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Systemic epothilone D improves hindlimb function after spinal cord contusion injury in rats

Beatrice Sandner[1] PhD, Radhika Puttagunta[1] PhD, Melanie Motsch[1], Frank Bradke[2] PhD, Jörg Ruschel[2] PhD, Armin Blesch[1, 3] PhD, Norbert Weidner[1, *] MD

[1]Spinal Cord Injury Center, Heidelberg University Hospital, Schlierbacher Landstrasse 200 a, 69118 Heidelberg, Germany

[2]Axonal Growth and Regeneration, German Center for Neurodegenerative Diseases, Ludwig-Erhard-Allee 2, 53175 Bonn, Germany

[3] Stark Neurosciences Research Institute, Indiana University School of Medicine Department of Neurological Surgery and Goodman Campbell Brain and Spine,
320 West 15th Street, NB 500B, Indianapolis, IN 46202

*Corresponding author:
Norbert Weidner, MD
Spinal Cord Injury Center
Heidelberg University Hospital
Schlierbacher Landstrasse 200a
69118 Heidelberg
Germany
Tel.: +49-6221-5626322
Fax: +49-6221-5626345
Email: norbert.weidner@med.uni-heidelberg.de

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Abstract

Following a spinal cord injury (SCI) a growth aversive environment forms, consisting of a fibroglial scar and inhibitory factors, further restricting the already low intrinsic growth potential of injured adult central nervous system (CNS) neurons. Previous studies have shown that local administration of the microtubule-stabilizing drug paclitaxel or epothilone B (Epo B) reduce fibrotic scar formation and axonal dieback as well as induce axonal growth/sprouting after SCI. Likewise, systemic administration of Epo B promoted functional recovery. In this study, we investigated the effects of epothilone D (Epo D), an analog of Epo B with a possible greater therapeutic index, on fibrotic scarring, axonal sprouting and functional recovery after SCI. Delayed systemic administration of Epo D after a moderate contusion injury (150kDyn) in female Fischer 344 rats resulted in a reduced number of footfalls when crossing a horizontal ladder at 4 and 8 weeks post injury. Hindlimb motor function assessed with the BBB open field locomotor rating scale and Catwalk gait analysis were not significantly altered. Moreover, formation of laminin positive fibrotic scar tissue and 5-HT positive serotonergic fiber length caudal to the lesion site were not altered after treatment with Epo D. These findings recapitulate a functional benefit after systemic administration of a microtubule-stabilizing drug in rat contusion SCI.

Keywords:

epothilone D, microtubule stability, spinal cord injury, functional recovery, moderate contusion

Highlights

- Systemic Epo D injections are beneficial after moderate spinal cord injury

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- Epo D does not enhance serotonergic axon growth below the lesion
- Epo D has no neuroprotective effects

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Introduction

Traumatic spinal cord injury (SCI) results in sensorimotor and autonomic deficits due to the disruption of descending motor and ascending sensory pathways. After the initial insult, secondary degeneration involving various pathophysiological mechanisms leads to the formation of fluid filled cavities, loss of grey and white matter and the formation of a glial and fibrotic scar. Furthermore, retrograde degeneration including dieback of axons, formation of retraction bulbs and disorganization of microtubule exacerbate the primary injury (Erturk et al., 2007).

Stabilizing microtubules inhibits proliferation of cells, which has been exploited in drug development for cancer therapies. The stabilization of microtubules in neurons is vital for axonal transport and function (Brunden et al., 2010; Garcia and Cleveland, 2001; Goedert and Jakes, 2005). Therefore microtubule stabilizing drugs have been investigated in animal models of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and schizophrenia to abate degenerating axons and promote regeneration (Andrieux et al., 2006; Ballatore et al., 2012; Brunden et al., 2010; Cartelli et al., 2013; Daoust et al., 2014; Fournet et al., 2012).

The effect of the microtubule stabilizing anti-cancer drug paclitaxel on microtubule dynamics is concentration-dependent. High concentrations, which are typically used for cancer treatment, over-stabilize microtubules, thus blocking microtubule dynamics, which in turn suppresses axon elongation (Erturk et al., 2007; Sengottuvel and Fischer, 2011; Sengottuvel et al., 2011). In contrast, low concentrations of paclitaxel lead to moderate microtubule stabilization in axonal growth cones, which still allows microtubule polymerization at the plus end (Derry et al., 1995; Derry et al., 1997; Witte et al., 2008), enhances axonal growth in cultured neurons (Hellal et al., 2011; Sengottuvel et al., 2011) and

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preserves microtubule stability and bundling in injured spinal cord axons (Erturk et al., 2007). In rodent SCI and optic nerve injury models, paclitaxel inhibits fibrotic scarring and deposition of inhibitory CSPGs (Chondroitin sulfate proteoglycans) in the lesion site and promotes axonal regeneration as well as functional recovery (Erturk et al., 2007; Hellal et al., 2011; Perez-Espejo et al., 1996; Popovich et al., 2014; Sengottuvel et al., 2011). Moreover, low paclitaxel concentrations have been shown to at least transiently delay macrophage infiltration and astrocyte proliferation around the lesion site after optic nerve injury (Sengottuvel and Fischer, 2011; Sengottuvel et al., 2011).

In contrast to paclitaxel, which cannot cross the blood-brain barrier (BBB), some epothilones are BBB permeable while possessing comparable biological effects and sharing a common binding site (Giannakakou et al., 2000). Thus they are more suitable candidates for treatment of neurological disorders and injuries. Epothilones already have U.S. Food and Drug Administration (FDA) approval for cancer treatment (Brogdon et al., 2014; Cheng et al., 2008; Goodin et al., 2004).

Both analogs, epothilone B (Epo B) and epothilone (Epo D), act in a dose dependent manner on microtubule stabilization similar to paclitaxel (Altmann et al., 2000a; Altmann et al., 2000b; Ballatore et al., 2012; Cheng et al., 2008; Goodin et al., 2004; Muhlradt and Sasse, 1997). A recent study using an *in vitro* axotomy model showed that the exposure of neurons to Epo D increased axonal sprouting without affecting their viability or metabolic function (Brizuela et al., 2015). The application of Epo D in animal models of tauopathies such as Alzheimer's disease improved axonal transport, decreased tau neuropathology, reduced neuronal loss and axonal dystrophy paralleled by amelioration of cognitive deficits (Barten et al., 2012; Brill et al., 2016; Brunden et al., 2010; Lou et al., 2014; Zhang et al., 2012). The systemic administration of Epo B in a moderate rat spinal cord contusion injury reduced

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fibrotic scar formation by interfering with fibroblast migration to the lesion site, promoted axonal growth by stabilizing microtubules and reduced the number of footfalls when crossing a regular spaced horizontal ladder (Ruschel et al., 2015).

Given that clinical trials Epo D reported a better safety profile than Epo B including a greater therapeutic index (Chou et al., 1998; Fumoleau et al., 2007; Goodin et al., 2004) we investigated potential effects of Epo D in a rat contusion SCI model. We observed that systemic administration of Epo D results in improved skilled hindlimb function after a moderate spinal cord contusion injury.

Methods

Animal subjects

Adult female Fischer 344 rats (Charles River Deutschland GmbH, Sulzfeld, Germany, Envigo, Cambridgeshire, UK; Janvier Labs, Saint-Berthevin Cedex, France) weighing 160-180g were used for all *in vivo* experiments. Experiments were carried out in accordance with the European Union Directive (2010/63/EU) and institutional guidelines. Animals had *ad libitum* access to food and water throughout the study.

Surgical procedures and treatment

For all surgical procedures, animals were anesthetized using a cocktail of ketamine (62.5 mg/kg; Medistar, Ascheberg Germany), xylazine (3.175 mg/kg; Bayer, Leverkusen, Germany), and acepromazine (0.625 mg/kg; Sanofi-Ceva, Düsseldorf, Germany) in 0.9% sterile saline solution. A total of 34 rats received a spinal cord contusion injury at midthoracic level Th9 (representing spinal level Th11) using the Infinite Horizon (IH) Impactor SCI device (Precision Systems & Instrumentation, Lexington, KY, USA) with an impact force of 150 kilodynes (kDyn) as previously described (Scheff et al., 2003). Postoperatively, animals

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were kept warm, and buprenorphine (0.03 mg/kg; Reckitt Benckiser, Mannheim, Germany, for 2 days postoperatively twice a day) and ampicillin (167 mg/kg; Ratiopharm, Ulm, Germany, twice a day) was given subcutaneously as long as manual bladder evacuation was necessary. Manual bladder evacuation was performed twice a day until reflex bladder function returned, usually 7-10 days post-injury. Animals received either intraperitoneal (i.p.) injections of Epo D (*Abcam, Cambridge, UK,* cat.no.: ab143616) (1.5 mg/kg body weight) dissolved in DMSO (3 mg/ml) and diluted 1:1 with prewarmed saline immediately prior to injections (Epo D group, n= 18) or vehicle (1:1 mixture of DMSO and saline, control group**,** n=16) on day 1 and 15 post-injury (at an equivalent time of the day as the SCI surgery was performed on the previous day) by a blinded, unbiased experimenter, who was not involved in any of the behavioral testing and histological analysis. The Epo D concentration used in this study was based on data for Epo B and Epo D in clinical cancer studies and recent dosing regimes in rodents (Brunden et al., 2010). Epo B is administered in a dose range between 2.5 and 50 mg/m^2, whereas Epo D is administered in a dose range between 50 and 100 mg/m^2 (Beer et al., 2007; Lee and Kelly, 2009). Based on these data doubling of the Epo B dose used in the previous study (0.75 mg/kg body weight) with two administration time points (day 1 and 15) (Ruschel et al., 2015) seemed most promising.

One animal of each cohort was excluded due to inadequate force impact curves upon spinal cord contusion. Based on the variability in spinal cord displacement and the importance of displacement on functional parameters, animals were divided into 2 groups dependent on the injury-induced spinal displacement prior to Epo D administration and any functional testing. We chose a cut off at a displacement value of 1000 µm allowing for equal distribution of animals into 2 cohorts (low displacement; **low dis** <1000 µm and high displacement; **high dis** >1000 µm). These animals were then assigned to receive either vehicle or Epo D treatment

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(**control low dis**; n=8; **Epo D low dis**: n=8; **control high dis**: n=7; **Epo D high dis**: n=9) (**Fig. 1**). Animals were sacrificed 8 weeks post injury.

Behavioral assessment

Hindlimb motor function was evaluated according to the Basso, Beattie, Bresnahan (BBB) open field locomotor rating scale (Basso et al., 1995). BBB scores range from 0 to 21 (0 = no observable hindlimb movement, 21 = normal locomotion). All animals had a score of 0 at day 1 post-injury (BBB assessment was always performed in the morning one day prior to the Epo D or vehicle injection on day 0 and day 14). From this time point, animals were scored once a week during the first 4 weeks and thereafter every two weeks by two independent blinded observers. A 7-point BBB subscore was additionally included for the 4 and 8 week time points, to evaluate higher motor functions (toe clearance, paw position, trunk stability and tail point (Lankhorst, 1999). However, only a selected number of the animals were evaluated for the parameters required for the BBB subscore (toe clearance, paw position, trunk stability and tail position): at 4 weeks: **control low dis**: n=6; **Epo D low dis**: n=7; **control high dis** n=2; **Epo D high dis**: n=9; at 8 weeks: **control low dis**: n=8; **Epo D low dis**: n=8; **control high dis** n=5; **Epo D high dis**: n=8.

To examine more subtle differences in locomotor function, the horizontal ladder walking test was performed. Animals were first trained to cross a 100-cm-long horizontal ladder with unevenly spaced rungs (1-3 cm) toward the home cage one week prior to the first testing. The test was carried out in the afternoon at 4 and 8 weeks post-injury (animals had a minimum of 3 h between BBB testing and the horizontal ladder test to keep fatigue to a minimum). Additionally, rats were tested on a horizontal ladder with evenly spaced rungs (1 cm spacing) 8 weeks post injury. The animals were recorded using a digital video camera, and performance was subsequently analyzed in slow motion by a blinded observer. The total

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number of steps and the number of footfalls below the plane of the ladder (failure to place the hindlimbs on the rungs) were quantified in five uninterrupted crossings. The number of missteps per trial was counted, given as a percentile of the total number of steps, and averaged for five trials, by a blinded observer. Naive animals show typically < 10% missteps on the horizontal ladder with unevenly spaced rungs versus < 1% with evenly spaced rungs.

For gait analysis, rats were tested 8 weeks after injury on an automated gait analysis system (CatWalk; Noldus, Oberreifenberg, Germany). Recorded footprints of 5 compliant runs were processed automatically using device specific software quantifying step distance of hindlimbs (stride length) and interpaw coordination (regularity index: the observed difference from normal step sequence patterns; base of support (BOS): average width between either the front paws or the hind paws).

Tissue processing and immunohistochemistry

Animals were transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer at 8 weeks post injury. The spinal cords were dissected, post-fixed overnight and cyroprotected in 30% sucrose. An 8 mm long block of the thoracic spinal cord including the lesion site and an adjacent 6 mm long block of the thoracolumbar spinal cord were cut into 30 μm thick coronal sections and directly mounted onto superfrost slides and processed for immunohistochemistry. Sections were collected in 14 series resulting in equally spaced sections with a distance of 420 μm between adjacent sections on each slide. Three animals (one from the **Epo D low dis**, one from the **control low dis** and one from the **control high dis** cohort) had to be excluded from all histological analysis, due to technical problems during cryo-sectioning.

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Eriochrome cyanine stain for spared myelin

Eriochrome cyanine (EC) staining was used to identify myelinated areas in the thoracic spinal cord (Kigerl et al., 2007). Briefly, spinal cord sections previously stored at −80°C were air-dried for 1–2 h at 37 °C in a dry incubator. After incubation with acetone for 5 min, the slides were air-dried for 20 min and then stained in EC solution (0.2% eriochrome cyanine R; Merck Millipore), 0.5% H_2SO_4, 10% iron alum in distilled water for 30 min, followed by a 5-min rinse in tap water. Stained sections were then differentiated for 5-20 min in 5% iron alum, which was followed by a 10-min rinse in tap water. Afterwards, sections were placed in borax-ferricyanide solution (1% borax, 1.25% potassium ferricyanide in distilled water) for 10 min. After another 5 min rinse in tap water slides were dehydrated through a graded ethanol series and coverslipped using Neomount (Merck Millipore, Darmstadt, Germany). The total cross-sectional area of the spinal cord and the inner border of spared white matter were measured by an observer blinded to group identity on digitized images of the cord using an open image analysis program (Fiji2) (Schindelin et al., 2012). The digital images of EC-stained sections were obtained using an Olympus BX53 microscope equipped with a XC camera. White matter was defined as the area that was stained for EC and considered spared if the myelin stain was dense, contiguous, and primarily normal in appearance with absence of cavities and minimal gliosis. The lesion area was identified by the loss of EC staining as well as severe tissue disruption. The injury epicenter was defined visually as the spinal cord section with the smallest visible rim of spared myelin. The spared white matter area (WMA) was measured in every 14^{th} section with a distance of 420 μ m between adjacent sections. To calculate the volume of spared white matter, the area of WMA of all sections was added before multiplying it with the series (14) and the thickness of the cut sections (30 µm).

Immunohistochemistry

Double/triple immunofluorescence labeling techniques were performed with direct-mounted

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sections to assess fibrotic scaring and total length of 5-HT positive fibers *in vivo*. Sections were washed in TBS, blocked in TBS/5% donkey serum (GE Healthcare)/0.25% Triton X-100 for 1 h and incubated with primary antibodies in TBS/1% donkey serum/0.25% Triton X-100 overnight at 4 °C. The following day, sections were rinsed in TBS/1% donkey serum and incubated with Alexa Fluor 594 or Alexa Fluor 488 donkey secondary antibodies for 2.5 h (1:300; Life Technologies; cat. no.: A21207 and A21202 respectively). Sections were cover-slipped with Fluoromount G (Southern Biotech, Birmingham, AL, USA). The following primary antibodies were used: mouse anti-GFAP for astroglia (1:1000; Merck Millipore; cat. no.: MAB360), rabbit anti-laminin (1:800; Sigma-Aldrich, Taufkirchen, Germany; cat. no.: L9393), rabbit-anti 5-HT (serotonin, 1:2000; Immunostar, Hudson, USA; cat. no.: 20080), mouse anti-ßIII-tubulin (1:1000; Promega, Mannheim, Germany; cat. no.: G7121), rabbit anti-fibronectin (1:500, Abcam; Cambridge, UK; cat. no.: ab2413) and DAPI as a nuclear counterstain.

Measurement of fibrotic scar area

Fibrotic scarring was quantified by measuring the laminin immunolabeled area in a complete series (interval between analyzed sections 420 µm). The laminin positive areas of all sections from the series were summed up. Based on the thickness of sections (30 µm) and the number of sections (14), the laminin positive volume was calculated (summed area x 30 µm x 14 = volume). The summed area was calculated either for the whole distance of the 8 mm block (4200 µm rostral to 4200 µm caudal), and shorter distances around the lesion center (2520 µm, 840 µm and 420 µm rostral to caudal).

Measurement of 5-HT positive fibers

The total length of 5-HT positive fibers in the ventral horn was measured caudal to the injury site and the mean of the right and left ventral horn was taken. Three sections with a 420 µm

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interval between sampled sections were taken from a caudal spinal cord block 2-4 mm caudal to the lesion epicenter. In addition, a spinal cord block located 4 to 10 mm caudal to the lesion epicenter corresponding to spinal level T12-L1, identical to the region examined previously, (Ruschel et al., 2015) was sectioned and 7-8 sections with 840 µm interval between sampled sections were analyzed. Images of the entire right and left ventral horn were taken using a 20x magnification and a zoom factor of 1.5x with a confocal microscope (FluoView FV1000, Olympus) with an optical thickness of 15 µm (7.5 µm above and 7.5 µm below the middle of the section). The images were saved as merged z-stacks. Images were further processed in Fiji2 by applying the Robust Automatic Threshold Selection (RATS) before skeletonizing the images. In these images the total skeleton length was automatically measured.

Statistics

All data are presented as mean ± standard error of the mean (SEM). Differences between groups were investigated using a parametric unpaired *t*-test (Student's t-test). A two-way ANOVA was performed for analyzing data from BBB and irregular horizontal ladder testing, followed by post hoc Bonferroni's multiple comparisons test. All statistical analyses were performed using PRISM6 software (GraphPad, San Diego, CA, USA).

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Results

Differential tissue displacement generates distinct injury severity cohorts

Following a 150 kDyn T9 contusion injury in adult female Fischer 344 rats, displacement curves were examined for consistency. The Infinite Horizon (IH) impactor is controlled by force usually resulting in consistent displacement of a given directed force, although some variation can occur. Because both force and displacement lead to anatomical

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changes and as a consequence affect functional outcomes after contusive SCI (Ghasemlou et al., 2005), it is important to examine tissue displacement in addition to the actual force applied (Scheff et al., 2003; Zhang et al., 2008). In the present study, a 150 kDyn moderate contusion injury with the IH impactor generated two distinct displacement cohorts, one with less than 1000 µm displacement (**low dis**) and one with more than 1000 µm displacement **(high dis)**. Prior to the first Epo D injection and prior to functional testing of animals, subjects were matched based on the displacement and divided into animals receiving Epo D or vehicle (control) treatment (**Fig. 2 A**). The actual force applied was not found to be significantly different between the cohorts (**Fig. 2 B**).

Post-hoc analysis revealed significant differences in functional impairment between the control low dis and control high dis cohorts. Scores for hindlimb motor function assessed by BBB were always higher in the control low dis cohort, but were only significantly different at 3 week post injury (**Fig. 2 C**), However, horizontal ladder testing as well as the histological parameter for spared white matter were significantly worse in the high dis control cohort compared to the low dis control cohort (**Fig. 2 D-E**), confirming that an average difference in displacement of 177 µm indeed results in two distinct injury severities.

Systemic Epo D administration promotes improvement in locomotor function

Systemic i.p. administration of 1.5 mg/kg of Epo D at 1 and 15 days post-injury did not yield any obvious adverse effects, which is in line with previous Epo B and Epo D studies (Barten et al., 2012; Brunden et al., 2010; Ruschel et al., 2015; Zhang et al., 2012). After a transient injury-induced weight loss, animal weight increased over time in the treatment as well as the control groups irrespective of displacement severity (**Supplementary Fig. 1**).

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Locomotor function was assessed by rating over-ground locomotion in an open field (BBB) or analysis of sensorimotor coordination by quantifying the number of footfalls across a regularly and irregularly spaced horizontal ladder. Moreover, instrumented over-ground gait analysis was performed with the Noldus Catwalk system. BBB locomotor scoring revealed complete hindlimb paralysis at day 1 post-injury. Thereafter, significant spontaneous improvement of hindlimb motor function was observed over time in all cohorts. Epo D treatment did not result in a superior recovery in BBB scores in either of the displacement cohorts although a slight trend towards better recovery was evident in Epo D cohort with the high displacement (**Fig. 3 A, B**).

To evaluate recovery of higher motor functions such as toe clearance, paw position, trunk stability and tail position we determined the BBB subscore for a subset of animals. However, the BBB subscore showed no difference between groups similar to what we observed with the overall BBB score **(BBB subscore 28 days**: **control low dis:** 3.17 ± 0.95; **Epo D low dis:** 4.5 ± 0.89, p = 0.18; **control high dis:** 2.5 ± 0.50; **Epo D high dis:** 0.31 ± 0.06 mm^3, p = 0.45; **BBB subscore 54 days control low dis:** 4.25 ± 0.41; **Epo D low dis:** 4.81 ± 0.60, p = 0.45; **control high dis:** 2.8 ± 0.73; **Epo D high dis:** 3.4± 0.35, p = 0.39).

Irregular ladder crossing, reflecting a more complex walking task, did reveal significant differences between control and Epo D treated animals within the high dis cohort at 4 and 8 weeks post-injury (**Fig. 4 C, D**). Epo D treated animals made fewer missteps in comparison to control animals on the irregular ladder (**control high dis:** 4 weeks: 64.74 ± 4.98%; 8 weeks: 51.32 ± 3.36%; **Epo D high dis:** 4 weeks: 48.59 ± 5.88%, 8 weeks: 32.34 ± 3.78%, $*p < 0.05$, **Fig. 4 C**). In the low dis cohort, significant changes were not detected at either time point (**control low dis:** 4 weeks: 32.72 ± 4.86%; 8 weeks: 24.49 ± 3.31%; **Epo D low dis:** 4 weeks: 31.76 ± 6.5%; 8 weeks: 20.09 ± 4.29%, **Fig.4 A**).

Injured animals made very few missteps on the less challenging horizontal ladder test

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with regularly spaced rungs at 8 weeks post injury. In the low dis cohort, less than 1% of the steps across the ladder were recorded as footfalls in both the Epo D and control cohorts (**Fig. 4 B**). In the high dis cohort, the Epo D treated animals displayed 68% fewer footfalls compared to the control cohort (**control high dis** 8.8 ± 2.73%; **Epo D high dis** 2.79 ± 0.7, $*p < 0.05$, **Fig. 4 D**).

In the gait analysis using the Catwalk system, which was performed at the end of the study at 8 weeks post-injury, no differences in commonly applied gait parameters such as the *regularity index* (the observed difference from normal step sequence patterns), *stride length* and *base of support* were observed between the Epo D or controls in either displacement cohort (**Supplementary Fig. 2**).

Systemic Epo D does not modify structural changes occurring after contusion SCI

To better understand which changes led to the functional benefits observed with Epo D, the pathological hallmarks of tissue sparing, fibrotic scarring, lesion extent and raphespinal innervation, which were previously positively modulated by paclitaxel and Epo B (Hellal et al., 2011; Ruschel et al., 2015), were examined.

As a measure of neuroprotection, white matter sparing was analyzed in eriochrome cyanine (EC)-stained coronal sections spanning the lesion site over a distance of 4 mm rostral and 4 mm caudal to the epicenter. White matter sparing at the epicenter was not significantly different between Epo D treated animals compared to control animals in either of the displacement cohorts (**Fig. 5 A, B, E, F, G, J**). White matter sparing at different rostro-caudal distances in respect to the epicenter and the volume of spared white matter over the entire length of the lesion also remained unchanged in Epo D versus control cohorts, irrespectively of the displacement cohort (**Fig. 5 C, D, H, I**). Taken together, Epo D administration did not

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improve white matter sparing although there seems to be a slight trend of higher white matter sparing at the epicenter in EpoD treated animals in the high displacement cohort.

To investigate if Epo D modulates the fibrotic scar, the laminin positive area at the lesion site was analyzed. Again, a displacement effect was observed, since the laminin immunoreactive area/volume in the low dis cohort was reduced compared to the high dis cohort (**Fig. 6 A-H**). However, Epo D treatment did not change the laminin immunolabeled area in the epicenter (**control low dis:** 0,262 ± 0,06 mm^2; **Epo D low dis:** 0,205 ± 0,03 mm^2, p = 0,416; **control high dis:** 0,322 ± 0,07 mm^2; **Epo D high dis:** 0,234 ± 0,05 mm^2, p = 0,305) or the overall laminin positive volume analyzed over a distance of 4 mm rostral and 4 mm caudal to the epicenter (**Fig. 6 C, D, G, H**). We further performed an exploratory cut off, where we calculated laminin immunoreactivity within fractions of the total volume, namely in a volume confined to the lesion core (420 µm rostral and caudal to the epicenter). From there, we expanded to two larger volumes covering 840 µm and 2520 µm rostral and caudal to the epicenter, respectively. None of these fractioned volumes yielded significant differences between control and Epo D treated animals (**420 µm cut off**: **control low dis:** 0.291 ± 0.08 mm^3; **Epo D low dis:** 0.264 ± 0.04 mm^3, p = 0.75; **control high dis:** 0.422 ± 0.07 mm^3; **Epo D high dis:** 0.31 ± 0.06 mm^3, p = 0.246; **840 µm cut off**: **control low dis:** 0.465 ± 0.09 mm^3; **Epo D low dis:** 0.408 ± 0.06 mm^3, p = 0.62; **control high dis:** 0.406 ± 0.11 mm^3; **Epo D high dis:** 0.50 ± 0.09 mm^3, p = 0.19; **2520 µm cut off**: **control low dis:** 0.66 ± 0.13 mm^3; **Epo D low dis:** 0.534 ± 0.032 mm^3, p = 0.45; **control high dis:** 1.006 ± 0.21 mm^3; **Epo D high dis:** 0.748 ± 0.14 mm^3, p = 0.31). Additional characterization of the fibrous scar with fibronection immunohistochemistry yielded specific and circumscribed labeling of the meninges but very diffuse staining in the spinal parenchyma, which did not differ qualitatively in between groups (**Supplementary Fig. 3)**. Moreover, astroglial morphology

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around the lesion center was not overtly altered after EpoD treatment (**Supplementary Fig. 4**).

To examine potential effects on axonal sprouting or regeneration, serotoninergic (5-HT) axon length was quantified caudal to the lesion epicenter, where a respective treatment effect would be most likely detectable. However, Epo D did not alter the total length of 5-HT axons compared to control animals directly caudal to the lesion in either displacement cohort (**Fig. 7 A-J**). It should also be noted that 5-HT fiber length was not significantly different in the control animals of the high dis cohort compared to the low dis cohort, unlike all other parameters measured (**Fig. 7 E**, **J**). To find out whether 5-HT sprouting in more caudal gray matter areas may have accounted for the observed functional improvement after Epo D treatment in the high dis cohort, 5-HT immunolabeled fibers were analyzed in the ventral gray matter of a more caudal spinal cord block corresponding to spinal level L1 containing rostral motoneurons projecting to hindlimb muscles. Again, the 5-HT fiber length was not found to be significantly different between Epo D treated and control animals (**control high dis:** 0.114 ± 0.02 mm; **Epo D high dis:** 0.09 ± 0.02 mm, p = 0.5077).

Discussion

In addition to the disruption of ascending and descending pathways, secondary injury mechanisms involving Wallerian degeneration and the formation of a fibroglial scar contribute to the functional deficits after SCI. Previous studies indicate that the administration of the microtubule-stabilizing drugs (paclitaxel and Epo B) result in reduced fibrotic scar formation, less axonal dieback and improvements in functional recovery after moderate SCI (Hellal et al., 2011; Ruschel et al., 2015).

The present study demonstrates a clear improvement in skilled hindlimb locomotor function after systemic administration of Epo D in a moderate rat contusion SCI model. This

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study aimed to examine the robustness of the effects of Epo D treatment, an Epo B analog with a better safety profile, following a spinal cord contusion injury. For comparison of our study with previous work, we utilized similar structural and functional readouts as previous reports (Ruschel et al., 2015). We retained the study length (8 weeks), timing of drug delivery (i.p. injection at 1 and 15 days post injury) and the injury model (150 kDyn contusion at the Th9 vertebral level). Due to variability in spinal cord displacement, our study was also able to examine the effects of Epo D on two distinct lesion severities. In addition to the structural outcomes (tissue preservation, fibrous scar formation and serotoninergic (5-HT) axon density) and the functional assays (regular ladder testing and Catwalk analysis) examined previously, we also evaluated BBB locomotor scores and a more complex irregular ladder task.

To establish the concentration of Epo D to be administered in the current study (1.5 mg/kg), previous work in a mouse model of tauopathy served as a starting point. Weekly i.p. injections of 3 mg/kg BW of Epo D for 3 months (Brunden et al., 2010), a dose equivalent to about 10 % of what was used in phase II clinical trials (Beer et al., 2007), were found to be more effective than a lower dose of 1 mg/kg BW. Furthermore, Epo D was found to cross the blood brain barrier and remains detectable in the brain 10 days after a single i.p. injection, whereas it is cleared rapidly from plasma (Brunden et al., 2011; Brunden et al., 2010). Even treatment with a higher dose or long-term application of Epo D for 15 months did not result in obvious side effects (Andrieux et al., 2006; Barten et al., 2012; Brunden et al., 2010). With consideration for allometric scaling, 3 mg/kg BW in the mouse would be equivalent to 1.5 mg/kg BW in the rat (Nair and Jacob, 2016). Taken together, these findings suggest that the administered Epo D dose is within an efficient range without incurring secondary effects. In fact, obvious adverse effects such as weight loss were not observed in the current study.

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Analysis of spinal cord displacement records for each contused animal revealed considerable variability despite the equal force that was applied. Consistent with previous findings, rats with a similar impact force but a lower displacement (<1000 µm) showed better hindlimb sensorimotor function (BBB score) (Ghasemlou et al., 2005). Because the extent of displacement greatly affects injury severity (Ghasemlou et al., 2005), animals were divided into two cohorts dependent on injury-induced spinal displacement with a cut off at 1000 µm. A careful review of surgical records for all animals did not reveal any systematic errors, which could explain the variability in spinal cord displacement. All surgeries were performed by the same experimenter and anesthesia was kept constant for all animals. There was no bias in respect to time or day when contusions were performed. Animals with a low dis performed significantly better on the horizontal ladder and had significantly more spared white matter tissue compared to animals with a high dis. These results suggest that in an impactor-induced spinal cord contusion model, both force and displacement are important to determine the lesion severity and thus the functional outcome, further justifying our a priori separation into two distinct cohorts.

Even though all animals revealed a spontaneous improvement of hindlimb motor function over time in the BBB score, no differences could be detected between Epo D treated and control animals, irrespective of the displacement severity. More subtle locomotor deficits, which are not detectable by the BBB score can be evaluated with the horizontal ladder test, in particular with unevenly spaced rungs. Indeed, Epo D treatment elicited superior performance (37% less missteps) in irregular spaced ladder crossing in the high dis cohort at 8 weeks post injury. As opposed to previous work investigating Epo B effects after SCI (Ruschel et al., 2015), this functional benefit was not paralleled by increased serotonergic axon sprouting (raphespinal tract) caudal to the lesion. In the study by Ruschel et al. animals had to cross an evenly spaced ladder enabling them to utilize a given gait possibly representing a less

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challenging locomotor task (Metz and Whishaw, 2009), which would require limited suprasegmental modulation due to minor perturbations. However, crossing an irregular ladder as in the present study depends upon not only descending propriospinal and supraspinal pathways but also upon sensory input via ascending tracts to adjust for perturbations caused by the unevenly spaced rungs (Filli et al., 2014; Kjell and Olson, 2016; Metz and Whishaw, 2009; Schucht et al., 2002; Vogelaar and Estrada, 2016).

The present study cannot confirm sprouting of 5-HT fibers - either at the caudal border of the lesion site or even further caudal in the region of motor neuronal pools relevant for locomotor function – as a structural correlate of the observed functional improvement on the horizontal ladder in contrast to previous studies (Hellal et al., 2011; Ruschel et al., 2015). Although many studies have associated sprouting of serotonergic fibers with sensorimotor recovery after SCI, ablation of serotonergic fibers with 5,7-dihydrotryptamin in spinal cord injured rats did not significantly alter locomotor function, measured by the BBB score, even though the study indicates that parts of the raphespinal system might still be important for some of these locomotor functions (Li et al., 2004). Other studies have shown that different descending tracts are also involved in crossing a horizontal ladder. Not only ventrolateral tracts, which are important for inter-limb coordination, but also reticulospinal axons to initiate stepping and corticospinal together with rubrospinal tracts for the control of voluntary movement are involved in performing such a complex task (Brustein and Rossignol, 1999; Jordan, 1998; Metz et al., 2000; Muir and Whishaw, 2000; Schucht et al., 2002). Thus, descending pathways other than raphespinal axons (Brustein and Rossignol, 1999; Jordan, 1998; Metz et al., 2000; Muir and Whishaw, 2000) as well as newly formed propriospinal relays (Bareyre et al., 2004; Courtine et al., 2008; Filli et al., 2014; van den Brand et al., 2012) might have contributed to the observed improvement. However, respective pathways were not analyzed in the present study. Thus, a structural neuroanatomical correlate for the

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observed functional improvement cannot be presented at this point. In the low dis cohort neither the regular nor the irregular spaced ladder detected a difference between Epo D versus control animals, which is likely attributable to ceiling effects caused by near complete spontaneous functional recovery.

One other important factor that might influence structural and functional outcomes after microtubule stabilization is the difference in sensitivity of various axonal tracts to Epo D. It has been previously shown that CNS neurons react differently to epothilone than PNS neurons (Jang et al., 2016), suggesting that different degrees of microtubule stabilization might be needed in different axons for optimal axonal growth. Indeed, hyperstabilization of microtubules may lead to deficits rather than increased regeneration (Erturk et al., 2007; Sengottuvel et al., 2011). More work needs to be done to define the dose of Epo D best suited for our high dis lesion and whether other concentrations might provide more benefit after other lesion severities.

Species and strain differences may also account for different outcomes between the current and previous studies. Rat strains and even sub-strains differ in terms of functional recovery and tissue sparing after SCI (Kjell and Olson, 2016; Mills et al., 2001; Webb et al., 2003). Given our data that Epo D treatment results in different outcomes depending upon the lesion severity it can be hypothesized that with the same contusion force in rats ranging in a 10-30% difference in animal weight (in the present study inbred Fischer 344 rats weighing around 160-180 g were used, whereas in the previous study outbred Sprague Dawley rats weighing around 200-250 g were used) may affect injury severity and thus may result in a differential response to treatment.

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Consistent with previous findings, where treatment with Epo B or paclitaxel did not affect the overall lesion size after contusion SCI (Hellal et al., 2011; Popovich et al., 2014; Ruschel et al., 2015), we did not observe any effect of Epo D on white matter sparing in Eriochrome cyanine-stained sections.

As opposed to paclitaxel and Epo B (Hellal et al., 2011; Popovich et al., 2014; Ruschel et al., 2015), Epo D did not modify laminin expression around the lesion site. In the present study, laminin immunoreactive areas were measured in equidistant coronal sections over a distance of 8 mm covering the lesion site as well as the intact area beyond the lesion site, whereas in studies investigating paclitaxel and Epo B (Hellal et al., 2011; Popovich et al., 2014; Ruschel et al., 2015), sagittal sections were analyzed only covering the lesion site (about 5 mm), which may have contributed to the observed differences. However, even after analyzing more confined volumes, approximating the 5 mm distance analyzed previously, laminin expression was still not significantly altered by Epo D. Alternatively, as mentioned above, strain differences or differential drug effects/concentrations may account for the differential effects on laminin expression. Finally, the effect of the drug may vary depending on the injury severity as described previously (Popovich et al., 2012a; Popovich et al., 2012b).

Conclusions

Overall, the present study revealed functional improvement after Epo D administration, comparable to the benefit observed in previous studies investigating the microtubule stabilizing drugs Epo B and paclitaxel (Hellal et al., 2011; Perez-Espejo et al., 1996; Ruschel et al., 2015). However, the underlying structural correlates could not be clearly identified. Therefore, future experiments will expand the focus to relevant descending and ascending axonal pathways other than serotoninergic axons that may play a role in the functional recovery we observed. Subsequent experiments also need to investigate varying

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concentrations, in conjunction with pharmacokinetic analysis and biomarker analysis to correlate spinal cord drug exposure with drug efficacy.

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Figure Legends

Figure 1: Experimental timeline.

Rats received a spinal cord contusion injury at midthoracic level T9 using the Infinite Horizon (IH) Impactor SCI device with an impact force of 150 kDyn. At day 1 and 15 post injury, Epo D (1.5mg/kg) or vehicle (control) was injected i.p.. Starting one day post injury, animals were scored weekly using the BBB open field locomotor rating scale. At 4 and 8 weeks post injury, the horizontal ladder test was performed (4 weeks only irregular and 8 weeks irregular and regular spacing of rungs). At 57 days post injury, gait analysis using the Catwalk system was performed and animals were perfused afterwards.

Figure 2: Cohort distributions.

(**A**) Animals were a priori divided into 2 cohorts dependent on the injury-induced spinal displacement (displacement of >1000 µm (**high dis**) or <1000 µm (**low dis**)). Animals with matching displacements were assigned to receive either vehicle or Epo D treatment (**control low dis**; n=8; **Epo D low dis**: n=8; **control high dis** n=7; **Epo D high dis**: n=9). Grey dotted line indicates the cut off at 1000 µm. Student's t-test of low dis vs. high dis: ****p<0.0001. (**B**) Means of the actual force applied for the contusion injury revealed no overall differences in impact forces. Student's t-test of low dis vs. high dis: p=0.11. (**C**) BBB testing showed an improvement over time in both control cohorts (low dis or high dis). Control animals with a low dis have higher BBB score at all time points compared to control animals with a high dis, which did only reach significance at 21 days post injury (2-way ANOVA ****p<0.0001 for time, *p<0.05 for group comparison, post hoc Bonferroni's multiple comparisons test, * p<0.05). (**D**) Horizontal ladder walking test crossing an irregular ladder was performed at 8 weeks post injury. Control animals with a low dis made significantly fewer missteps compared to control animals with a high dis (Student's t-test: *** p<0.001). (**E**) Spared white matter area at the epicenter was measured using eriochrome cyanine (EC) stained coronal

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sections at 8 weeks post injury. Control animals with a low dis showed significantly more spared white matter area compared to control animals with a high dis (Student's t-test: ** $p<0.01$).

Figure 3: Assessment of over-ground gross locomotor function.

Hindlimb motor function was evaluated according to the BBB open field locomotor rating scale. BBB testing did not reveal any group differences. BBB scores are shown in the (**A**) low dis (**control low dis**; n=8; **Epo D low dis**: n=8: 2-way ANOVA ****$p<0.0001$ for time, $p=0.85$ for group comparison) and (**B**) high dis cohort (**control high dis** n=7; **Epo D high dis**: n=9: 2-way ANOVA ****$p<0.0001$ for time, $p=0.15$ for group comparison) up to 8 weeks post injury.

Figure 4: Assessment of skilled walking on the regular and irregular ladder tests.

Functional outcome on the horizontal ladder test were evaluated 4 and 8 weeks post injury (**A**, **B**: low dis: **control low dis**; n=8; **Epo D low dis**: n=8; **C**, **D**: high dis: **control high dis** n=7; **Epo D high dis**: n=9). Bar graphs showing the number of missteps given as a percentile of the total number of steps for the horizontal ladder with (**A**, **C**) irregularly or (**B**, **D**) regularly spaced rungs. Epo D treated animals with a high dis showed a significant reduction in the number of missteps in the irregular horizontal ladder at 4 and 8 weeks post injury (2-way ANOVA, ****$p<0.0001$ over time and ***$p<0.001$ for overall group differences, post hoc Bonferroni's multiple comparisons test, * $p<0.05$). Note that animals made fewer mistakes when crossing the regular compared to the irregular ladder. Epo D treated animals with a displacement of >1000 µm showed a significant reduction in missteps in the regular horizontal ladder test (Student's t-test, *$p<0.05$).

Figure 5: Tissue sparing and representative images of spinal cord cross-sections at the

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lesion center.

(**A**, **B**, **F**, **G**) Tissue sparing was measured using eriochrome cyanine (EC) stained coronal sections at the lesion center 8 weeks post injury (**A-E**: low dis: **control low dis**; n=7; **Epo D low dis**: n=7; **F-J**: high dis: **control high dis** n=6; **Epo D high dis**: n=9). Animals with a displacement <1000 µm have a greater rim of spared tissue compared to animals with a displacement greater than 1000 µm. Dorsal is to the top of the images. Scale bar: 250 µm. No apparent differences are visible between control animals (**A**, **F**) and Epo D-treated animals (**B**, **G**), independent of the displacement. (**C**, **H**) The area of spared white matter is shown between 4200 µm rostral and caudal to the epicenter. The lowest amount of tissue sparing is seen at the epicenter, gradually increasing with further distance from the epicenter. (**D**, **I**) Volume of spared white matter and (**E**, **J**) area of spared white matter at the epicenter did not differ between Epo D-treated and control animals at 8 weeks post injury (Student's t-test, low dis: $p = 0.33$; high dis $p = 0.11$).

Figure 6: Fibrotic scar formation.

(**A, B, E, F**) Laminin (red) and glial fibrillary acidic protein (GFAP; green) immunolabeling of coronal sections at the lesion center 8 weeks post injury (**A-D**: low dis: **control low dis**; n=7; **Epo D low dis**: n=7; **E-H**: high dis: **control high dis** n=6; **Epo D high dis**: n=9). Dorsal is to the top of the images. Scale bar, 250 μm. (**C**, **G**) The laminin positive area up to 4200µm rostral and caudal to the epicenter decreases with further distance from the epicenter. (**D, H**) Scatter plot showing the laminin positive volume compared to control animals at 8 weeks post injury. Animals with a displacement greater than 1000 µm have more laminin positive area than animals with displacement <1000 µm. Epo D treated animals show slightly reduced laminin positive volume, which does not reach significance (Student's t-test, low dis: $p = 0.61$; high dis $p = 0.22$).

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Figure 7: Total length of serotonergic (5-HT)-positive fibers in the ventral horn.

(**A-J**) Serotonin (5-HT) immunolabeling 8 weeks post injury (**A-E**: low dis: **control low dis**; n=7; **Epo D low dis**: n=7; **F-J**: high dis: **control high dis** n=6; **Epo D high dis**: n=9). (**A-D**, **G-I**) Coronal sections of the spinal cord after spinal contusion injury. Note the beaded 5-HT positive fiber appearance in higher magnification images of the boxed area of (**A, B, F, G**) shown in (**C, D, H, I**), respectively. Dorsal is to the top of the images. Scale bar (**A, B, F, G**): 250 µm; (**C, D, H, I**): 100 µm. (**E**, **J**) Bar graphs showing the total length of 5-HT positive fibers in the ventral horn of evenly spaced sections caudal from the lesion epicenter. The values of each the right and left ventral horn of 3 consecutive sections were averaged and plotted as means ± SEM. The total length of 5-HT positive axons was not significantly increased in Epo D treated animals in either displacement cohort (Student's t-test, low dis: $p = 0.31$; high dis $p = 0.73$).

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Author Contributions

F.B. initiated the continuation study and shared the Epo D results of his lab prior to initiation of this study. F.B. and J.R. helped to design the experiments. J.R. advised the Heidelberg laboratory on drug dosing, administration and on the contusion injury procedure. F.B and J.R. were not present during the experiments and did not participate in the data analysis. B.S and A.B. performed all surgeries and administered the drug. B.S. performed the behaviorial testing and analysis, perfusions, sectioning, staining and of the tissues as well as writing the manuscript. M.M. helped perform all behavioral testing as well as analyzing the horizontal ladder data. R.P. helped with data analysis and writing the manuscript. A.B. and N.W. helped with experimental design and writing the manuscript.

Author Disclosure Statement

H. Witte, A. Ertürk, F. Hellal, and F.B. filed a patent on the use of microtubule-stabilizing compounds for the treatment of lesions of CNS axons (European Patent no. 1858498; European patent application EP 11 00 9155.0; U.S. patent application 11/908,118). The authors declare no competing financial interests.

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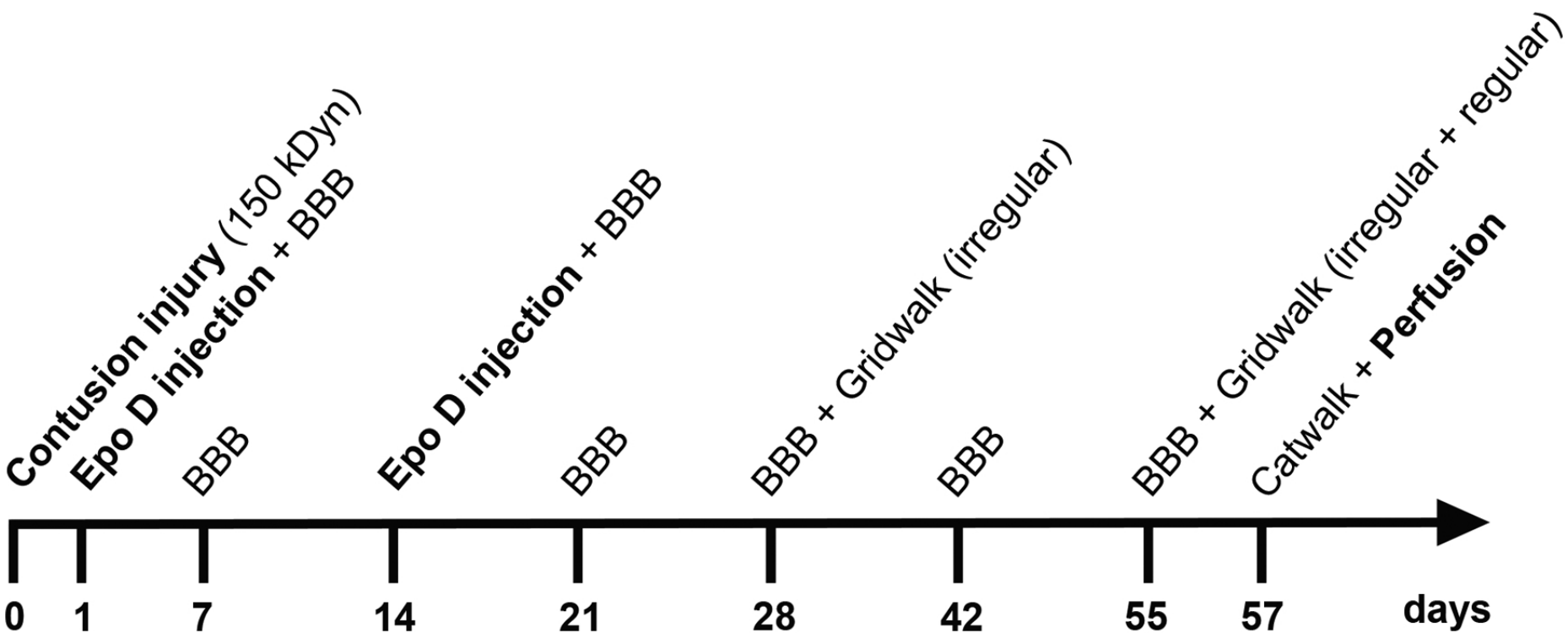
Contusion injury (150 kDyn)
Epo D injection + BBB
BBB
Epo D injection + BBB
BBB
BBB + Gridwalk (irregular)
BBB
BBB + Gridwalk (irregular + regular)
Catwalk + Perfusion
0
1
7
14
21
28
42
55
57
days

Figure 1

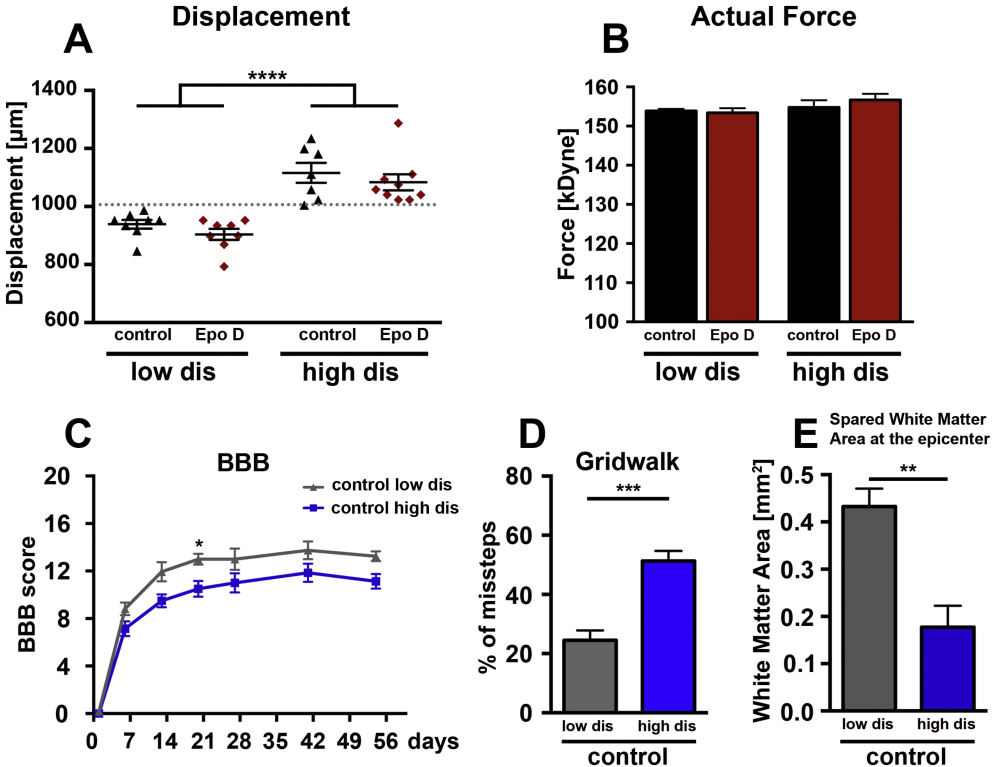
A
Displacement
****
Displacement [µm]
1400
1200
1000
800
600
control
Epo D
control
Epo D
low dis
high dis
B
Actual Force
Force [kDyne]
160
150
140
130
120
110
100
control
Epo D
control
Epo D
low dis
high dis
C
BBB
control low dis
control high dis
BBB score
20
16
12
8
4
0
*
0 7 14 21 28 35 42 49 56 days
D
Gridwalk
***
% of missteps
80
60
40
20
0
low dis
high dis
control
E
Spared White Matter
Area at the epicenter
**
White Matter Area [mm²]
0.5
0.4
0.3
0.2
0.1
0.0
low dis
high dis
control

Figure 2

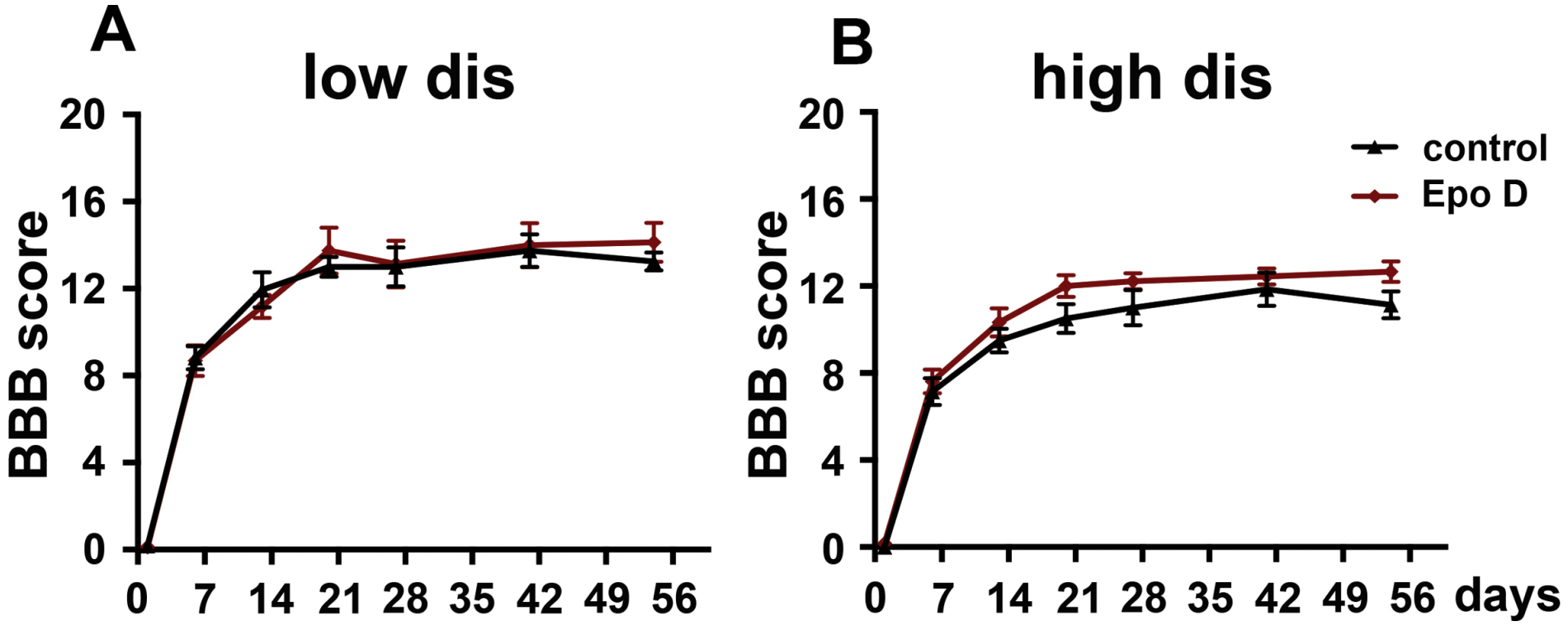
A
low dis
B
high dis
BBB score
20
16
12
8
4
0
0 7 14 21 28 35 42 49 56
days
control
Epo D

Figure 3

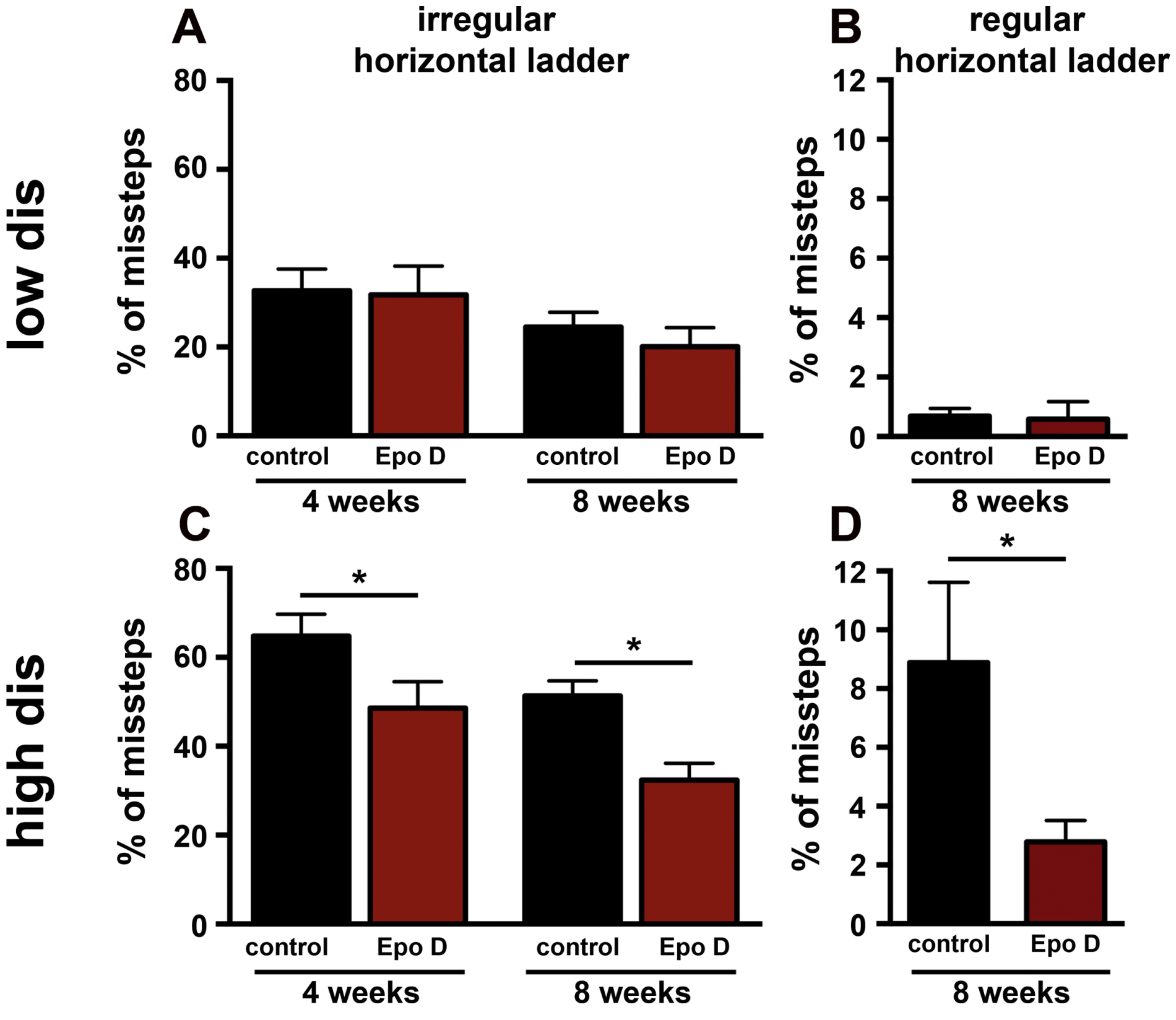
low dis
high dis
A
irregular
horizontal ladder
B
regular
horizontal ladder
C
D
% of missteps
control
Epo D
4 weeks
8 weeks
*

Figure 4

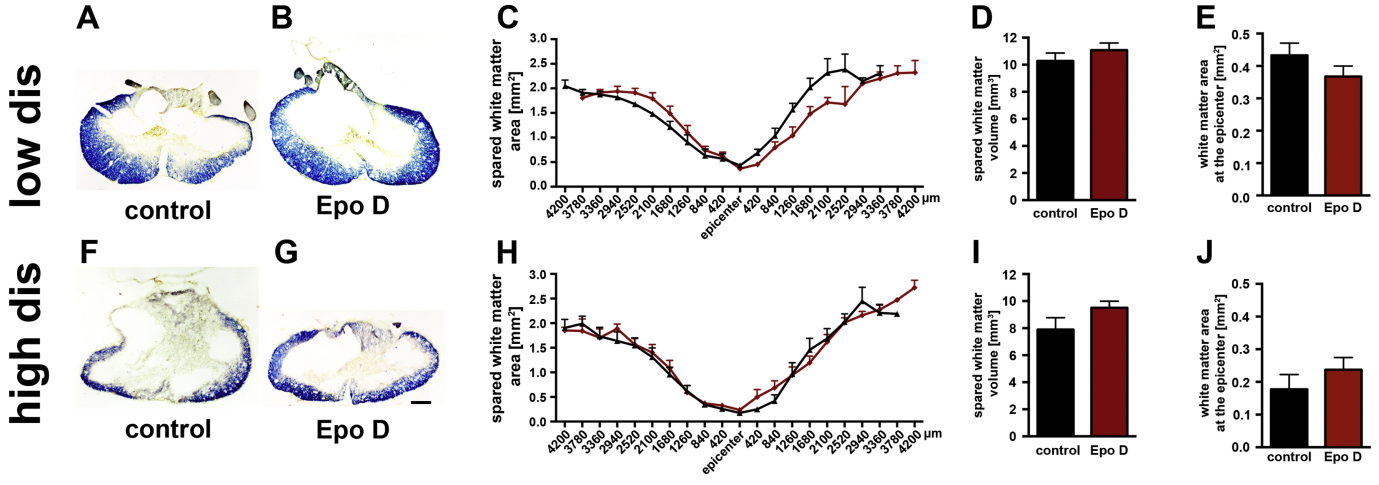
low dis
high dis
A
B
C
D
E
F
G
H
I
J
control
Epo D
spared white matter area [mm²]
spared white matter volume [mm³]
white matter area at the epicenter [mm²]
4200 3780 3360 2940 2520 2100 1680 1260 840 420 epicenter 420 840 1260 1680 2100 2520 2940 3360 3780 4200 µm

Figure 5

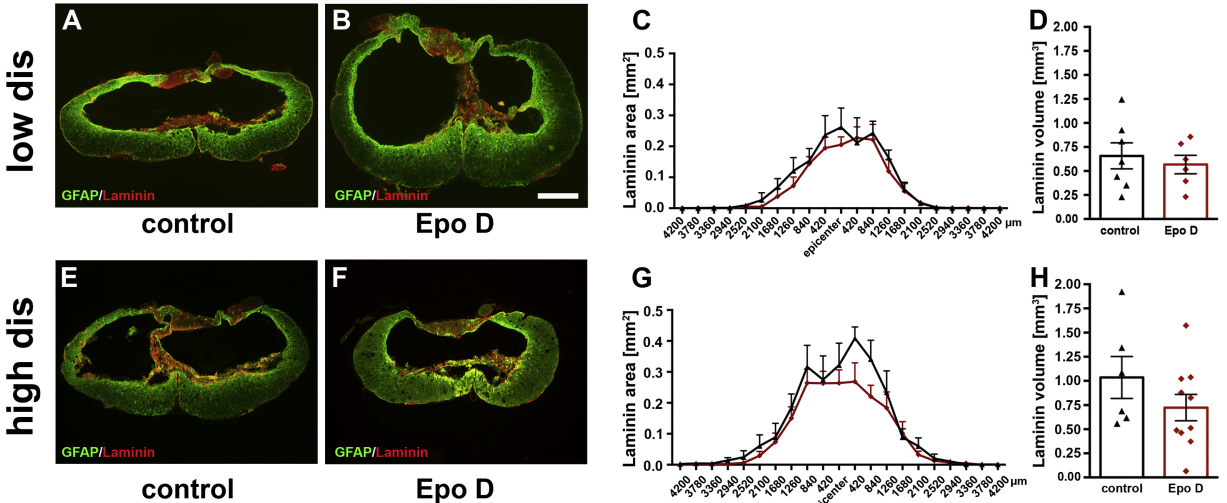
low dis
high dis
A
B
C
D
E
F
G
H
GFAP/Laminin
control
Epo D
Laminin area [mm²]
Laminin volume [mm³]
epicenter
µm

Figure 6

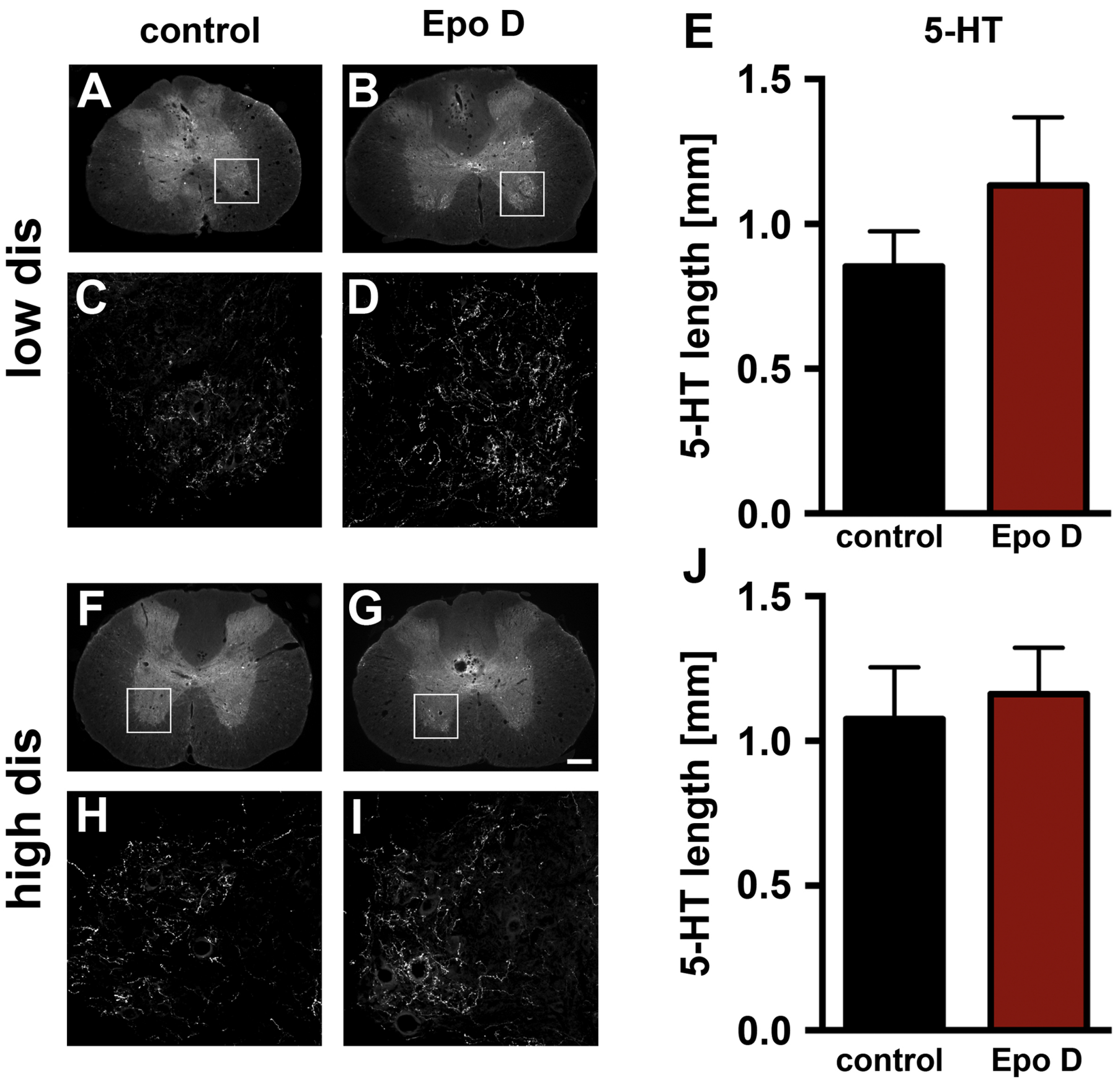
control
Epo D
A
B
C
D
low dis
F
G
H
I
high dis
E
5-HT
5-HT length [mm]
1.5
1.0
0.5
0.0
control
Epo D
J
5-HT length [mm]
1.5
1.0
0.5
0.0
control
Epo D

Figure 7

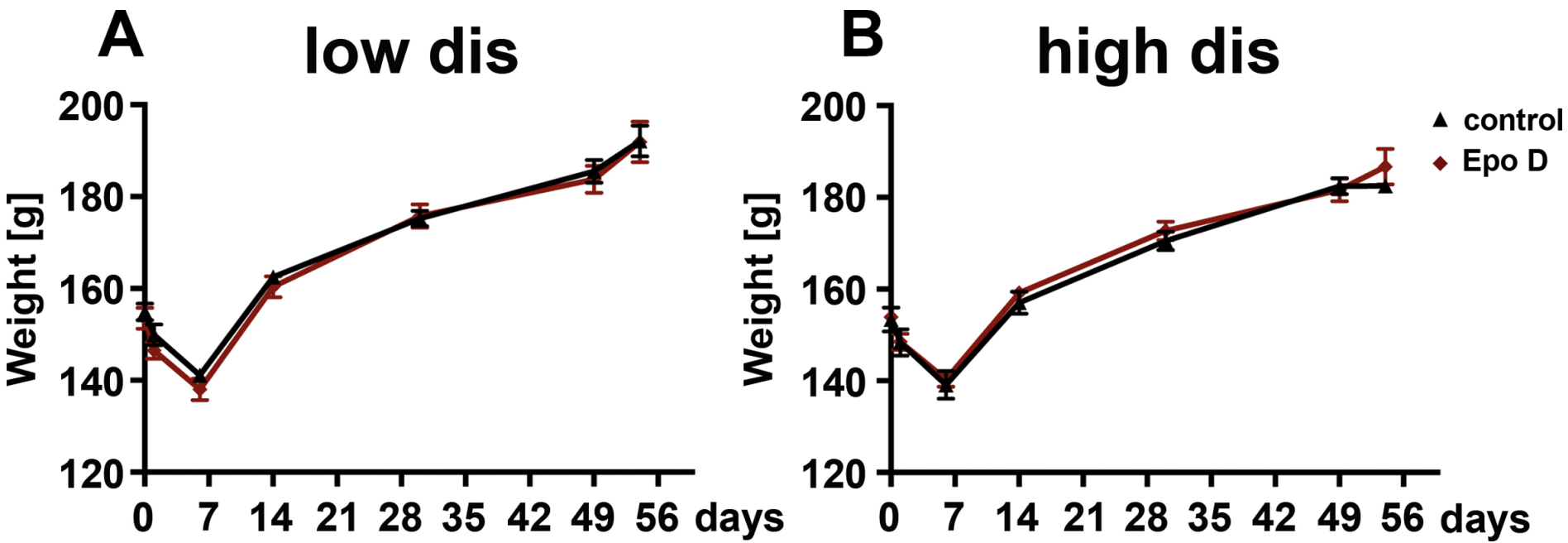
A
low dis
B
high dis
Weight [g]
200
180
160
140
120
0
7
14
21
28
35
42
49
56
days
control
Epo D

Figure 8

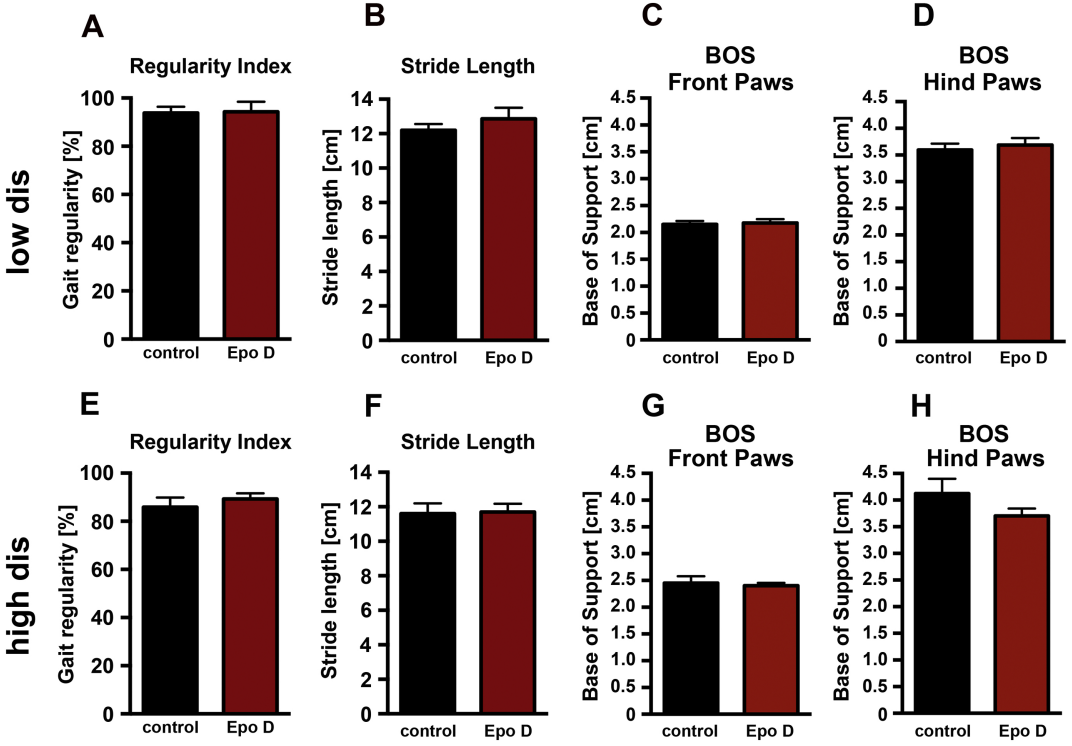
low dis
high dis
A
Regularity Index
Gait regularity [%]
B
Stride Length
Stride length [cm]
C
BOS
Front Paws
Base of Support [cm]
D
BOS
Hind Paws
Base of Support [cm]
E
Regularity Index
Gait regularity [%]
F
Stride Length
Stride length [cm]
G
BOS
Front Paws
Base of Support [cm]
H
BOS
Hind Paws
Base of Support [cm]
control
Epo D

Figure 9

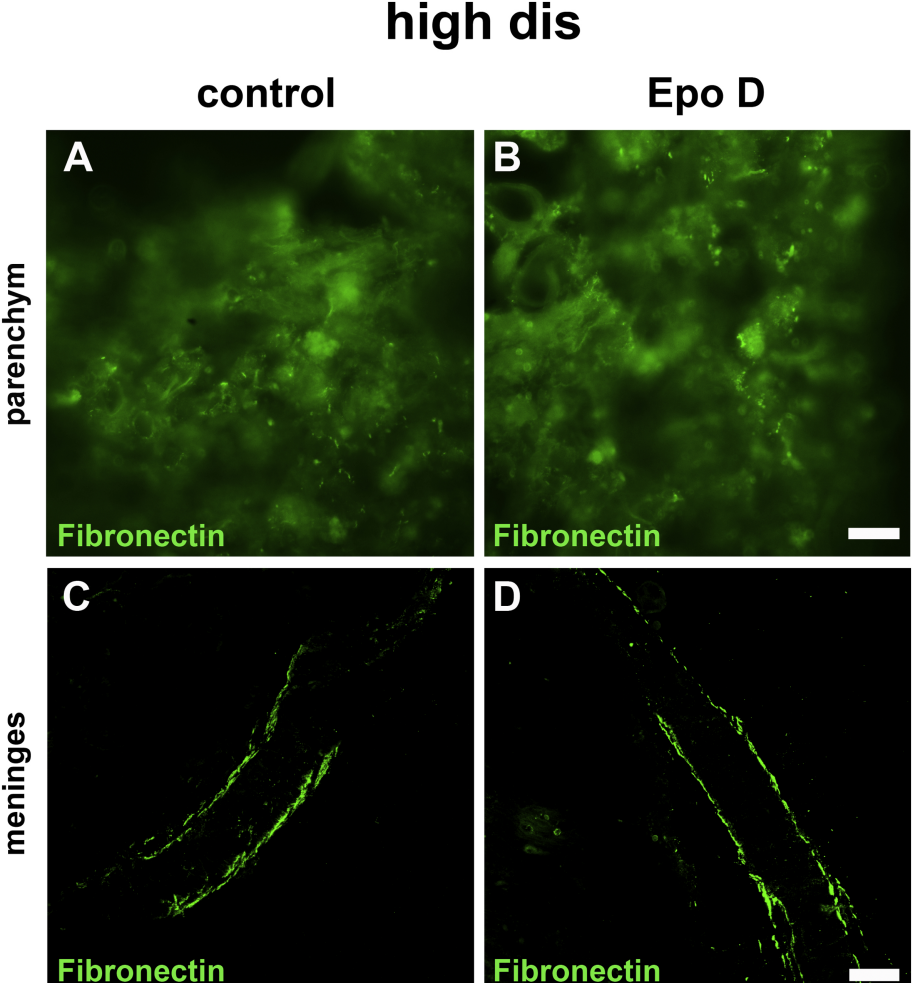
high dis
control
Epo D
parenchym
meninges
A
B
C
D
Fibronectin
Fibronectin
Fibronectin
Fibronectin

Figure 10

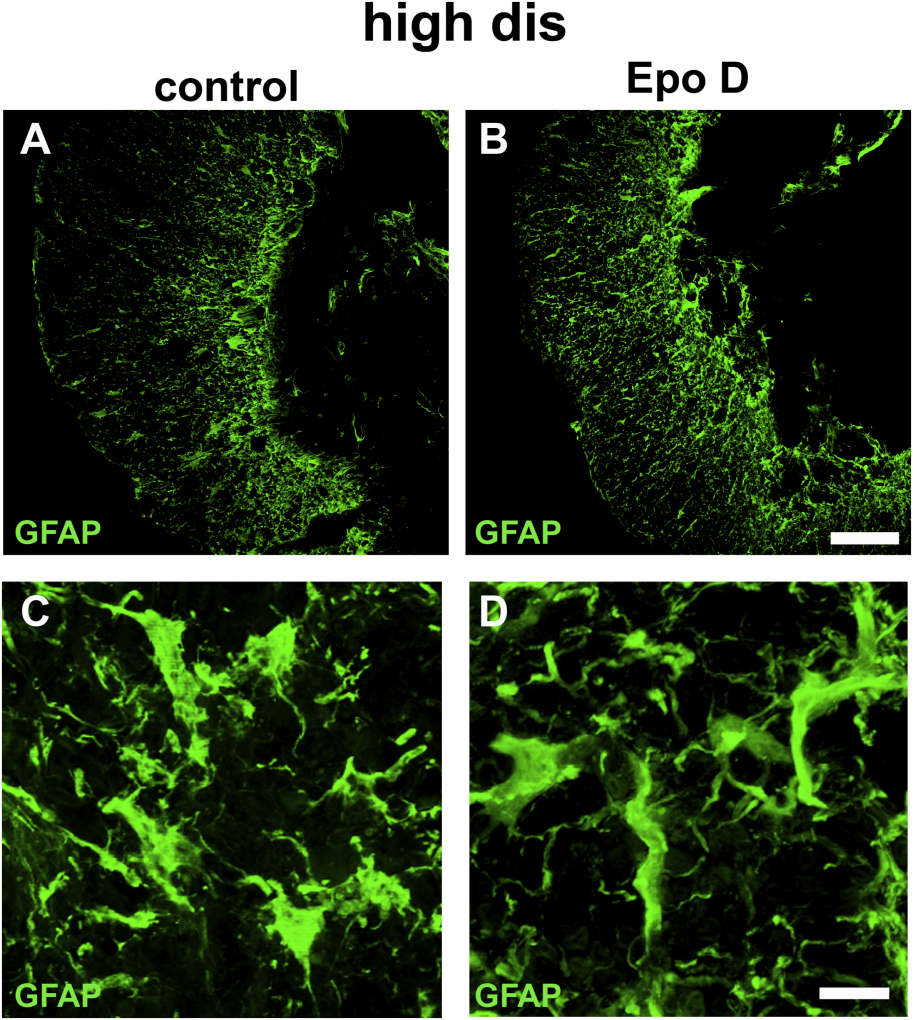

Figure 11