Neuroscience 219 (2012) 290–301

ERYTHROPOIETIN EFFECT ON SENSORIMOTOR RECOVERY AFTER CONTUSIVE SPINAL CORD INJURY: AN ELECTROPHYSIOLOGICAL STUDY IN RATS

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Abstract—Spinal cord injury (SCI) is a debilitating clinical condition, characterized by a complex of neurological dysfunctions. It has been shown in rats that the acute administration of recombinant human erythropoietin (rhEPO) following a contusive SCI improves the recovery of hindlimb motor function, as measured with the locomotor BBB (Basso, Beattie, Bresnahan) scale. This scale evaluates overall locomotor activity, without testing whether the rhEPO-induced motor recovery is due to a parallel recovery of sensory and/or motor pathways. Aim of the present study was to utilize an electrophysiological test to evaluate, in a rat model of contusive SCI, the transmission of both ascending and descending pathways across the damaged cord at 2, 5, 7, 11, and 30 days after lesion, in animals treated with rhEPO ($n = 25$) *vs* saline solution ($n = 25$). Motor potentials evoked by epicortical stimulation were recorded in the spinal cord, and sensory-evoked potentials evoked by spinal stimulation were recorded at the cortical level. In the same animals BBB score and immunocytochemical evaluation of the spinal segments caudal to the lesion were performed. In rhEPO-treated animals results show a better general improvement both in sensory and motor transmission through spared spinal pathways, supposedly via the reticulo-spinal system, with respect to saline controls. This improvement is most prominent at relatively early times. Overall these features show a parallel time course to the changes observed in BBB score, suggesting that EPO-mediated spared spinal cord pathways might contribute to the improvement in transmission which, in turn, might be responsible for the recovery of locomotor function.

Key words: spinal cord injury (SCI), erythropoietin, motor evoked potential, sensory evoked potential, BBB scale, locomotor pathway.

INTRODUCTION

Spinal cord injury (SCI) is a debilitating and often life-threatening clinical condition, characterized by a complex of motor, sensory and autonomic dysfunctions. The severity of SCI depends upon the extent of gray and white matter damage and the tissue reorganization at the injury site. Lesions following SCI induced with different experimental approaches (trauma, ischemia and localized inflammation) have been investigated mainly with morphological methods (Matis and Birbilis, 2009). At present the functional evaluation of recovery is based, in rodents, upon the locomotor BBB (Basso, Beattie, Bresnahan) scale, which applies a range of scores to the different degrees of hindlimb locomotor deficits evaluated by visual inspection (Basso et al., 1995; Webb and Muir, 2000; Gorio et al., 2002, 2005). It has been observed that the extent of the neurological deficits caused by SCI depends on disruption of ascending and descending pathways traveling in the white matter (Gorio et al., 2007; Bottai et al., 2008), occurring in two phases after injury. The extent of fiber loss depends initially on the severity of the mechanical trauma, then is greatly amplified by a secondary phase (over days and weeks after SCI) of tissue injury involving the fibers spared by the initial impact (Crowe et al., 1997) and eventually leading to chronic demyelination (Totoiu and Keirstead, 2005). The preservation of white matter from secondary injury seems to be critical for limiting neurological deficits (Gorio et al., 2002). At present, the gold standard therapeutic intervention for SCI (Rabchevsky et al., 2011) is the glucocorticosteroid methylprednisolone, despite the lack of significant efficacy. Interestingly, it has been recently shown that the acute administration of recombinant human erythropoietin (rhEPO) in a rat model of contusive SCI significantly improves functional outcome, as measured with the BBB scale (Gorio et al., 2002, 2005). rhEPO has a neuroprotective effect by promoting cell survival (Bittorf et al., 2001; Digicaylioglu and Lipton, 2001; Sirén et al.,

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Abbreviations: 5HT, 5-hydroxy tryptophan; ANOVA, analysis of variance; BBB, Basso, Beattie, Bresnahan; BF, Biceps Femoris; CPG, Central Pattern Generator; N1, first negative; P1, first positive; PBS, phosphate-buffered saline; QL, Quadratus Lumborum; rhEPO, recombinant human erythropoietin; SC, Splenium Capitis; SCI, spinal cord injury; SD, standard deviation; SEP, sensory evoked potential; TH, tyrosine hydroxylase.

0306-4522/12 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2012.05.041

2001a) together with an anti-oxidative (Sela et al., 2001) and anti-inflammatory action (Brines et al., 2000 and Sirén et al., 2001b). The administration of rhEPO after contusive SCI acts mainly through the limitation of secondary damage and attenuation of gliosis and microglia/macrophage activation (Vitellaro-Zuccarello et al., 2007, 2008). However it does not prevent the loss of the dorsal long propriospinal and corticospinal tracts at the lesion epicenter. Therefore the recovery of hindlimb motor function induced by rhEPO and observed with the BBB scale could depend on the preservation of the descending and segmental pathways contributing to locomotor function, but traveling in the lateral and ventral funiculi (Loy et al., 2002; Ballermann and Fouad, 2006). This hypothesis cannot be verified with anatomical measurements or behavioral tests of locomotor function. Locomotion is indeed accomplished at spinal level by a complex neural network, the Central Pattern Generator (CPG), which includes motoneurons and interneurons. CPG activity depends on the peculiar membrane properties of its neural elements, fed by reflexes and descending systems such as the reticulo-spinal system (see for review, Guertin, 2009). Estimating the overall locomotor function, the BBB score does not allow separating the different components. In this light, the better locomotor score obtained in EPO-treated animals in previous studies could be mainly due to plasticity changes occurring in the cord below the injury contributing in an important manner in the re-expression of locomotion after partial SCI in quadruped mammals (Rossignol et al., 2001, 2008). The only way to shed light on the role of the descending systems on motor performance is to measure whether rhEPO also produces an improvement in transmission through the spinal tissue spared from the lesion. In the present study electrophysiological investigations were carried out in a rat model of contusive SCI (Gorio et al., 2002, 2005) to evaluate, in animals treated with rhEPO, the transmission throughout both ascending and descending pathways across the damaged cord at different time intervals after the lesion. In the same animals, a behavioral evaluation by means of the BBB scale, as well as an immunocytochemical analysis of the spinal segments caudal to the lesion were also performed. With the latter technique the degree of myelination of ventral-lateral and dorsal white matter of EPO-treated rats and 5-hydroxy tyrosine (5HT)- and tyrosine hydroxylase (TH)-positive innervations were quantitatively assessed.

EXPERIMENTAL PROCEDURES

Animal treatments

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 those of the European Community (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 Institutes of Health. Adult female Sprague–Dawley rats (Charles River Laboratories Inc., Calco, LC, Italy) weighing 200–220 g were kept under standard housing conditions (22 ± 2 °C, 65% humidity, lights on from 6.00 a.m. to 8.00 p.m.), with standard lab chow and water freely available. Traumatic SCI was performed as previously described (Gorio et al., 2002, 2005), using the IH device (Precision System and Instrumentation LLC), at the core of which is a 2.3-mm diameter stainless steel rod that is precisely driven into the spinal cord with specified force and displacement. The movement and impact were 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, records, and manages the data. The impounding piston was positioned 1 mm above the exposed cord at T9. A force of 100 Kdyne is applied to carry out the lesion, followed by the automatic return of the impaction rod. Animals were maintained under chloral hydrate anesthesia and, before awakening, were treated with buprenorphine (0.03 mg/kg) for pain management, and penicillin G (10 000 U/kg) to prevent infection. Fifty-lesioned animals were randomly divided into two groups of 25 each. Within 30 min after injury, rats of the first group received a single dose of 5000 units/kg of rhEPO (Epoietin Alpha, Ortho Biotech, Milan) administered via an i.p. injection while rats of second group were i.p. injected with a comparable volume of saline solution. This dosage and modality of application of rhEPO improved recovery of function after rat spinal cord injury as described by Gorio et al. (2002, 2005). After SCI, the rats were housed two per cage and underwent manual bladder evacuation three times daily. Five uninjured rats matched for age and weight were added as normal controls.

Both electrophysiological tests and quantitative morphology were performed blind to the treatment each animal had received, and to the time elapsed from the lesion.

Functional assessment

Basal motor activity was evaluated the day before the lesion (day-1) to ensure that the animals enrolled in the study had no initial motor deficits. Lesioned rats were divided in five groups (10 rats for each group, five treated with rhEPO and five with saline solution) each one tested at a different time interval from the lesion, i.e. at 2, 5, 7, 11, and 30 days post-injury.

In each animal motor function was examined by four observers blinded to the treatment, and values for each animal were averaged across observers using the methodology described by Basso and colleagues (Basso et al., 1995).

In each group the average BBB score was expressed as the mean ± standard deviation (SD) of the scores obtained by all the animals of the group. Multiple group comparisons of the differences in quantitative measurements were made by 2-way analysis of variance (ANOVA) (between group factors: ‘‘day after lesion’’ and ‘‘treatment’’). Statistical significance was accepted for a $P < 0.05$.

Electrophysiology

The electrophysiological study aimed at evaluating the extent of ascending and descending transmission through the lesioned cord. The electrophysiological investigations were performed in the same 50-lesioned rats immediately after the BBB evaluation and in the five-control healthy rats. The same test with the same experimental protocol was performed on all animals.

Surgical preparation. Animals were anesthetized with Zoletil (a 50% association of two active compounds: Tiletamine hydrochloride, Zolazepam hydrochloride; total dosage 40 mg/kg im), additional doses (20 mg/kg) being administered when necessary. Local infiltrations of Lidocaine HCl (20 mg/ml) were injected along the surgical site. Spontaneous respiratory rate was monitored and temperature maintained at 36–38° throughout the whole procedure. Physiological solution was used to prevent drying of the eyes. L2–L3 vertebrae were exposed. One Ag Teflon-insulated wire (bare diameter 200 μm, coated 280 μm, A-M System Carlsborg, WA, USA) exposed at the tip (cathode), was introduced