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Single exposure to erythropoietin modulates Nerve Growth Factor expression in the spinal cord following traumatic injury: Comparison with methylprednisolone

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Abstract

Acute lesions of the spinal cord lead to dramatic changes in neuronal function. In the present study, we examined the possible involvement of neurotrophic factors in the action of the drug of choice for the treatment of such an emergency, i.e. the glucocorticoid methylprednisolone is compared to erythropoietin, a cytokine recently shown to markedly shorten the time necessary for motor recovery following injury [Gorio, A., Gokmen, N., Erbayraktar, S., Yilmaz, O., Madaschi, L., Cichetti, C., Di Giulio, A.M., Vardar, E., Cerami, A., Brines, M., 2002. Recombinant human erythropoietin counteracts secondary injury and markedly enhances neurological recovery from experimental spinal cord trauma. Proc. Natl. Acad. Sci. 99, 9450–9455]. We found that methylprednisolone reduces the lesion-enhanced Nerve Growth Factor (NGF) mRNA levels 3 h after injury in the trauma epicenter and caudal section of the cord whereas erythropoietin reinforced the NGF gene expression. Three days after the occurrence of the lesion, erythropoietin, but not methylprednisolone, significantly up-regulated the NGF gene expression both caudally and rostrally to the lesion site, an effect that, based on the chemo-attractant properties of neurotrophin, might facilitate the growth of injured axons toward NGF-rich sites and contribute to the enhancement of the regenerative process. The differences between the effects of methylprednisolone and erythropoietin dissipate 7 days after the lesion when they both enhance NGF mRNA levels at the epicenter. These data show that methylprednisolone and erythropoietin display a different pattern of activation of the neurotrophin NGF which is strictly dependent on the portion of the cord examined and the time elapsed from the injury. Based on our results, we suggest that the higher increase of NGF expression mediated by erythropoietin soon after the injury might explain, at least in part, the improved recovery of motor functions produced by erythropoietin compared to methylprednisolone and saline.

Keywords: Regeneration; Neuroplasticity; Neurotrophic factor; Gene expression

1. Introduction

Spinal cord injuries impair vital functions in humans. The response develops in two well defined steps, the first being the trauma itself leading to direct damage of the nervous tissue while the secondary injury is characterized by events that amplify the early primary lesion. Spinal cord injuries lead to complex responses spanning from massive inflammatory reactions and formation of glial scarring to changes in the mechanisms of intracellular signaling which may contribute to the deleterious outcome that eventually results in the functional blockage of descending and ascending pathways with the consequent inability to move (for a review, see Frigon and Rossignol, 2006).

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Table 1
Motor scores of animals assessed for 28 days after a single injection of saline, erythropoietin or methylprednisolone

Days from impact	Saline	MP	rhEPO
0	21±0	21±0	21±0
1	0±0	0±0	0.142±0.244
3	0±0	0.143±0.244	2.071±0.449
7	0.937±0.18	0.643±0.475	6.5±0.478[a]
15	5.5±0.378	5.214±0.172	12±0.763[b]
21	7.375±0.353	7.428±0.345	13±0.5[b]
28	9.475±0.517	9.035±0.224	13.928±0.534[b]

Mean±S.E.M.
[a]$P<0.01$ and [b]$P<0.001$ vs. all the other groups.
Motor neurological functions of the rats were evaluated by using the locomotor rating scale of Basso et al. (1995). In this scale, animals are assigned a score ranging from 0 (no observable hindlimb movements) to 21 (normal gait). The rats were tested for functional deficits at 1 and 3 h as well as 1, 2, 3 and 4 weeks after injury by the 4 different examiners who were blind to the treatment each animal had received.

Neurotrophic factors play an important role in the Central Nervous System (CNS) both during development and adulthood by influencing neuronal survival and maintaining a correct cell homeostasis (Thoenen, 1995; Abe and Saito, 2001; Frebel and Wiese, 2006). Beyond their potent neurotrophic activity, these molecules can act as "protective" agents by rescuing neuronal cells after injuries of specific neuronal pathways (Deller et al., 2006) or by preventing excitotoxic damage (Wong et al., 2005; Bemelmans et al., 2006). To this end, several lines of evidence indicate that their biosynthesis can be modulated through the activation of specific neuronal pathways or the interaction with neurotransmitters and cytokines (for a review see Reichardt, 2006) raising the possibility that manipulation of neurotrophic factor production within the CNS might represent an "alternative" therapeutic intervention for neurodegenerative disorders (Fumagalli et al., 2006a,b; Zuccato et al., 2005a,b).

In recent years, it has been shown that lesions to the spinal cord modify the expression of neurotrophic factors (Bregman et al., 1997; Blesch and Tuszynski, 2002). Among others, Nerve Growth Factor (NGF) and Brain Derived Neurotrophic Factor (BDNF) expression were especially altered (Brunello et al., 1990; Follesa et al., 1994; King et al., 2000; Nakamura and Bregman, 2001; Ikeda et al., 2001; Brown et al., 2004; Qin et al., 2006; Li et al., 2007) thus suggesting the possible involvement of neurotrophic factors in spinal cord plasticity and repair after injury. In addition, it was shown that exogenous delivery of such neurotrophins to injured animals promotes robust axonal growth (Grill et al., 1997; Vavrek et al., 2006; Iarikov et al., 2007).

Nowadays, the drug of choice for the emergency treatment of spinal cord injuries is represented by methylprednisolone, a glucocorticoid whose beneficial effects could be due, at least in part, to glucocorticoid-induced regulation of trophic factors (Riva et al., 1995a,b; Gonzalez et al., 2005). However, we have recently demonstrated that the cytokine recombinant erythropoietin markedly improves motor performances in injured animals (Gorio et al., 2002), an effect that is neutralized by

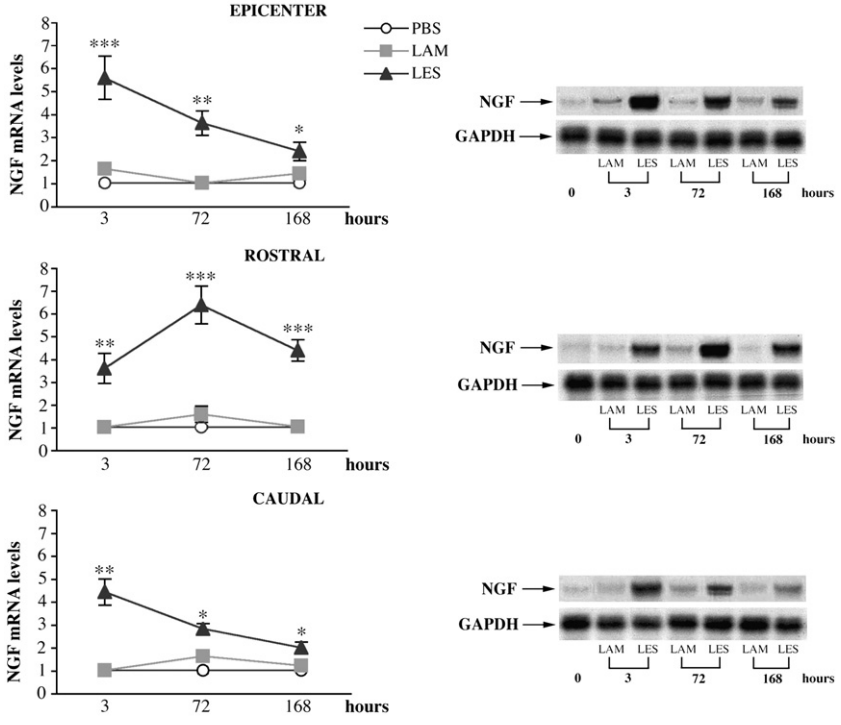

Fig. 1. Quantitative analysis of NGF mRNA levels in different portions of the spinal cord (epicenter, rostral and caudal sites). The left panel shows the graph with the temporal profile (3, 72 and 168 h) of NGF induction. The results, expressed as arbitrary units, represent the mean±S.E.M. of at least 8 independent determinations. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs. control rats (PBS), (one way ANOVA with Dunnett's t-test). The right panel shows a prototypical experiment of RNase Protection Assay. 8 μg of total RNA were used for the determination. Data show the levels of NGF mRNA in control (0), laminectomized (LAM) and injured (LES) animals. 4 μg of total RNA were used for the determination. The autoradiographic film was exposed at −70 °C with an intensifying screen for 8 h (NGF) or 3 h (GAPDH).

methylprednisolone (Gorio et al., 2005). Among the various mechanisms that might contribute to the effects of erythropoietin, the regulation of trophic factor biosynthesis may well be considered in light of recent results showing that erythropoietin is able to protect primary hippocampal neurons by increasing the expression of BDNF (Viviani et al., 2005).

Thus, our working hypothesis is that the recovery of motor functions after spinal cord lesions might, at least in part, depend on a different regulation of neurotrophic factors, which exert a crucial role on neuronal development and repair. Our analysis included the epicenter of the lesion as well as the caudal and rostral sites in order to evaluate a possible regional selectivity by the drugs under investigation. To this end, the process of recovery from injury was monitored by behavioral experiments and molecular analyses of trophic factor expression in an attempt to detect a possible correlation between erythropoietin-induced improvement in motor scores and trophic factor production, comparing these data with those obtained with methylprednisolone.

2. Materials and methods

2.1. Materials

General reagents were purchased from Sigma (Milan, Italy) and molecular biology reagents were obtained from Cellbio (Pero, Milan, Italy) and Promega (Milan, Italy).

2.2. Animals

Adult Sprague–Dawley rats (Charles River Laboratories) weighing 240–260 g were used. The animals were kept under standard housing conditions (22±2 °C, 65% humidity, and lights from 6:00 a.m. to 8:00 p.m.) and were fed a standard dry diet; water was freely available. All experimental protocols were approved by the Animal Review Committee of the University of Milan and met the Italian guidelines for laboratory animals which conform to the European Communities (EEC Council Directive 86/609 1987) and the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by United States National Institute of Health.

2.3. Spinal cord injury with UTS-impactor and drug treatment

Traumatic spinal cord injury was performed by means of the UTS-impactor, as previously described (Gorio et al., 2002). The core of the UTS-impactor is a 2.3 mm diameter stainless steel rod that is precisely driven into the spinal cord with a specific force and displacement. The movement and impact is monitored by means of a miniaturized piezoelectric dynamometer present within a section of the impacting rod and linked to a computer that drives the device and records and manages the data. The impounding piston was positioned 1 mm above the exposed cord at T9 and set for an excursion of 3 mm. A force of 1 N for 1 s was applied, followed by the automatic return of the impaction rod. Animals were maintained under halothane anesthesia and positioned over a mat kept at the temperature of 38 °C and, before awakening, were treated with buprenorphine [0.03 mg/kg] for pain and penicillin G (10,000 U/kg) as an antimicrobial agent. Each experimental group contained at least eight animals. After spinal cord injury, the rats were housed two per cage and underwent manual bladder evacuation three times daily. Recombinant erythropoietin (Epoietin Alpha, Ortho Biotech, Milan) was administered as a single treatment at the dose of 1000 U/kg-body weight by intraperitoneal injection within 30 min after injury; methylprednisolone sodium succinate (Sigma) was administered at a dose of 30 mg/kg by intraperitoneal injection as well (Gorio et al., 2002).

2.4. Functional assessment

Recovery of motor functions was examined at different time points after the lesion (0, 1 and 3 h; 1, 2, 3 and 4 weeks) in blinded fashion by four investigators and averaged using the methodology of Basso et al. (1995). For these experiments, eight animals for each time point were used.

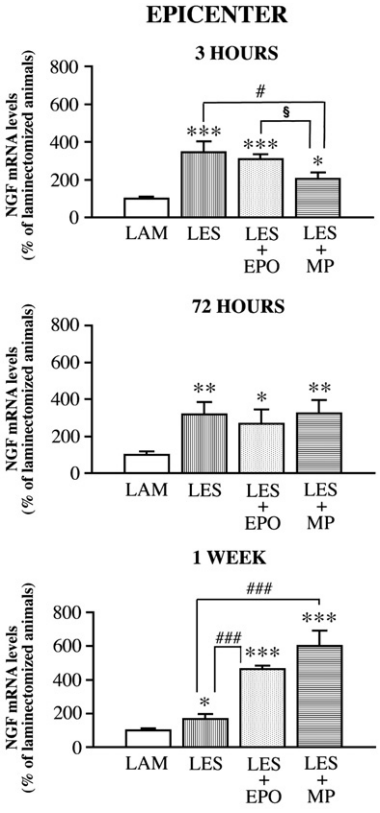

Fig. 2. Quantitative analysis of NGF mRNA levels at the epicenter of the lesion. Animals were injured (LES) and then divided in groups receiving erythropoietin (EPO) or methylprednisolone (MP), respectively. Since no differences were observed in the expression of NGF between control and laminectomized (LAM) animals, the results were expressed as % of LAM rats. Drugs were injected 30 min after the lesion and animals were sacrificed at different time points (3, 72 and 168 h). Data represent the mean±S.E.M. of at least 8 independent determinations. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. LAM animals; $^{\#}P<0.05$ and $^{\#\#\#}P<0.0001$ vs. LES animals; $^{\S}P<0.05$ vs. LES+MP (one way ANOVA with Dunnett's *t*-test).

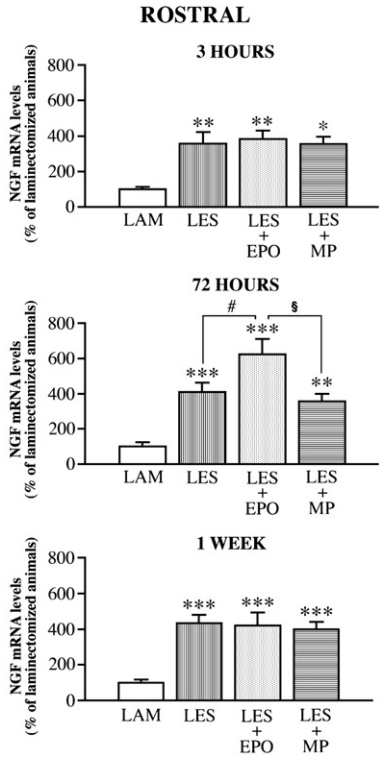

Fig. 3. Quantitative analysis of NGF mRNA levels at the site rostral to the epicenter of the lesion. Animals were injured (LES) and then divided in groups receiving erythropoietin (EPO) or methylprednisolone (MP), respectively. Since no differences were observed in the expression of NGF between control and laminectomized (LAM) animals, the results were expressed as % of LAM rats. The drugs were injected 30 min after the trauma and animals were sacrificed at different time points (3, 72 and 168 h). Data represent the mean±S.E.M. of at least 8 independent determinations. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs. laminectomized (LAM); $^{\#}P<0.05$ vs. LES animals; $^{\S}P<0.05$ vs. LES+MP (one way ANOVA with Dunnett's *t*-test).

2.5. RNA preparation

Different portions of the spinal cord of the control, laminectomized and lesioned animals were homogenized in 4 M guanidinium isothiocyanate (containing 25 mM sodium citrate pH 7.5, 0.5% sarcosyl and 0.1% 2-mercaptoethanol) and total RNA was isolated by phenol–chloroform extraction. Quantification was carried out by spectrophotometric analysis and RNA aliquots were re-precipitated in ethanol for RNase protection assays.

2.6. cRNA probes and RNase protection assay

A transcription kit (MAXIscript, Ambion) was used to generate cRNA probes and [^{32}P]-CTP was used as a radiolabelled nucleotide. The RNase protection assay was performed on an 8 µg sample of total RNA as described previously (Fumagalli et al., 2005), using a rat neurotrophin multiprobe template set (catalogue number 556148, Becton Dickinson, Milan) (which included NGF, BDNF, GDNF, CNTF). The plasmid RObFGF503 containing a 1016 bp portion of the rat FGF-2 cDNA was added to the hybridization solution. The FGF-2 cRNA probes and its relative protected fragment (p.f.) were the following: FGF-2=524, p.f.=477 which fitted on the top of the trophic factors included in the multiprobe template set.

Briefly, after ethanol precipitation, total RNA, was dissolved in 20 µl of hybridization solution containing 150,000 cpm of [^{32}P]-labelled neurotrophin cRNA multiprobe. After being heated at 85 °C for 10 min, the cRNA probes were allowed to hybridize the endogenous RNAs at 45 °C overnight. At the end of hybridization, the solution was diluted with 200 µl of RNase digestion buffer containing a 1/400 dilution of an RNase cocktail (RNase A and RNase T1) and incubated for 30 min at 30 °C. Proteinase K and SDS were then added to the sample and the mixture was incubated at 37 °C for an additional 15 min. At the end of incubation, the sample was extracted with phenol/chloroform and ethanol-precipitated. The pellet, containing the RNA:RNA hybrids, was dried and re-suspended in loading buffer, boiled at 95 °C for 5 min and separated on 5% polyacrylamide gel under denaturing conditions.

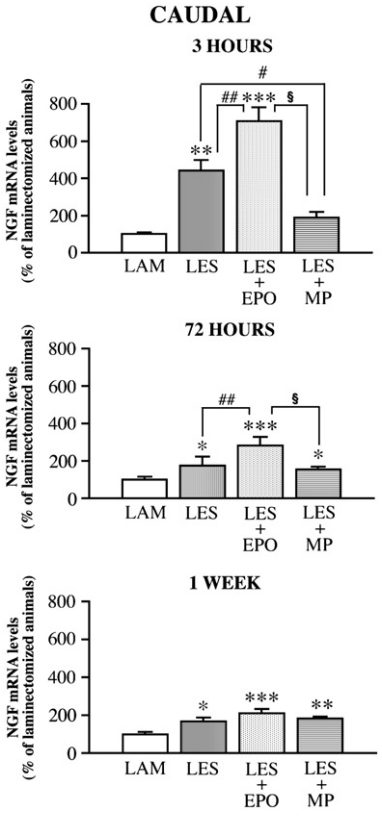

Fig. 4. Quantitative analysis of NGF mRNA levels at the site caudal to the epicenter of the lesion. Animals were injured (LES) and then divided in groups receiving erythropoietin (EPO) or methylprednisolone (MP), respectively. Since no differences were observed in the expression of NGF between control and laminectomized (LAM) animals, the results were expressed as % of LAM rats. Drugs were injected 30 min after the trauma and animals were sacrificed at different time points (3, 72 and 168 h). Data represent the mean±S.E.M. of at least 8 independent determinations. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs. LAM animals; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. LES animals; $^{\S}P<0.001$ vs. LES+MP at 3 h and $^{\S}P<0.05$ vs. LES+MP at 72 h (one way ANOVA with Dunnett's *t*-test).

Table 2
Effect of erythropoietin or methylprednisolone on lesion-induced increase of BDNF mRNA levels

	3 h			72 h			168 h		
	Epicenter	Rostral	Caudal	Epicenter	Rostral	Caudal	Epicenter	Rostral	Caudal
LAM	100±11	100±9	100±15	100±15	100±11	100±13	100±7	100±19	100±5
Lesion	195±10[a]	352±41[a]	190±10[a]	96±7	84±4	67±37	58±13[a]	116±17	106±10
LES+EPO	159±17	393±47	239±10	91±7	99±7	106±44	44±9	125±12	104±12
LES+MP	169±17	369±27	181±23	86±7	90±6	76±30	42±6	103±5	140±9

Mean±S.E.M.
[a] $P<0.05$ vs. laminectomized animals.

2.7. RNA calculation

The levels of mRNA for neurotrophins or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were calculated using the Quantity One software from Biorad. In order to ensure that the autoradiographic bands were in the linear range of intensity, different exposure times were used. GAPDH was employed as the internal standard for RNase protection assay as its expression was not regulated by laminectomy or lesion.

2.8. Statistical analysis

Data are expressed as the mean±S.E.M. Multiple group comparisons of the differences in quantitative measurements were made by one way analysis of variance (ANOVA), followed by Dunnett's *t*-test. Statistical significance was accepted at $P<0.05$.

3. Results

We have previously demonstrated that, following contusive trauma between vertebrae T9 and T10, recombinant erythropoietin promoted a significant neurological improvement (Gorio et al., 2002), that was counteracted by methylprednisolone (Gorio et al., 2005), the current drug of choice for traumatic spinal cord lesion. In line with previous reports (Gorio et al., 2002, 2005) we here confirm that the effect of methylprednisolone on the recovery of locomotor activity is not distinguishable from saline whereas erythropoietin significantly improved such scores (Table 1), indicating that erythropoietin clearly improves motor performance as compared to methylprednisolone.

In order to find a molecular correlate that could, at least in part, explain the difference existing between methylprednisolone and erythropoietin in motor function recovery following spinal cord injury, we sacrificed the animals at different time points (3 h, 72 h and 168 h after the lesion) and analyzed neurotrophic factor gene expression under these extreme experimental conditions. Since both erythropoietin and methylprednisolone, when administered alone in non-injured animals, do not affect basal expression of NGF at any time, we have not reported these data in the figures. Fig. 1 shows that NGF gene expression is markedly and significantly up-regulated in the lesion epicenter as well as in caudal and rostral sites, an effect that is significant with respect to control and laminectomized animals. Interestingly the temporal pattern of NGF induction varies in the different parts of the cord. There is a similar pattern of NGF induction in the lesion epicenter and in the caudal site, with marked increase within the first 3 h that gradually decreases with time, although it is still significantly elevated 7 days after the trauma (Fig. 1). Conversely, in the section rostral to the epicenter, NGF gene expression peaks 72 h after injury but is significantly higher than controls throughout the experimental period.

Fig. 2 shows the effect of drug treatment (erythropoietin or methylprednisolone) on NGF mRNA levels at the epicenter of the lesion. Since NGF gene expression in the laminectomized animals does not statistically differ from the control animals, we present hereafter the results with respect to laminectomy. NGF mRNA levels are markedly enhanced (+380%, $P<0.0001$) in comparison to laminectomized rats 3 h after injury; a similar increase is observed in erythropoietin-treated rats (+364%, $P<0.0001$) whereas such up-regulation is significantly reduced in methylprednisolone-treated rats (+200%, $P<0.01$), indicating a difference between the drugs that is statistically significant ($P<0.05$). At 72 h after trauma there is no difference among the three groups (lesion=+303%, $P<0.01$; lesion+erythropoietin=+288%, $P<0.01$; lesion+methylprednisolone=310%, $P<0.01$). This profile is totally changed 7 days after injury when the lesion-promoting effect is almost exhausted (+170%, $P<0.05$) and both erythropoietin and

Table 3
Effect of erythropoietin or methylprednisolone on lesion-induced increase of FGF-2 mRNA levels

	3 h			72 h			168 h		
	Epicenter	Rostral	Caudal	Epicenter	Rostral	Caudal	Epicenter	Rostral	Caudal
LAM	100±9	100±29	100±7	100±38	100±30[a]	100±13	100±13	100±12	100±18
Lesion	182±31[a]	295±50[a]	240±21[a]	197±9[a]	188±37[a]	184±10[a]	137±25	198±13[a]	171±21[a]
LES+EPO	162±16	358±32	254±20	185±30	193±13	191±15	185±29	192±38	212±20
LES+MP	183±25	291±32	205±35	319±57	197±27	161±17	137±15	200±29	228±12

Mean±S.E.M.
[a] $P<0.05$ vs. laminectomized animals.

Table 4
Effect of erythropoietin or methylprednisolone on lesion-induced increase of CNTF mRNA levels

	3 h			72 h			168 h		
	Epicenter	Rostral	Caudal	Epicenter	Rostral	Caudal	Epicenter	Rostral	Caudal
LAM	100±7	100±21	100±15	100±10	100±8	100±9	100±9	100±11	100±16
Lesion	209±24[a]	128±15	108±8	122±10	108±6	114±4	113±6	109±3	112±28
LES+EPO	217±26	162±28	120±9	100±11	109±1	121±4	127±14	118±12	134±15
LES+MP	225±33	159±29	108±13	147±21	116±5	117±8	119±18	95±7	133±19

Mean±S.E.M.
[a] $P<0.05$ vs. laminectomized animals.

methylprednisolone produce the maximum increase (lesion+erythropoietin=+470%; lesion+methylprednisolone=+580%, $P<0.001$) (Fig. 2).

Rostrally to the lesion site, the extent and pattern of NGF gene expression after injury is different (+380%, $P<0.01$). Neither drug affects NGF mRNA lesion-induced levels at 3 h (lesion+erythropoietin=+393%, $P<0.01$; lesion+methylprednisolone=377%, $P<0.05$) and 1 week (lesion=+421%, $P<0.01$; lesion+erythropoietin=+416%, $P<0.01$; lesion+methylprednisolone=404%, $P<0.01$) after the injury, whereas at 72 h there is a marked enhancement by erythropoietin treatment (lesion=+402%, $P<0.001$; lesion+erythropoietin=+618%, $P<0.001$) which is not observed in methylprednisolone-treated rats (lesion+methylprednisolone=384%, $P<0.01$), again pointing to a significant difference between the drugs ($P<0.05$) (Fig. 3).

Caudally to the lesion site, there is a marked enhancement of NGF expression 3 h after the lesion (+421%, $P<0.01$). This is significantly enhanced by erythropoietin (+70%, $P<0.01$) and reduced by methylprednisolone as it is observed in the lesion epicenter (lesion+methylprednisolone=+180%, $P<0.05$), revealing a significant difference between the effects of the drugs ($P<0.001$). At 72 h erythropoietin promotes a significant increase of NGF expression over the injured animals (lesion=+172%, $P<0.05$; lesion+erythropoietin=+308%, $P<0.001$) that is not observed with methylprednisolone (lesion+methylprednisolone=166%, $P<0.05$), further highlighting a different effect between the drugs in the regulation of NGF mRNA levels after injury ($P<0.05$) (Fig. 4). Seven days after injury, both drugs do not alter lesion-induced NGF mRNA increase (lesion=+191%, $P<0.05$; lesion+erythropoietin=+204%, $P<0.001$; lesion+methylprednisolone=198%, $P<0.01$).

In order to evaluate the specificity of regulation of NGF expression produced by these drugs, we analyzed other trophic factors, namely BDNF, basic Fibroblast Growth Factor (FGF-2), Ciliary Neurotrophic Factor (CNTF) and Glial-Derived Neurotrophic Factor (GDNF) that are known to be induced by spinal cord lesion. Our results show that the expression of BDNF is significantly enhanced at the lesion epicenter and in the adjacent portions of the cord 3 h after injury, with no effect from erythropoietin or methylprednisolone (Table 2). Whereas no changes were observed at 72 h post-lesion in all the experimental conditions, BDNF expression was reduced at the epicenter after 1 week, an effect that was not modified by either drug (Table 2). Table 3 shows that FGF-2 expression is enhanced by the lesion in all experimental conditions (with the exception of the epicenter of the lesion at 1 week) but such up-regulation is not altered by the drugs. GDNF is expressed only at the epicenter of the lesion (no signal was detected in the rostral or caudal sites under any of the experimental conditions used in this study) and its expression is highly induced at the different time points investigated (Table 4). Interestingly, such enhancement was affected by both drugs only 7 days after injury (Table 5).

Table 5
Effect of erythropoietin or methylprednisolone on lesion-induced increase of GDNF mRNA levels at the epicenter

	3 h	72 h	168 h
LAM	100±8	100±7	100±7
Lesion	652±68[a]	163±19[a]	137±10
LES+EPO	609±103	172±38	208±17[b]
LES+MP	491±51	158±7	201±20[b]

Mean±S.E.M.
[a] $P<0.05$ vs. laminectomized animals.
[b] $P<0.05$ vs. lesioned animals.

4. Discussion

In this report we show that the present drug of choice for the emergency treatment of spinal cord injury, the glucocorticoid methylprednisolone, and the cytokine recombinant erythropoietin, recently shown to attenuate secondary degeneration and improve recovery of motor functions after traumatic injury of the cord (Gorio et al., 2002), differently modulate injury-induced up-regulation of NGF gene expression in an anatomical- and temporal-dependent fashion.

Both methylprednisolone and erythropoietin must be administered within 8 h from the lesion to elicit the most beneficial effects (Bracken et al., 1992; Gorio et al., 2002; Ehrenreich et al., 2002). Our data show that, 3 h after the injury, erythropoietin significantly enhanced injury-induced NGF gene expression at the caudal site whereas methylprednisolone reduced the neurotrophin expression both at the caudal site and at the epicenter. The first hours after trauma seem to be critical for recovery and, therefore, early up-regulation of NGF mRNA levels produced by erythropoietin at the caudal site might somehow contribute to the recovery of injured motoneurons by enhancing neuronal sprouting and synaptic rearrangement, in line with the pro-regenerative features of the neurotrophin (McCallister et al., 2004; Kim et al., 1996; Tuszynski et al., 1997; Murakami et al., 2002; Li et al., 2007). We cannot ignore the evidence that NGF is known to support sprouting of nociceptive sensory fibers inducing pain following

injury (Krenz et al., 1999). However, the evidence that pain develops between 2 and 4 weeks after injury of the cord is not in agreement with the early changes in NGF expression promoted by erythropoietin observed in our manuscript.

The portions of the cord distal from the lesion epicenter represent an environment strongly permissive to the regeneration whereas the epicenter itself is refractory to axon growth (Davies et al., 1999), thus adding further importance to the early enhancement of NGF gene expression caudally produced by erythropoietin. In addition, erythropoietin, but not methylprednisolone, significantly enhanced lesion-induced increase of NGF mRNA levels both caudally and rostrally 72 h after injury, in line with the permissive nature of the regions surrounding the epicenter. Keeping NGF expression elevated in the sites adjacent to the epicenter might be a critical feature of erythropoietin because of the chemo-attractant properties exerted by the neurotrophin: We hypothesize that such increase in the expression of NGF at distal sites would facilitate injured axons to grow toward the NGF source (Hagg and Varon, 1993). If this holds true, the lack of effect produced by methylprednisolone on NGF mRNA levels at distal sites would delay, or even oppose, axonal growth.

Notably, in the spinal cord, NGF gene expression is barely detectable under basal conditions while markedly up-regulated after trauma, at variance with other trophic factors, namely FGF-2 and CNTF, which are constitutively expressed. Thus, it is possible that FGF-2 and CNTF contribute to the basal trophic support at the spinal level whereas NGF comes into play only as a consequence of the lesion, implicating a different degree of trophic support subserved by these factors and conferring specificity as well as complexity to the trophic response set in motion by the lesion. Since NGF is widely known to possess neuroprotective activity (Ramer et al., 2000; Romero et al., 2001), our data suggest that the lesion-induced increase of the neurotrophin might represent a defensive strategy of the injured cell.

A different picture can be drawn for BDNF. We found that injury produced an early up-regulation (3 h) and a late down-regulation (168 h) of the neurotrophin, changes that were not modulated by either drugs suggesting that, after lesion of the cord, BDNF is not a target of these drugs. This observation is in apparent contrast with the results of Viviani et al. (2005) who found that erythropoietin was neuroprotective via regulation of BDNF expression: however, these authors examined neuronal cultures after neurotoxicant insults, i.e. a markedly different experimental condition that may have a very limited comparability to the traumatic lesion of the cord.

The different effects produced by methylprednisolone or erythropoietin on NGF gene expression early after injury or after 7 days might depend on the cellular composition at the epicenter of the lesion and in the neighboring areas. Early after injury, NGF expression is mainly observed in reactive astrocytes and meningeal cells whereas, later, the neurotrophin seems to be expressed also by neurons and invading cells (Widenfalk et al., 2001; Brown et al., 2004, 2007). The effect produced by methylprednisolone is in line with data from our laboratory showing that, in cortical astrocytes, NGF gene expression is reduced following administration of the glucocorticoid analogue dexamethasone as early as 2 h after the injection (Riva et al., 1995a,b), thus identifying glial cells as the likely cellular source of the early response to injury and, consequently, as a putative site of methylprednisolone action. Conversely, the evidence that the neurotrophin expression is up-regulated by both drugs at the epicenter seven days after the lesion is indicative of long-lasting effects of both drugs on probably the same type of cells, since both treatments are administered only once within 30 min after injury. To this end, Ahmed et al. (2005) have shown that Schwann cells penetrate within the first ten days in the injury site and are able to produce NGF; in addition, the administration of genetically modified Schwann cells over-expressing NGF was found effective in the treatment of severe spinal cord injury (Feng et al., 2005). Furthermore, macrophages also invade the injured site gradually and might be involved in nerve regeneration process via NGF up-regulation (Mitsuma et al., 2004). These results suggest that, among others, Schwann cells and macrophages might be targets of both erythropoietin or methylprednisolone therapy even prior to their entry into the cord where they are effective long after the lesion occurs. The long-lasting effects of the single administration of erythropoietin and methylprednisolone is also confirmed by the higher expression of GDNF measured 7 days after injury.

The neuroprotective activity of erythropoietin has been previously demonstrated. It stimulates angiogenesis, thereby restoring an adequate blood flow after injury (Squadrito et al., 1999), and it also reduces inflammatory response via regulation of cytokines and members of NFκB family (Villa et al., 2003; Digicaylioglu and Lipton, 2001); in addition, erythropoietin modulates the expression of myelin proteins (Vitellaro-Zuccarello et al., 2007) or the activity of inducible nitric oxide synthase (Calapai et al., 2000). Our data, illustrating modulation of NGF mRNA levels after injury, provide novel information as to how erythropoietin might exert its neuroprotective action.

Nowadays, methylprednisolone represents the only available therapy for acute lesions of the spinal cord and recent data further support its use (Leypold et al., 2007) although the improvement is indeed limited. Our recent preclinical work has strongly pointed to erythropoietin as an important option that should be considered in clinical trials (Gorio et al., 2002), in light of the evidence that the beneficial effect of erythropoietin on motor scores, as opposed to the negative action of methylprednisolone, persists up to 28 days after the injection (Gorio et al., 2002; present report) thus revealing the long-lasting effect of the cytokine. In addition, we have demonstrated that methylprednisolone neutralizes the beneficial effects of erythropoietin in experimental spinal cord lesion (Gorio et al., 2005), at variance with Cetin et al. (2006) who have shown that the combination of both drugs improves functional recovery in the experimental animal; however, these authors limited their evaluation to the 72 h time point whereas our study extended the analysis up to the end of the 4th week post-lesion, suggesting that a longer time frame is needed to reveal differences between these drugs.

In line with the above considerations, our results suggest a putative mechanism to explain, at least in part, the improvement in motor functions produced by erythropoietin as opposed to methylprednisolone. Further studies are needed to elucidate the mechanism of action of erythropoietin which might indeed represent a valuable therapeutic alternative in acute spinal cord injury.

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