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KAINATE AND METABOLIC PERTURBATION MIMICKING SPINAL INJURY DIFFERENTIALLY CONTRIBUTE TO EARLY DAMAGE OF LOCOMOTOR NETWORKS IN THE *IN VITRO* NEONATAL RAT SPINAL CORD

G. TACCOLA,[a,b] G. MARGARYAN,[a] M. MLADINIC[a] AND A. NISTRI[a,b,c]*

[a]*Neurobiology Sector, International School for Advanced Studies, Via Beirut 2-4, 34014 Trieste, Italy*

[b]*Spinal Person Injury Neurorehabilitation Applied Laboratory, Istituto di Medicina Fisica e Riabilitazione, 33100 Udine, Italy*

[c]*Fondazione STAND Onlus, 37100 Verona, Italy*

Abstract—Acute spinal cord injury evolves rapidly to produce secondary damage even to initially spared areas. The result is loss of locomotion, rarely reversible in man. It is, therefore, important to understand the early pathophysiological processes which affect spinal locomotor networks. Regardless of their etiology, spinal lesions are believed to include combinatorial effects of excitotoxicity and severe stroke-like metabolic perturbations. To clarify the relative contribution by excitotoxicity and toxic metabolites to dysfunction of locomotor networks, spinal reflexes and intrinsic network rhythmicity, we used, as a model, the *in vitro* thoraco-lumbar spinal cord of the neonatal rat treated (1 h) with either kainate or a pathological medium (containing free radicals and hypoxic/aglycemic conditions), or their combination. After washout, electrophysiological responses were monitored for 24 h and cell damage analyzed histologically. Kainate suppressed fictive locomotion irreversibly, while it reversibly blocked neuronal excitability and intrinsic bursting induced by synaptic inhibition block. This result was associated with significant neuronal loss around the central canal. Combining kainate with the pathological medium evoked extensive, irreversible damage to the spinal cord. The pathological medium alone slowed down fictive locomotion and intrinsic bursting: these oscillatory patterns remained throughout without regaining their control properties. This phenomenon was associated with polysynaptic reflex depression and preferential damage to glial cells, while neurons were comparatively spared. Our model suggests distinct roles of excitotoxicity and metabolic dysfunction in the acute damage of locomotor networks, indicating that different strategies might be necessary to treat the various early components of acute spinal cord lesion.

*Correspondence to: A Nistri, Neurobiology Sector, International School for Advanced Studies, Via Beirut 2–4, 34014 Trieste, Italy. Tel: +39-040-3756518; fax: +39-040-3756502.
E-mail address: nistri@sissa.it (A Nistri).

Abbreviations: BSA, bovine serum albumin; CCF, cross-correlation function; CTRL, control; CV, coefficient of variation; DAPI, 4′,6-diamidino-2-phenylindole; DR, dorsal root; FFT, fast Fourier transformation; L, lumbar (with number); NGS, normal goat serum; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; PM, pathological medium; PM+KA, pathological medium plus kainate; SCI, spinal cord injury; SNP, sodium peroxynitrite; T, threshold; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; VRs, ventral roots.

Key words: locomotion, spinal cord injury, excitotoxicity, burst, motoneuron.

Acute spinal cord injury (SCI) is a major cause of morbidity and mortality with approximately 10,000 new cases/year in the USA (McDonald and Sadowsky, 2002; Fehlings and Perrin, 2006). This health problem largely affects children as well, because pediatric SCIs are 1–2% of all pediatric fractures and are associated with the highest mortality rate of all orthopaedic injuries in infants (Leonard et al., 2007).

Regardless of its origin, SCI evolves into secondary damage affecting apparently-spared areas, magnifying the disability and leading to spinal neurodegeneration which requires neurorehabilitation and neurorepair (McDonald and Sadowsky, 2002; Dobkin and Havton, 2004; Edgerton et al., 2004; Ramer et al., 2005; Thuret et al., 2006). It is, thus, clear that early intervention is important to prevent further damage. As the high-dose methylprednisolone treatment remains controversial (Sayer et al., 2006), better understanding of the initial pathophysiological changes may help to devise new approaches based on neuroprotection (Thuret et al., 2006; Faden and Stoica, 2007).

Despite its heterogeneous causes, the early stage of SCI is believed to start with excitotoxic damage due to massive release of glutamate (Hall and Springer, 2004; Park et al., 2004; Rossignol et al., 2007) together with a pathological cascade comprising nitric oxide (NO; Hall and Springer, 2004; Pacher et al., 2007), free oxygen radicals, and metabolic dysfunction due to ischemia/hypoxia, energy store collapse, acidosis and edema triggered by loss of vascular tone autoregulation (Dumont et al., 2001; Hall and Springer, 2004; Norenberg et al., 2004). This scenario, however, leaves several unclear issues. Is excitotoxicity per se sufficient to damage spinal networks, especially the circuits responsible for locomotion which are intrinsic to the lumbar spinal cord (Grillner, 2006; Kiehn, 2006)? Can metabolic disruption contribute to the damage without a primary excitotoxic stimulus? These important questions cannot be readily answered with clinical studies or *in vivo* animal SCI models (Dietz and Curt, 2006) because of the complexity of the intertwined mechanisms.

As a model for investigating acute SCI, our study used the rat spinal cord *in vitro* subjected to distinct paradigms. The neonatal rat spinal cord preparation is advantageous to study fictive locomotion (Kiehn, 2006), intrinsic rhythmicity (disinhibited bursting; Bracci et al., 1996a,b; Taccola and Nistri, 2007), synaptic transmission at segmental level,

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and motoneuron properties. In addition, the neonatal preparation might offer insights into the phenomena underlying pediatric acute SCI. Our first protocol relied on the excitotoxicity of kainate formerly used to lesion brain (Wang et al., 2005; Pinheiro and Mulle, 2006) and spinal (Hugon et al., 1989; Magnuson et al., 1999) neurons. The second one included a pathological medium (PM) that comprised NO, H_2O_2, low Mg^{2+}, acidosis, aglycemia, hypoxia, edema to resemble the dysmetabolism of acute SCI. The third protocol combined the first two.

Monitoring spinal network electrophysiology enabled us to follow functional damage evolution during and after those paradigms, and correlated it with a histological map of damaged cells. This approach provided novel data on acute experimental lesion with a focus on the vulnerability of locomotor networks.

EXPERIMENTAL PROCEDURES

Rat spinal cord preparation

In accordance with the National Institutes of Health guidelines and the Italian act Decreto Legislativo 27/1/92 n. 116 (implementing the European Community directives n. 86/609 and 93/88), experiments were performed on thoraco-lumbar spinal cord preparations isolated from neonatal Wistar rats (0–2 days old) under urethane anesthesia (0.2 ml i.p. of a 10% w/v solution).

All efforts were made to reduce the number of animals used and to minimize animal suffering. The experimental setup is as described by Taccola and Nistri (2006a). Full details about laboratory procedures have been previously published (Bracci et al., 1996a,b, 1997; Beato and Nistri, 1999). In brief, the neonatal rat spinal cord was superfused (7.5 ml min^{-1}) with Krebs solution composed as follows (in mM): NaCl, 113; KCl, 4.5; $MgCl_2 7H_2O$, 1; $CaCl_2$, 2; NaH_2PO_4, 1; $NaHCO_3$, 25; glucose, 11; gassed with 95% O_2 5% CO_2; pH 7.4 at room temperature.

Experimental lesion protocols

Clinical studies have shown that, under the most favorable conditions, the average admission time to hospital emergency care for acute SCI is about 3 h (Bracken et al., 1990; Pointillart et al., 2000; Hall and Springer, 2004). While attempting to mimic *in vitro* the early pathophysiological events of SCI and assuming that prehospital management with stabilization of metabolic conditions should start earlier (Bernhard et al., 2005), we elected to apply toxic agents for 1 h with distinct protocols for producing spinal cord damage. This approach was also consistent with the excitotoxicity evoked by 1 h microdialysis of glutamate in the *in vivo* rat spinal cord and observed 24 h later (Liu et al., 1999b). Control preparations were kept for the same time *in vitro* (sham preparations).

In one protocol aimed at inducing excitotoxic damage to spinal networks, the glutamate analog kainate was applied for 1 h at 1 mM concentration and then washed out, while its effects were monitored for up to 24 h. This concentration of kainate was chosen because it was empirically found to be adequate for irreversible loss of fictive locomotion. Preliminary studies based on electrophysiological recordings indicated that longer times (3 h) of kainate application did not intensify the spinal network functional damage.

A more complex protocol (that was introduced because mere application of kainate left substantial spinal network survival; see Results) mimicked the conditions thought to occur in the chemical environment at the time of neuronal injury and contributing to secondary spinal damage (see Supplementary Table 1 and references therein). Thus, we applied (1 h) 1 mM kainate in a solution lacking Mg^{2+}, glucose and oxygen. In accordance with the notion that simple arrest of oxygen delivery to a spinal preparation *in vitro* produces severe hypoxia (Carlin and Brownstone, 2006), we measured (with a miniature O_2 electrode) the pO_2 values in the experimental bath under our conditions and found to be 196±2 mm Hg (n=11; 46±0.5% with respect to normal oxygenated Krebs solution). With this solution, $NaHCO_3$ was omitted and Hepes added to reach pH 6.75–6.80 (with 0.1 N NaOH). Osmolarity was 230–240 mOsm, thus recreating the typical acid and hypo-osmotic milieu surrounding an acute lesion (Dumont et al., 2001; Norenberg et al., 2004). H_2O_2 plus sodium peroxynitrite (SNP; a NO donor) were also added to produce toxic free radicals (Supplementary Table 1). This solution was termed pathological medium plus kainate (PM+KA). Preliminary experiments were run to identify the minimum concentrations of kainate, H_2O_2 and SNP capable of causing cellular death: the simplest end point was the ability to irreversibly suppress the robust disinhibited bursting induced by 1 μM strychnine and 20 μM bicuculline (Bracci et al., 1996a,b). With such a criterion, 1 mM kainate, 10 mM H_2O_2 and 500 μM of SNP were then employed as a routine for experimental injury protocols.

An additional protocol included the use of kainate-free PM to explore the consequences of metabolic disruption without a primary excitotoxic agent.

In all three protocols, in order to assess the preparation viability 1 day after treatment, electrophysiological re-recording from the spinal cord was resumed 24 h later after overnight washout in standard oxygenated Krebs solution.

Electrophysiological recording

In view of the need to record fictive locomotor rhythms for a long time, the majority of experiments were based on DC-coupled recordings from lumbar ventral roots (VRs) using tight-fitting suction electrodes (Taccola and Nistri, 2006a). As a routine, we recorded from lumbar (L) 2 (denoting the spinal segment) VRs which express mainly flexor motor signals to the hind-limb muscles, and from L5 VRs which convey mainly extensor motor commands to the same limbs. The alternation of these discharges between flexor and extensor motor pools and between left (l) and right (r) sides represents the hallmark of fictive locomotion (Kiehn, 2006; Taccola and Nistri, 2006b). Original records of these extracellular responses are calibrated in terms of time only, as their amplitude was a variable dependent on the electrode resistance, in turn directly related to the tightness of the seal between the root and the electrode itself. In a few experiments aimed at clarifying the effect of drugs on root axons, recording of the compound action potential from isolated VRs or DRs was carried out by using suction electrodes to stimulate (2× threshold, T; 0.03 Hz; 0.1 ms) one end of the root and to record the electrical response from the other end. Data were averaged (three responses) and their peak amplitude and time to peak from the stimulus artifact were measured.

Intracellular recordings (from L3–L5 motoneurons) were obtained using sharp electrodes filled with 3 M KCl (30–60 MΩ resistance; Taccola and Nistri, 2006a). The input resistance of motoneurons was measured by delivering hyperpolarizing current steps (0.1–0.9 nA, 30–50 ms) through the intracellular electrode and constructing I/V curves which were linear within the voltage range considered. All data were acquired with pClamp software (version 9.2; Molecular Devices, Sunnyvale, CA, USA) and analyzed off-line (Taccola and Nistri, 2006a).

Parameters of spinal network activities

Dorsal root (DR) electrical stimuli were employed to elicit either single VR responses (recorded from the ipsilateral VR of the same segment once every 60 s) or cumulative depolarization (1–30 V range; 0.1 ms duration; 30 pulse trains at 1–2 Hz). Stimuli were considered low T when their intensity did not exceed 2× stimulus