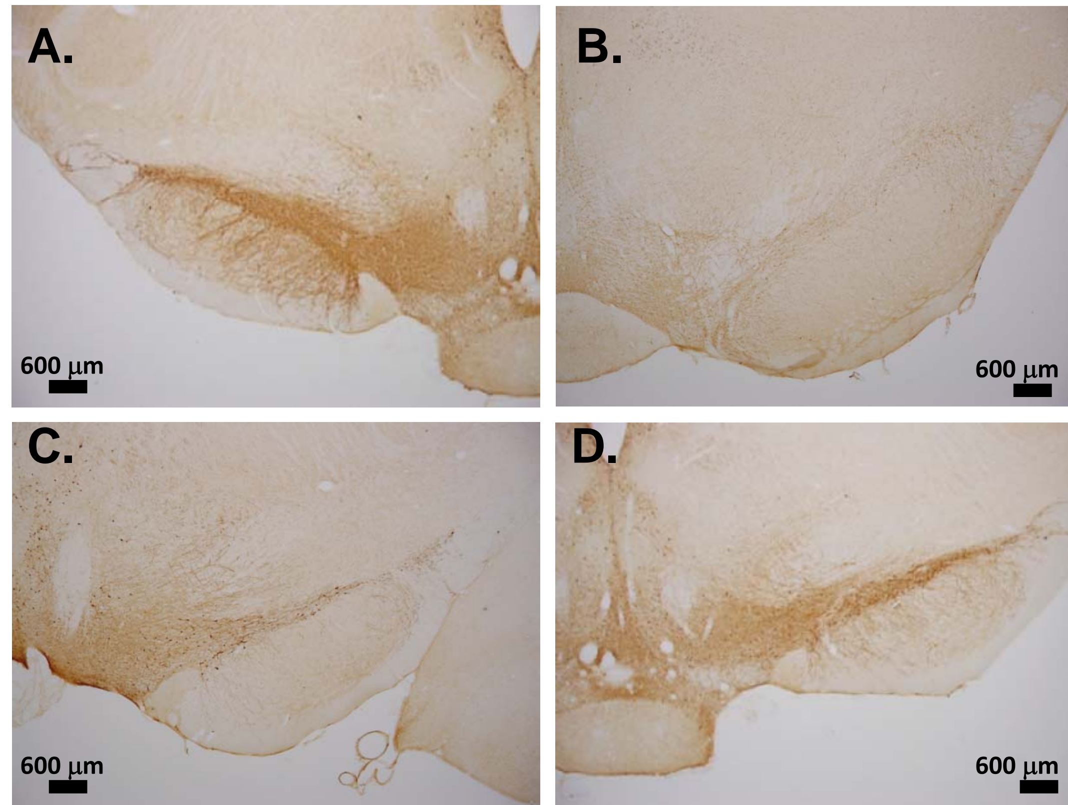


Fig. 4



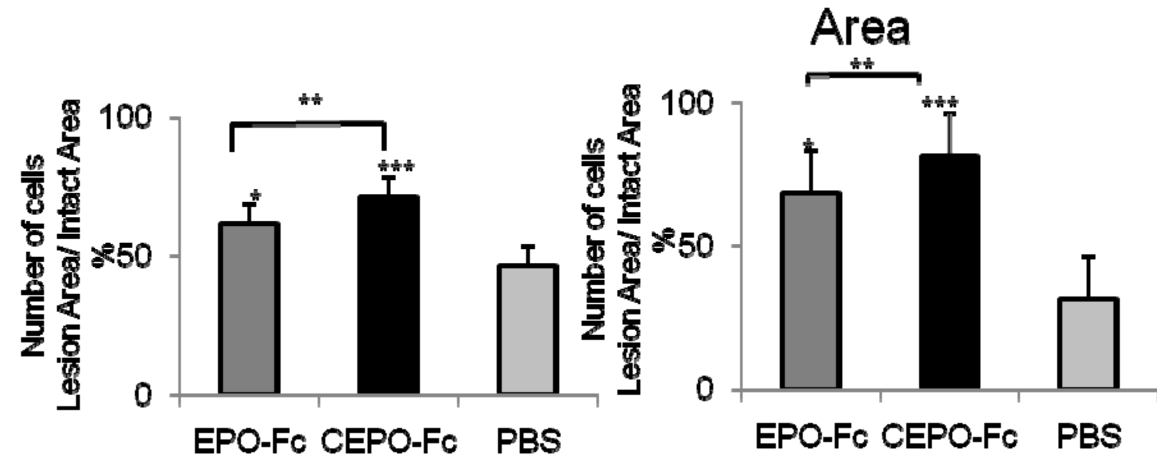
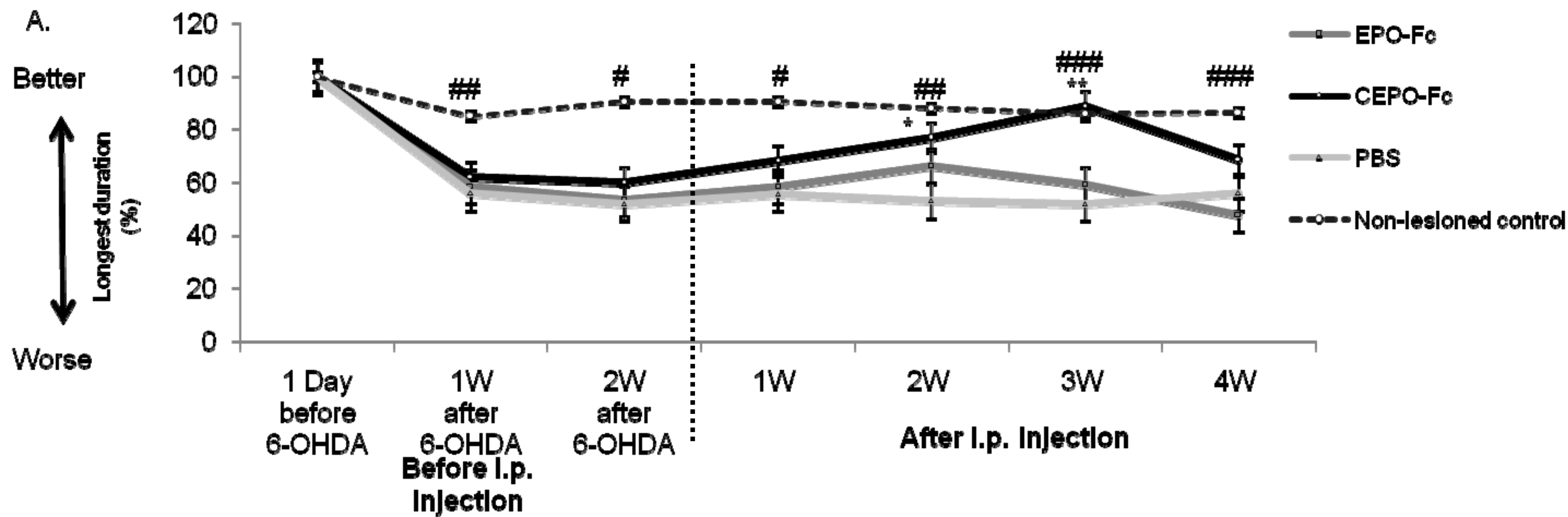
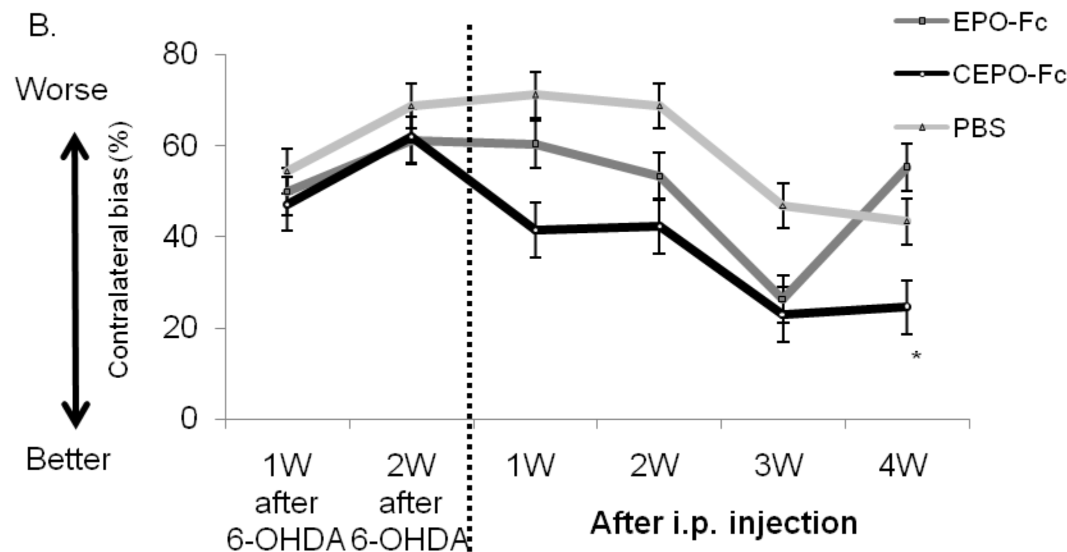


Fig. 5





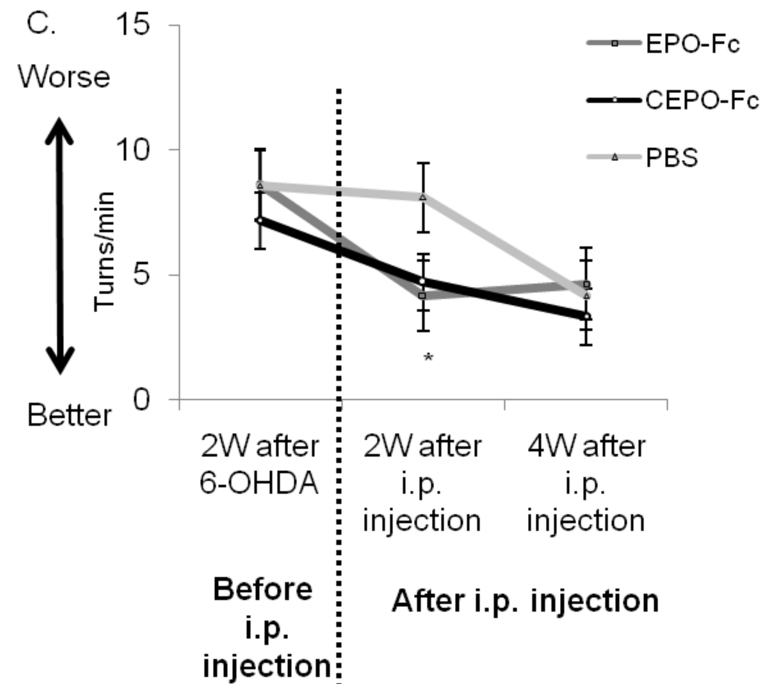
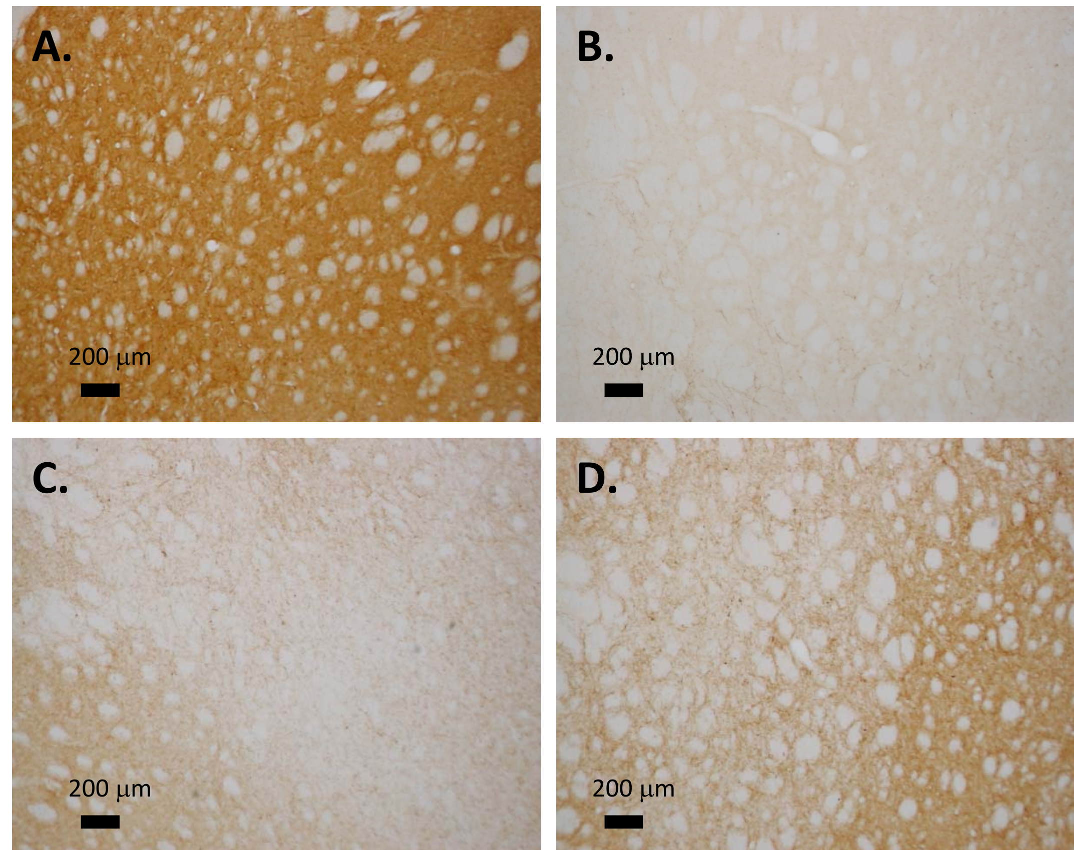


Fig. 6



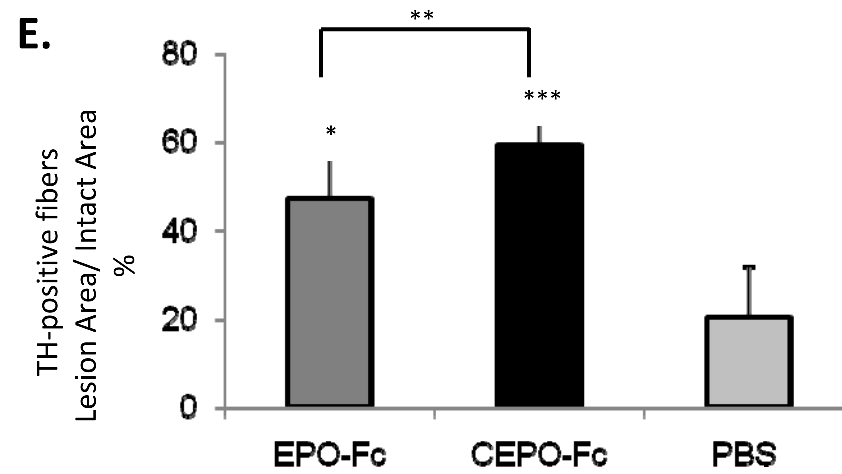
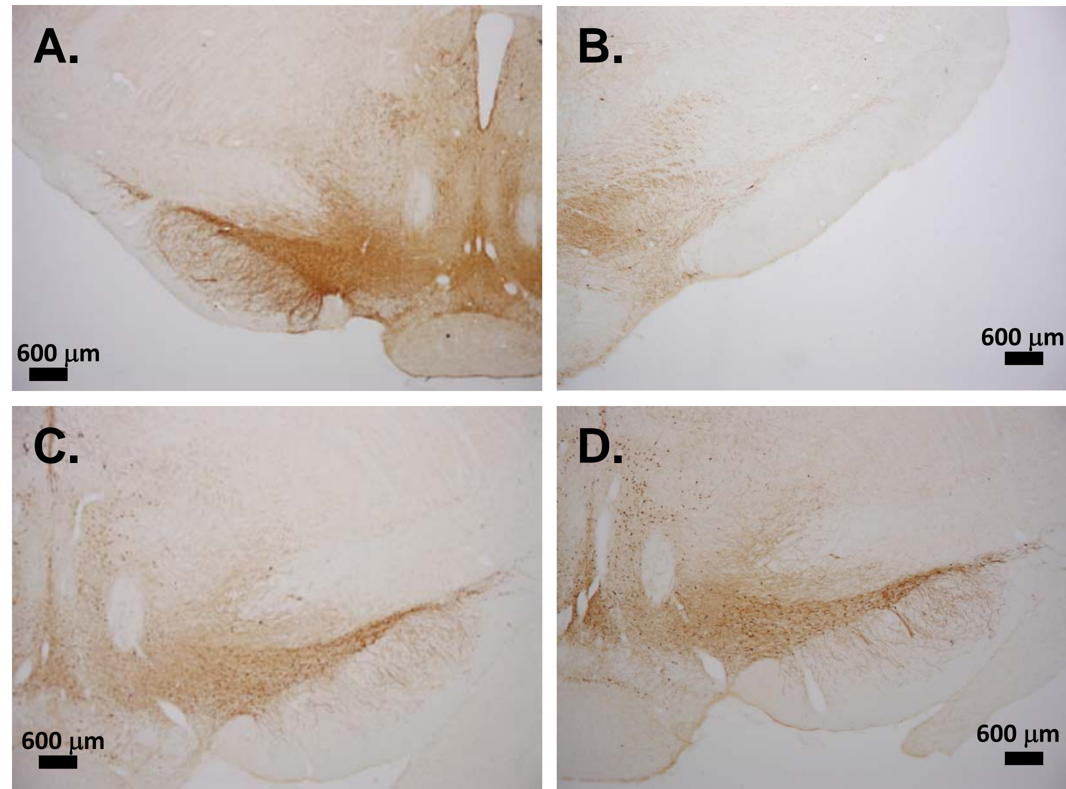


Fig. 7



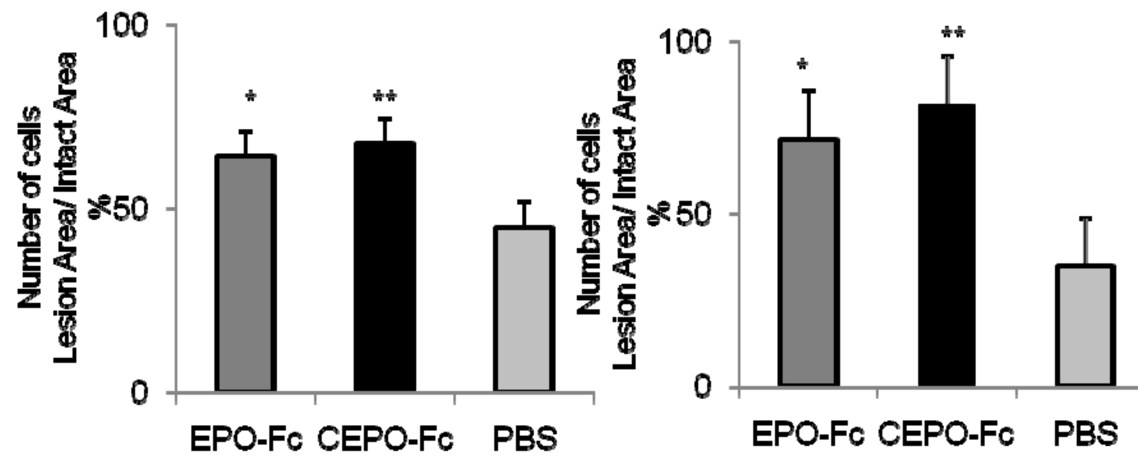


Fig. 8