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A STUDY OF METHYLPREDNISOLONE NEUROPROTECTION AGAINST ACUTE INJURY TO THE RAT SPINAL CORD *IN VITRO*

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Abstract—Methylprednisolone sodium succinate (MPSS) has been proposed as a first-line treatment for acute spinal cord injury (SCI). Its clinical use remains, however, controversial because of the modest benefits and numerous side-effects. We investigated if MPSS could protect spinal neurons and glia using an *in vitro* model of the rat spinal cord that enables recording reflexes, fictive locomotion and morphological analysis of damage. With this model, a differential lesion affecting mainly either neurons or glia can be produced via kainate-evoked excitotoxicity or application of a pathological medium (lacking O_2 and glucose), respectively. MPSS (6–10 μM) applied for 24 h after 1-h pathological medium protected astrocytes and oligodendrocytes especially in the ventrolateral white matter. This effect was accompanied by the return of slow, alternating oscillations (elicited by NMDA and 5-hydroxytryptamine (5-HT)) reminiscent of a sluggish fictive locomotor pattern. MPSS was, however, unable to reverse even a moderate neuronal loss and the concomitant suppression of fictive locomotion evoked by kainate (0.1 mM; 1 h). These results suggest that MPSS could, at least in part, contrast damage to spinal glia induced by a dysmetabolic state (associated to oxygen and glucose deprivation) and facilitate reactivation of spinal networks. Conversely, when even a minority of neurons was damaged by excitotoxicity, MPSS did not protect them nor did it restore network function in the current experimental model.

Key words: motoneuron, glial cells, glutamate, metabolic perturbation, excitotoxicity, fictive locomotion.

INTRODUCTION

The incidence of new cases of spinal cord injury (SCI) has remained at a high, stable level throughout the last decade (Jain et al., 2015) with poor long-term outcome for neurological recovery (Chen et al., 2013; Furlan et al., 2013). The acute phase of SCI (due to the primary insult of mechanical, vascular or dysmetabolic nature) rapidly evolves into secondary damage, characterized by excitotoxicity caused by massive release of glutamate, in turn triggering a complex pathophysiological cascade generating toxic compounds (Dumont et al., 2001; Park et al., 2004; Rowland et al., 2008; Forder and Tymianski, 2009; Fatima et al., 2014). Thus, neuroprotection against secondary injury is a major therapeutic target (York et al., 2013; Cox et al., 2014) to preserve the spinal gray (Lipton, 2006; Sámano et al., 2012) and white matter containing the long-fiber tracts (Kanellopoulos et al., 2000; Lee et al., 2008; Margaryan et al., 2010; Sun et al., 2010; Cox et al., 2014). Large-scale clinical trials proposed the early i.v. administration of the glucocorticosteroid methylprednisolone sodium succinate (MPSS) in high doses as the mainstay treatment especially directed to white matter protection (Bracken et al., 1990, 1992; Bracken, 2012). This approach stems from the potent anti-inflammatory and antioxidant properties of MPSS to reduce lipid peroxidation, and to alter gene transcription (Oudega et al., 1992; Hall, 1993).

After the publication of the National Acute Spinal Cord Injury Study trials (NASCIS I, II and III; Bracken et al., 1990; Bracken, 1992), the MPSS treatment has become controversial because the neurological improvements were modest (Harrop, 2014; Fehlings et al., 2014) and coupled to important side effects (Bydon et al., 2013; Harrop, 2014). Notwithstanding the debate about the clinical use of MPSS, this drug is still used in several centers as first approach to SCI (Nicholas et al., 2009; Bracken, 2012; Druschel et al., 2013; Miekisiak et al., 2014; Cheung et al., 2015). A large-scale clinical survey has very recently re-examined the usefulness of MPSS administration to SCI patients with debatable outcome in terms of short- or long-term motor control (Evaniew et al., 2015). Animal models of SCI have shown protection especially for white matter oligodendrocytes (Oudega et al., 1999; Lee et al., 2008; Xu et al., 2009; Sun et al., 2010). These observations raise the issue

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Abbreviations: ANOVA, analysis of variance; ChAT, choline acetyltransferase; DAPI, 4′,6-diamidino-2-phenylindole; DR, dorsal root; GABA, γ-aminobutyric acid; GFAP, glial fibrillary acidic protein; KA, kainate; l, left; L, lumbar; LWM, lateral white matter; MPSS, methylprednisolone sodium succinate; NeuN, Neuronal-specific nuclear protein; NMDA, N-methyl-D-aspartate; PBS, Phosphate-buffered saline; O4, oligodendrocyte progenitor marker; r, right; PM, pathological medium; RIP, oligodendrocyte and myelin sheath marker; ROI, region of interest; SCI, spinal cord injury; SD, standard deviation; SEM, standard error of the mean; S100, Ca^{2+}-binding protein; SMI-32, non-phosphorylated neurofilament H; VR, ventral root; VWM, ventral white matter; 5-HT, 5-hydroxytryptamine.

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whether certain types of SCI might receive more benefit from MPSS than others.

In recent years we have developed an *in vitro* model of SCI in which transient application of the potent glutamate agonist kainate or a ''pathological medium" (PM; mimicking the dysmetabolic conditions occurring after a vascular dysfunction) evokes distinct alterations in locomotor networks with primary damage to gray or white matter, respectively (Taccola et al., 2008; Nasrabady et al., 2012). Useful features of this model are the limited tissue damage in analogy to the majority of the new SCI cases that are incomplete (Jain et al., 2015), and the study of how delayed drug administration (mimicking the clinical setting) might work. The model offers the distinctive advantage of direct investigation of locomotor spinal networks that express alternating motor patterns recorded from lumbar ventral roots (Grillner, 2006; Kiehn, 2006). Thus, the aims of the present study were to find out if, in our *in vitro* model, any neuroprotection by MPSS could be observed and if it might be differentially effective on white or gray matter (Taccola et al., 2008; Margaryan et al., 2009; Kuzhandaivel et al., 2010a,b).

EXPERIMENTAL PROCEDURES

Rat spinal cord preparation

The experiments were carried out on neonatal Wistar rats (ages 1–2 days). All efforts were directed toward minimizing the number of animals used for the experiments and their suffering. Under urethane anesthesia (0.2 ml i.p. of a 10% w/v solution), the spinal cords were carefully dissected out, superfused (7.5 ml/min) with Kreb's solution containing (in mM): 113 NaCl, 4.5 KCl, 1 $MgCl_2 \cdot 7H_2O$, 2 $CaCl_2$, 1 NaH_2PO_4, 25 $NaHCO_3$ and glucose 11; gassed with O_2 95%, CO_2 5%, pH 7.4 at room temperature (22 °C) (Taccola et al., 2008; Margaryan et al., 2009). The experiments were performed in accordance with the ethical guidelines for the care and use of laboratory animals of National Institutes of Health (NIH). All treatment protocols were approved by the Scuola Internazionale Superiore di Studi Avanzati (SISSA) ethics committee and are in accordance with the European Union guidelines on animal experimentation.

Electrophysiological recordings

In order to study reflexes and fictive locomotor rhythms, the experiments were based on DC coupled recordings with tight-fitting suction electrodes from lumbar (L) ventral roots (VRs) (Taccola et al., 2008). Signals were routinely recorded from L2 and L5 VRs carrying mainly flexor and extensor motor signals to hind limb muscles, respectively (Kiehn, 2006). Further analysis of recorded signals was done using pClamp software (version 9.2; Molecular Devices, Sunnyvale, CA, USA).

VR responses were evoked by stimulating a single ipsilateral dorsal root (DR) via a bipolar suction electrode once every 60 s. First, the minimum stimulus intensity was estimated to produce a VR threshold response homolaterally which is considered equivalent to 1× threshold to induce monosynaptic reflexes (Marchetti et al., 2001). Three times higher values of threshold were used to elicit polysynaptic responses (Baranauskas and Nistri, 1995). Generally a train of DR stimuli (30 pulse trains at 2-Hz frequency, 0.1-ms pulse duration) was given to electrically induce fictive locomotion. The responses were calculated by averaging the peak amplitude and area of 3–5 events. Alternatively, rhythmic cycles were recorded by application of N-methyl-D-aspartate (NMDA; 3–6 μM) and 5 hydroxytryptamine (5-HT; 10 μM) (Kiehn, 2006). The periodicity and amplitude of cycles were measured from 20 continuous oscillations as already described by Taccola et al. (2008). Disinhibited bursting was induced by continuous bath application of blockers of γ-aminobutyric acid (GABA)-A and glycine receptors, bicuculline (20 μM) and strychnine (1 μM), respectively. Burst parameters were analyzed in accordance with Bracci et al. (1996).

Protocols for spinal cord lesion and neuroprotection

Two experimental protocols were used: in the first one, spinal lesioning of the gray matter (with loss of fictive locomotion) was induced with 1-h application of the excitotoxic agent kainate (KA, 0.1 mM) in standard Krebs' solution (Taccola et al., 2008; Margaryan et al., 2009; Mazzone et al., 2010).With this approach white matter lesions are usually quite limited (Taccola et al., 2008).

The second protocol mimicked the condition of anoxia/aglycemia that mainly damages the white matter of the spinal cord and is thought to better simulate what occurs after a non-traumatic lesion (Taccola et al., 2008; Margaryan et al., 2009; Kuzhandaivel et al., 2010a). Thus, spinal cord preparations were subjected (for 1 h) to PM, namely a modified Kreb's solution containing 10 mM H_2O_2, 500 μM sodium nitroprusside (SNP), and lacking extracellular Mg^{2+}, glucose and oxygen (replaced by N_2). $NaHCO_3$ was replaced by 1 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) to reach pH 6.75–6.80 (with 0.1 N NaOH), while the osmolarity was lowered to 230–240 mOsm. This solution is known to induce a pathological condition that includes locomotor network depression and spinal damage (Taccola et al., 2008; Margaryan et al., 2009).

With either protocol, we investigated any neuroprotection by MPSS (Pfizer, Italy) after the experimental (1 h) injury. MPSS was diluted with distilled sterile water to get a stock solution of 100 mM, from which test concentrations of 6 or 10 μM were made. The MPSS solution was used within 24 h, since the solution is unstable after 48 h as instructed by the manufacturer. The selection of MPSS concentrations (6 or 10 μM) was based on published clinical guidelines (Sauerland et al., 2000) and former reports with oligodendrocytes cell culture and an organotypic-based model of spinal cord damage (Guzmán-Lenis et al., 2009; Sun et al., 2010). After the application of kainate or PM was terminated with standard Krebs's solution washout, MPSS treatment (6 or 10 μM) started immediately and lasted for up to 24 h. Longer observation periods were prevented by the spontaneous deterioration of the spinal