INTERNATIONAL SCHOOL OF ADVANCED STUDIES

STUDIES OF LOCOMOTOR NETWORKS OF THE NEONATAL RAT SPINAL CORD AFTER ACUTE SPINAL INJURY IN VITRO

Thesis submitted for the degree of "Doctor Philosophiae"

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NOTE

Part of the data reported in the present thesis have been published in the articles listed below. In all cases the candidate personally performed the experimental work and data analysis, and contributed to paper writing. Histological examination was performed by Chiara Mattioli and Miranda Mladinic.

Taccola G, **Margaryan G**, Mladinic M, Nistri A (2008) Kainate and metabolic perturbation mimicking spinal injury differentially contribute to early damage of locomotor networks in the in vitro neonatal rat spinal cord. Neuroscience 155:538-555.

Margaryan G, Mladinic M, Mattioli C, Nistri A (2009) Extracellular magnesium enhances the damage to locomotor networks produced by metabolic perturbation mimicking spinal injury in the neonatal rat spinal cord in vitro. Neuroscience 163:669-682.

Margaryan G, Mattioli C, Mladinic M, Nistri A (2009) Neuroprotection of locomotor networks after experimental injury to the neonatal rat spinal cord in vitro. Neuroscience (Acceptable subject to revision).

Nistri A, Taccola G, Mladinic M, **Margaryan G**, Kuzhandaivel A (2009) Deconstructing locomotor networks with experimental injury to define their membership. Ann N Y Acad Sci (Submitted).

ABSTRACT

Spinal cord injury (SCI) represents a significant health problem associated with life-long disability and a broad range of secondary complications. Acute spinal cord injury evolves rapidly within hours and days after the initial trauma, producing secondary damage even to initially spared areas. The early pathophysiological mechanisms affecting spinal networks remain largely obscure despite widespread incidence of this condition and its social consequences. Regardless of their etiology, spinal lesions are believed to include combinatorial effects of excitotoxicity and severe metabolic perturbations. The present study used an in vitro spinal cord model from the neonatal rat to investigate the relative contribution by excitotoxicity and toxic metabolites to dysfunction of locomotor networks, spinal reflexes and intrinsic network rhythmicity. Preparations were treated (1 h) with either kainate or a pathological medium (containing free radicals and hypoxic/aglycemic conditions), or their combination. Damage was measured by taking as outcome locomotor network activity for up to 24 h after the primary insult. Kainate led to irreversible suppression of fictive locomotion, while intrinsic bursting induced by synaptic inhibition block persisted. The pathological medium slowed down fictive locomotion and intrinsic rhythmicity. Combination of kainate with pathological medium evoked extensive, irreversible damage to the spinal cord. This phenomenon was associated with loss of fictive locomotion and intrinsic bursting as well as polysynaptic reflex depression. Thus, while suggesting distinct roles of excitotoxicity and metabolic dysfunction in the acute damage of locomotor networks, our model indicates that different strategies might be necessary to treat the various early components of acute spinal cord lesion.

Next, we investigated the role of extracellular Mg^{2+} in the lesion evoked by pathological medium, as the recent clinical trials to treat this condition with i.v. Mg^{2+} to stabilize its extracellular concentration provided disappointing results. Pathological medium in 1 mM Mg^{2+} solution (1 h) largely depressed spinal reflexes and suppressed fictive locomotion on the same and the day after. Conversely, pathological medium in either Mg^{2+}-free or 5 mM Mg^{2+} solution evoked temporary network depression and enabled fictive locomotion the day after. Although the excitotoxic damage elicited by kainate was insensitive to extracellular Mg^{2+}, 1 mM Mg^{2+} potentiated the effect of combining pathological medium

with kainate at low concentrations. These results indicate that preserving Mg^{2+} homeostasis rendered experimental spinal injury more severe.

Treatment to block the pathophysiological processes triggered by acute spinal injury remains unsatisfactory as the underlying mechanisms are incompletely understood. We further investigated the feasibility of neuroprotection of lumbar locomotor networks by the glutamate antagonists CNQX and APV against acute lesions induced by either pathological medium or excitotoxicity. Inhibition of fictive locomotion by pathological medium was contrasted by simultaneous and even delayed (1 h later) co-application of CNQX and APV. Delayed neuroprotection was accompanied by increased survival of ventral horn premotoneurons and lateral column white matter. Neither CNQX nor APV alone provided neuroprotection. Kainate-mediated excitotoxicity always led to loss of fictive locomotion. CNQX and APV co-applied with kainate functionally protected 1/3rd of preparations, although they failed when their application was delayed. Our data suggest that locomotor network neuroprotection was possible when introduced very early during the pathological process of spinal injury, but also showed how the borderline between presence or loss of locomotor activity was a very narrow one that depended on the survival of a certain number of neurons or white matter elements. The present report provides a model not only for preclinical testing of novel neuroprotective agents, but also for estimating the minimal network membership compatible with functional locomotor output.

INTRODUCTION

1. Spinal cord Injury (SCI)

1.1 First reports

Spinal injuries have been diagnosed and treated since antiquity and are still one of the most severe injuries, which lead to handicap and disability. Until the 1940s, spinal cord injury (SCI) was essentially a death sentence: "If the injury itself did not prove fatal, then the complications became fatal" (Kreutz, 2004), while after the World War II, the life prognosis and living conditions of spinally injured persons have significantly improved due to the supportive techniques and appropriate rehabilitation.

The oldest case of the spinal cord injuries is described in the Edwin Smith Surgical Papyrus, dating from the sixteenth century B.C. The manuscript reveals the anatomical observations, diagnosis and treatment of 48 types of medical problems, including collections of instructions for crushed cervical vertebra or a dislocation of a vertebra. The egyptian physician accurately described the clinical features of traumatic tetraplegia and revealed an awareness of the awful prognosis with the chilling advice: "an ailment not to be treated." This view prevailed until the early years of last century.

Further evidence of spinal cord injuries is described in Hippocratic orthopedic textbook *On Articulations,* where based on the spine deformity and direction of displacement, he categorized spinal injuries into three different types. Hippocrates invented a long-lasting device, the traction table, which was used for nearly every spinal deformity.

The descriptions of spinal cord injuries made further by Greek physician Galen of Pergamon were similar to those of Hippocrates. Additionally, he performed experiments on spinal cord of primates and having already a good knowledge of neurological topography was able to diagnose the level of the injury by observing the paralyzed muscles and the loss of sensation in different areas.

At the end of antiquity, Paulus of Aegina, the last of the great Byzantian physicians performed the first laminectomies in cases when posterior elements were fractured and pushed into cord.

While the Middle Ages were practically devoid of any advancement of spinal injury treatment, the studies of Andreas Vesalius in the Renaissance, led to a better understanding of spinal anatomy.

In the 19th century general anesthesia started with William Morton and the diagnostic relevance of X-rays discovered by Wilhelm Conrad Roentgen.

In the early 1950s, major progress was made by neurosurgeon Ludwig Guttmann who started an improved treatment based on rehabilitation and sports activities for the spinally injured, which dramatically decreased mortality. In World War I, 80% of patients with spinal cord injuries died within the first 3 years, while in World War II this rate fell to about 7%.

Since then, the development of antibiotics, the advances in rehabilitation medicine and in technologies, improvements in general medical care have resulted in gains in patient survival, care, and life expectancy.

1.2 Paralysis: facts and figures

Acute spinal cord injury (SCI) is a major cause of morbility and mortality (McDonald and Sadowsky, 2002; Fehlings and Perrin, 2006). Based on conservative average, the worldwide annual incidence of spinal cord injury is 22 per million of population and over 130,000 survivors of traumatic spinal cord injury each year who begin a "new and different life" bound to a wheelchair for 40 years or more (Rossignol et al., 2007). In comparison to Europe the incidence of SCI in the US is higher, mainly due to gunshot wounds (DeVivo, 1997; McDonald, 1999; The National SCI Statistical Center, 2001). About 40% of patients suffer from quadriplegia, 60% from paraplegia.

SCI primarily affects young adults in their mid-20s, mainly occurring among males (about 80% of cases). This raises important personal, social and economic costs, greatly varying according to the severity of injury. Although life expectancy for an individual with a spinal cord injury has improved dramatically, it remains lower than that for the general population (Charlifue and Lammertse, 2002). Mortality rates are significantly higher during the first year after injury than during subsequent years, particularly for severely injured persons.

This health problem largely affects children as well, because paediatric SCIs are 1-2 % of all paediatric fractures and are associated with the highest mortality rate of all orthopaedic injuries in infants (Leonard et al., 2007). Spinal cord injuries (about 5 %)

occur in children, usually as a result of traffic accidents or falls (Swain and Grundy, 2002).

Since 2005, motor vehicle crashes result for 42.1% of reported SCI cases. Another most common cause of SCI is falls, followed by acts of violence (primarily gunshot wounds), and recreational sporting activities (Fig. 1). Recent trends, however, show a decrease in work-related causes of injuries and an increase in sports and recreational causes (Sekhon and Fehlings, 2001).

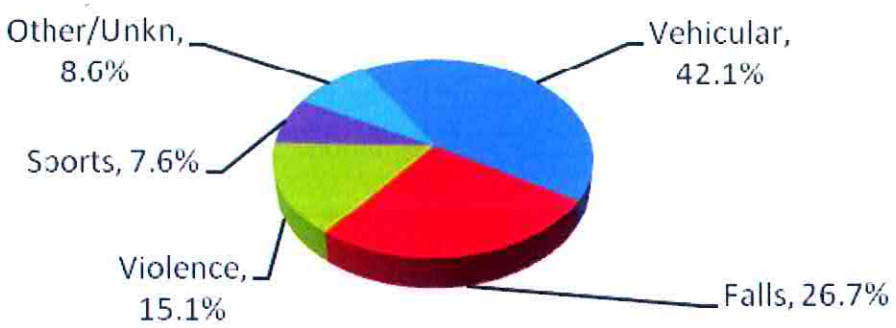


Figure 1. Etiology of SCI; from Foundation for Spinal Cord Injury Prevention, Care & Cure.

1.3 Classification of SCI

The nature and extent of spinal cord injuries vary widely, depending on the site of the injury and its type and severity. SCI patients suffer from some important handicaps that seriously diminish their quality of life (paralysis, sensory loss, intractable pain, pressure sores, and urinary and other infections). Each individual's experience is unique in terms of the degree of paralysis and pain, the extent of spasticity, and the therapies involved in stabilizing autonomic system dysfunction. Hence, how a spinal cord injury impacts a person's life is highly individualized. Injuries to the upper sections of the spine can result in quadriplegia (also termed tetraplegia), with the individual losing motor and sensory functions in the arms and legs, as well as bowel, bladder, chest, abdominal, and diaphragm function.

Injuries occurring in the lower areas of the spine may result in paraplegia (loss of movement and sensation in the lower body) or the loss of specific function.

In case of the absence of both sensory and motor functions below the level of injury, an injury is categorized as complete, and incomplete, when anyone of such functions is preserved.

The most frequent neurological category is incomplete quadriplegia (34.3%), followed by complete paraplegia (25.1%), complete quadriplegia (22.1%) and incomplete paraplegia (17.5%) (National Spinal Cord Injury Statistical Center - NSCISC, 2004). Recovery of some function and sensation largely depends on the extent and severity of injury (Levi, 2004). Persons with incomplete paraplegia or tetraplegia have higher rates of improvements in motor function that the ones with complete injury (Ditunno et al., 2000).

Clinicians have long used a clinical scale to grade severity of neurological loss. First devised at Stokes Manville before World War II and made popular by Frankel in the 1970's, the original scoring approach segregated patients into five categories, i.e. no function (A), sensory only (B), some sensory and motor preservation (C), useful motor function (D), and normal (E).

The American Spinal Injury Association (ASIA) defined an international classification based on neurological levels, touch and pinprick sensations tested in each dermatome, and strength of ten key muscles on each side of the body. The ASIA Impairment Scale

follows the Frankel scale but differs from the older scale in several important respects. These changes significantly improved reliability and consistency of the classification. According to ASIA and International Spinal Cord Injury Classification System, traumatic spinal cord injury is classifies into 5 types as shown in Table 1.

ASIA Grade	Level of Impairment
A	No motor or sensory function preserved in the lowest sacral segments (S4 and S5)
B	Sensory but no motor function preserved, including the lowest sacral segments (S4-S5)
C	Motor function present below the injury, but the strengths of more than half of the key muscles are graded < 3 of 5
D	Motor function present below the injury, but the strengths of more than half of the key muscles are graded ≥ 3 of 5
E	Motor and sensory functions in key muscles and dermatomes are normal

Table 1. ASIA Impairment Scale

This scale provides clinicians with a standard way of grading the functional severity of a spinal cord injury:

A indicates complete sensorimotor loss below injury level.

B indicates complete motor loss, sensory incomplete, including sacral levels (to S4-S5) Usually it is a transient phase and individuals recovering any motor function below the neurological level basically show an incomplete motor lesion, i.e. ASIA C or D.

C indicates motor incomplete, but half the key muscles below the injury have less than functional muscle strength (muscle grade < 3 / 5).

D indicates motor incomplete with more than half the key muscles below injury having functional muscle strength (muscle grade ≥ 3/5).

E indicates normal motor and sensory function. It is possible to have spinal cord injury and neurological deficit with completely normal motor and sensory scores.

1.4 Phases of SCI

Injury to the spinal cord triggers a cascade of biological processes that unfold within seconds and proceed for months or even years. These processes affect three major bodily systems: the nervous system, the immune system, and the vascular system. These three systems interact dynamically as they respond to injury. Though some injurious responses heal and promote the recovery of function, others leave a wave of tissue damage that expands well beyond the original site of injury.

The pathological processes after SCI are divided into three distinct time stages: the acute, intermediate and chronic phases.

The acute phase (seconds to minutes after the injury) is caused by direct mechanical damage and marked by systemic as well as local events (Tator et al., 1998; Hulsebosch, 2002). The primary systemic event is a prolonged decrease in blood pressure (hypotension) that coincides with a decrease in blood volume. A condition, known as spinal shock and resulting in temporary failure or depression of all or most spinal reflex below the level of the injury activity for the first 2 to 24 hours after injury, is known to occur in the acute stage and has still unclear origin and mechanisms (Atkinson and Atkinson, 1996; Ditunno et al., 2004).

Cell death of the affected neural and endothelial tissues at this stage is immediate and, therefore, not suited for therapeutic intervention.

The intermediate phase takes place from minutes to weeks after SCI. Most motor deficits that follow the primary mechanical injury are exacerbated by secondary mechanisms. First, damage expands vertically, through the gray matter, afterwards it spreads further horizontally within the white matter. Consequently the paralysis can affect the higher segments. Due to the delayed nature of these events, the secondary phase of SCI is the most appropriate target stage for therapeutic intervention.

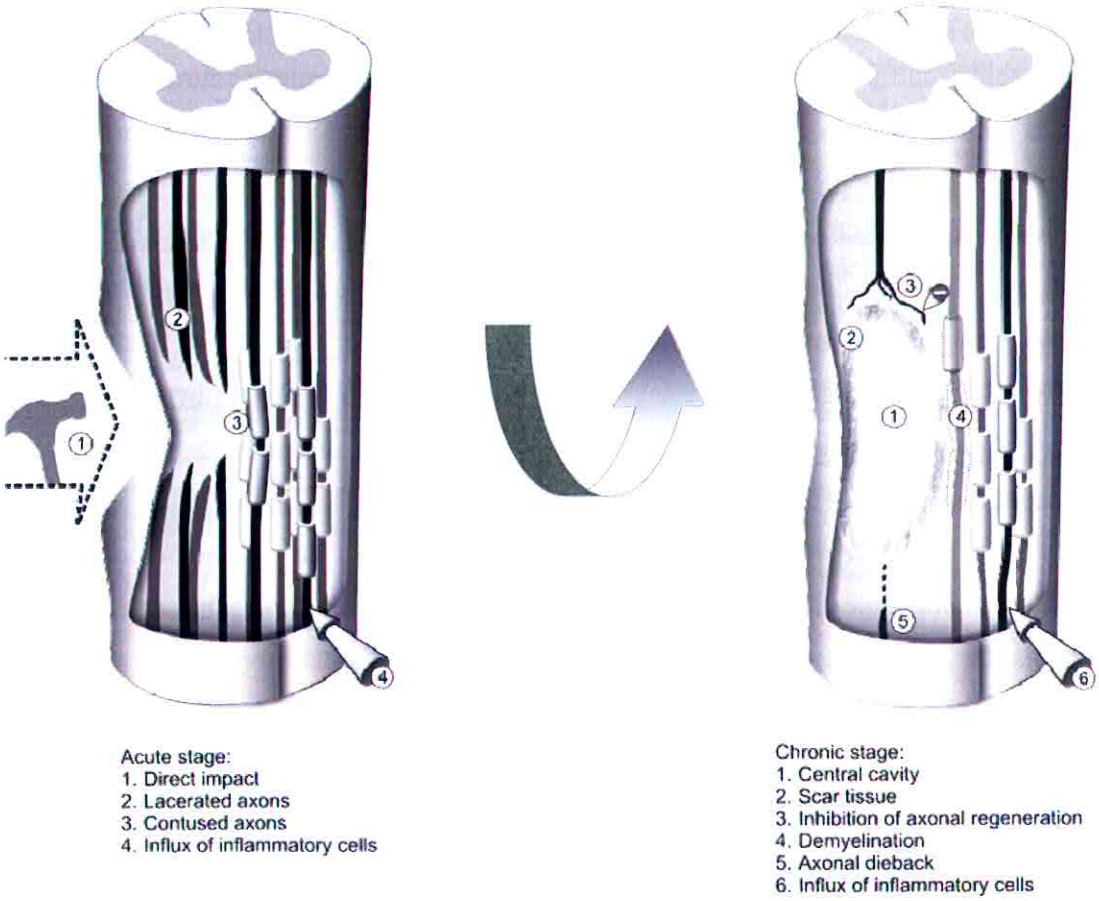


Figure 2. Acute and chronic stages of SCI (Ronsyn et al., 2008)

The chronic phase starts days after injury, lasting up to years. It is characterized by ongoing demyelination and apoptosis of cells along the spinal cord and brain and marked by the emergence of a new type of pathology such as formation of fluid-filled cavities or glial scar (Fitch and Silver, 2008). The cavity creates a physical gap that blocks axon regrowth, whereas the glial scar contains substances that inhibit axon regrowth. (Schwab et al., 2006).

Such a process of degradation of neuronal activities with time has clinical consequences as it questions the beneficial effect of future regeneration attempts in chronic patients with SCI (Dietz and Muller, 2004).

1.5 Secondary injury

Although the development of the secondary injury is not well understood, it involves a cascade of intricate processes that ultimately lead to neuronal cell death by mechanisms

of necrosis and apoptosis (Fig. 3). Usually it affects apparently-spared areas, magnifying the disability and leading to spinal neurodegeneration which requires neurorehabilitation and, hopefully, neurorepair (McDonald and Sadowsky, 2002; Dobkin and Havton, 2004; Edgerton et al., 2004; Ramer et al., 2005; Thuret et al., 2006).

The early stage of SCI starts 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 involving 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).

Early on, after a few minutes following mechanical damage, the membrane potential is lost and thereby the neuronal and glial cells become depolarized. This activates the presynaptic, voltage-dependent Ca^{2+} channels and results in the release of excitatory amino acids (glutamate, aspartate, homocysteate, etc.) into the extracellular space. Within minutes after SCI, glutamate levels are amplified six- to eightfold. In addition to the mechanical disruption of cells by the primary insult, synaptic and nonsynaptic transport processes also contribute to the accumulation of glutamate, which finally becomes cytotoxic. The release of excitatory amino acids then activates the N-methyl-D-aspartate receptors (NMDA-R), inducing a further neuronal Ca^{2+} overload, which finally results into a massive neuronal Na+ influx and a K+ efflux. It has also been reported that chloride homeostasis may be affected after adult SCI (Vinay et al., 2006). One of the most damaging consequences of intracellular Ca^{2+} accumulation is the activation of phospholipase A2 and release of arachidonic acid (AA), which is then converted by cyclooxygenases (COX 1, 2) to a number of deleterious prostanoids, as well as activation of the calcium-activated cysteine protease calpain, which results in cytoskeletal degradation (Fig. 4).

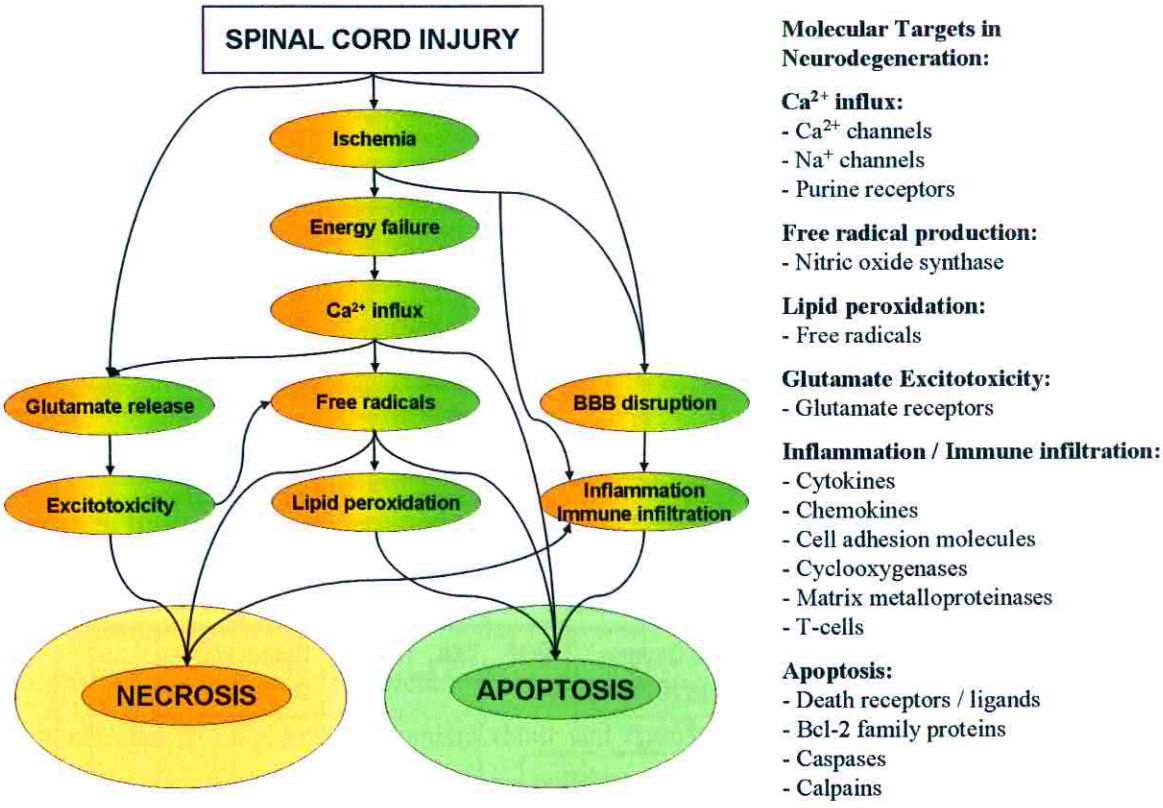


Figure 3. Neurotoxic events following SCI (Klussmann and Martin-Villalba, 2005)

Vasospasms and damage to the vasculature lead to local ischemia in the area surrounding the lesion epicenter. Insufficient oxygen supply induces a shift of metabolism from aerobic respiration towards anaerobic glycolysis, leading to exhaustion of further ATP production. The following energy depletion due to the rising metabolic dysfunction results in expansion of the damage through ischemic cell death.

The process of free-radical formation begins in the mitochondria and the perturbation of the Ca^{2+} homeostasis after injury results in mitochondrial respiratory electron transport defects (Young, 1992). Free-radical formation, usually from oxygen atoms, leads to a series of pathological reactions inside cells, including the breakdown of lipids in the cell membrane, a process known as lipid peroxidation. Reactive oxygen species can also attack membrane enzymes, distort ion gradients across the cell membrane, and damage genes.

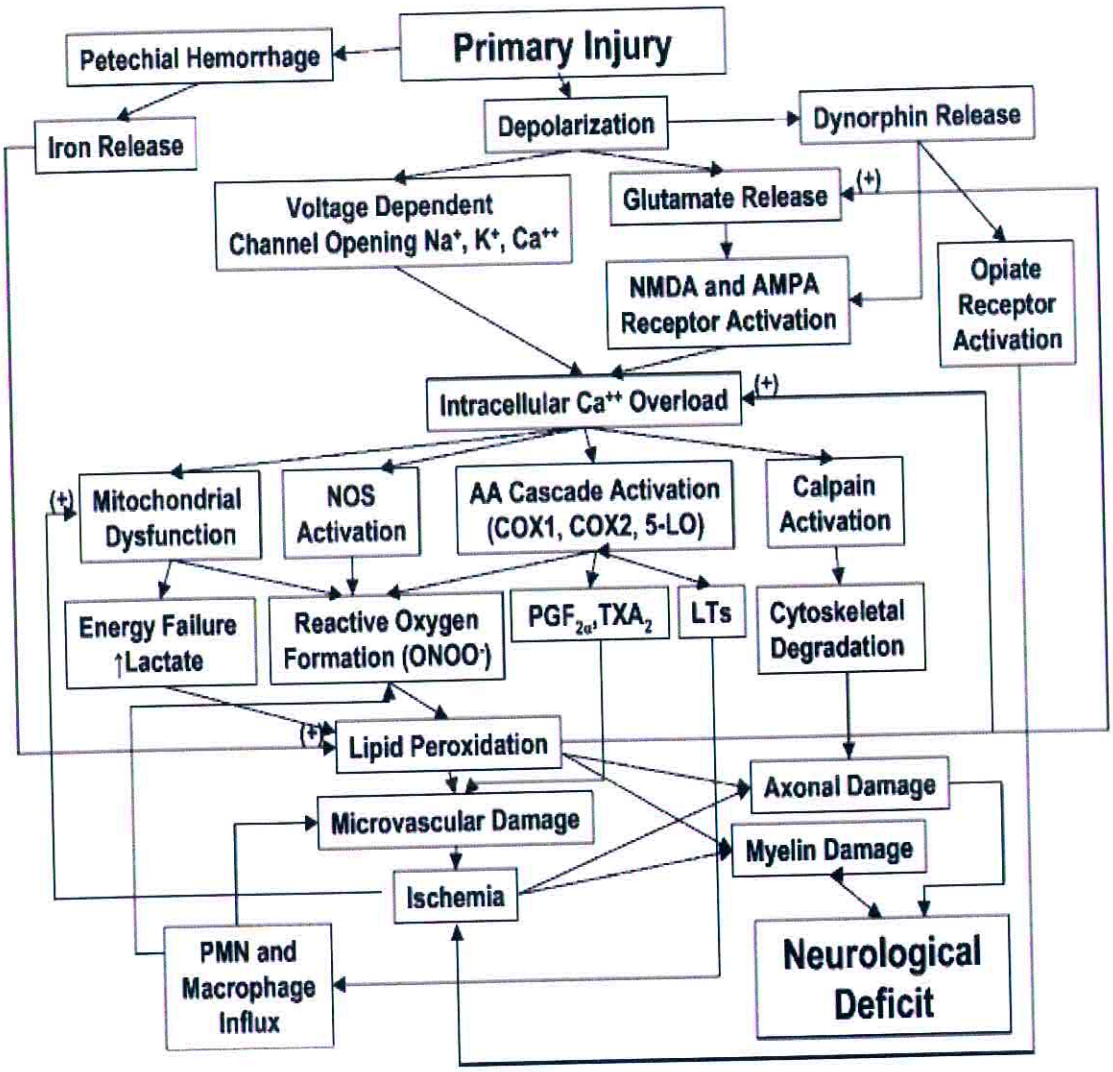


Figure 4. Pathophysiology of secondary injury (Hall and Springer, 2004)

Among ROS, hydrogen peroxide and its derived hydroxyl radical are known to play an important role in posttraumatic oxidative damage. Another free radical that has been implicated in the development of damage after SCI is nitric oxide (NO). The expression of nitric oxide synthase (NOS), especially of the inducible isoform, iNOS, is strongly increased after injury. Recently, Beckman has introduced the theory that the principal ROS involved in producing secondary tissue injury is peroxynitrate (Xiong et al., 2007), formed by reaction between nitric oxide and super oxide radical (Fig. 5). It is also believed that the potent oxidizing ability of peroxynitrate is actually due to its

decomposition products that possess potent free radical characteristics (Xiong et al., 2007).

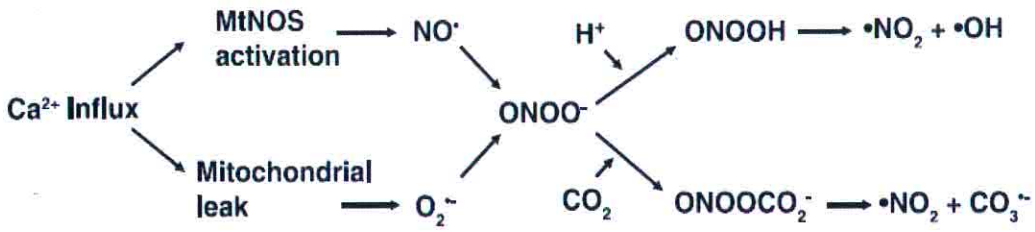


Figure 5. Biochemistry of peroxynitrate formation (Xiong et al., 2007).

Peroxynitrite can trigger cellular damage by a variety of mechanisms, whereas cell membrane (plasma and organelle) lipid peroxidation (LP) has been conclusively demonstrated to be a key mechanism (Hall and Braughler, 1989; Hall and Braughler, 1993; Hall 1996). Nonetheless, iron is also a powerful catalyst, which accelerates the propagation of LP reactions. Endogenous opiates, such as dynorphin A, can exacerbate the secondary injury process by stimulating NMDA receptors and by activating opiate receptors, thus leading to vascular dysfunction, and ionic and metabolic disturbances (Faden and Salzman, 1992; Faden 1997).

Inflammation is a hallmark of the secondary phase of injury. Immune cells secrete a set of proteolytic enzymes (proteases), which degrade the proteins of the cytoskeleton, e.g., actin and spectrin, and extracellular matrix proteins like laminin. Thereby, the function of the blood–brain barrier (endothelial integrity, basal lamina) is impaired resulting in propagated edema formation. These cells surround the damaged area, eliminating debris and releasing a host of powerful regulatory chemicals that are both beneficial and harmful.

The inflammatory response to injury involves four major categories of immune cells: neutrophils, monocytes, microglia, and T-lymphocytes (Schnell et al., 1999; Bareyre and Schwab, 2003). The neutrophils are the first immune cells to arrive at the site of injury, which release cytokines, proteases, and free radicals, activating other inflammatory and glial cells and leading to neuronal injury or death. Microglia, residing within the spinal cord, responds over the next 24 hours of injury in earnest. As the monocytes penetrate the spinal cord tissue from the circulatory system, they differentiate into macrophages and

start to eliminate degenerating fiber tracts and other tissue debris by phagocytosis. They also secrete several cytokines, free radicals, and growth factors, which, in turn, influence nearby cells in positive and negative manner (Lindholm et al., 1992; Schnell et al., 1999; Anderson, 2002). Both free radicals and proinflammatory cytokines contribute to extension of the lesion, worsening the impact of the injury. Activation of macrophages and microglia continues over the course of weeks. The role of lymphocytes in spinal cord injuries is somewhat controversial. Some argue that one type of lymphocyte (autoreactive T-lymphocyte) has destructive properties (Popovich and Jones, 2003), while others insist that this lymphocyte protects myelin-insulated neurons (Schwartz and Kipnis, 2001; Kipnis et al., 2002).

Until recently, most cell death in spinal cord was attributed to necrosis, primarily during the acute phase of injury, as the cells swell and break open upon mechanical insult. Recent experiments have shown that same cells die as a result of apoptosis, a programmed cell death. The most affected ones are oligodendrocytes, which reach highest levels at about 1 week after injury (Liu et al., 1997; Beattie et al., 2000). Afterwards, several neurons die in previously unaffected remote areas like degenerating fiber tracts of the spinal cord or in the motor cortex of the brain.

Apoptosis has been detected in humans (Emery et al., 1998) and lasts for about one month in animal models (Beattie et al., 2000). With apoptosis, cells condense and break apart into small fragments in a very orderly process that requires energy and protein synthesis. Caspases, a family of cysteine-aspartate proteases, play a key role in the execution of programmed cell death. With extracts from injured spinal cords it was shown that caspase-3 quickly becomes activated after SCI and cleaves several of its downstream targets leading to apoptosis.

As there are multiple factors involved in the development of secondary damage, the ideal therapy should act at different levels. Whereas most treatment approaches are based on rational design with the idea to interfere with one or the other component of the overall pathophysiology, it is thus important to better understand the initial pathophysiological cascade of changes following spinal injury in order to establish therapeutic strategies and to devise new approaches based on neuroprotection (Thuret et al., 2006; Faden and Stoica, 2007).

1.6 Animal models of SCI: from the laboratory to the clinic

Animal models permit deep investigation of the anatomical, physiological and molecular changes that occur in response to a spinal cord injury at a level of detail that would not be possible or ethical with human studies. These approaches are critical for the design and interpretation of the results of studies with humans, as without the knowledge coming from animal studies, the spinal cord would remain the equivalent of a black box and therapies aimed at restoring function would be restricted. Animal models are developed in a way that mimics different attributes associated with spinal cord injuries. Each type of experimental lesion model has advantages and limitations.

Maiman has suggested the following criteria for evaluating the SCI model: "First of all, a spinal cord injury model should be physiologic: that is, it should accurately reproduce the anatomic and functional changes seen in clinical injury. Second, it must be both quantifiable and reproducible, so that the effects of modifying agents can be determined" (Maiman et al., 1983).

On the basis of pathology, there are at least four general types of spinal cord injuries: contusion, compression, laceration, and solid cord injuries. Contusion is the most common type of spinal injury. The spinal cord is bruised, but not severed; the consequence is inflammation and bleeding from the vessels near the injury site. Compression is caused by pressure on the spinal cord. Laceration results in severing or tearing of the spinal cord, typically from gunshot or knife wounds. Solid cord injuries count for 17% of all spinal injuries resulting in axonal injury and demyelination (Bunge et al., 1993, 1997; Harper et al., 1996; Hulsebosch, 2002).

Injury models produced by complete or partial transection of the spinal cord have been studied extensively (de la Torre 1984; Young 1989) as they can reveal structure-function relationship (Goldberger et al., 1990). This model is more appropriate for those studies which evaluate strategies targeting axon regeneration or which require implantation of a specific device. However, this model is limited as it is not commonly encountered in clinical settings (Young 1989).

Because compression and contusion types of SCI are the most commonly seen injuries in humans, contusion models may be more appropriate for assessment of acute management strategies (Hughes 1988; Young 1989). The first attempt to mimic these forms of injury experimentally was the weight-drop trauma model developed by Allen in 1911. A number of alternative methods for external compression have been further devised to

injure the spinal cord, ranging from application of a surgical spring-loaded clip or balloon to the computer-controlled reproducible impact contusion devices (Metz et al., 2000; Borgens 2001; Stokes and Jakeman, 2002). Surgical clip or balloon compressions simulate the displacement and continual pressure to the spinal cord (Rivlin and Tator, 1978). Their advantages include the ability of precise control of the length and magnitude of the compressive injury. In addition, balloon compression can be used percutaneously and does not require a laminectomy to create the injury. Currently, the most popular animal models of spinal cord injury are hemisection, temporary application of an aneurysm clip or graded contusion injuries delivered to the thoracic spinal cord (Wrathal et al., 1985; Anthes et al., 1991; Gruner 1992). Such models principally examine the functional deficits resulting from the interruption of ascending and descending white matter tracts, and recovery of function in these models must therefore rely heavily on axonal regeneration and remyelination. For certain experimental paradigms, a combination of models might also be useful (Talac et al., 2004).

Small animals such as rats and mice are preferred for many experimental studies as they have short life cycle, are inexpensive to purchase and easy to maintain in limited space. Depending on the treatment, it may be worthwhile to examine the efficacies of some therapies in primate models. However, there are also restrictions to the use of non-human primates for mimicking human responses; for example, metabolism of anti-rejection drugs in non-human primates and human is essentially different. As a result, rodents have been used as the favored model to study the efficacies of new therapeutic agents, also because the metabolism of humans and rodents is very similar. In addition, rabbits and cats, which have larger spinal cords and are less expensive and easier to maintain than primates, are also used for experimental purposes.

Although it is important to test therapeutic interventions in animals, animal models have both advantages and disadvantages and are continuing to be a matter of discrepancies between scientists and clinicians. The term ''model'' implies deviation from reality, usually by simplifying and reducing variables (Hartung 2008). Genetic differences between animal species can potentially result in different responses to spinal cord injuries or treatments. The human spinal cord is more than four times as long as the rat's entire CNS (brain and spinal cord). The diameter of the human spinal cord is also much larger than that of the rat spinal cord. Twenty slices of a rat spinal cord can fit inside one slice of a human cord (Dobkin and Havton, 2004). A contusion or transection trauma in humans can affect upwards of 2 to 3 centimeters of the spinal cord, which is

approximately 10 times the length of the 1 to 3 millimeters often affected by contusion injuries in rats (Metz et al., 2000). There are basic differences between rats and human beings concerning the mode of locomotion (ie, bipedal versus quadrupedal) and the autonomic nervous system function, which has a much greater role in man than in rats (Dietz and Curt, 2006). In rats with SCI, a basic form of automatic selftraining prevails, which facilitates recovery of locomotor function (de Leon et al., 1999a). Despite these limitations, laboratory models have proven relevant to human SCI. A recent review by the Nuffield Council on Bioethics concluded that "animal research has been, and can potentially be, scientifically valid, in that it is possible to extrapolate from animal models to humans (or other animals)" (Nuffield, 2005).

1.7 Preclinical and Clinical Testing of New Therapies

There are several prescribed series of steps that validate and ensure the safety and efficacy of any therapeutic intervention designed in the laboratory before being approved for patient use. Although these steps in drug development and approval are time-consuming and expensive, they are designed and regulated to ensure patient safety. Before pharmaceutical companies start clinical trials on a drug, they conduct extensive preclinical studies. Efficient preclinical tests can ensure that the most promising potential therapies proceed rapidly to clinical testing. It takes at least 3 years, and often longer than 7 years, for a potential therapeutic compound to be identified and for the preclinical research to be conducted. Randomized, controlled clinical trials are the gold standards for revealing the benefits and negative aspects of a particular therapy.

Clinical trials involving new drugs are commonly classified into four phases. Each phase of the drug approval process is treated as a separate clinical trial.

	Phase		Description
Pre-Clinical Trials	Laboratory toxicology		Gain information on toxicity and doses
	Regulatory toxicology		**A** – Repeated dose-toxicity testing
			B – Long term toxicity and carcinogenicity tests
Clinical Trials	**Phase 1**	**A**	Single dose trials in healthy volunteers
		B	Repeated dose trials in healthy volunteers
	Phase 2	**A**	Small number of patients
			Decision to proceed based on trial information this far
		B	Larger number of patients-determines dose regimes
	Phase 3		Large-scale International Randomised Controlled Trials (RCIs)
			Licensing and Marketing
	Phase 4		On-going observation after marketing

Table 2. Drug development phases (English, 2009)

Phase I clinical trials determine safety, treatment dosage and regimen of the intervention. Phase II and phase III clinical estimate the efficacy of the new intervention and examine side effects in studies with larger populations. A novel drug, entering a phase I clinical trial has approximately 30 to 40 percent chance of successfully completing a phase III clinical trial and being approved by the Food and Drug Administration (FDA) (Harding, 2004). Phase IV clinical trials are required by the FDA for additional analysis of long-term risks and benefits.

In 2001, the American Society for Neural Transplantation and Repair developed a series of guidelines that recommend safety studies to be conducted with the best available model—or in the case of spinal cord injuries, multiple models—before the therapy is tested on humans (Redmond et al., 2001). Exclusively animal models should be used to examine potential toxicities and harmful complications (Dietrich, 2003).

1.8 Current Interventions into SCI therapy

Numerous human clinical trials of possible neuroprotective therapies after SCI were tested in the 1980s and 1990s (Mirza and Chapman, 2001); yet, none of them conclusively demonstrated a benefit for increasing function after a spinal cord injury. Current clinical guidelines propose the administration of high dose i.v. methylprednisolone within first 8 hours after the spinal injury, aimed at reducing the formation of cytotoxic edema, inflammation and the release of glutamate and free radicals, while its efficacy and safety in human injury is highly controversial (Short 2000; Hurlbert 2001; Sayer et al., 2006). It has been stated that the data describing improved recovery from treatment with this corticosteroid are weak and that the improvements observed may represent random events (Hurlbert, 2000). In the United States this therapy is frequently applied, on the contrary not all European centers are using methylprednisolone because of its serious side effects, such as higher infection rates, respiratory complications, pneumonia and gastrointestinal hemorrhage. In rodent models of SCI, treatment with methylprednisolone resulted in only a weak (or no) increase in functional recovery when compared with other therapeutic strategies (Rabchevsky et al., 2002; Takami et al., 2002). The usefulness of this treatment remains very controversial (Sayer et al., 2006) and the need for more beneficial pharmacological agents aimed at neuroprotection is widely perceived (Thuret et al., 2006; Faden and Stoica, 2007).

An additional pharmacological therapy, the ganglioside GM-1, a lipid that is abundant in mammalian central nervous system membranes, was also reported to show improvement in animal models, but it has not been found to be useful in humans. The potential therapeutic value of this substance is attributed to its ability to prevent apoptosis and to induce neuronal sprouting in animal models. Conversely, the findings from a large-scale clinical trial were negative when the results for the treated group were compared with individuals who received placebo.

Aside from cyclooxygenase inhibitors (Hurley et al., 2002; Schwab et al., 2004), which have an anti-inflammatory effect, reducing the formation of free radicals, recently erythropoetin (EPO) has been discovered as a neuroprotective agent (Goldman and Nedergaard, 2002; Gorio et al., 2002). Besides its anti-inflammatory action (Agnello et al., 2002), EPO also possesses protective characteristics, normalizes the disrupted autoregulation of the vessel tone and suppresses expression of the inducible nitric oxide synthase. Unfortunately, it interferes with rheology and causes an augmented hematocrite

and an increased aggregation of thrombocytes. It has also been found that systemic application of minocycline, a CNS permissive tetracycline antibiotic derivate, was able to reduce the gliotic response (microgliosis, astrocytosis), to diminish apoptotic cell death of oligodendrocytes, and finally to attenuate axonal dieback improving functional outcome following SCI (Stirling et al., 2004; Teng et al., 2004). The opiate receptor antagonist naloxone was also tested in the Second National Acute Spinal Cord Injury Study (NASCIS II) based upon the demonstration of its beneficial effects in SCI models. Although it did not show a significant overall effect, some evidence of efficacy was seen in incomplete (i.e., paretic) patients (Hall and Springer, 2004). Similarly, experiments with rodents (Behrmann et al., 1994) and cats (Faden et al., 1981) have demonstrated that thyrotropin-releasing hormone (TRH) can significantly improve long-term motor recovery after a spinal cord injury, while again a large-scale randomized clinical trial examining the effects of TRH analogs on individuals with acute spinal cord injuries was not fully completed (Pitts et al., 1995), and such an evaluation has not been revised.

The K+ blocker 4-aminopyridine (4- AP), which inhibits the activation of the fast, voltage-dependent K+ channels and increases membrane resistance at axon sites with an insufficient myelin sheath, has been reported to restore some sensorimotor functions in patients with spinal lesions (Waxman 1993; Grijalva et al., 2003). However, a recent phase III study failed to prove its clinical efficacy. Attempts to protect central nervous tissue by blocking excitotoxicity with glutamate antagonists or free radical scavengers have also yielded disappointing results that have even lead to dismiss the possibility of clinical neuroprotection against trauma or ischemia (Thuret et al., 2006; Faden and Stoica, 2007; Savitz and Fischer 2007). Their failure has been mainly attributed to inadequate preclinical models and, in particular, poor understanding of the timecourse of the damage development.

Magnesium has been shown to have neuroprotective properties in experimental brain injury and spinal cord ischemia (Robertson et al., 1986; Simpson et al., 1994; Heat and Vink, 1999; Lang-Lazdunski et al., 2000; Ustun et al., 2001). Indeed, a large multicenter trial of Mg^{2+} infusion into acute brain lesion patients has been completed with very unsatisfactory outcome (Maas and Murray, 2007; Temkin et al., 2007). Such clinical results have actually shown that many patients receiving Mg^{2+} infusion fared worse than untreated patients, a result unexpectedly found also for stroke patients (IMAGES, 2004).

Neural prostheses present another approach for improving the quality of life after spinal cord injury. These devices, such as hand-grasp prostheses, connect with the nervous

system to supplement or replace lost motor or sensory function. They electrically stimulate peripheral nerves, either through surface electrodes attached to the skin or through electrodes implanted in close proximity to nerves. Implantable neural prosthesess have also been developed to restore limb movements. Epidural spinal cord stimulators and deep brain stimulators are routinely implanted to control pain, spasticity, tremor and rigidity.

Rehabilitation can greatly improve SCI patients' health and quality of life. In spinal animals training for a motor task stimulates reorganization of spinal locomotor networks, thus generating locomotor pattern (Edgerton et al., 1997; Pearson 2000; Barriere et al., 2008). Reorganization of spared neural pathways can lead to the considerable recovery of locomotor activity in SCI mammals (Curt et al., 2004; Edgerton et al., 2004). Stepping can be performed more successfully in trained spinalized cats than in ones in which it is not practiced (Lovely et al., 1986, 1990). Upon intensive training, even in the absence of supraspinal input, cat neuronal networks below the level of the lesion can adapt to generate locomotor activity (De Leon et al., 1998a, b). Similar beneficial effects of training have been observed in acute complete paraplegic patients (Wirz et al., 2001).

Using specific training approaches, such as robotic devices to induce controlled stepping movements can lead to the plasticity of spinal neuronal circuits in SCI patients (Dietz et al., 2002). For assisted walking, the driven gait orthosis (DGO) 'Lokomat' (Hocoma AG, Zurich) mounted on a treadmill is used (Dietz and Muller, 2004). DGO provides the drives for hip and knee joint movements of each leg, whereas the dorsiflexion of the ankles during the swing phase is achieved by passive foot lifters (elastic straps) (Fig. 6). Unloading is achieved by a parachute harness connected to counterweights. The DGO is fixed to the subjects with straps around the waist, the hip, the thigh and the shank. However, only incomplete paraplegic patients profit from locomotor training on a treadmill with partial body unloading (Wernig et al., 1992, 1995; Barbeau and Rossignol, 1994; Dietz et al., 1995; Kojima et al., 1998), whereas complete or severely affected patients do not profit from locomotor training (Wirz et al., 2001). In these patients, the recovery of walking might perhaps be improved with a combination of such training approaches and some regeneration of injured spinal tract fibres (Dietz and Muller, 2004)

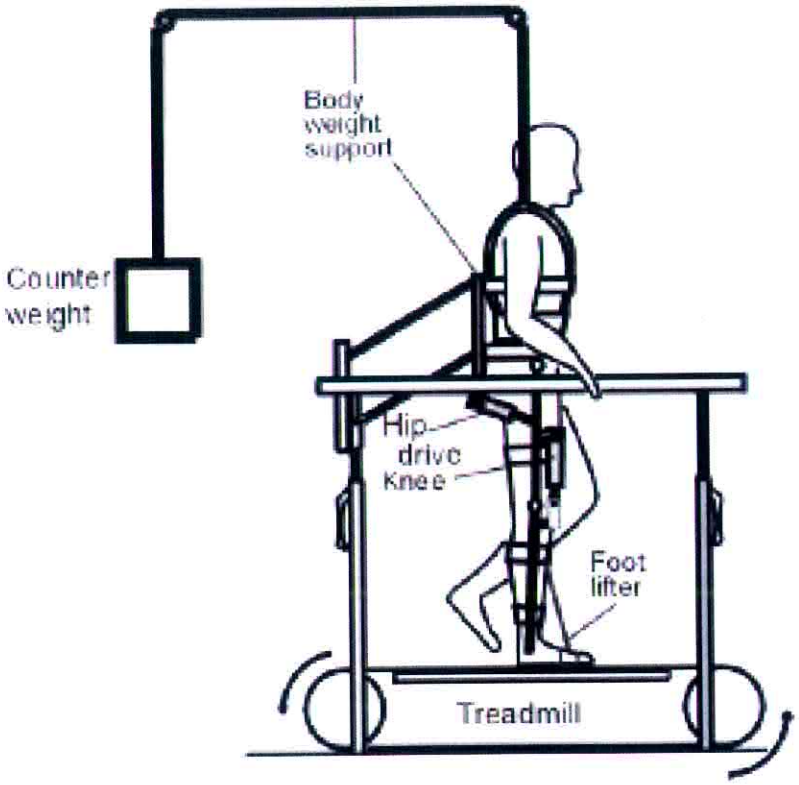


Figure 6. Experimental set-up, locomotion in a treadmill within a DGO (Dietz and Muller, 2004).

A recent study has shown that the levels of leg extensor muscle activity recorded in clinically complete SCI subjects significantly improved over the course of several weeks of step training (Wirz et al. 2001). Interestingly, the levels of extensor muscle activity decreased over a three-year period following the training program. Globally, these results point toward a use-dependent phenomenon that may exist for the human spinal cord like the one reported for spinal cats (de Leon et al. 1999a, b). In complete SCI subjects stepping can improve in response to training, but so far the improvement has not reached a level that allows complete independence from assistance during full weight-bearing.

2. Neurophysiological principles behind Central Pattern Generators (CPGs)

2.1 Introduction to CPG

The brain and spinal cord neuronal systems involved in the initiation of locomotion have been extensively studied over the last several decades. For the large number of vertebrate species it is now indisputable that the neuronal networks in the spinal cord known as central pattern generators (CPGs) generate much of the timing and pattern of the rhythmic, coordinated muscle activities even in the absence of sensory inputs or descending signals from supraspinal structures. Although animals typically exhibit quite varied patterns of motor behavior, such as breathing, chewing, swimming, scratching and walking, the CPGs underlying locomotion are recruited as a component of most goal-directed patterns of vertebrate behavior.

As early as 1911, the idea, favored by Sherrington, that complex motor behaviors, including locomotion, were generated by chains of reflex actions, was countered by Brown, who provided evidence that intrinsic networks in the spinal cord can generate rhythmic locomotor-like patterns of activity with alternation of flexor and extensor muscles pools. This observation gave rise to the concept of the CPG. Due to the vast complexity of the mammalian CNS (central nervous system), to understand the general principles of CPG organization it has become necessary to use simple, experimentally amenable model systems with relatively small circuits, containing few neurons. The lamprey has been used as a model for vertebrate CPGs, as its nervous system has a vertebrate organization, sharing many positive characteristics with invertebrates (Grillner et al., 1995). Considerable knowledge have arisen also from invertebrate preparations, including leech heartbeat system, the crustacean stomatogastric nervous system, Tritonia and variety of insect preparations in which rhythmic motor patterns are activated by different neuromodulators (Marder and Calabrese, 1996). CPGs contribute to locomotion in higher animals and humans. Calancie claimed to have witnessed the first well-defined example of a central rhythm generator for stepping in the human adult (Calancie et al., 1994). Further evidence for human CPG comes from experiments, in which specific sites of spinal cord were electrically stimulated, demonstrating that tonic stimulation of

completely injured spinal cord can produce locomotor-like pattern of activity (Dimitrijevic et al., 1998). Although it is likely that the human pattern generator for walking has retained some (or many of the) characteristics found in other vertebrates, it is not a foregone conclusion (Capaday 2002; Nielsen 2003). The behavior of the pattern generator for walking was further studied in human infants, by supporting them to step on a split-belt treadmill, and have concluded that the pattern generator for each limb is autonomous, but it interacts with its complement for the contralateral limb (Yang et al., 2005).

The human CPG is very adaptable and robust. Networks responsible for human walking adapt to short and long timescales as well as to different gait patterns and walking contexts (Choi and Bastian, 2007). Forward and backward walking can be controlled independently as well as networks controlling each leg can adapt independently and be trained to walk independently (Choi and Bastian, 2007).

2.2 Operation of CPG

In quadruped mammals significant advance in our understanding of the CPG operation has been obtained by combining experimental data with a model in which the CPG is thought to comprise two main components, namely a "clock" that starts the program, sets its locomotor frequency and is hierarchically above the "pattern" formation responsible for producing the correct phase and sequence activation of motoneurons (Lafreniere-Roula and McCrea, 2005). Such a bilayered structure of CPG was proposed as a result of examining deletions of rhythmic motoneuron activity during fictive locomotion and scratch (Fig. 7). Deletions are reductions or absences of one or more expected rhythmic bursts of activity in multiple agonist motoneuron pools, which can occur spontaneously and during rhythmic fictive locomotion and scratch. Pattern formation modules comprise the circuitry for reciprocal inhibition of antagonist motor pools and control the activity of subsets of motoneurons. Deletions, including a change in the locomotor rhythm, affect the rhythm generator network, whereas deletions in which cycle timing is maintained are assumed to occur by disturbances at the level of the pattern formation networks and may affect individual pattern formation networks differentially. The experimental evidence for Lafreniere-Roula and McCrea's model is: (a) afferent perturbation can alter motor pattern activity without changing the rhythm (Burke et al., 2001), (b) a change of amplitude can occur independent of a change in period (Kriellaars et al., 1994), (c) deletions of muscle

activity can occur in individual muscles without resetting changes in locomotor frequency.

In the classic half-center model of the CPG, proposed by Brown in 1911, the same network is responsible for both the generation of cycle timing and the excitation of motoneurons. In this model, populations of interneurons in the flexor and extensor half-centers excite flexor and extensor muscles throughout the limb and their simultaneous activity is prevented by mutual inhibitory interconnections between the half-centers. A high level of excitability within the half-center generates activity that is restricted by a fatigue process, which continuously repeats and the system oscillates. A failure of the fatigue process would result in the system's being locked in its current state of flexion or extension and a continued inhibition of the antagonist halfcenter. A key aspect of the half-center model is that the interneurons comprising the half-centers are in charge for both the generation of timing as well as the excitatory drive to agonist motoneuron pools.

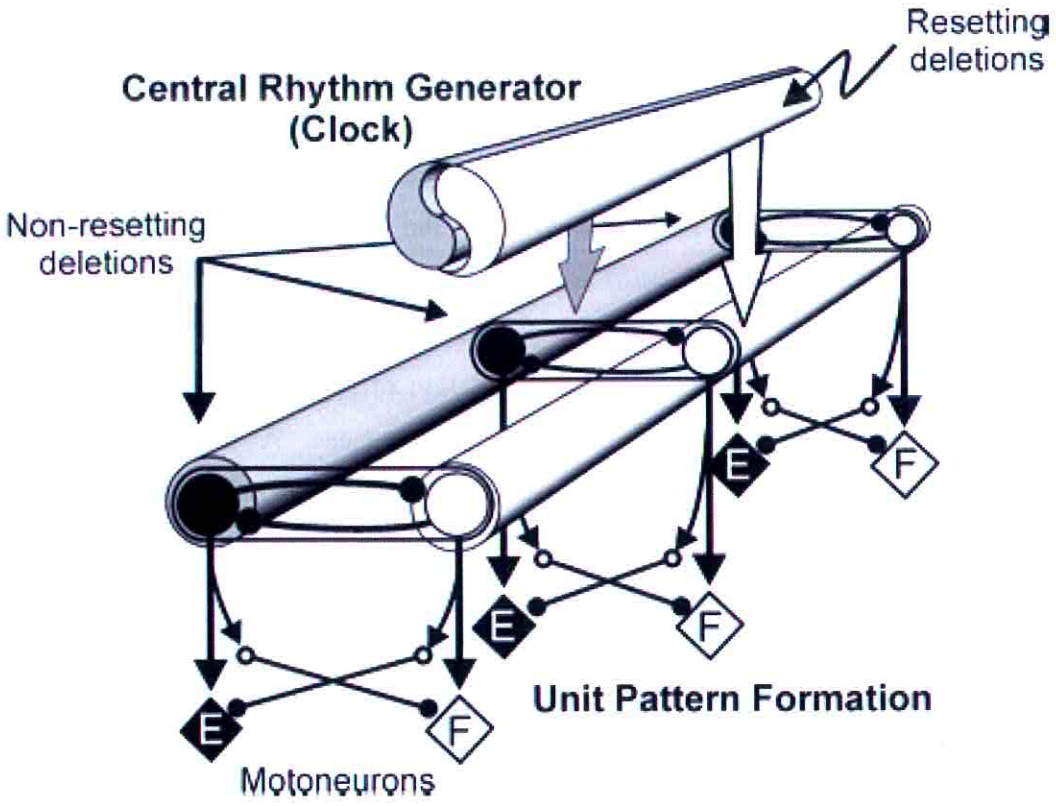


Figure 7. Schematic representation of the proposed architecture for the CPG, which consists of separate networks for rhythm generation (clock) and for pattern formation (Lafreniere-Roula and McCrea, 2005).

The advantages of a mammalian CPG organization for locomotion and scratch consisting of multiple oscillators and displaying independent rhythmicity have been also reported (Grillner 1981; Stein and Smith 1997; Stein 2005), while in this organization, oscillators, referred to as unit burst generators, would operate on a limited number of motoneuron pools (Grillner 1981). The structure represented in Fig. 6 has a single rhythm–generation module along with several pattern-formation modules. It could be described as a Central Rhythm Generator controlling several Unit Pattern Generator modules each responsible for excitation and inhibition of subsets of motoneurons.

A clear understanding of the CPG structure and mode of operation still remains an ambitious goal that is especially important to elucidate the pathophysiology of spinal injury.

2.3 The structure and localization of CPG

The crucial features of walking, integrated in a fully functioning CPG are (1) the rhythm, (2) the ipsilateral coordination of flexors and extensors, (3) left/right coordination (Kiehn 2006). The current consensus regarding the localization of the CPG responsible for locomotor activity in the mammalian spinal cord suggests that the rhythmogenic capacity of hindlimb locomotor CPG is distributed along the lumbar cord with a rostrocaudal excitability gradient (Fig. 8; Kjaerulff and Kiehn, 1996). One cause of this gradient is a greater proportion of intraspinal inputs to rostral segments than to caudal (Berkowitz 2004). Another possibility is differential modulation of rhythmogenic networks in the rostral and caudal cord by neuromodulatory substances. A different picture of longitudinal distribution of rhythmogenic capacity in mammals emerged from studies in the neonatal rat using a partitioning setup, selectively exposing upper and lower lumbar segments to rhythmogenic agents (Cazalets et al., 1995; Bertrand and Cazalets, 2002). Such observations point out that spinal interneurons directly involved in producing rhythmic activity are restricted to the upper lumbar segment, while the lower segments have no rhythmogenic capacity. Studies regarding the distribution of CPG elements in the transverse plan are less controversial. Activity-labeling studies demonstrated that key elements for generation of locomotion are concentrated in the ventral gray matter: laminae VII, VIII, and X (Kjaerulff et al., 1994; Cina and Hochman, 2000; Dai et al., 2005). This idea has been further confirmed with microlesion studies in the rodent (Bracci et al., 1996a; Kjaerulff and Kiehn, 1996).

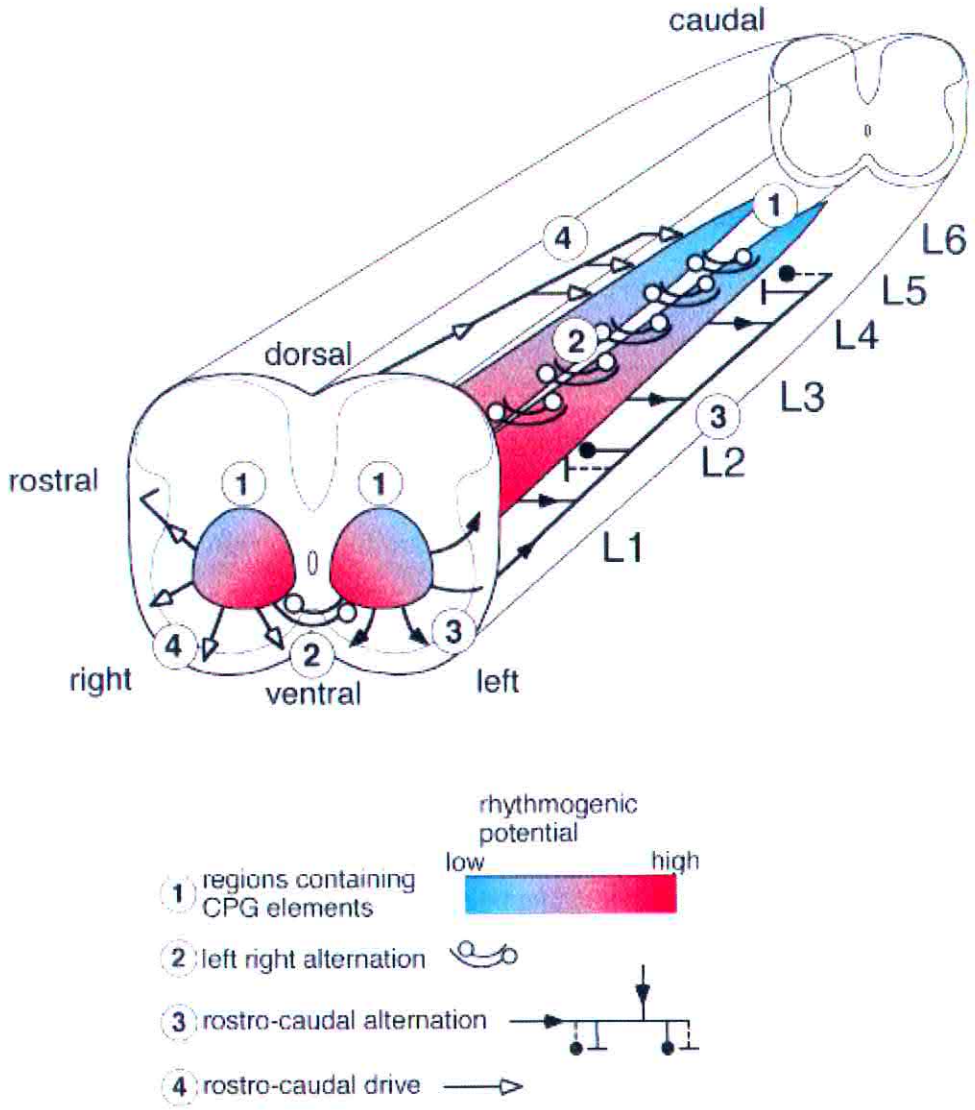


Figure 8. Summary of the lesion experiments: (1) The rhythm-generating network in L1–L6 is shown distributed along the cord as two medial columns. The taper and the color gradient indicate the high rostral and lower caudal ability to generate rhythmic activity. (2) The localization of the pathways mediating left/right alternation in the ventral commissure. (3) The pathways mediating rostrocaudal alternation are shown widely distributed in the lateral and ventral funiculus on the left side of the preparation. (4) The rostrocaudal drive is indicated on the right side of the cord (Kjaerulff and Kiehn, 1996).

Appropriate alternation between flexor and extensor motor neurons on the same side requires both excitatory and inhibitory networks. Flexor and extensor motor neurons receive rhythmic glycinergic inhibition, which alternates with rhythmic glutamatergic excitation (Kiehn et al., 1997). Ipsilateral inhibitory networks are strongly involved in flexor-extensor coordination: when inhibition is blocked, flexors and extensors are activated in synchrony (Cowley and Schmidt, 1995; Beato and Nistri, 1999). Another class of neurons, named commissural interneurons (CINs), has been uniquely identified anatomically and physiologically because of axons projecting across the midline via the ventral commissure. Such neurons connect locomotor circuits on the two sides of the spinal cord, and represent the known neural substrate for left-right coordination. CINs in mammalian spinal cord are localized in Rexed laminae VII, VIII, and X and can be subdivided into two major categories based on their axonal projection: intrasegmental and intersegmental CINs. Intersegmental CINs are further subdivided into (a) ascending CINs, (b) descending CINs, and (c) bifurcating CINs (Hoover and Durkovic, 1992; Eide et al., 1999; Nakayama et al., 2002; Bannatyne et al., 2003; Matsuyama et al., 2004).

2.4 Control of locomotion

Locomotion in mammals is controlled by networks of spinal neurons constituting a central pattern generator (CPG), and can be initiated by certain pathways that originate in the brainstem and descend to the spinal cord. There is both supraspinal and spinal automaticity in neuromotor control systems that initiate and generate locomotor pattern. Fig. 9 summarizes some of the main structures of the brain, which are thought to be involved in the locomotion initiation. The basal ganglia exert a tonic inhibitory influence on different motor centers. Once a pattern of motor behavior is selected, the inhibition is released, allowing in this case the locomotor center in the brainstem to be activated. Input form the lateral hypothalamus, the mesencephalic locomotor region (MLR) and the cerebellum are thought to activate reticulospinal cells directly. Whereas a pathway from the medial hypothalamus and periaqueductal gray (PAG) can activate reticulospinal cells either directly or indirectly (through certain components of MLR).

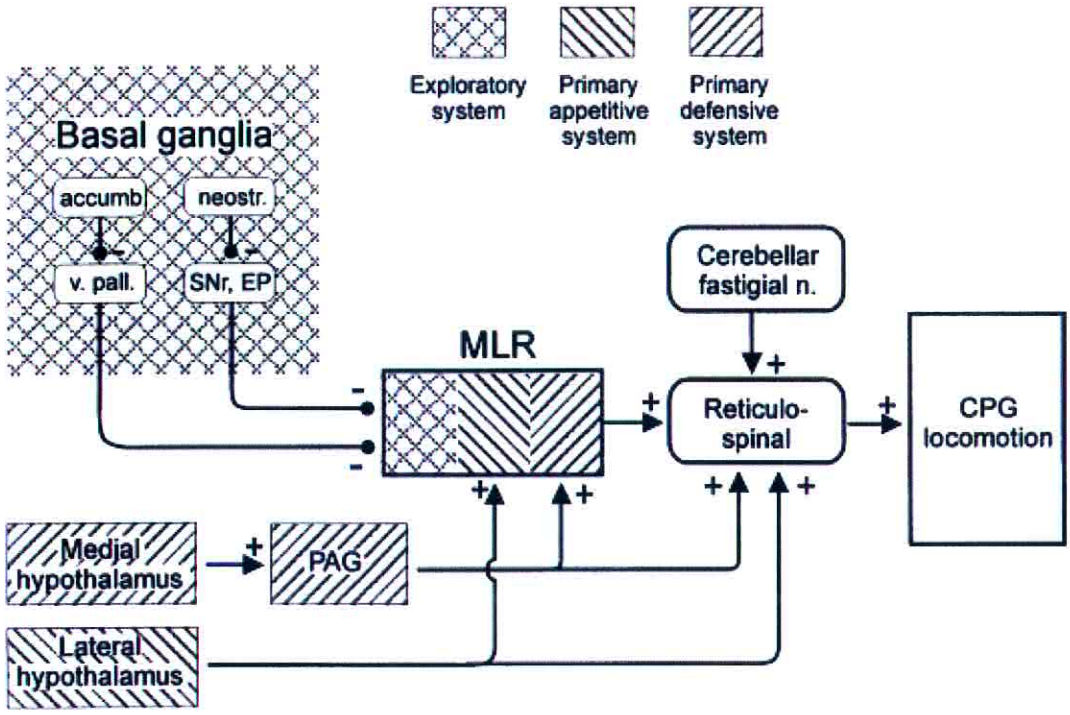


Figure 9. Brain structures important for initiation of locomotion in mammals (Jordan 1998).

Different subsets of the nuclei within the MLR appear to be activated during locomotion produced in different behavioral contexts. The locomotor nuclei can be classified into areas associated with exploratory, appetitive and defensive locomotion. According to Sinnamon's theory, locomotion serves different roles in these three systems: (1) in the appetitive systems, locomotion functions in a way to bring the organism in contact with consummative stimuli, (2) in the defensive system, locomotion functions to increase the distance between the organism and threatening or painful stimuli, (3) in the exploratory system, locomotion is aimed at distal stimuli that comprise the features of an environment (Jordan 1998).

In all classes of vertebrates the overall locomotor control system is designed in a similar way. The same meso-pontine and diencephalic centers initiate locomotor activity both in lamprey and in primates, via an activation of lower brainstem reticulospinal neurons (Grillner et al., 1998). Besides the slow and fast descending pathways controlling locomotion, other types of input are also essential for the behaviorally-adequate

locomotor pattern. Thus, postural adaptation involves cerebellar control via vestibular, reticular, and rubro-spinal pathways, while visuomotor coordination is mediated via the corticospinal pathways (Grillner and Wallen, 2002).

The brain, in turn, also receives feedback information from spinal networks, for instance in lamprey spinal cord there are neurons projecting to the brainstem, informing the reticulospinal neurons about spinal CPG activity (Vinay and Grillner, 1992). Further studies in cat have concluded that the spino-rubral pathway may transmit both somatosensory information and corollary discharges associated to the activity of the spinal CPG for locomotion (Vinay et al., 1993).

Sensory feedback acting on the CPG is an integral part of the control system and helps to adapt the motor pattern to external events (Fig. 10). Afferents originating from the skin and muscles, with their cell bodies in the dorsal root ganglion, can influence the CPG. They may impinge on the CPG or terminate on neurons, which are under the influence of the CPG activity. The phenomenon, in which the CPG during fictive locomotion is entrained either by cutaneous (Rossignol et al., 1981) or proprioceptive stimuli (Rossignol 1996; Rossignol et al., 1988), indicates strong influence on the timing of CPG. Electrical stimuli during swing phase can activate flexor muscles; the same stimulus during stance may excite or inhibit extensor activity (Rossignol et al., 1988).

Fig. 10 demonstrates that there is a two-level automatism control system for locomotion: One provides nonspecific tonic input, determining the speed of locomotion, the other is responsible for making fine adjustments in the control of the limbs, including maintaining equilibrium, by interacting with multiple modes of sensory information such as proprioceptive, visual, and auditory inputs (Shik and Orlovsky, 1976). Proprioceptive reflexes are state-dependent: inputs from some proprioceptors, like the Golgi tendon organ which are inhibitory at rest, can become excitatory during locomotion (Gossard et al., 1994; Guertin et al., 1995). The propriospinal pathways in the mammalian spinal cord include both local circuits and long projection fibers, by which motor activity propagates along the spinal cord and ensures postural behaviors and the appropriate coordination of the body parts (Cazalets 2005).

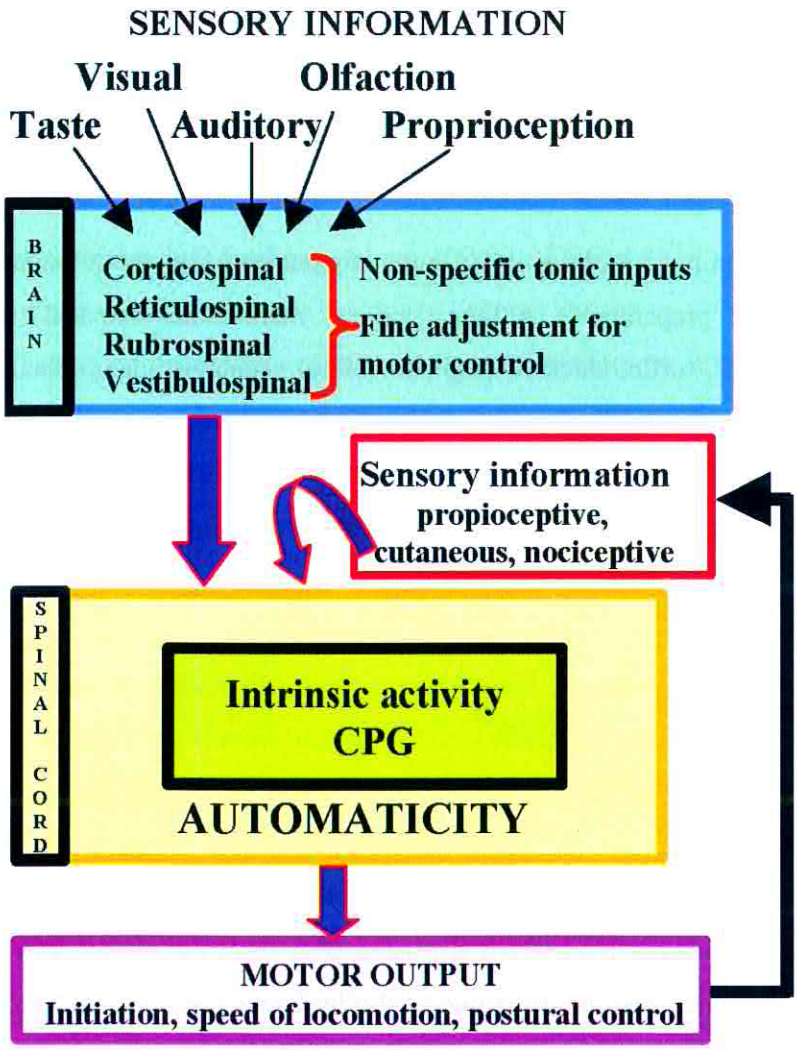


Figure 10. Automaticity is a key feature of neural motor control systems. Many sources of input to the brain (blue box) and spinal cord (yellow box) are continuously processed as we accommodate to the complexities of an ever-changing physical environment. An extensive level of sensory processing and integration by the brain and spinal cord is automatic, resulting in reasonably "smart" and predictable motor output (pink box) that is appropriate for the current state (Edgerton 2004).

3. The spinal cord as an in vitro preparation

3.1 The isolated mammalian spinal cord

There has been a dramatic increase in the study of electrophysiology of isolated *(in vitro)* mammalian CNS preparation. Among various mammalian isolated spinal cord preparations (mouse, turtle, chicken, frog and feline) neonatal rodent spinal cord is the most widely adopted one (Kerkut and Bagust, 1995; Clarac et al., 2004). There are advantages and disadvantages for using such a preparation. Here I list some of them with a focus that addresses the use of isolated spinal cord preparation.

Advantages;

1. The spinal cord can be easily and rapidly dissected, while in vivo stereotaxic experiments can take several hours to set up and require controlled anaesthetics.
2. The preparation remains alive for many hours at room temperature in an appropriate physiological medium that reproduces the composition of the cerebrospinal fluid. Recently it has been also shown a good survival of spinal networks for at least 24 h in vitro with electrophysiological and morphological characteristics analogous to those observed shortly after dissection in isolated spinal cord preparation of neonatal rat (Taccola et al., 2008).
3. Such preparations do not demonstrate mechanical movements due to respiration or blood pressure pulses, which are present in in vivo studies, thus it is feasible for extracellular and intracellular recordings to proceed for several hours.
4. There is no blood-brain barrier; pharmacological agents can be easily added to the artificial cerebrospinal fliud (ACSF).
5. There is improved visualization, therefore positioning of stimulating and recording electrodes is facilitated.
6. The oxygen and carbon dioxide concentrations in the ACSF are controlled, in whole animals it can depend on the rate and volume of respiration.

Disadvantages;

1. As a result of dissection, there is a mechanical damage to the nervous tissue.
2. *In vivo* CNS has a good oxygen supply with blood capillaries within almost 1 mm of every nerve cell, whereas *in vitro* ACSF has oxygen in solution (approximately 0.5 ml oxygen per 100 ml ACSF at 37^0 C). Thus, it is possible for nerve cells in the core regions to become anoxic.
3. There is no descending or afferent inputs, therefore the activity of the isolated spinal cord can be reduced.
4. The ACSF has a simple chemical composition, while important organic compounds such as hormones, growth factors and neuropeptides are missing.

Despite these disadvantages, the usefulness of such preparation is rather significant, justifying its widespread use. The spinal cord of P0-P7 rat is less sensitive to anoxia, presumably due to its small size, and demonstrates good electrical activity. The preparation was first introduced by Otsuka and Konishi (1974).

Finally, the isolated spinal cord preparation provides the opportunity to study the overall structure of spinal locomotor networks as well as the identification of CPG neurons, which can be an important factor for developing new neurorehabilitative strategies for SCI persons. It can be also used as an advantageous tool for preclinical testing of new pharmacological agents proposed for the treatment of SCI.

3.2 Fictive locomotion

The term fictive locomotion implies the manifestation of rhythmic oscillations alternating between left (l) and right (r) as well as flexor and extensor motoneuronal pools in a spinal cord preparation, which is deprived from supraspinal control (paralized decerebrated animals, in vitro preparations). Such pattern can be evoked electrically, by delivering a train of electrical stimuli, or chemically, via application of excitatory aminoacid agonists.

The double alternation of these discharges between flexor and extensor motor pools and between (l) and (r) sides represents the hallmark of fictive locomotion (Butt et al., 2002; Kiehn 2006; Taccola and Nistri, 2006b). Some studies have examined the dissected

spinal cord preparation connected to the hindlimbs in order to correlate neuronal activity with muscle output.

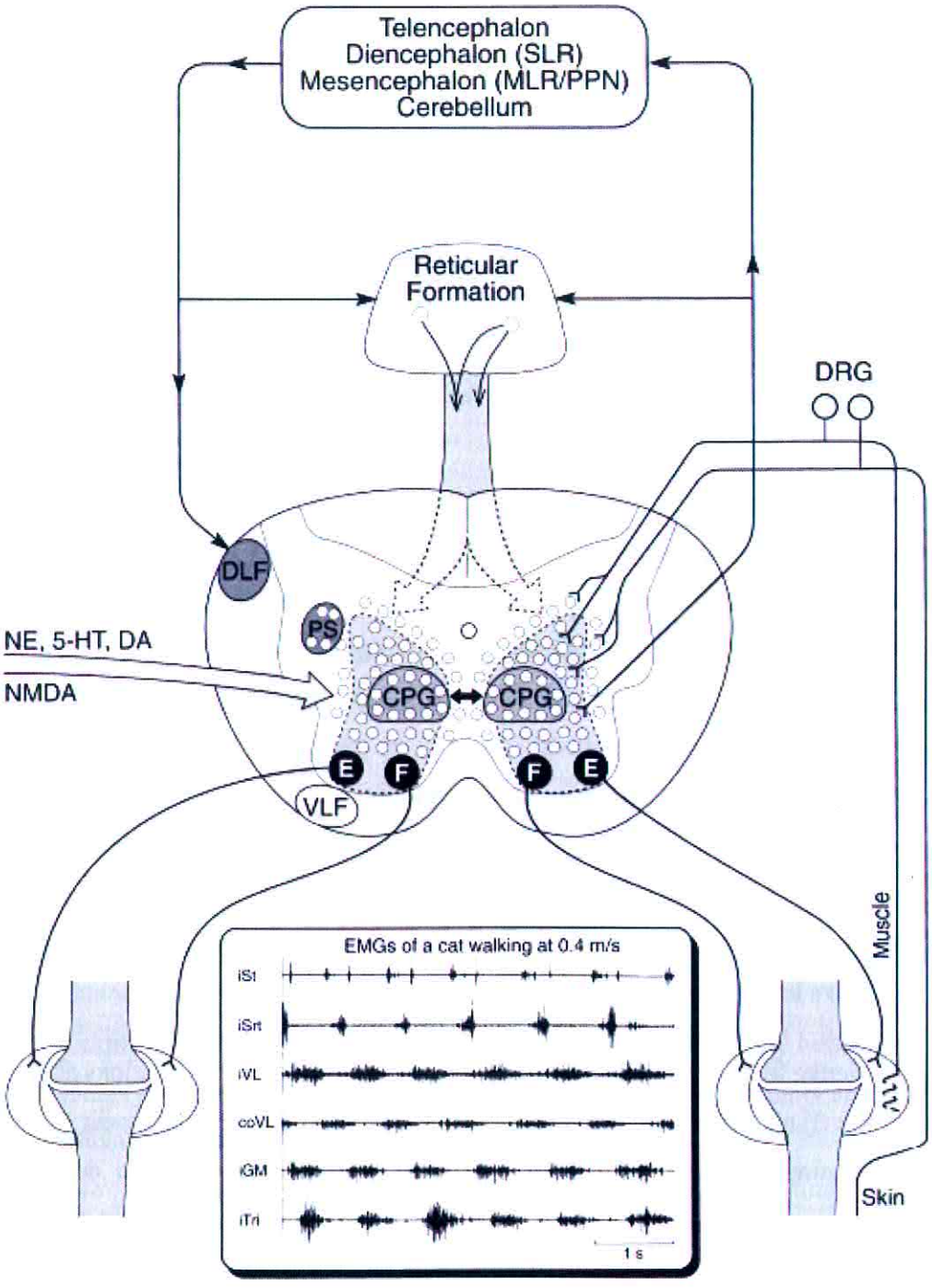


Figure 11. Summary of structures involved in the initiation and control of locomotion (Barbeau et al., 1999)

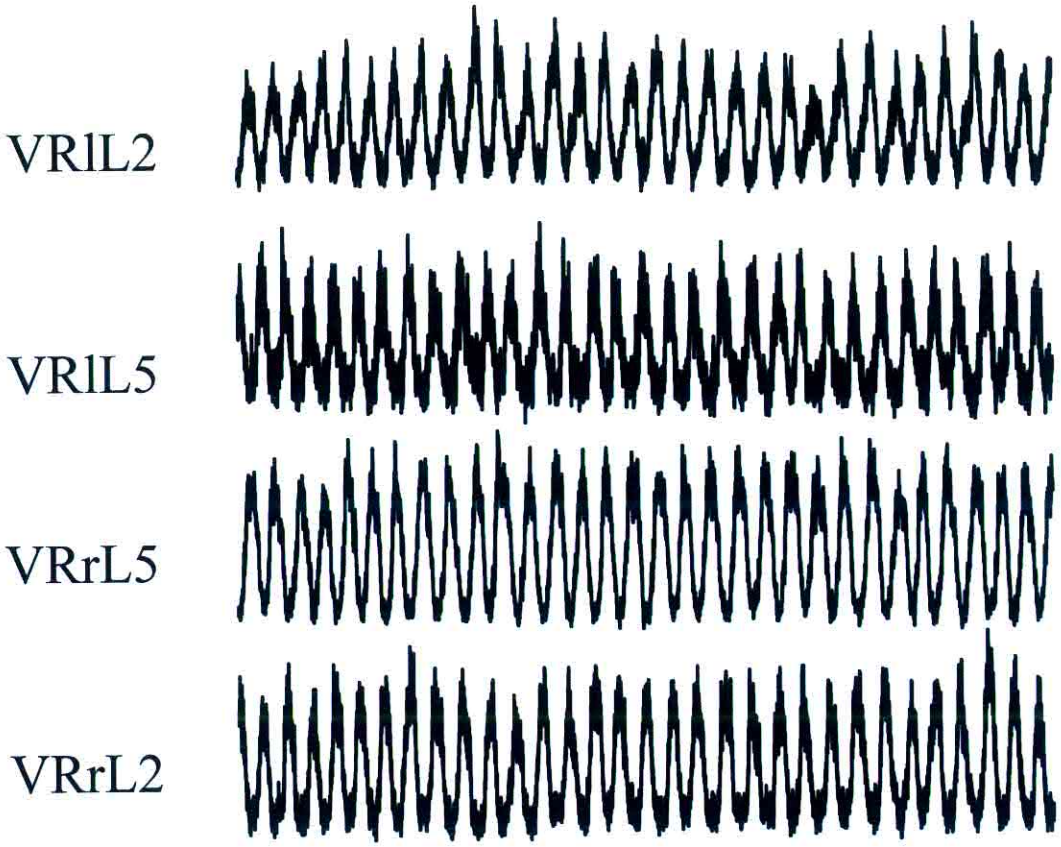
By pharmacologically inducing the locomotor pattern, it is possible to get real alternation of hindlimbs (Atsuta et al., 1988; Cazalets et al., 1992). Lumbar L2 VRs mainly express flexor motor signals to the hind-limb muscles, while L5 VRs primarily convey extensor motor commands to the same limbs (Kiehn 2006). Fig. 11 illustrates how the spinal circuitry (CPG) can produce rhythmic alternating patterns via excitation and inhibition of groups of interneurons as well as flexor and extensor motoneurons, which in turn activate muscles. Electrical impulses or various neurotransmitter systems (noradrenaline, 5-HT, dopamine, NMDA) can also trigger or modulate the locomotor pattern. The inset to the Fig. 11 shows an electromyogram of walking cat where electrical discharges alternate between the flexor and extensor motoneuronal pools.

3.3 Pharmacologic triggering of locomotion.

The pharmacological activation of the locomotor program has been started by the pioneering studies of Swedish school (Jankowska et al., 1967; Grillner and Zangger, 1979). In paralyzed spinal cats they have shown that L-DOPA together with a monoamino oxidase inhibitor can activate a fictive locomotor pattern. A similar pattern was recorded from the hindlimbs of acute spinal cats by application of the noradrenergic agonist clonidine. Excitatory amonoacids, such as glutamate and aspartate, as well as agonists, like NMDA and kainate, can also elicit locomotor pattern of activity in rat spinal cord (Kudo and Yamada 1987; Cazalets et al., 1992). AMPA can not induce the fictive locomotion because of fast desensitizing properties of this receptor (Dingledine et al., 1999).

In the isolated spinal cord preparation the rhythmic locomotor-like patterns can be elicited by bath applied NMDA (Kudo & Yamada, 1987), 5-HT (Cazalets et al., 1992; Beato et al., 1997) or high levels of potassium (Bracci et al., 1998). Although NMDA receptor activation is important for the generation of locomotion, it was also shown that the alternating activity persisted in the presence of NMDA receptor antagonist AP5 (Cowley et al., 2005). It was previously shown that both NMDA and 5-HT induce locomotor- like activity with a characteristic dose-response curve (Cazalets et al., 1992). When they were combined, no further increase in the cycling activity was observed, but the period was set at an intermediate value between the periods induced by each of these components (Sqalli-Houssaini et al., 1993).

A **NMDA 5μM + 5-HT 10μM (P1 rat)**



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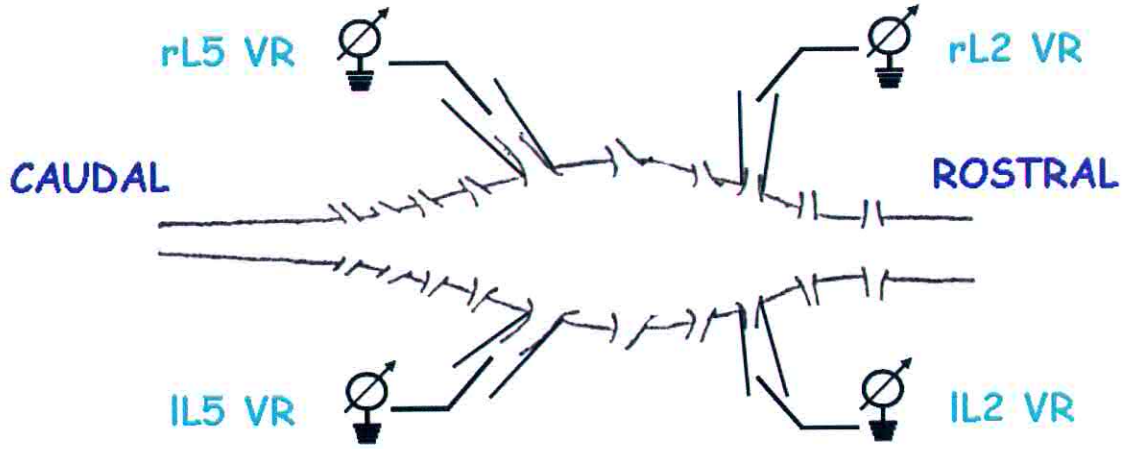

Figure 12. A; Rhythmic discharges alternating between l and r as well as flexor extensor motoneuronal pools recorded from isolated spinal cord preparation from P1 rat during continuous bath application of NMDA and 5-HT, B; Experimental setup used to characterize fictive locomotor pattern. Ventral root recordings were obtained from the r and l side both at the L2 and L5 levels (Margaryan, unpublished data).

Combination of acetylcholine with the acetylcholinesterase inhibitor edrophonium generates sustained rhythmic activity characterized by l-r alternation and non-locomotor co-activation of ipsilateral flexor and extensor discharges (Cowley and Schmidt, 1994). Combination of NMDA and 5-HT generates very stable and long-lasting fictive locomotor pattern in isolated spinal cord of the rat (Fig. 12).

3.4 Electrically evoked locomotion

Besides chemically induced locomotion it is possible to elicit the locomotor-like pattern electrically, via application of trains of electrical stimuli to the spinal cord preparation (Fig. 13). In an *in vitro* neonatal rat spinal cord preparation, an alternating pattern of activity is evoked by delivering repeated trains of stimuli to one of dorsal roots (DRs) (Marchetti et al., 2001). The oscillations induced by DR stimulation present the typical phase alternation at segmental and intersegmental levels, which is a hallmark of fictive locomotion evoked by chemical substances (Kiehn et al., 1997). Tonic stimulation of the dorsal roots can evoke fictive locomotion also in decerebrate paralyzed cats (Budakova 1971). In chronic spinal cats, simply moving the treadmill belt is sufficient to initiate locomotion (Barbeau and Rossignol, 1987). In less active cats perineal stimulation is a powerful stimulus to induce locomotion. While electrical stimulation of the descending ventrolateral funiculus can induce fictive locomotor patterns (Magnuson et al., 1995; Magnuson and Trinder, 1997), there is only a preliminary report (Smith et al., 1988) that stimuli applied to a skin flap still attached to the rat isolated spinal cord can produce similar effects.

Notwithstanding the precise mechanism underlying the generation of fictive locomotor-like patterns by DR stimuli in the isolated spinal cord of the rat, it seems likely that rhythmogenesis was caused by enhanced release of excitatory transmitters from spinal neurones activated by DR fibre stimulation. From the other hand, the application of single pulses is not sufficient to trigger either fictive locomotion or oscillatory activity.

Most probably repetitive stimulation causes persistent neuronal depolarization which, in turn, (through membrane depolarization and facilitation of transmitter release) recruits a larger population of neurons to threshold for locomotor-like electrical behaviour. Thus, there is a strong nonlinearity of the input/output operation of the CPG network, which is based on the weak dependence of oscillations on stimulus intensity and their lack of dependence on stimulus frequency (Marchetti et al., 2001).

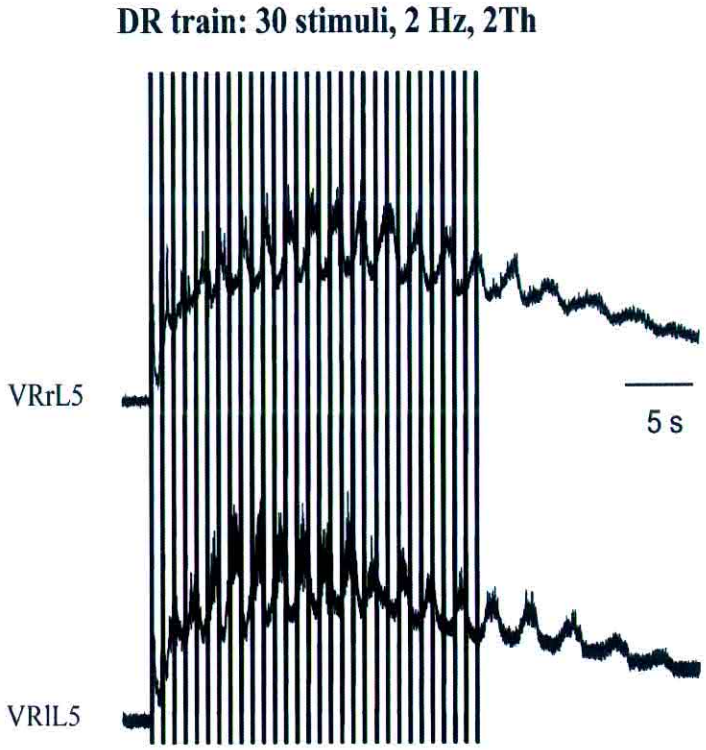

Figure 13. Locomotor-like pattern induced by the electrical stimulation of L2 right DR and alternating between l and r VR L5 (Margaryan et al., 2009).

As it is shown in Fig. 13, electrically evoked oscillations (unlike the ones induced chemically) are transient. Loss of rhythmicity can be a result of either fatigue or stimulus-dependent release of neurotransmitter, which inhibit the operation of CPG.

3.5 Disinhibited bursting

In order to understand the elementary processes of rhythmic discharges in rodent spinal networks it is useful to employ a simplified preparation in which synaptic inhibition is pharmacologically blocked and the influence of descending inputs removed by maintaining the isolated spinal cord in vitro (Bracci et al., 1996a,b). Such a model is obtained by blocking glycinergic and GABAergic synapses with strychnine and bicuculline, respectively and is characterised by the emergence of a regular bursting pattern arising spontaneously and lasting for many hours (Streit 1993; Bracci et al., 1996a,b; Streit et al., 2001). However, it is important to note that the disinhibited rhythmicity is not a physiological process.

The rhythm, as shown in Fig. 14, is synchronous among VRs and indicates a manifestation of the rhythmicity of spinal networks supported by intrinsically active neurons via the interplay of their voltage dependent conductances (Bracci et al., 1996a; Darbon et al., 2002, 2003). Disinhibited bursting requires spontaneous activity via recurrent excitatory collaterals that spread depolarization until neuronal recruitment triggers a burst (Bracci et al., 1996a,b, 1997; Ballerini et al., 1999; Tscherter et al., 2001). Despite the progress made to understand the mechanisms responsible for burst generation and duration (Rozzo et al., 2002; Taccola et al., 2004; Cazalets, 2005; Yvon et al., 2005), current understanding on this issue remains incomplete. Recent studies have demonstrated the role of persistent Na+ current to support long bursting and enhanced excitability of motor circuits (Darbon et al., 2004; Kuo et al., 2006; Pace et al., 2007). The role of L-type Ca^{2+}currents underlying large plateau potentials in spinal neurons may be another contributing mechanism (Hounsgaard and Mintz, 1988).

A notable property of disinhibited bursting is to reduce burst duration in response to increased frequency of afferent signals (Bracci et al., 1997; Streit et al., 2001; Tabak et al., 2001; Darbon et al., 2002). After surgical ablation of the dorsal laminae, it is still possible to evoke disinhibited rhythmicity by blocking synaptic inhibition (Bracci et al., 1996 a). The question whether the fictive locomotion and disinhibited bursting belong to the same network is still unclear. Evidence favouring the hypothesis of the same network is supported by the experimental data that (a) both patterns can be blocked by ionotropic glutamate receptors (Beato et al., 1997., Bracci et al., 1996a), (b) they both have similar anatomical location, mainly localized to the ventral horn area. In addition, disinhibited rhythm can be accelerated by 5-HT (Bracci et al., 1996a), known to activate locomotor

pattern (Cazalets et al., 1992). However, disinhibited bursting requires a circuitry simpler than fictive locomotion and can be detected even in single quadrant of spinal cord as well as in organotypic spinal cultures (Bracci et al., 1996a,b; Darbon et al., 2002).

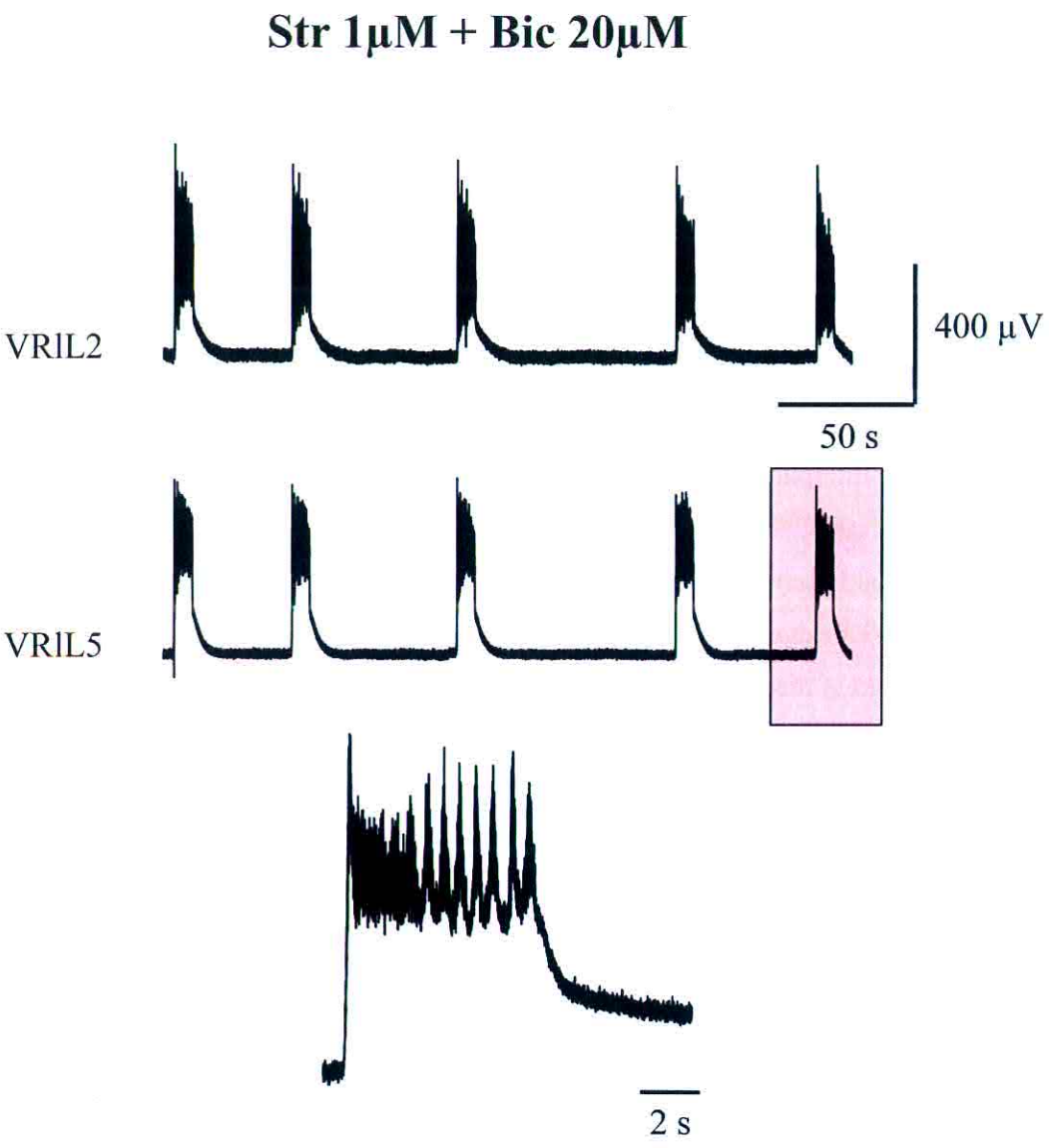

Figure 14. Disinhibited rhythm evoked by pharmacological blockade of synaptic inhibition, recorded from l VR L2 and L5; single burst is shown on a faster time scale (Margaryan, unpublished data).

AIMS OF THE STUDY

Despite its extensive occurrence, the pathophysiological mechanisms responsible for SCI remain largely ambiguous. Several experimental systems have been employed to investigate the pathophysiology of SCI and to test the effects of neuroprotective agents in the laboratory. A reasonable number of such studies are susceptible to a number of inherent flaws in experimental design that impair their ability to simulate human SCI.

The aim of the present study is directed towards following issues:

1. To set up conditions to produce an irreversible damage to the spinal cord in vitro in order to understand the basic mechanisms responsible for the early pathophysiology of spinal injury and to investigate:
   - What are the in vitro conditions necessary to induce an irreversible lesion?
   - How does the locomotor CPG operate after a lesion?
   - Is excitotoxicity per se sufficient to damage spinal networks, especially the circuits responsible for locomotion which are intrinsic to the lumbar spinal cord?
   - Can metabolic disruption contribute to the damage without a primary excitotoxic stimulus?

2. To use varied extracellular Mg^{2+} concentrations, ranging from a nominally-Mg^{2+} free medium to one containing 20 mM Mg^{2+} together with pathological medium (PM), kainate or their combination in order to investigate:

- If the attempts to preserve extracellular Mg^{2+} at (or very near) the standard CNS concentration during PM administration (and thus lesion development), could influence the outcome of locomotor network operation?
- If the changes in extracellular Mg^{2+} influence the effect of PM on locomotor network electrophysiology and histology?
- If the changes in extracellular Mg^{2+} also affect the excitotoxic action of kainate?

3. To investigated the feasibility of neuroprotection of lumbar locomotor networks by application of the glutamate antagonists CNQX and APV against acute lesions induced by either PM or excitotoxicity with kainate:

- To find out if proof of principle of neuroprotection could be obtained with our in vitro model:
- To compare whether excitotoxicity or metabolic perturbation posed equivalent challenges to neuroprotection
- To understand what time window after the lesion was compatible with neuroprotection

NEUROPROTECTION OF LOCOMOTOR NETWORKS AFTER EXPERIMENTAL INJURY TO THE NEONATAL RAT SPINAL CORD IN VITRO

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Key words: central pattern generator, kainate, hypoxia, oxidative stress, CNQX, APV

LIST OF ABREVIATIONS

5-HT (5-hydroxytryptamine)

aoi (area of interest)

AU (arbitrary units)

BSA (bovine serum albumin)

CNQX (6-cyano-7-nitroquinoxaline-2,3-dione)

CPG (central pattern generator)

CTR (control)

CV (coefficient of period variation)

DAPI (4', 6-diamidino-2-phenylindole)

D-APV (D-aminophosphonovalerate)

DR (dorsal root)

FCS (fetal calf serum)

GFAP glial fibrillary acidic protein

l (left)

L (lumbar)

NMDA (N-methyl-D-aspartate)

PM (pathological medium)

r (right)

S.D. (standard deviation)

SNP (sodium nitroprusside)

VRs (ventral roots)

Abstract

Treatment to block the pathophysiological processes triggered by acute spinal injury remains unsatisfactory as the underlying mechanisms are incompletely understood. Using as a model the in vitro spinal cord of the neonatal rat, we investigated the feasibility of neuroprotection of lumbar locomotor networks by the glutamate antagonists CNQX and APV against acute lesions induced by either a toxic solution (pathological medium to mimic the spinal injury hypoxic-dysmetabolic perturbation) or excitotoxicity with kainate. The study outcome was presence of fictive locomotion 24 h after the insult and its correlation with network histology. Inhibition of fictive locomotion by pathological medium was contrasted by simultaneous and even delayed (1 h later) co-application of CNQX and APV with increased survival of ventral horn premotoneurons and lateral column white matter. Neither CNQX nor APV alone provided neuroprotection. Kainate-mediated excitotoxicity always led to loss of fictive locomotion and extensive neuronal damage. CNQX and APV co-applied with kainate protected 1/3rd of preparations with improved motoneuron and dorsal horn neuronal counts, although they failed with delayed application. **Our data suggest that locomotor network neuroprotection was possible when introduced very early during the pathological process of spinal injury, but also showed how the borderline between presence or loss of locomotor activity was a very narrow one that depended on the survival of a certain number of neurons or white matter elements.** The present report provides a model not only for preclinical testing of novel neuroprotective agents, but also for estimating the minimal network membership compatible with functional locomotor output.

Introduction

Acute spinal cord injury usually produces severe damage to locomotor networks with ensuing paralysis even when the lesion is incomplete (McDonald and Sadowsky, 2002; Klussmann and Martin-Villalba, 2005; Schwab et al., 2006). Regardless of its etiology (trauma, vascular deficit, invasive cancer, infective disease), the primary damage is compounded by secondary damage spreading to areas not initially affected by the lesion and probably caused by multifactorial pathological processes that include excitotoxicity (Hall and Springer, 2004; Park et al., 2004; Rossignol et al., 2007), hypoxia, ischemia, local edema and generation of toxic radicals (Dumont et al., 2001; Hall and Springer, 2004; Norenberg et al., 2004; Xiong et al., 2007). Current clinical guidelines propose the use of i.v. methylprednisolone to treat this condition preferably within 4 h from its onset. The usefulness of this treatment is, however, very controversial (Sayer et al., 2006) and the need for more beneficial pharmacological agents aimed at neuroprotection is widely perceived (Thuret et al., 2006; Faden and Stoica, 2007).

Attempts to protect central nervous tissue by blocking excitotoxicity with glutamate antagonists or free radical scavengers have yielded disappointing results that have even lead to dismiss the possibility of clinical neuroprotection against trauma or ischemia (Thuret et al., 2006; Faden and Stoica, 2007; Savitz and Fischer, 2007). Failure has been largely attributed to inadequate preclinical models and, in particular, poor understanding of the timecourse of the damage development. Studies of stroke management show that "time is brain" (Hill and Hachinski, 1998), namely the quickest the treatment administration, the better the outcome is. It seems likely that limiting acute damage to locomotor networks requires clarification of its early dynamics preferably with a model that can allow on-going monitoring of locomotor activity in relation to histological damage. **Although this goal would be best attained with in vivo animal**

models, it is, however, very difficult to monitor locomotor network activity before, during and after acute injury, also in view of the fact that general anaesthesia is necessary to avoid animal suffering. To clarify the cellular and network mechanisms underlying acute spinal lesions to locomotor networks it is, therefore, advantageous to use in vitro models. To this end, we have developed in vitro models of acute spinal injury by using the isolated spinal cord of the neonatal rat, a preparation extremely useful to investigate the structure and function of mammalian locomotor networks (Kiehn, 2006; Goulding, 2009) because it readily generates locomotor-like patterns (fictive locomotion) following chemical or afferent stimuli. We have shown distinct forms of damage produced by either excitotoxicity or metabolic perturbation mimicking the biochemical derangement accompanying an acute spinal lesion (Taccola et al., 2008; Margaryan et al., 2009). The present study pursued three aims: 1. to find out if proof of principle of neuroprotection could be obtained, at least with our in vitro models; 2. to compare whether excitotoxicity or metabolic perturbation (maximized in the presence of 1 mM Mg^{2+}; Margaryan et al., 2009) posed equivalent challenges to neuroprotection; 3. to understand what time window after the lesion was compatible with neuroprotection. In our experiments the outcome was presence of fictive locomotion one day after the experimental lesion protocol, or, failing that, generation of disinhibited bursting as an index of basic network rhythmicity (Bracci et al., 1996). To simplify our approach at this exploratory stage, we elected to study conventional glutamate receptor antagonists like CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) and APV (D-aminophosphonovalerate).

Materials and methods

Rat spinal cord preparation

All experiments were carried out on neonatal Wistar rats (0-2 day old) in accordance with the guidelines of the National Institutes of Health and the Italian act D.Lgs. 27/1/92 n. 116 (implementing the European Community directives n. 86/609 and 93/88). All efforts were directed toward reducing the number of animals used and minimizing their suffering. Under urethane anesthesia (0.2 ml i.p. of a 10 % w/v solution), spinal cords were dissected out and placed in a recording chamber (at room temperature) continuously superfused (7.5 ml min^{-1}) with Krebs solution (in mM): NaCl, 113; KCl, 4.5; $MgCl_27H_2O$, 1; $CaCl_2$, 2; NaH_2PO_4, 1; $NaHCO_3$, 25; glucose, 11; gassed with 95 % O_2 5 % CO_2; pH 7.4. The experimental setup is fully described by Taccola and Nistri (2006a), Taccola et al. (2008) and Margaryan et al. (2009).

Electrophysiological recording

Experiments were based on DC-coupled recordings from lumbar (L) ventral roots (VRs) using tight-fitting suction electrodes. As a routine, signals were recorded from left (l) and right (r) L2 VRs generating mainly flexor motor-pool commands to the hindlimbs, and l and r L5 VRs conveying mainly extensor motor-pool commands to the same limbs (Kiehn and Kjaerulff, 1998). Signals were captured, digitized and analyzed with pClamp software (version 9.2; Molecular Devices, Sunnyvale, CA, USA).

Single electrical stimuli were delivered to one dorsal root (DR) via a bipolar suction electrode to elicit ipsilateral homosegmental VR responses (once every 60 s). Repeated pulses (1-30 V stimulus range; 0.1 ms duration; 30 pulse trains at 1-2 Hz) were used to induce cumulative depolarization and fictive locomotor patterns. The stimulation strength was 2 x threshold referred to the VR response amplitude. Peak amplitude and area of such DR stimulation-induced responses were calculated after averaging at least seven events.

Chemically-evoked fictive locomotion was induced by continuous bath application of N-methyl-D-aspartate (NMDA; 4 or 5 μM) plus 5-hydroxytryptamine (5-HT; 10 μM; see Kiehn and Kjaerulff, 1996; Butt et al., 2002). The double alternation of between homosegmental l and r VRs, and between L2/L5 on the same side was taken as a typical indicator of fictive locomotion (Butt et al., 2002). For rhythmic discharges we measured their period, namely the time interval between the onset of two oscillatory cycles calculated after averaging at least 20 consecutive cycles, and their regularity indicated by the coefficient of period variation (CV; given by standard deviation [S.D.] $mean^{-1}$).

Disinhibited bursting (Bracci et al., 1996, 1997) was induced by continuously bath-applied strychnine (1 μM) and bicuculline (10 μM). All parameters concerning the definition of bursts and their measurements (duration, cycle period, number and frequency of intraburst oscillations) were in accordance with Bracci et al. (1996).

Protocols for spinal cord lesion and neuroprotection

The first protocol was employed to mimic the biochemical conditions believed to occur at the site of the lesion and that (as a consequence of strong metabolic perturbation) induce a pathological state comprising locomotor network dysfunction and morphological spinal cell damage (Margaryan et al., 2009). In keeping with our recent observations, we routinely used 1 mM Mg^{2+} in the toxic solution to enhance such a damage (Margaryan et al., 2009). As discussed previously (Taccola et al., 2008; Margaryan et al., 2009), our aim was to generate an experimental model which might mimic the acute clinical lesion when the metabolic insult is temporary followed by intensive care treatment to correct the metabolic derangement **and the associated ischemic insult**. Hence, we applied (for 1 h) a solution containing 10 mM H_2O_2,

1mM Mg^{2+}, 500 µM sodium nitroprusside (SNP) **without adding either oxygen or glucose since we wished to reproduce the experimental conditions of previous studies showing that simple arrest of oxygen delivery to a spinal preparation in vitro produces severe hypoxia (Carlin and Brownstone, 2006): this medium was prepared to simulate the acute ischemia that has been reported to occur in the injured human spinal cord (Norenberg et al., 2004). We also omitted $NaHCO_3$ that was replaced by** Hepes to reach pH 6.75-6.80 (with 0.1 N NaOH); osmolarity was lowered to 230-240 mOsm. For the sake of simplicity, this solution was termed pathological medium (PM) and it **corresponds to PM+1mM Mg^{2+} in our previous study (Margaryan et al., 2009).**

The second protocol for spinal lesion relied on the 1 h application of the strong excitotoxic agent kainate (1 mM) in standard Krebs solution (Taccola et al., 2008; Margaryan et al., 2009). In this case excitotoxicity is associated with complete loss of fictive locomotion and substantial network damage throughout the spinal cord, though white matter lesions are usually limited (Taccola et al., 2008).

With either protocol, the toxic solution was washed out after 1 h and standard Krebs solution was superfused again for up to 24 h. Before, during and after applying the toxic solution, spinal cords were tested for reflex activity, fictive locomotion evoked by neurochemical or DR stimulus trains, or disinhibited bursting. Electrophysiological responses were monitored for up to 24 h.

With either toxic protocol, we attempted two neuroprotection strategies, both based on the use of CNQX (10 µM) and APV (50 µM).The selection of each drug concentration was based on former reports for the IC_{50} value related to suppression of synaptic transmission in the isolated spinal cord of the rat. Thus, as CNQX IC_{50} is 1 µM (Long et al., 1990), we applied a ten fold increase in this concentration to prevent potential displacement of this antagonist by glutamate

(or kainate). As for D-APV the IC_{50} value is 1.4 μM (Evans et al., 1982), we used this antagonist at 50 μM concentration (also in view of the high affinity of glutamate for NMDA receptors; Dingledine et al., 1999). Our previous investigation has indicated that 10 μM CNQX and 50 μM APV co-applied to the neonatal rat spinal cord fully suppress locomotor network activity (Beato et al., 1997).

In the present investigation we adopted two experimental strategies: in one case, glutamate receptor antagonists (dissolved in the same solution containing either PM or kainate) were co-applied with the toxic medium via continuous superfusion to test their ability to provide early onset neuroprotection. **Alternatively, such antagonists (dissolved in standard Krebs)** were applied for 2 h after washing out PM or kainate to study their delayed potential to limit spinal cord damage. After 2 h antagonist application, standard Krebs superfusion recommenced. Sham experiments were performed by keeping preparations for analogous times in the same experimental set-up without applying a toxic medium. The need for sham preparations for long term comparisons stems from our previous study (Taccola et al., 2008) that indicated that synaptic transmission properties of the rat spinal cord spontaneously decline after 12 h in vitro in accordance with the early report on the protracted use of such preparations in vitro (Evans, 1978). Thus, to obtain meaningful analysis of the effects of neuroprotective drugs 24 h later, comparisons of their action had to be taken vs untreated sham preparations.

At the end of the experiments, preparations were fixed as previously reported (Taccola et al., 2008) and processed for histology as detailed below.

Immunofluorescence

After electrophysiological recording, spinal cords were fixed in 4 % paraformaldehyde (12-24 h), cryoprotected for 24 h (30 % sucrose), sectioned with a sliding microtome (30 μm sections) and

stored in phosphate buffer saline for further use. For free-floating immunofluorescence, sections were incubated in blocking solution (5 % fetal calf serum (FCS)/5 % bovine serum albumin (BSA)/0.3 % Triton) for 1 h at room temperature and overnight at 4°C in a solution containing 1 % FCS/1 % BSA/0.1 % Triton and the primary antibody. We used monoclonal NeuN (1:50 dilution, Chemicon, Temecula, CA, USA) for neurons, and SMI-32 (Sigma, St. Louis, MO, USA) for motoneurons. To analyze astrocytes we used monoclonal mouse glial fibrillary acidic protein (GFAP; 1:100, Sigma, St. Louis, MO, USA), or polyclonal rabbit S100 (1:100, Dako Cytomation, Denmark); for oligodendrocyte precursors we performed immunostaining with monoclonal mouse O4 (1:100, RandD Systems, Minneapolis, USA) or polyclonal rabbit NG2 chondroitin sulfate proteoglycan (1:100, Chemicon). The primary antibody was tagged using secondary fluorescent anti-mouse Alexa Fluor 488 antibody (1:500; Invitrogen, Carlsbad, CA, USA). Sections were analyzed with MetaVue (Molecular Devices, Sunnyvale, CA, USA) software. Five different spinal cords were analyzed and for each spinal cord, 3 or 6 different sections were counted for NeuN positive cells, **taking samples from L1-L2 lumbar segments**. The NeuN positive cells were counted in a 170x170 μm area of interest (aoi) of dorsal gray matter (Rexed laminae I-IV), 170x170 μm aoi of central gray matter (Rexed laminae V-VIII and X) and 450x450 μm aoi of ventral gray matter (Rexed laminae IX). To count motoneurons we analyzed Rexed laminae VIII and IX.

Quantification of dead cells

Dead or dying cells after experimental insult were identified and quantified as previously reported (Taccola et al., 2008) **using the same L1-L2 lumbar segments employed for neuronal counts**. Pyknotic nuclei were counted using 4', 6-diamidino-2-phenylindole (DAPI)

nuclear staining and "eCELLence" (Glance Vision Tech, Trieste, Italy) software. For each experimental condition, two to five different spinal cords were analyzed and for each spinal cord, 3 or 6 different 30 μm cross-sections from lumbar segments were examined. For each section, four different aoi of the spinal cord were investigated: dorsal gray matter (Rexed layers I-IV), central gray matter (Rexed layers V-VIII and X), ventral gray matter (Rexed layer IX) and lateral white matter. For each aoi, 3-7 fields of 280 x 280 μm (gray matter) or 100 x 280 μm (white matter) area were analyzed by counting pyknotic nuclei using eCELLence software. Our previous study has shown that about $1/3^{rd}$ of cells visualized with DAPI staining is represented by neurons (Margaryan et al., 2009).

Data analysis

All data were expressed as means ± S.D. where n indicates the number of spinal cord preparations. After using the normality test to distinguish between parametric and non-parametric data, parametric values were analyzed with Student's *t*-test (paired or unpaired) for two groups, **as we avoided multiple comparisons within groups**. For non-parametric values, the Mann-Whitney test was used for two groups. For electrophysiological data related to early effects (same day) of any treatment, comparison was carried out between data observed after treatment versus control (CTR) data from the same preparation. To statistically evaluate data obtained from the second experiment day, in view of the reported changes in responses after 24 h in vitro, results were compared with those from companion experiments in which no protection was attempted or, whenever necessary, sham preparations kept for the same time length in vitro (Margaryan et al., 2009). The accepted level of significance was always $P < 0.05$.

Drugs

Kainate was purchased from Ascent Scientific (Weston-super-mare, UK) while NMDA, CNQX, and APV were obtained from Tocris (Bristol, UK). 5-HT, SNP and strychnine were purchased from Sigma (Milan, Italy). H_2O_2 was obtained from Carlo Erba Reagents (Milan, Italy) and bicuculline methiodide from Fluka (Milan, Italy).

Results

Glutamate receptor antagonists limit damage to locomotor networks produced by metabolic perturbation

Our recent investigation has shown that 1 h application of PM (**including 1mM Mg^{2+}**) induced irreversible damage to locomotor spinal networks with suppression of fictive locomotion and substantial death of premotoneurons and white matter elements (Margaryan et al., 2009). In the present study we investigated whether this network deficit included an early component caused by excitotoxicity induced by local release of glutamate and subsequent neuronal loss (Hall and Springer, 2004; Park et al., 2004; Rossignol et al., 2007). To examine this issue, we co-applied (for 1 h) the PM solution with or without 10 μM CNQX and 50 μM APV (see methods).

Fig. 1 compares the evolution of locomotor network activity after either administration of PM (Fig 1 A) or with PM + glutamate antagonists (Fig.1 C). Fictive locomotion evoked by NMDA and 5-HT in CTR condition (Fig. 1 A, left) could not be elicited 1 h after PM had been washed out (Fig. 1 A, middle), and did not recover 24 h later (Fig. 1 A, right), even if disinhibited bursting was present on all four VRs with 131±39 s period and 12±7 s burst duration (Fig. 1 B). Conversely, when PM was applied with CNQX and APV, fictive locomotion was present already at 1 h washout of this solution, and persisted after 24 h (Fig. 1 C). While fictive locomotion

period was not significantly different from CTR after this combined treatment (Fig. 1 E; P = 0.178 for the same day and P = 0.152 vs. sham for the second day), cycle amplitude was substantially reduced (Fig. 1 F; P = 0.04) on the same day and remained at low level like the one of sham preparations 24 h later. As exemplified in Fig. 1 D, disinhibited bursting (24 h later) was fully expressed with 61±28 s period (CV=0.39±0.17; n=4) that was faster (P=0.001) than PM treated preparations (159±24 s); burst duration (11±1 s; n=4) was unchanged. In 3 preparations no VR depolarization was observed during co-application of PM plus CNQX and APV, while in one 1.45 mV depolarization was recorded.

After application of PM plus glutamate antagonists, VR reflexes elicited by DR stimulation were largely decreased in peak amplitude (Fig. 1 G; P = 0.042 for the same day; n=4), suggesting impairment in mono and polysynaptic transmission. Reflex area size observed 24 h later did not differ from the one of sham preparations (Fig. 1 H).

Fictive locomotor cycles induced by stimulating one DR with pulse trains (2 Hz; 30 pulses) did not reappear 24 h after PM plus CNQX and APV in two preparations, while short-lasting oscillatory cycles were detected in other two preparations (see supplemental Fig. 1). On average, the amplitude of cumulative depolarization fell from 0.60±0.21 mV to 0.14±0.03 mV (on the same day; P = 0.021) and was 0.23±0.21 mV the day after (P = 0.023 vs sham).

Twenty-four h after PM plus glutamate antagonists, we also assessed the extent of cell death by counting pyknotic cell nuclei in four distinct areas of the spinal cord as indicated by the scheme in Fig. 2. Damage to the white matter was almost as large (53±3 %; n=4) as the one (**66±14** %) observed with just PM (**+ 1 mM Mg^{2+};** see Fig. 5 C in Margaryan et al., 2009). **It was difficult to identify on the basis of pyknosis, the cell type predominantly affected by the experimental lesion. In fact, cells with pyknotic nuclei could not be co-labeled with cell-type**

specific immunofluorescent markers such as NeuN or GFAP, indicating their death and loss of intracellular antigens. Examples of pyknosis are shown in Fig. 2 B, C.

Although the number of pyknotic nuclei in the other areas was very small (Fig. 2), it is interesting that pyknotic ventral horn cells were significantly (P=0.004) fewer **(1±0.3 %) than those reported earlier after PM (+1mM Mg^{2+}; 7±2 %;** Margaryan et al., 2009).

The results suggest that PM-dependent damage comprised an early component attributable to excitotoxicity as it was limited by blocking ionotropic glutamate receptors: the residual impairment in fictive locomotion and synaptic transmission was associated with modest cell loss with the exception of the white matter region.

Relative contribution of AMPA/kainate receptors versus NMDA receptors to neuroprotection

We investigated if either APV or CNQX was sufficient to preserve fictive locomotion in the presence of PM. Fig. 3 compares the effectiveness of each one of these two antagonists. APV alone could not protect spinal networks as fictive locomotion was absent 1 or 24 h after washout of PM plus APV (Fig. 3 A, middle and right). Disinhibited bursting was poorly expressed after 24 h (Fig. 3 B) with 114±44 s period (CV=0.33±0.12) and 31±18 s burst duration (n=7). On average, out of 9 preparations, seven did not express fictive locomotion, one generated alternating oscillations on two VRs only (1.95±0.30 s period) and only on the same day of treatment, and one produced oscillatory cycles in just one VR the day after (2.50±0.49 s period). Reflex peak amplitude and area values 24 h after PM plus APV were 0.35±0.12 mV and 245±167 mV/s, (P = 0.272 and P = 0.025 vs sham preparations) respectively.

Fig. 3 C shows an example of the effect of PM plus CNQX alone: in this case fictive locomotion was present 1h after washout of the toxic medium (Fig. 3 C, middle), a finding obtained in 4/5 preparations with standard periodicity (see Fig. 3 E) and cycle amplitude (Fig. 3 F). Twenty-four h later 3/5 preparations could express fictive locomotion (see example in Fig. 3 C) although not on all four VRs. Furthermore, the pattern was elicited by lower concentrations of NMDA (2 or 3 μM rather than the standard 5 μM concentration that induced irregular firing only) with periodicity (Fig. 3 E) similar to control ($P > 0.05$ for the same day and for the day after vs. sham); however, fictive locomotion cycle amplitude was smaller (Fig. 3 F; $P = 0.023$ for the same day and $P = 0.268$ for the day after vs. sham). **As indicated by the example in Fig. 3 D**, all five preparations exhibited disinhibited bursting 24 h later with 73±35 s period (CV=0.47±0.08) and 10±2 s burst duration. VR reflexes had reduced amplitude ($P = 0.040$ for the same day and $P = 0.065$ for the day after vs. sham) and area ($P = 0.035$ for the same day and $P = 0.033$ for the second day vs. sham) as shown in Fig. 3 G, H. DR pulse trains failed to elicit locomotor-like oscillations either on the same or the day after treatment with PM plus either CNQX or APV.

Thus, neither CNQX nor APV alone could efficiently protect spinal networks from PM: CNQX was somewhat better than APV, but the functional outcome was inferior to the result observed with the combination of the two antagonists.

Delayed application of glutamate antagonists could still provide neuroprotection

While the former data suggested that co-applied glutamate antagonists could provide a degree of neuroprotection against PM, it would be interesting to know if their effect could be observed even after the administration of PM. Hence, our protocol was to apply CNQX and APV

immediately after PM washout (1 h exposure) and then continuously superfused them for 2 h, after which standard Krebs solution was resumed.

Fig. 4 shows an example of this experiment. Three h after the end of PM administration (and 2 h of CNQX plus APV), fictive locomotion was evoked by NMDA and 5-HT with periodicity similar to control (Fig. 4 D, middle; P = 0.383) and halved amplitude (Fig. 4 E; P = 0.048) in 4/5 spinal cords. The day after, all 5 preparations displayed fictive locomotion with periodicity and cycle amplitude similar to the sham ones (P > 0.05; Fig. 4 A, right, D, E). These data were observed on all four VRs of the five preparations tested. Disinhibited bursting was also present (n=5) with 147±65 s period (CV=0.27±0.15) and 25±7 s burst duration.

Fig. 4 C shows that DR stimulus train generated alternating VR oscillations (period 1.67±0.23 s) in control solution. After delayed application of CNQX and APV, cumulative depolarization was strongly decreased (from 0.61±0.10 to 0.12±0.06 mV on the same day, n=5; 0.20±0.13 mV 24 h later). Nevertheless, the day after treatment, oscillatory cycles were present in 2/5 preparations (Fig. 4 C) although only on a few VRs (1-2 roots). Their average period was 2.97±0.37 s. Fig. 4 F, G shows the average fall in VR reflex amplitude (P = 0.012 for the same day and P = 0.019 for the day after vs. sham) and area (P = 0.047 for the same day and P = 0.070 for the day after vs. sham) on the same day or the day after PM followed by CNQX and APV.

Histological analysis of these preparations indicated that, in comparison with PM (**+1 mM Mg^{2+}; Margaryan et al., 2009**), delayed co-application of CNQX and APV significantly (P=0.003) reduced the number of white matter pyknotic elements (**from 66±14 in PM+1mM Mg^{2+}** to 47±3 % in neuroprotected preparations; Fig. 5A, B), while the number of pyknotic cells in the other areas remained consistently low **(dorsal 0.8±2.1, central 0.2±0.1, ventral 7±2 %; Fig. 5 B)**. We further investigated numbers of surviving neurons (NeuN positive cells) and motoneurons (large,

ventral horn cells immuno-positive to SMI-32) after the delayed administration of CNQX and APV. The neuronal number in the ventral horn area (**151±46/aoi**; Fig. 5 C, D), was significantly (P=0.003) higher than the corresponding average without glutamate antagonists (**105±20/aoi in PM+1mM Mg^{2+}**; Margaryan et al., 2009). Neuronal counts in the dorsal and central areas (**see Fig. 5 D**) were, however, similar to those for PM (+ 1 mM Mg^{2+}; Margaryan et al., 2009) **as indicated by the example of Supplemental Fig. 2**. Likewise, the number of motoneurons (n=13±3/aoi; Fig. 5 E, F) was similar to the one found after PM (**+1mM Mg^{2+}; 15±2/aoi;** Margaryan et al., 2009).

Supplemental Fig. **3** shows the distribution of glial markers among ventral and lateral gray matter areas after PM followed by CNQX and APV. **There was a significantly stronger staining (362±32 arbitrary units, AU) of astrocytes (with GFAP) after PM followed CNQX and APV, in comparison with PM (297±29 AU; Margaryan et al., 2009).**

These results suggest that delayed neuroprotection with CNQX and APV was feasible and was chiefly directed to white matter elements **(such as astrocytes)** and ventral horn premotoneurons that enabled persistence of fictive locomotion.

Limited efficiency of glutamate antagonists against kainate excitotoxicity

We have previously shown that kainate (1 h) produces extensive spinal network lesion with loss of fictive locomotion, and histological damage primarily affecting premotoneurons and motoneurons (Taccola et al., 2008; Margaryan et al., 2009). In the present investigation we examined the possibility that CNQX and APV might contrast these toxic effects. Fig. 6 shows that co-application of kainate with CNQX and APV did not eliminate the depolarization of motor pools detected from lumbar VRs. On average, this depolarization was 2.74±1.35 mV (n=6)

versus 2.68±1.25 mV elicited by kainate alone (n=6). The latency for kainate evoked depolarization was 82±17 when co-applied with the antagonists, or 93±14.0 s ($P > 0.05$) when applied alone. This result shows that the onset of the depolarizing action of kainate was much faster than the slow pharmacokinetics of APV and CNQX block of glutamate receptors in the rat spinal cord in vitro (requiring up to 10 min; Evans et al., 1982; Long et al., 1990). Thus, the VR depolarization produced by kainate in the presence of CNQX and APV suggested that any potential action by these agents against kainate would have been delayed and unrelated to the initial network depolarization. One h after washing out kainate plus CNQX and APV, VR reflexes were fully blocked, although there was slight recovery in peak amplitude and area 24 h later (Fig. 6 D, E; $P = 0.024$ for peak and $P = 0.019$ for area). Likewise, DR stimulus trains always failed to induce cumulative depolarization on the same day after washout (Fig. 6 C, middle), although, 24 h later, low amplitude cumulative depolarization was observed in all preparations (on average 0.23±0.09 mV vs 0.63±0.3 sham; n=5). Notwithstanding one preparation in which sparse, low-amplitude cycles were seen (Fig. 6 C, right), there were no oscillatory cycles typical of locomotor activity.

Poor neuroprotection was observed even when fictive locomotion was studied with NMDA and 5-HT application. On the same day of washout of co-applied kainate plus CNQX and APV, there was no reappearance of fictive locomotion (Fig. 7 A, B; middle panels; n=6). Nevertheless, 24 h later, two preparations generated fictive locomotion as exemplified in Fig. 7 A (right; see also expanded record) with slow period (5.4±0.1 s; CV=0.13±0.02) and small amplitude (0.04±0.007 mV). The other four preparations were unable to express fictive locomotion as only irregular firing was detected from VRs (see example in Fig. 7 B and expanded record). In either case,

disinhibited bursting was present with similar characteristics (92±34 s period; CV=0.36±0.09; 12±4 s burst duration; n=6).

Fig. 8 shows data related to the histological damage induced by kainate in the presence of CNQX and APV. It seemed interesting to examine separately the data from preparations displaying fictive locomotion 24 h later and those that did not. Fig. 8 A indicates that the main difference between these groups in terms of cell pyknosis was a lesser degree of cell loss (P=0.003) affecting the dorsal horn area when fictive locomotion was still expressed.

Fig. 8 B shows that, when histological analysis was extended to identifying survived cell types, a larger number of neurons (P=0.001) was present in the dorsal horn area when fictive locomotion was preserved. Furthermore, a significantly larger (P=0.015) number of motoneurons was found in the preparations with fictive locomotion.

These observations suggest that co-applied CNQX and APV had limited ability to protect spinal networks from kainate damage.

When the application of CNQX and APV commenced on the washout of kainate and continued for 2 h, there was actually no recovery in fictive locomotion (n=6) with NMDA plus 5-HT or DR stimulus trains as exemplified in Fig. 9 A, B either on the same (middle) or the following day (right). Cumulative depolarization was suppressed (Fig. 9 B) and reflexes recorded from VRs severely attenuated 24 h later (Fig. 9 C; peak amplitude = 0.08±0.04 mV; area 61±31 mV/s; n=6). It was, however, possible to record disinhibited bursting as shown in Fig. 9 D: on average, period value was 77±12 s (CV=0.42±0.16) and burst duration 8±5 s (n=6).

Discussion

The principal finding of the present report is that, using the in vitro model of the neonatal rat spinal cord widely employed for studies of mammalian locomotor networks (Kiehn, 2006; Goulding, 2009), it was possible to demonstrate functional neuroprotection against a powerful metabolic insult by applying glutamate receptor antagonists either together with or after the toxic medium. Less effective neuroprotection was observed against kainate-evoked excitotoxicity. Because neuroprotection was associated with distinctive changes in spinal network histology, it was possible to estimate the minimal membership of spinal networks compatible with expression of locomotor patterns.

CNQX and APV as potential neuroprotective drugs

CNQX and APV are canonical antagonists of AMPA/kainate and NMDA receptors, respectively (Dingledine et al., 1999), and were tested simply as prototypic agents to investigate if these receptor classes might play an important role in the overall scenario of spinal network deficit. Previous reports have shown that glutamate antagonists have limited usefulness against long term recovery from spinal injury in vivo perhaps for reasons like the clinical predictiveness/relevance of the animal models, the adequacy of pharmacological methodology, and the outcome measures used (Faden and Stoica, 2007).

The in vitro preparation allowed us **to study, in more detail, the mechanisms underlying** two potential pathological conditions: 1. the antagonists were co-applied with the experimental insult (PM or kainate) to ascertain the contribution of glutamate receptor activation to damage, where it was produced, and to what degree damage prevention might be achieved; 2, the antagonists were given after removing the primary insult, in an attempt to mimic the consequences of early

treatment of acute spinal injury with intensive care that should start as short as possible after the primary lesion event (Bracken et al., 1990; Pointillart et al., 2000; Hall and Springer, 2004).

In our model, the study outcome was always presence of fictive locomotion on the day after the primary lesion and its relation to spinal network histological damage. Furthermore, by monitoring the expression of disinhibited bursting, we had the opportunity to assess the lesion severity and survival of the minimal circuitry sufficient to generate basic rhythmicity (Bracci et al., 1996). This approach prompted us to make certain predictions regarding the extent of network loss compatible with persistence of function. As far as network synaptic transmission was concerned, the present data show that, after applying CNQX and APV, reflex amplitude and area, and cycle amplitude were often similar to those normally found for sham preparations in view of their expected decline after 24 h in vitro (Evans, 1978; Taccola et al., 2008). It should also be noted that reflex activity is not a good predictor of locomotor patterns 24 h after experimental lesion (Taccola et al., 2008) in accordance with standard clinical practice where abnormal reflexes often represent pathological responses rather than network physiology.

CNQX and/or APV have been tested before in experiments in vivo to find out their ability to block glutamate receptors and spinal functional responses either in terms of locomotion or pain reflexes. Intrathecal infusion of either antagonist (1 mM) blocks locomotion in cats (Douglas et al., 1993) and spinal reflexes in rats (Al-Chaer et al., 1996): these doses are clearly largely higher than those used in vitro by us, although it is very difficult to estimate the effective drug concentration in the extracellular space of the spinal cord in vivo. CNQX and APV (1-4 mg/kg i.p.) show neuroprotective effect on neonatal rat motoneurons after sciatic nerve transection (Iwasaki et al., 1995): such a dose of CNQX would provide an estimated plasma concentration of 5-20 μM, with comparable estimate for APV dosage.

Thus, our data with 10 µM CNQX and 50 µM APV are based on concentrations that can be approximated to those efficacious in animal experiments and are related to pharmacological parameters like antagonist IC_{50} values (Evans et al., 1982; Long et al., 1990). Of course, these drug doses cannot be construed to be safe for human use and can only provide an indication on whether glutamate receptor antagonism is a feasible option for neuroprotection of the spinal cord tissue.

PM induced spinal damage: antagonism by CNQX and APV

Co-application of CNQX and APV with PM consistently enabled fictive locomotion 24 h later. Disinhibited bursting periodicity was also faster than in PM-treated preparations, suggesting improved network function. This condition was accompanied by a smaller loss of ventral horn cells with unchanged values for other areas. Conversely, neither antagonist administered alone could provide a satisfactory degree of protection. The simplest interpretation is that, within the protocol timeframe, damage due to PM comprised a broad excitotoxic component.

Because global cell loss was not extensive, it appeared interesting to test whether neuroprotection could still be obtained with the same agents applied after the primary insult. This approach provided neuroprotection in terms of fictive locomotion elicited by NMDA and 5-HT, and partly even for oscillatory cycles induced by DR stimulus trains. The latter finding was unexpected in view of the fact that, in previous models of in vitro spinal damage, it had not been possible to retain electrically-stimulated fictive locomotion (Taccola et al., 2008; Margaryan et al., 2009).

Histological analysis indicated that delayed application of CNQX and APV enabled a larger population of glial cells of the lateral column area to survive, taking the average number of dead

white matter elements to below 50 % of the total population. This observation closely accords with the delayed white matter degeneration described as a secondary mechanism of in vivo spinal cord injury and comprising an excitotoxic process (Park et al., 2004). When white matter damage affected ≥50 % elements, electrically-induced fictive locomotion was not possible, yet chemically-induced fictive locomotion could persist. We reckon that nearly 50 % loss was close to the upper limit of white matter damage tolerability to express rudimentary electrically-stimulated VR oscillations, as long as there were sufficient gray matter elements necessary for rhythm generation. Viceversa, the more lesion-resilient NMDA and 5-HT evoked patterns probably relied on propriospinal connectivity, of which the white matter lateral columns were not the fundamental component. Our in vitro data accord with observations on in vivo rats subjected to graded contusion injury to the spinal cord (Cao et al., 2005). The extent of changes affecting the white matter of ventral and lateral funiculi is believed to be closely related to the locomotor deficit caused by dysfunction of descending supraspinal inputs (Cao et al., 2005).

In our study neuronal survival data from the lumbar ventral horn, a region fundamental for expressing the locomotor rhythm (Kiehn, 2006; Goulding, 2009), showed that, while motoneuron numbers were unchanged by late application of CNQX and APV, premotoneurons were detected in significantly larger number (on average 151 neurons/aoi), a value almost identical to the one (149/aoi) found with neuroprotection by 5 mM Mg^{2+}, likely due to inhibition of network synaptic transmission (Margaryan et al., 2009). Thus, block of either presynaptic release of glutamate by high Mg^{2+} or of its postsynaptic actions by receptor antagonists appears to be an effective strategy to limit early spinal damage in vitro.

Our former study demonstrated that PM (+1mM Mg^{2+}) led to a mean survival of 105 ventral horn neurons/aoi (with much less damage to other gray matter areas; Margaryan et al., 2009).

Hence, it is tempting to hypothesize from the consequences of PM application and neuroprotection that, on average, the ability of spinal lumbar networks to produce locomotor patterns requires a premotoneuron membership of (at least) 150 neurons/aoi and, in any case, larger than 105 neurons/aoi.

The causes for the differential cell vulnerability (premotoneurons vs motoneurons) to PM-evoked damage remain unclear even though neuron-selective vulnerability to ischemia, chronic seizures or brain trauma has been observed in the rat (Papp et al., 2008) and human (Toth et al., 2007) hippocampus, and in the rat cerebellum (Park et al., 2006). Identification of the mechanisms predisposing premotoneurons to PM damage requires future investigation in view of its potential importance for the treatment of acute spinal injury.

Kainate induced spinal damage: antagonism by CNQX and APV

In keeping with former observations (Taccola et al., 2008), kainate consistently abolished fictive locomotion and strongly depressed reflex activity. This effect is typically accompanied by large neuronal loss affecting ventral horn premotoneurons and motoneurons, while white matter is slightly affected (Taccola et al., 2008). It should, however, be noted that despite the strong excitotoxic challenge and the resulting functional damage, loss of ventral horn premotoneurons is 43%, while loss of motoneurons is 37% (Taccola et al., 2008). Thus, kainate administration is not equivalent to massive destruction of spinal networks that retain their property to generate disinhibited bursting.

In the present study, CNQX and APV co-applied with kainate produced partial protection of locomotor networks, as NMDA and 5-HT could still activate this rhythm in 1/3rd of preparations. Despite the large survival of white matter elements, electrically-induced fictive locomotion was

poorly protected and only few oscillatory cycles were sporadically present even if some recovery of cumulative depolarization was detected. It is likely that the large neuronal loss meant disappearance of those neurons necessary to integrate afferent inputs into the locomotor networks. Our observations, thus, accord with the demonstration that, following in vivo intraspinal injection of kainate, gray matter loss can result in significant functional deficits, including paraplegia, in the absence of the disruption of major descending pathways typically caused by trauma (Magnuson et al., 1999).

The slow pharmacokinetic properties of CNQX and APV, that require a much longer time to block glutamate receptors of the rat spinal cord in vitro (Evans et al., 1982; Long et al., 1990) than kainate to activate them, likely account for the restricted neuroprotection and for the ineffective defense from kainate insult when the antagonist application was delayed. The most parsimonious explanation is that a substantial component of the kainate evoked lesion occurred rapidly and that more efficient damage inhibition should perhaps be sought in future studies by targeting downstream processes sequential to glutamate receptor overactivity.

The outcome of the kainate neuroprotection protocol (some preparations could produce fictive locomotion with NMDA and 5-HT, while others could not) led us to investigate any histological difference. Effective neuroprotection was accompanied by fewer dead cells in the dorsal horn (in keeping with the expression of kainate receptors on DR afferents; Agrawal and Evans, 1986), and more surviving motoneurons. Other lumbar neurons were, however, present in similar numbers, implying that the degree of neuronal loss tilting the balance between presence or absence of locomotor patterns was quite small, at the least in those areas in which the locomotor central pattern generator (CPG) is thought to be located (Kiehn, 2006; Taccola and Nistri, 2006b; McCrea and Rybak, 2008).

One might imagine diverse reasons for such a finding: perhaps the loss of few crucial neurons was below the resolution of our histological analysis and implied the existence of scattered cells strategic for rhythm generation. Their future identification might benefit from the availability of interneuron-selective genetic markers (Goulding, 2009). Alternatively, neuronal damage might have been mainly functional without actual cell death. Regardless of its origin, the kainate data allowed us to further refine the estimate (obtained from PM experiments) of the lowest number of lumbar ventral horn neurons necessary, on average, for locomotor patterns: in fact, this value can be approximated to ≥125 neurons/aoi. This reduced population seemingly still contained the basic elements of the CPG for rhythm generation and pattern formation (McCrea and Rybak, 2008).

Conclusions

The present study demonstrated the feasibility of protecting spinal locomotor networks in vitro from experimental damage evoked by PM or kainate by using the standard glutamate antagonists CNQX and APV. These data, therefore, suggest that excitotoxicity was an early and important component to spinal lesion and outline a minimal size of network membership to support locomotor patterns. We posit that these results may be useful to better understand the structure of locomotor networks especially if further developed with a detailed characterization of the neuronal phenotypes affected by the experimental lesion and integrated with recent data on the composition of locomotor networks obtained from mouse genetics (Kiehn, 2006; Goulding, 2009). Moreover, the present model may be viewed as an experimental tool to predict the suitability of novel neuroprotective agents for advanced preclinical testing.

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Figure Legends

Figure 1 Neuroprotection of locomotor network activity by CNQX and APV co-applied with PM. A: example of fictive locomotion evoked by 5 μM NMDA and 10 μM 5-HT recorded as alternating oscillations between L2 and L5 VRs (left). PM was applied for 1 h and fictive locomotor ability re-tested after 1 h wash without response (middle). Likewise, fictive locomotion could not be induced on the same spinal cord preparation the day after (right). B: Disinhibited bursting elicited by 1 μM strychnine and 20 μM bicuculline the day after application of PM. C: protocol similar to A with CNQX (10 μM) and APV (50 μM) applied together with PM for 1 h. Note persistence of fictive locomotion after 1 h washout (middle) and even 24 h later (right). D: disinhibited bursting recorded after 24 h from application of PM plus CNQX and APV. E, F: histograms showing average changes in period and amplitude of fictive locomotion in control conditions (CTR), at 1 h or 24 h wash from PM plus CNQX and APV. E: $P > 0.05$ vs same day control and vs. sham for the second day; F: * shows $P = 0.04$ vs same day control. G, H: histograms showing changes in DR-evoked VR reflex amplitude (G) and area (H) for the same protocols as in E, F. G: * shows $P = 0.042$ vs same day control. All data are from 4 preparations.

Figure 2 Quantification of pyknosis in spinal cords treated with PM co-applied with CNQX and APV. **A:** The histograms show the number of pyknotic nuclei (percent of the total number of DAPI-stained nuclei) in 30 μm cross-sections of lumbar segments of the rat spinal cord. The following aoi were analyzed: 280x280 μm in the dorsal gray matter (Rexed laminae I-IV; open column), the central gray matter (Rexed laminae V-VIII and X; dashed column), and the ventral gray matter (Rexed laminae IX; gray column). For lateral white matter the aoi was 100x280 μm

(filled column). Inset: schematic representation of the aoi. Dors: dorsal; cent: central; vent: ventral; wm: white matter. P = 0.004 for the difference related to ventral horn pyknosis vs data for PM (+ 1 mM Mg^{2+}; see Margaryan et al., 2009, Fig 5 C). **B: Example of the dorsal part of the spinal cord treated with PM + 1mM Mg to show the morphology of pyknotic nuclei with condensed chromatin, clearly distinguishable from normal cells. C: Higher magnification of the area within the box shown in B. Arrows indicate pyknotic nuclei. Scale bars = 50 μm.**

Figure 3 Effect of APV or CNQX on the block of locomotor activity produced by PM. A: co-application of PM with APV does not allow expression of fictive locomotion after either 1h (middle) or 24 h (right) wash. For further details see Fig.1 legend. B: Disinhibited bursting is observed after 24 h washout of co-applied PM with APV. C: co-application of PM with CNQX allows limited expression of fictive locomotion after either 1h (middle) or 24 h (right) wash. D: Disinhibited bursting is observed after 24 h washout of co-applied PM with CNQX. E, F: histograms showing average changes in period and amplitude of fictive locomotion in control condition (CTR), at 1 h (n=4) or 24 h (n=3) wash from PM plus CNQX. E: P > 0.05 vs same day control and sham for second day. F: * shows P = 0.023 vs same day control and P = 0.268 vs sham for the second day. G, H: histograms showing changes in DR-evoked VR reflex amplitude (G) and area (H; n=5) for the same protocols as in E, F. G: * shows P = 0.040 vs same day control and P = 0.065 vs sham for the second day. H: * shows P = 0.035 vs same day control and P = 0.033 vs sham for the second day.

Figure 4 Delayed application of CNQX and APV protects locomotor networks from PM damage. A: co-application (2 h) of CNQX and APV after 1 h application of PM allows expression of fictive locomotion evoked with NMDA and 5-HT and recorded at either 3 h (middle) or 24 h (right) washout. For further details see Fig.1 legend. B: Disinhibited bursting is observed at 24 h washout of PM followed by CNQX and APV. C: DR stimulus trains evoke cumulative depolarization with superimposed alternating oscillations in Krebs solution (left). This effect is lost at 3 h wash (middle) after PM followed by CNQX and APV. Limited presence of oscillations is observed at 24 h wash (right). D, E: histograms showing average changes in period and amplitude of fictive locomotion in control condition (CTR), at 3 h (n=4) or 24 h (n=5) wash from PM followed by CNQX and APV. D: $P > 0.05$ vs same day control and vs sham for the second day. E: * shows $P = 0.048$ vs same day control and $P = 0.874$ vs sham for the second day. F, G: histograms (n=5) showing the average change in VR reflex amplitude and area for the same protocol as in D, E. F: * shows $P = 0.012$ vs same day control and $P = 0.019$ vs. sham for the second day. G: * shows $P = 0.047$ vs same day control and $P = 0.070$ vs sham for the day after.

Figure 5 Quantification of spinal cord damage after 1 h PM followed by 2 h CNQX plus APV. A: DAPI staining of the ventral part of the rat lumbar spinal cord shows pyknosis in the lateral column white matter (30 μm section, lumbar region); B: Histograms showing the number of pyknotic nuclei counted in the dorsal (open column), central (dashed column), ventral (gray column) gray matter and in the lateral white matter (filled column) regions, after 1h treatment with PM followed by 2h CNQX and APV and washout. $P = 0.028$ and 0.003 for ventral and white matter aoi, respectively, vs PM (+ 1 mM Mg^{2+}; see Fig. 5 C of Margaryan et al., 2009).The

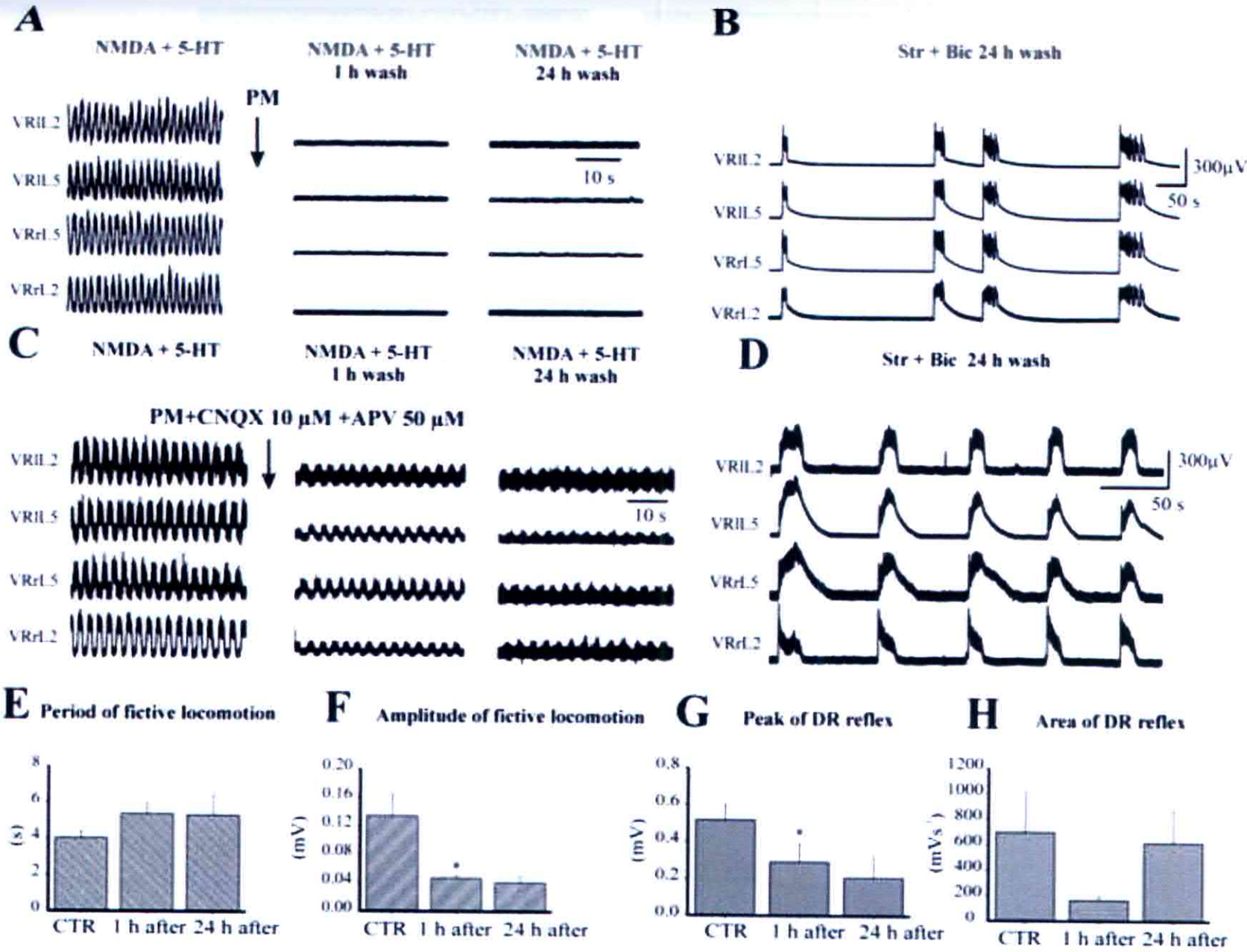
inset shows schematic representation of the aoi used for analysis. Dors: dorsal; cent: central; vent: ventral; wm: white matter. C: NeuN immunoreactivity of the ventral region **after 1 h PM followed by 2 h CNQX plus APV**. D: Histograms showing the number of NeuN positive neurons counted in a 170x170 μm aoi of the dorsal and central gray matter (left and central columns) and 450x450 μm aoi of the ventral gray matter (right column). P = 0.003 for neurons in the ventral aoi vs PM (Fig. 6 E of Margaryan et al., 2009). E: SMI32-positive motoneurons in the ventral area. F: Histogram showing the number of SMI32 positive motoneurons counted in Rexed laminae VIII and IX: this was the same as after PM. For data comparison with the effect of PM without CNQX and APV see Fig. 5 C and 6 E, F of Margaryan et al. (2009). Bar=100 μm for A, C, E.

Figure 6 Effects of co-application of kainate with CNQX and APV on network activity. A: recording from L2 and L5 VRs shows depolarization (with transient oscillations) evoked by kainate together with CNQX and APV applied at the arrow. B: DR-induced VR reflexes in control Krebs solution (left), after 1 h (middle) or 24 h (right) wash of kainate plus CNQX and APV. Note strong depression of synaptic transmission. C: DR stimulus trains evoke cumulative depolarization with superimposed alternating oscillations in Krebs solution (left). This effect is lost 1 h after washout of kainate plus CNQX and APV (middle). Partial recovery of cumulative depolarization and oscillatory activity is observed 24 h later (right). D, E: histograms showing average changes in VR reflex amplitude and area in control condition (CTR), at 1 h or 24 h wash from kainate plus CNQX and APV (n=6). * shows P = 0.024 for peak and P = 0.019 for area vs sham preparations.

Figure 7 Partial neuroprotection by CNQX and APV against kainate evoked suppression of fictive locomotion. A: fictive locomotion is induced by NMDA and 5-HT in control condition (left), at 1 h from washout of kainate plus CNQX and APV no locomotor patterns are seen (middle). However, 24 h later small amplitude locomotor like cycles reappear. Their oscillatory nature is demonstrated in the high gain, fast time base inset (bar indicates trace segment from which inset is taken). This pattern was observed in 2/6 spinal cords. B: experimental protocol like in A shows lack of fictive locomotion at 1 or 24 h washout of kainate plus CNQX and APV. NMDA and 5-HT produce irregular high frequency discharges from VRs (see expanded record in inset) only. This pattern was found in 4/6 preparations.

Figure 8 Quantification of kainate induced damage in the presence of CNQX and APV. A: Histograms showing the percent of pyknotic nuclei counted in 30 μm sections of spinal cords treated with kainate (KA) plus CNQX and APV. The cluster on the left shows fictive locomotion (evoked by NMDA plus 5-HT; FL) 24 h later (n=2), while the cluster on the right does not (n=4). Four aoi have been measured: dorsal (open column), central (dashed column), and ventral (gray column) gray matter and the lateral white matter (filled column). P = 0.003 for dorsal aoi between the two groups. B: Number of NeuN positive neurons counted in sections from treated spinal cords. Filled columns refer to preparations recovering fictive locomotion 24 h after the application of kainate plus CNQX and APV (n=2), while open columns refer to samples without locomotor recovery (n=4). P = 0.001 for the difference between dorsal horn neurons of the two groups. C: Number of SMI32 positive motoneurons, counted in 30 μm sections. The column on the left refers to preparations with locomotor recovery (n=2), while the column on the right to spinal cords without it (n=4). P = 0.015.

Figure 9. Failure by delayed application of CNQX and APV to inhibit damage induced by kainate. A: fictive locomotion evoked by NMDA and 5-HT in control Krebs (left) is suppressed at 3 h wash after 1 h kainate followed by 2 h CNQX and APV administration. No recovery is present even 24 h later. B: control electrically-evoked fictive locomotor patterns induced by DR stimulus trains (left) are fully blocked at 3 h wash after 1 h kainate followed by 2 h CNQX and APV administration. No recovery is observed even the day after. C: control VR reflex elicited by DR stimulation is likewise inhibited at 3 h wash after 1 h kainate followed by 2 h CNQX and APV administration and on the day after. D: disinhibited bursting is present 24 h after kainate application followed by 2 h CNQX and APV administration. This finding indicates persistence of elementary functional activity despite the treatment.



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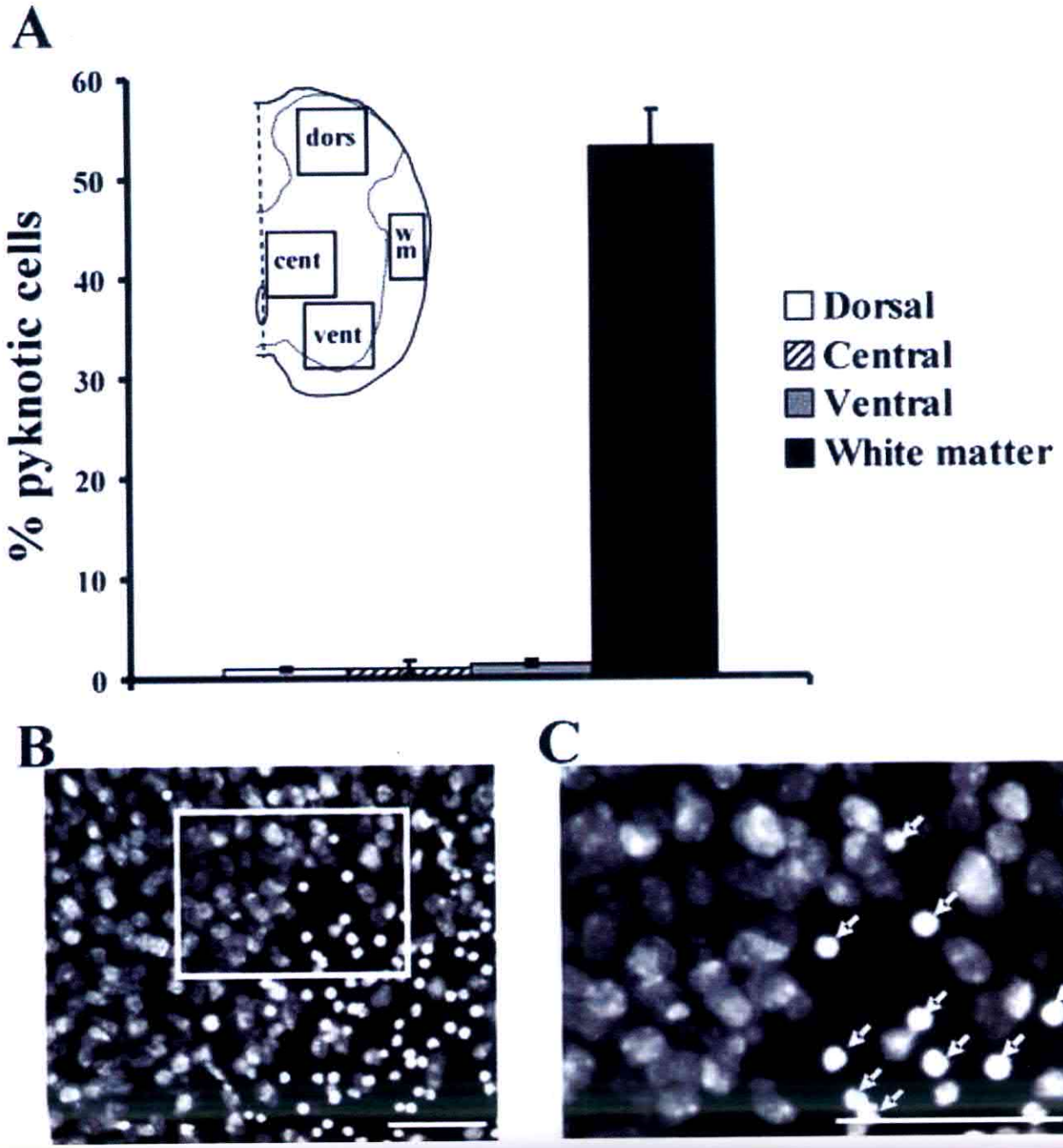

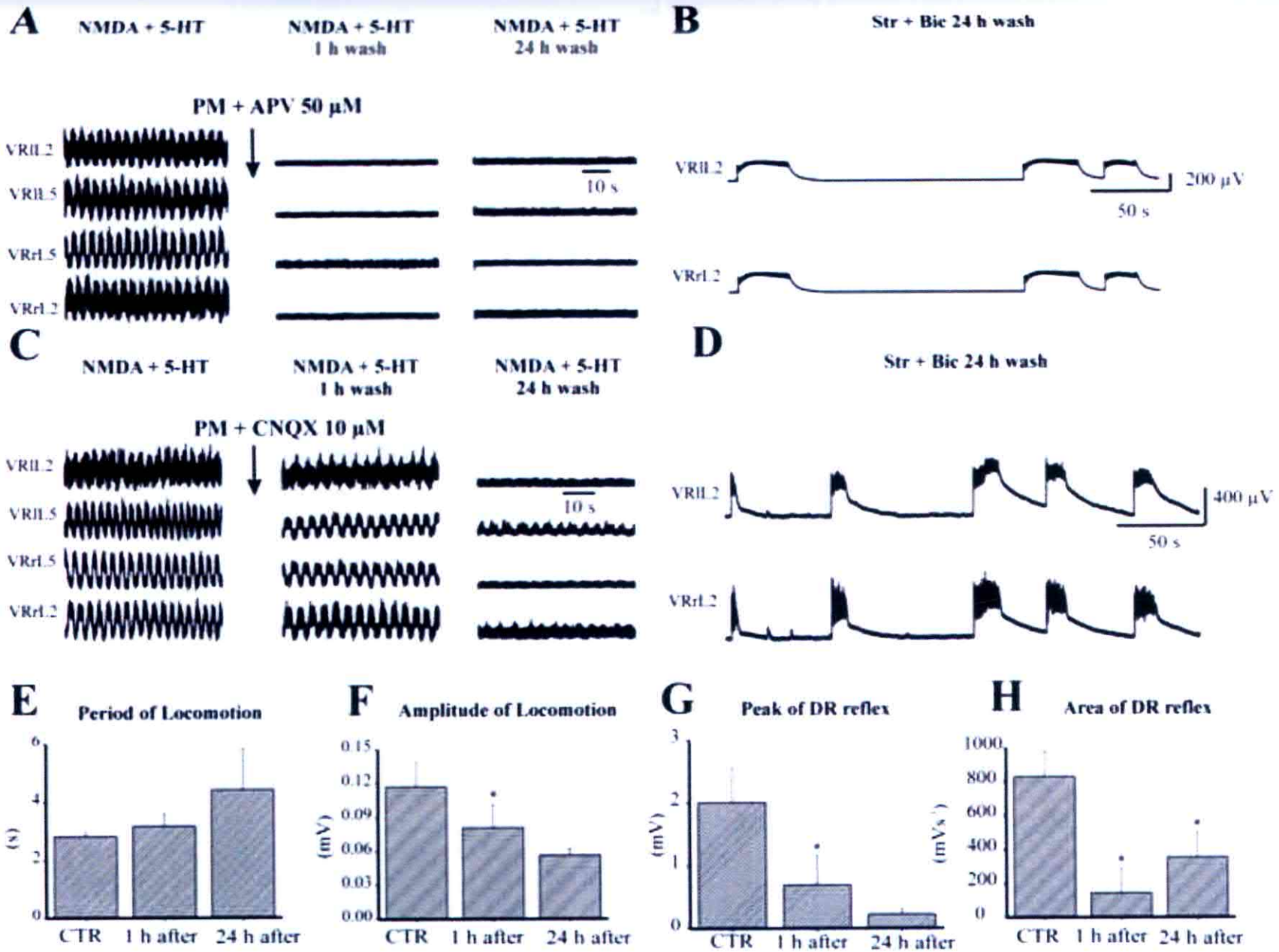

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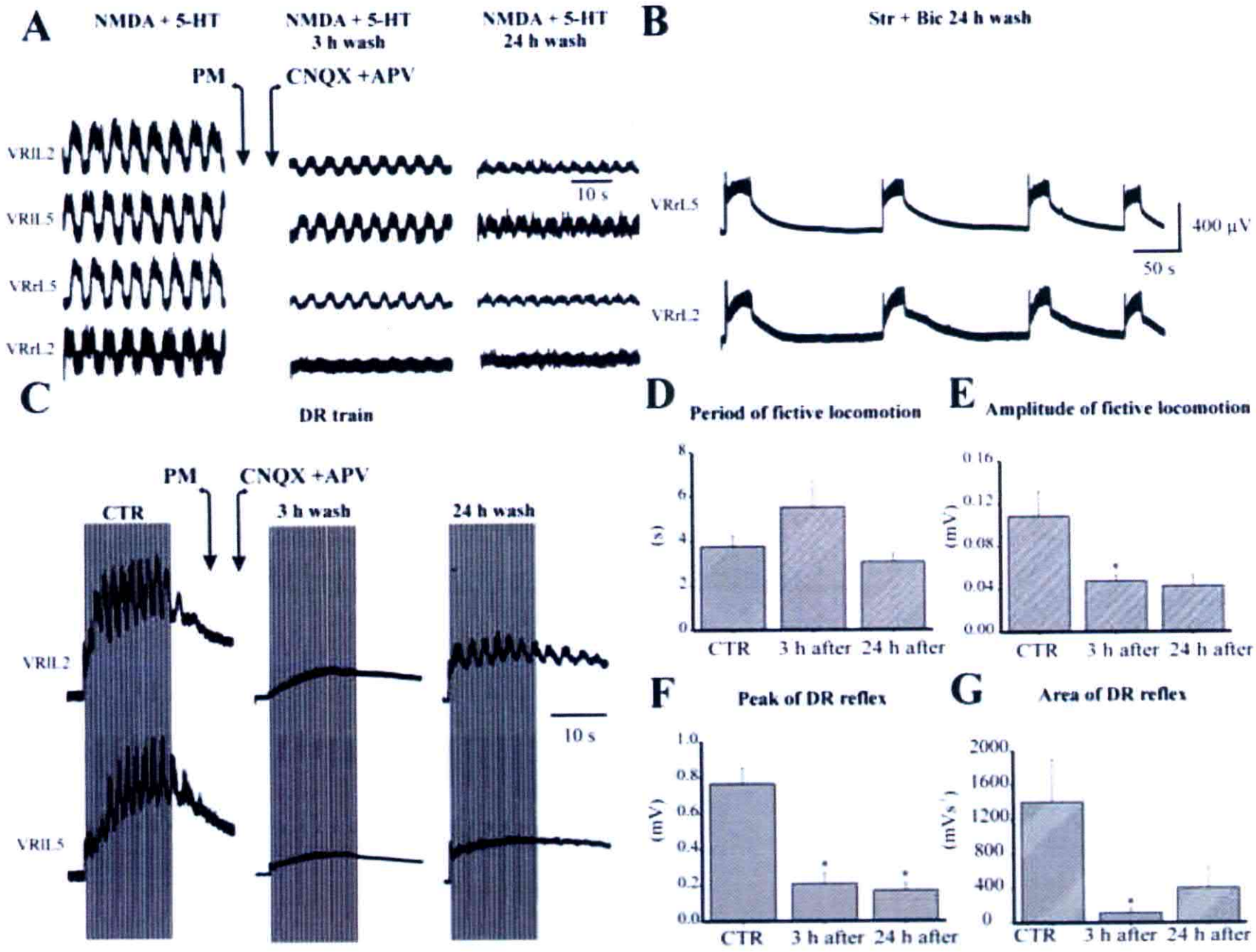

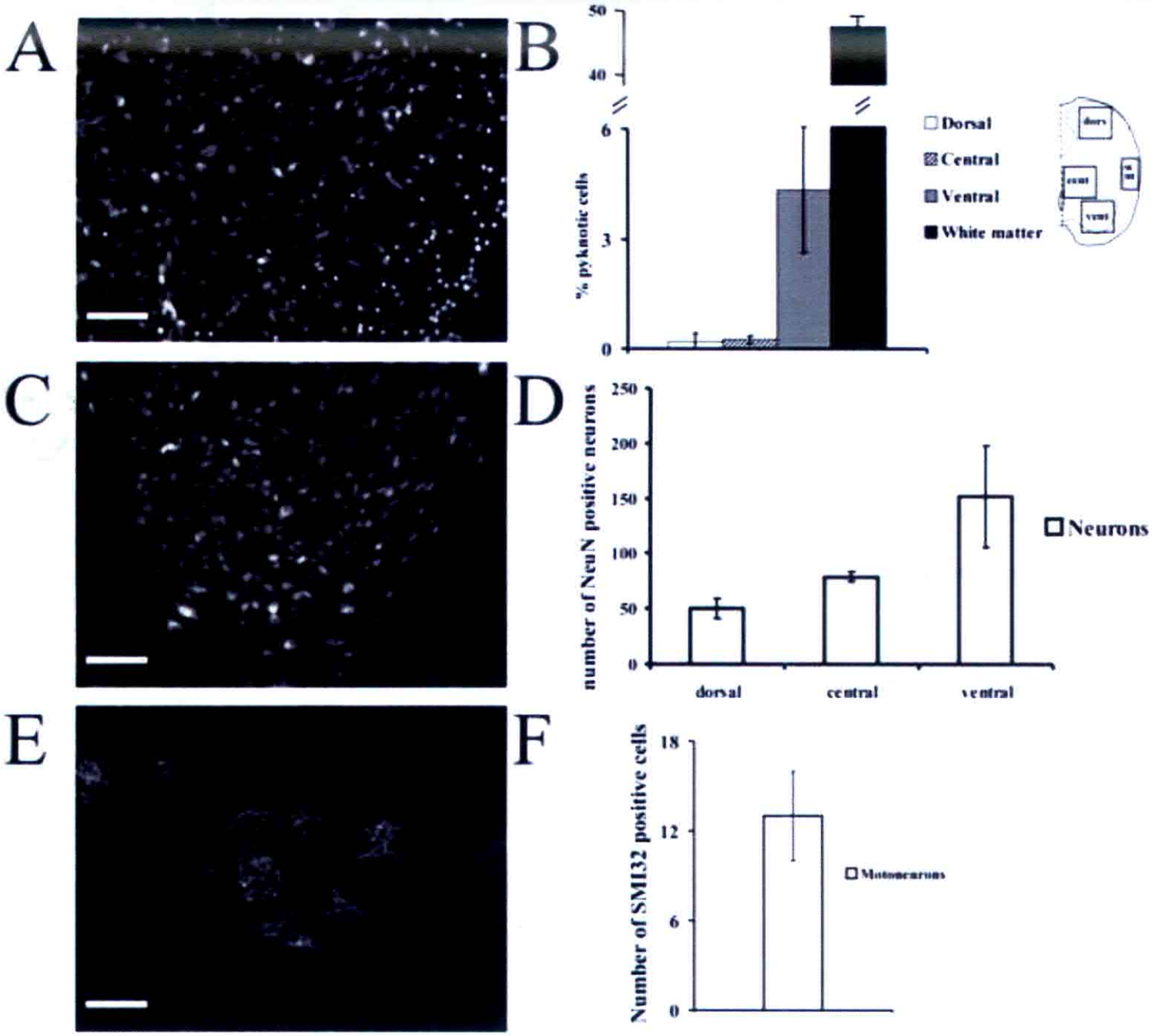

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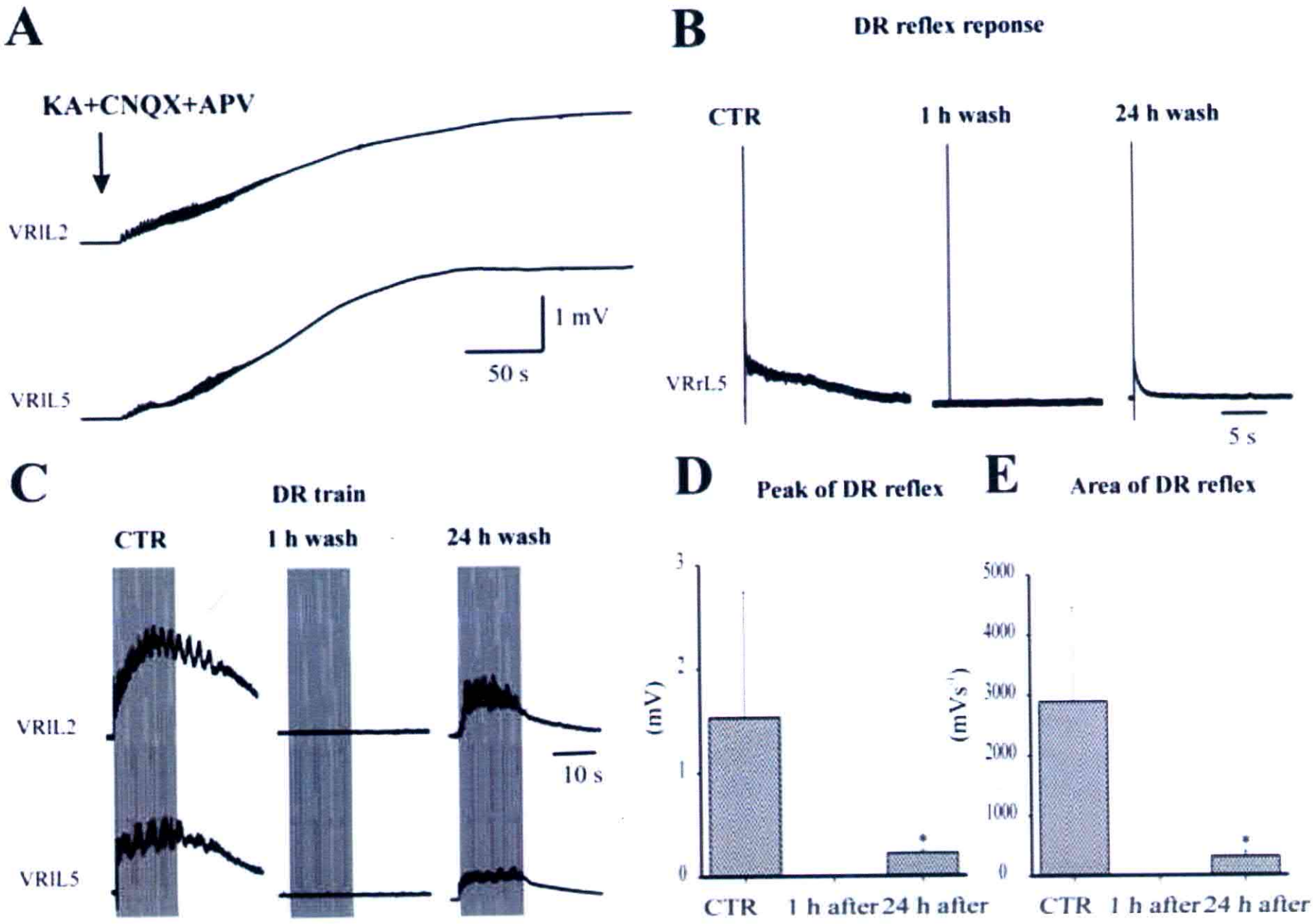

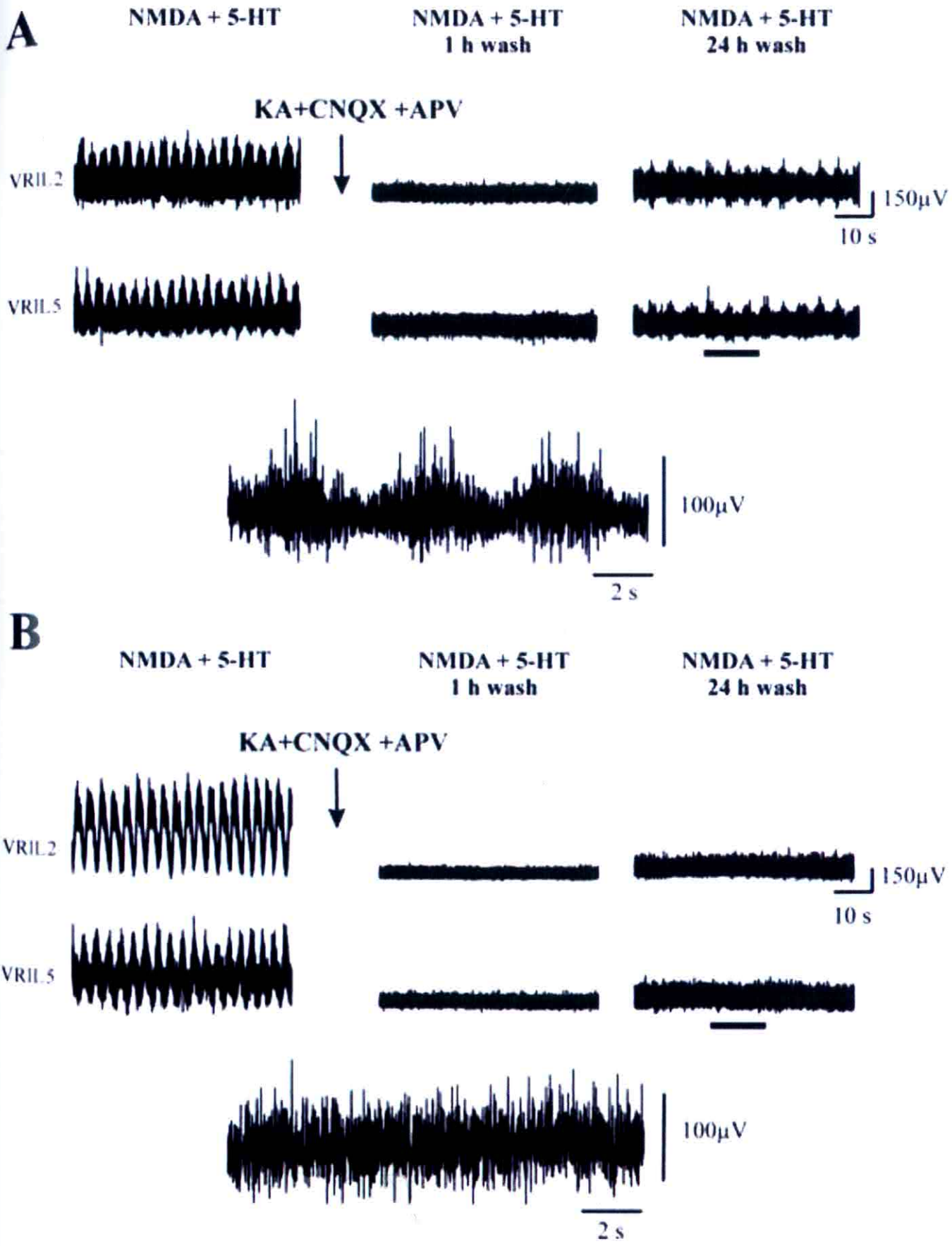

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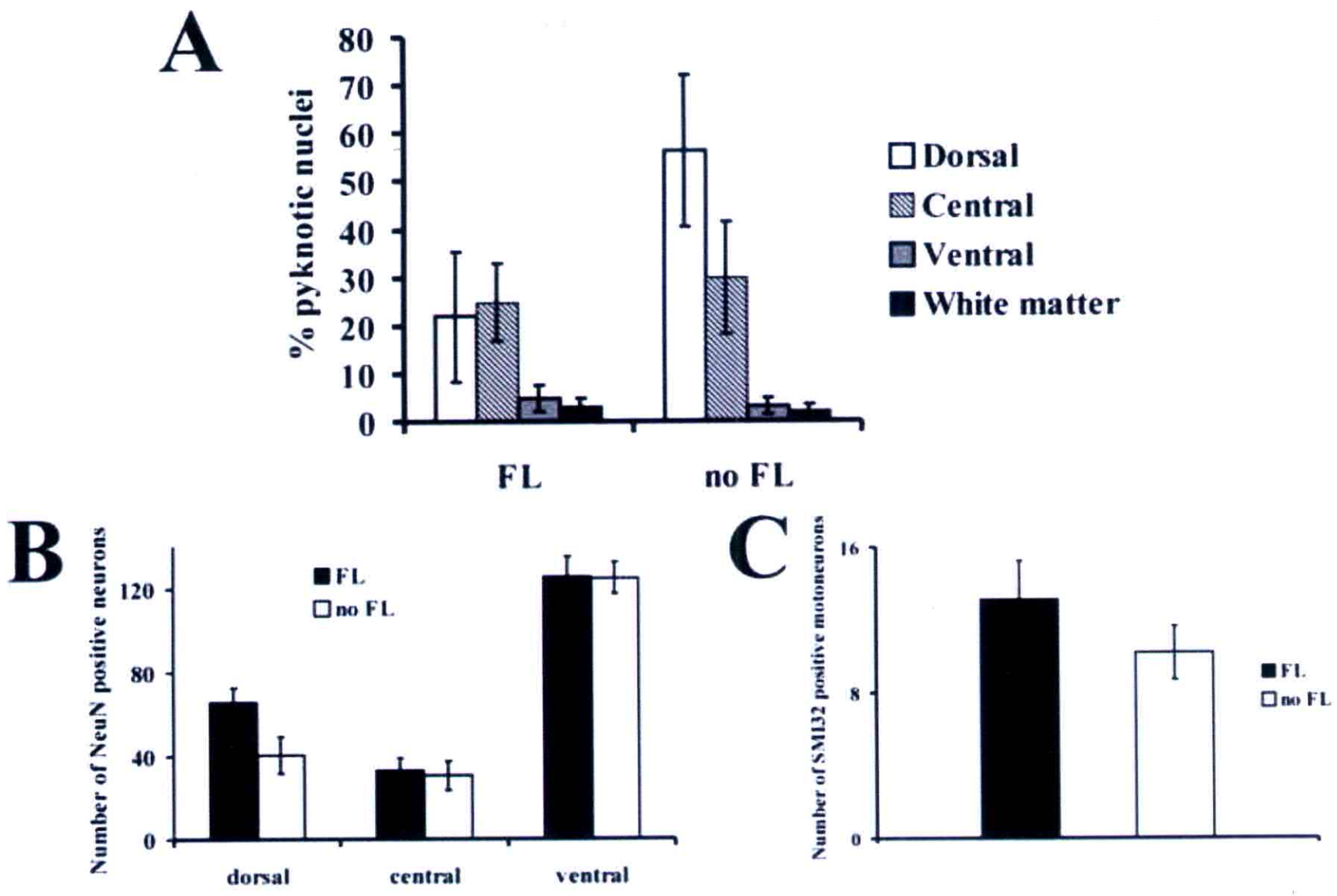
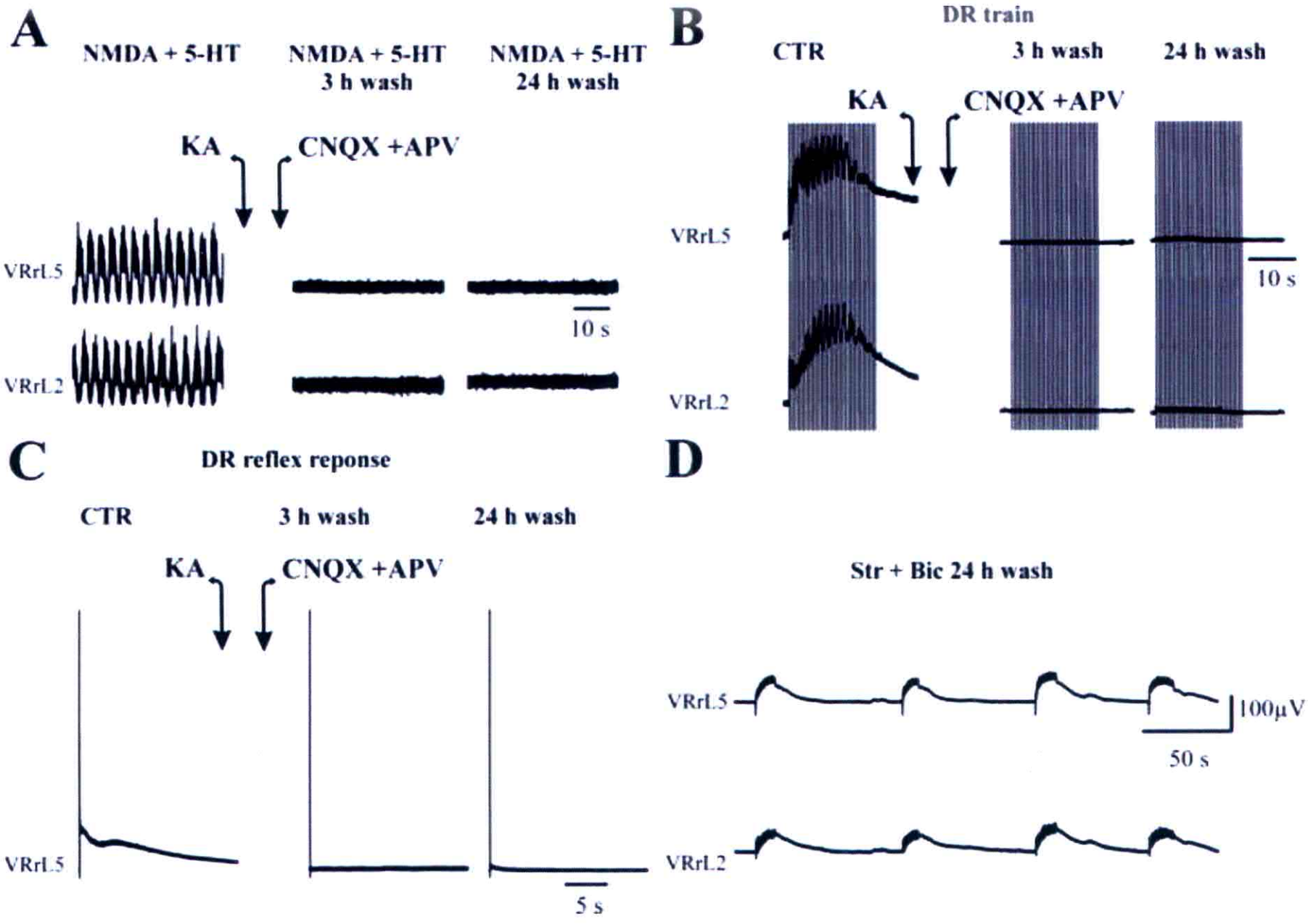
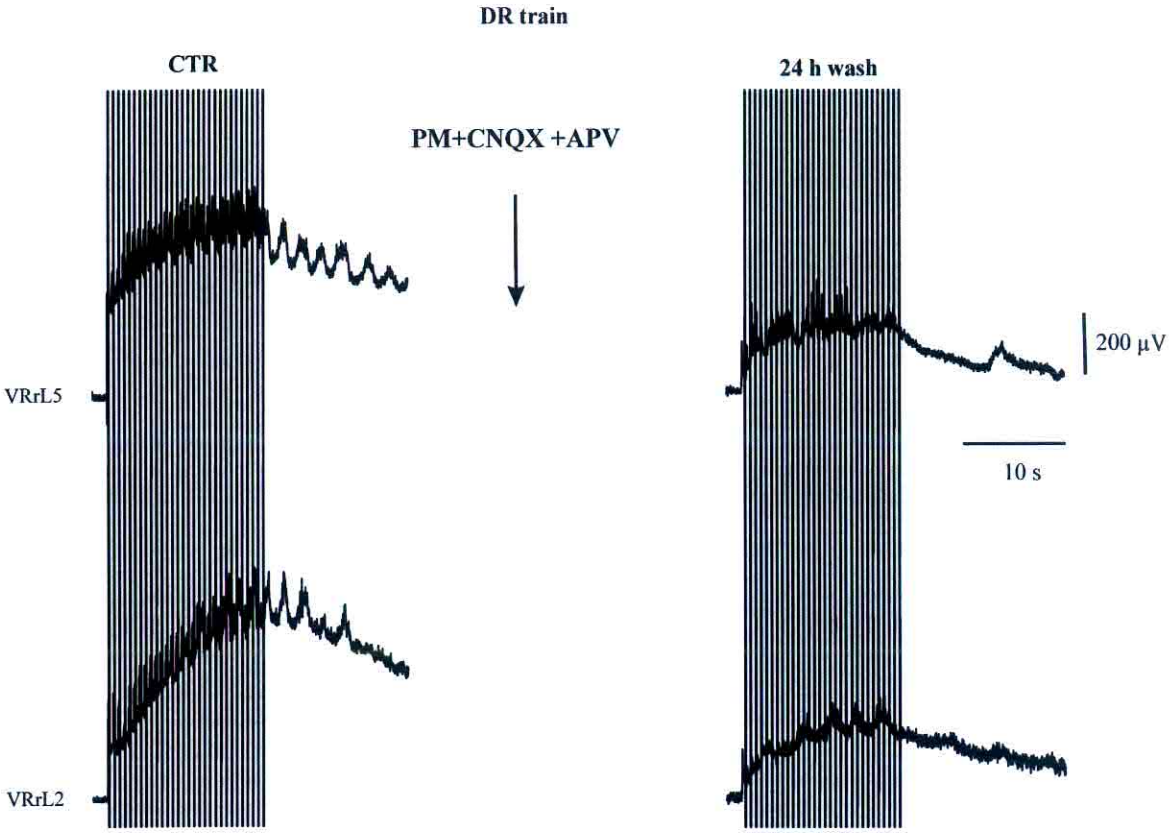


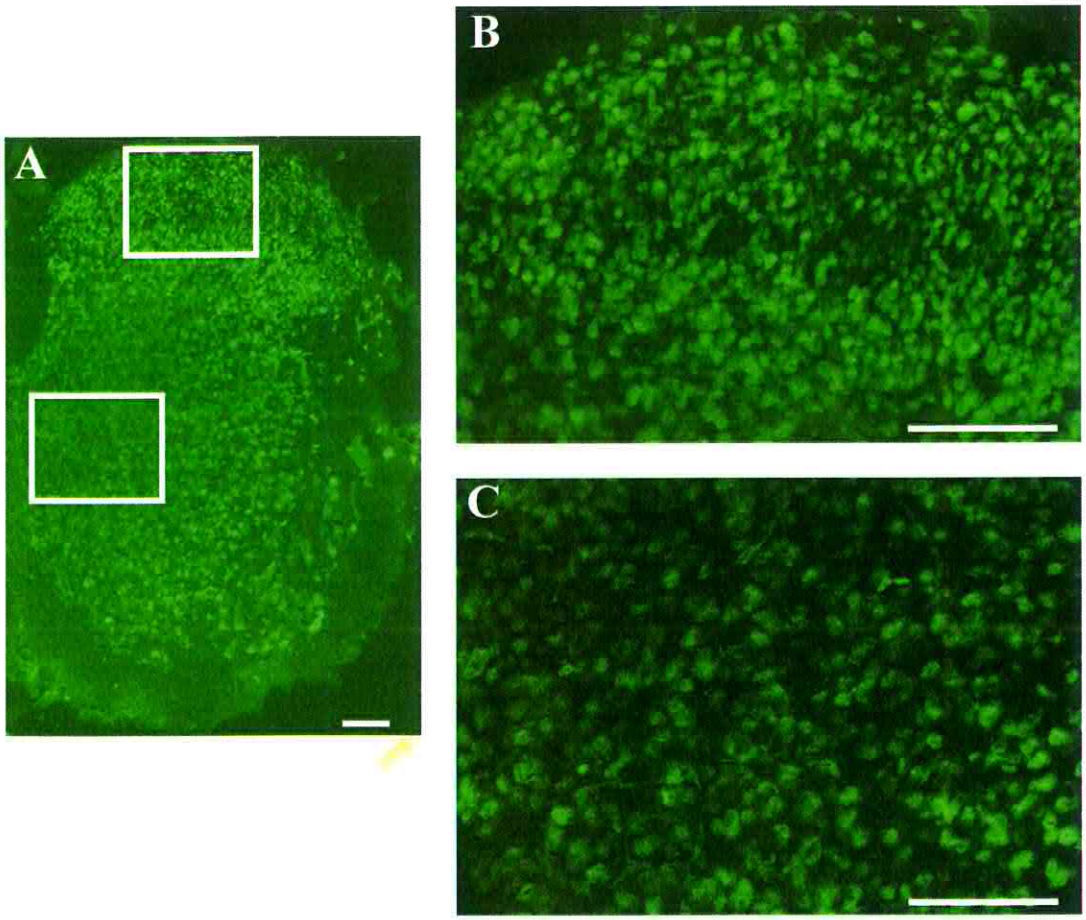


Supplementary Figure 1.



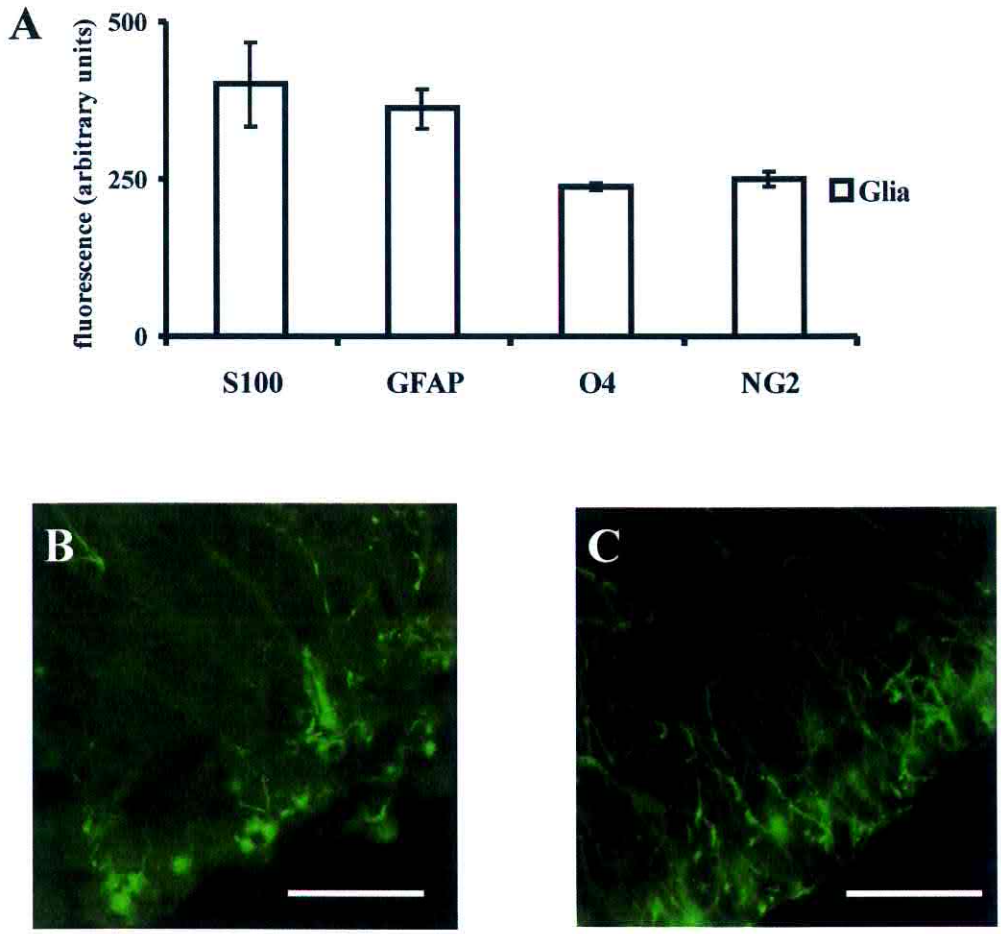
CNQX and APV co-applied with PM elicit partial protection of oscillatory cycles and cumulative depolarization induced by DR stimulus trains. Responses on the left show, for two lumbar VRs, control oscillatory cycles outlasting the stimulus train, while right hand side records indicate (24 h later) depression of cumulative depolarization and rudimentary oscillations following co-application of these agents. When PM is applied without glutamate antagonists, there is inhibition of oscillatory cycles and depression of cumulative depolarization (see Fig. 3 A of Margaryan et al 2009).

Supplementary Figure 2.



A: Example of neuron specific immunostaining with NeuN in 30 µm hemisection of the lumbar L1 segment of the spinal cord treated with PM+1mM Mg^{2+} and CNQX+APV. B and C: Higher magnification of the dorsal (B) and central (C) region of the spinal cord. Scale bars=100 µm.

Supplementary Figure 3.



A: Effect of PM+1mM Mg^{2+} followed by 2 h application of CNQX and APV on glial markers investigated the day after washout. Histograms show intensity of fluorescence signal (expressed in arbitrary units) of S100 (marker for protoplasmic astrocytes), GFAP (for astrocytes of the white matter), O4 and NG2 (for oligodendrocytes precursors) in the sections of rat spinal cord; n = 3. B: Example of GFAP immunostaining in the 30µm section of L1 segment of the spinal cord treated with PM+1mM Mg^{2+}. C: Example of GFAP immunostaining in the 30µm section of L1 segment of the spinal cord treated with PM+1mM Mg^{2+} and CNQX and APV. Scale bars=100 µm.

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Deconstructing locomotor networks with experimental injury to define their membership

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Short title: spinal injury in vitro

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Key words: excitotoxicity; oxygen glucose deprivation; reactive oxygen species; spinal injury; central pattern generator; rhythmogenesis

Abstract

Although spinal injury is a major cause of chronic disability, the mechanisms responsible for the lesion pathophysiology and their dynamic evolution remain poorly understood. Hence, current treatments aimed at blocking damage extension are unsatisfactory. To unravel the acute spinal injury processes, we have developed a model of the neonatal rat spinal cord in vitro subjected to kainate-evoked excitotoxicity or metabolic perturbation (hypoxia, aglycemia and free oxygen radicals) or their combination. The study outcome is fictive locomotion one day after the lesion and its relation to histological damage. Excitotoxicity always suppresses locomotor network activity together with large gray matter damage, while network bursting persists supported by average survival of nearly half premotoneurons and motoneurons. Conversely, metabolic perturbation simply depresses locomotor network activity as damage mainly concerns white rather than gray matter. Co-application of kainate and metabolic perturbation completely eliminates locomotor network activity. These results indicate distinct cellular targets for excitotoxic versus dysmetabolic damage with differential consequences on locomotor pattern formation. Furthermore, these data enable to estimate the minimal network membership compatible with expression of locomotor activity.

Introduction

The lumbar region of the spinal cord contains extensive neuronal networks which can generate coordinated locomotion independently from supraspinal or peripheral inputs[1, 2, 3]. Operationally this network arrangement is termed central pattern generator (CPG)[4].

The locomotor program consists of a series of motor commands (expressed by motoneurons) to flexor and extensor limb muscles to provide the swing and stance alternating pattern essential for over-ground gait. This is, indeed, a complex program that involves correct timing and phasing of the neuronal signals with rhythmic excitation and inhibition of reciprocal motor units. In fact, when synaptic excitation is blocked, locomotion is arrested[5], whereas block of synaptic inhibition converts locomotor patterns into synchronous, slow disinhibited bursting[6]. The latter activity is believed to express basic network excitability and is useful to monitor the persistence of elementary functional connectivity within the circuitry.

Locomotion requires the activation of the CPG by descending inputs, for example from the mesencephalic locomotor centre, or from appropriate sensory inputs, often supplemented by drugs[7, 8]. Because the locomotor CPG is believed to be fully contained in the lumbar spinal cord, in vitro preparations of the baby mouse or rat spinal cord produce rhythmically alternating motor patterns (readily recorded from ventral roots) when activated by neurochemicals (typically NMDA and serotonin, 5HT)[1] or dorsal root stimuli[9]. The observed pattern is termed "fictive locomotion" and is a very useful model to investigate the properties of the locomotor CPG.

In quadruped mammals considerable advance in our understanding of the CPG operation has been obtained by combining experimental data with a model in which the CPG is thought to comprise two main components, namely a "clock" that starts the program, sets its locomotor frequency and is hierarchically above the "pattern" formation responsible for producing the correct phase and sequence activation of motoneurons[2].

Molecular genetics have recently identified important classes of interneuron that provide a powerful contribution to the locomotor program[1,3]. Their role has been investigated with direct imaging and/or genetically-manipulated disruption or activation[10, 11, 12]. It is, however, difficult to identify the full membership (and their wiring) of the locomotor CPG since, to date, none of such cells has been proven to be absolutely essential to produce the full locomotor program. A clear understanding of the CPG structure remains an ambitious goal that is especially important to elucidate the pathophysiology of spinal injury. Once the blueprint of the locomotor CPG is obtained, strategies for repairing it or replacing lost elements can be better targeted to combat paralysis.

Pathophysiology of spinal injury

The damage occurring to the spinal cord can be temporally divided into various phases that start with the primary damage at the site of the lesion, followed by secondary damage spreading to uninjured areas and slowly evolving into a chronic condition[13, 14, 15]. Once the lesion is chronic, it is often accompanied by histopathological alterations like gliosis and formation of cysts that, in addition to aberrant network plasticity, make difficult to repair the damage. It is, therefore, a more attainable target to try to block the negative evolution of the secondary damage.

Current theories propose that, regardless of its initial cause (traumatic, vascular, infective etc.), the secondary damage develops because of a chain of deleterious cell processes that comprise, amongst the main players, excitotoxicity[16, 17, 18], namely cell destruction because of excessive glutamate release and overactivation of glutamate receptors, and severe metabolic perturbation with generation of toxic reactive oxygen species and free radicals[15, 19, 20, 21]. The basic pathophysiology of such events is difficult to investigate in patients as well as in animal models because of the need to continuously monitor the locomotor deficit and to correlate it with cell damage during a timeframe of minutes, hours and days without additional complicating factors arising from anaesthesia, cardiovascular changes, and intensive care support.

In vitro model of spinal cord injury

In the attempt to clarify these important issues, we thought that using an in vitro model of acute spinal injury might help to understand the early dynamics of this process. In particular, this model should enable us to correlate the ability to produce fictive locomotion with the extent of network histological damage and should, ideally, represent a simple test system to screen neuroprotective agents. Furthermore, it should be possible to compare the relative contribution of excitotoxicity versus metabolic perturbation. Finally, a focal lesion in vitro should allow studying the consequences for spinal

segments remote from the primary injury site. Of course, an in vitro model has limitations intrinsic to the experimental protocol, namely the lack of any vascular supply and the use of a neonatal preparation. Unfortunately, adult spinal cord preparations in vitro do not usually show survival beyond a few hours and are taken from non-locomotor (sacral) regions[22].

Clinical reports indicate that the shortest hospital admission time after acute spinal injury is under 3 h[16, 23, 24]. In hospital the first goal of intensive care medicine is to stabilize the patient and to correct his metabolic state prior to any surgical or pharmacological intervention. To try to mimic this series of events in vitro, we applied a toxic solution (either locally or to the whole isolated spinal cord preparation) for 1 h and then washed it out with standard oxygenated-buffered saline for 24 h[25,26]. The outcome of each experiment was whether fictive locomotion was still present 24 h after the lesion. To this end, we performed electrophysiological recording from various ventral roots to collect information from rear limb flexor and extension motor pools (namely, at L2 and L5 segmental level) during and after the lesion protocol, and correlated it with histological and histochemical analysis.
Preliminary tests with a blunt glass micropipette positioned in the spinal cord to simulate mechanical injury failed to induce a long-lasting suppression of fictive locomotion as preparations spontaneously recovered their activity 24 h later. This observation led us to devise chemically-evoked spinal lesions.
Three main lesion protocols were, therefore, investigated: 1. intense neurotoxicity evoked by the powerful glutamate agonist kainate; 2. metabolic perturbation induced by a pathological medium (thereafter termed PM) with hypoxia, aglycemia, H_2O_2, sodium nitroprussiate, hypo-osmotic, Mg^{2+} free medium and acid medium. 3. co-application of kainate and PM. The solution recipes can be found in[25,26].
We further refined our model by creating (with transverse plastic barriers) leak-proof compartments separately superfused with saline or toxic solution. This approach enabled us to restrict the application of the toxic medium (comprising kainate and PM) to 2-3 segments at the lower thoracic and upper lumbar segments (T12-L1), thus sparing the main L2-L5 segments containing the locomotor CPG. This experimental arrangement allowed us to study the downstream consequences of a lesion on apparently unaffected locomotor networks.

Excitotoxic damage to locomotor networks

Excitotoxicity stems from excessive activation of glutamate receptors with deleterious early and delayed effects on neuronal and glial survival. This process is thought to be important for a large number of pathological brain conditions like stroke, trauma and neurodegenerative diseases[27, 28, 29]. Likewise, in the case of spinal injury, excitotoxicity is considered to be an important component.
We investigated the effect of a saturating concentration of kainate (1 h application) on locomotor networks of the neonatal rat spinal cord in vitro[25]. Several considerations led to the choice of kainate: its stability in solution, lack of transport by glutamate carriers and ability to produce excitotoxicity[30]. Because of previous reports on the need to apply

high doses of kainate to lesion the rat spinal cord in vivo[31,32], we used a high concentration (1 mM) of this drug that exceeds the one required for maximal depolarization of spinal neurons[33], and approaches the glutamate levels detected after spinal trauma[34].

Fig. 1 summarizes the protocol employed for the kainate-evoked lesion of the lumbar spinal cord in vitro and its consequences on the rat spinal cord. First, typical fictive locomotion with alternating ventral roots discharges was induced by continuously-applied (see horizontal green bar) NMDA (5 μM) and 5HT (10 μM). After washout and return to control saline solution (blue bar), kainate was applied (red bar) to generate a large depolarization of motor pools (with transient oscillations) that persisted to a stable plateau throughout the application time. After washout, synaptic responses recorded from ventral roots following dorsal root stimulation were severely depressed. Fictive locomotion was not observed either on the same day after extensive kainate washout or the day after (see Fig. 1). It was, however, possible to evoke disinhibited bursting (magenta bar) after pharmacological block of synaptic inhibition with a combination of strychnine and bicuculline, suggesting that basic network rhythmicity and excitability were retained.

Histological analysis indicated substantial number of pyknotic nuclei (Fig. 1), showing cell death in the four selected regions (dorsal and ventral horns, area around the central canal and lateral white matter). Examination of immunoreactive neurons and motoneurons with NeuN and SMI-32, respectively, demonstrated that, in the ventral horn area, essential for the rat locomotor CPG[35], there was 43 % neuronal loss and 37 % motoneuronal loss.

To clarify the molecular mechanisms underlying the acute spinal cord dysfunction, we analyzed mRNA and protein content of the neonatal rat spinal cord at 4 h and 24 h following 1 h application of kainate. Gene expression levels of different cell type markers (GFAP for astroglia, NeuN for neurons), the neuronal injury marker ATF-3[36], and various genes involved in neuroinflammation (interleukin 1b, IL-1b[37]; Serpine-1[38]), and cell proliferation (Egr-1[39]) were studied using Real-Time PCR or large-scale Superarrays. Preliminary data indicate that, already at 4 h from treatment, there was upregulation of ATF-3, Egr-1, IL-1b and Serpine-1 expression.

Metabolic perturbation induced by PM

In addition to early excitotoxic damage due to massive release of glutamate, the acute stage of spinal cord injury is believed to comprise a pathological cascade that includes generation of nitric oxide (NO)[16], free oxygen radicals, and metabolic dysfunction due to ischemia/hypoxia, energy store collapse, acidosis and oedema triggered by loss of vascular tone autoregulation[16, 19, 20]. Thus, we tested a medium containing a free oxygen radical donor (H_2O_2), a NO precursor (sodium nitroprussiate) and metabolic perturbations (low Mg^{2+}, low osmolarity, acid pH, hypoxia, aglycemia) typically associated with spinal (and brain) lesions. This solution was termed PM and its effects are exemplified in Fig. 2. After obtaining standard fictive locomotion with NMDA and 5HT (green bar), application of PM (red bar) induced a slowly developing depolarization with irregular ventral root discharges. After approximately 1 h washout, fictive locomotion could be observed again although with smaller cycle amplitude and slower period[25]. This pattern

was present also 24 h later, together with the ability to produce disinhibited bursting (magenta bar). PM elicited extensive damage to the lateral white matter with relatively modest damage to interneurons and motoneurons (Fig. 2). In keeping with white matter damage we were unable to evoke fictive locomotion by electrical stimulation of dorsal root afferents.

While the results after either kainate or PM application showed some preservation of spinal networks, when kainate plus PM were applied together for 1 h, all electrophysiological responses were always lost and did not recover even one day later despite sustained washout. In this case, histological examination (24 h later) indicated widespread damage with large neuronal loss throughout the gray matter, substantial damage to the white matter, and strong loss of motoneurons. These data show that extensive damage to white matter, premotoneurons and motoneurons was likely responsible for the suppression of electrophysiological responses[25].

Furthermore, we unexpectedly found that the relatively restricted neuronal lesion produced by PM was largely intensified when the PM solution contained 1 or 2 mM Mg^{2+} (see ref.[26]). In fact, fictive locomotion was fully and irreversibly suppressed together with enhanced neuronal loss chiefly affecting premotoneurons of the ventral horn (decreased by approximately 50 %) against a relatively unchanged population of motoneurons. The damage to the white matter remained extensive. The mechanisms responsible for the deleterious action of Mg^{2+} are currently unknown, but they closely resemble the negative outcome of Mg^{2+} i.v. infusion in patients with acute brain trauma or stroke[40, 41,42].

Focal lesion induces remote effects on locomotor networks

The in vitro model of the neonatal rat isolated spinal cord is also suitable to investigate the process of secondary damage following acute experimental spinal injury. In fact, as indicated in Fig. 3, transverse barriers placed at the low thoracic-upper lumbar region allowed focal superfusion (1 h) of kainate plus PM to these segments only. In this way, the spinal lesion was restricted as shown by average histological data in Fig. 3: the number of cells with pyknotic nucleus was large in all areas of the segments comprised within the barriers, yet minimal in the segments below.

Fig. 3 shows the results of functional tests to explore locomotor network activity with this arrangement. From L2 and L5 segments fictive locomotion evoked by NMDA and 5HT was present in control conditions prior to the application of kainate and PM that generated a large and sustained ventral root depolarization of the L1 segment (between the barriers) and less intense and transient depolarization in segments above or below the barriers (not shown). Despite baseline recovery, L2 and L5 segments did not produce fictive locomotion 1 h later after washing out the kainate plus PM solution (Fig. 3). Nevertheless, after one day in vitro, recovery of fictive locomotion by NMDA and 5HT was observed as demonstrated in Fig. 3, although trains of stimuli applied via dorsal root afferents failed to elicit this pattern in spite of a large cumulative depolarization recorded from the L5 ventral root. The causes for the loss of electrically-induced fictive locomotion remain unclear especially in the absence of significant cell loss and suggest long-lasting plasticity changes perhaps triggered by neuromodulators like

endocannabinoids and nitric oxide[43] released during the strong depolarization evoked by the toxic solution that possibly led to persistent downregulation of certain synapses[44,45].

Basic processes of acute spinal injury

While our in vitro model suggests distinct roles (and cell targets) of excitotoxicity and metabolic dysfunction in the acute injury to locomotor networks, it is clear that synergy of action between the powerful excitotoxic agent kainate and deranged metabolism leads to irreversible damage[25].

It is interesting to compare the consequences of kainate or PM: kainate always abolished fictive locomotion, while PM induced only a deterioration of locomotor patterns (slow periodicity, small cycle size) observable when locomotor networks were directly activated by NMDA and 5HT. Electrical pulses, however, failed to produce fictive locomotion. Perhaps the strong loss of white matter elements elicited by PM led to impaired polysynaptic transmission that functionally isolated spinal networks from external inputs or reduced their ability to process them.

These results raise issues of potential relevance to the management of spinal injuries. In fact, if the early excitotoxicity could be pharmacologically blocked during the most acute phase, the metabolic perturbation developing as a consequence of trauma or vascular collapse might leave a network with enough structure for locomotion and whose potential further deterioration might be prevented once the cellular mechanisms are fully elucidated.

The toxic solutions used for the present investigation comprised a variety of substances and factors whose relative contribution will have to be studied in the future. It is, however, clear that, if excitotoxicity caused by kainate comprised generation of toxic metabolites, the latter process must have been of limited extent (or generated substances not included in the cocktail employed for our tests) because of the obvious differences in the effects due to kainate or PM.

The focal lesion protocol allowed us to reproduce the transient loss of locomotor activity which is typically seen immediately after an acute lesion in man ("spinal shock")[46]. Our data indicate that, below the lesion, the locomotor network output was regained when the CPG was activated by neurochemicals, and that 24 h after the primary lesion, neuronal cell loss in those segments was minimal. However, like in the case of lesions affecting the whole spinal preparation, there was a persistent inability to produce fictive locomotion with dorsal root stimuli: the loss of integration of sensory afferent signals into the locomotor CPG may pose a major challenge to gait recovery and to neurorehabilitation. We propose that this is a novel observation so far unavailable from in vivo animal experiments in which the general anaesthesia used together with the lesion protocol inevitably compounds the network synaptic transmission properties.

Elementary architecture of locomotor networks

Integrating our electrophysiological data with histological and immunohistochemical observations enables us to work out some estimates of the network size sufficient for locomotor pattern generation. Previous studies have shown that fictive locomotion

requires at least three intact segments of the most rhythmogenic spinal region (i.e. the upper lumbar one[47]). The present study expands this information by estimating, in a standard cross section of the lumbar spinal cord, the minimum number of surviving neurons and glia compatible with fictive locomotion.

Following application of PM, intersegmental coupling responsible for fictive locomotion induced by NMDA and 5HT was still operational even with the loss of approximately 50 % of the lateral white matter (though electrically evoked patterns were absent), suggesting that most intrinsic connectivity amongst cells of the locomotor CPG normally relied on proprioceptive wiring. The challenge will, therefore, be how to direct activation signals to the CPG when the sensory input integration is made inoperative by a lesion.

Focussing on gray matter elements and starting from the simplest form of synchronized rhythmicity, the network structure required the presence of at least 55 % premotoneurons and 40 % motoneurons to generate disinhibited bursting as indicated by the results with kainate[25]. This estimate is likely to be near the lowest sustainable membership because residual bursts were small and slow. Experiments with extracellular Mg^{2+} changes[26] show that fictive locomotion required a more complex circuitry including at least 65 % ventral horn premotoneurons and a nearly intact motoneuron population. It is noteworthy that the margin between neuronal numbers necessary for locomotor pattern expression and neuronal numbers for synchronized bursting is rather narrow.

Because the locomotor CPG appears to be contained within the ventral/central area of the lumbar spinal cord[35], the role played by dorsal horn neurons is probably less fundamental to the generation of this motor pattern. However, the large kainate-induced damage to the cells around the central canal (lamina X) which are part of the neuronal network generating locomotor activity[48] is likely to be an important contributing factor to the loss of fictive locomotion after excitotoxicity. Of course, our interpretation should be tempered by the fact that it derives from histological analysis and it does not include functional damage without obvious cell losses. In keeping with this notion, we also observed enhanced expression of genes involved in neuroinflammation (IL-1b; Serpine-1), cell proliferation (Egr-1) and motoneuron stress (ATF-3). Hence, the operational damage might even be more extensive than the one measured histologically, suggesting urgency of any post-lesional intervention to inhibit further network deterioration. Furthermore, these data indicate the minimal objective in terms of network numbers that any attempt for repair or regeneration should attain in order to recover locomotion.

In conclusion, the in vitro acute injury model offers novel data potentially interesting to the pathophysiology of spinal injury and its treatment/repair. In future, more emphasis should perhaps be placed on how to reinstate the function of the locomotor CPG especially when its basic structure remains viable after a lesion such the one due to dysmetabolic insult. Motoneuron losses, even if slight, have strong, negative impact on the locomotor program and leave little room for redundancy and compensation. Preserving their integrity or replacing them may be a daunting challenge. The large premotoneuronal network (which undoubtedly includes a significant number of cells unrelated to the locomotor CPG) may better tolerate damage, leaving thus open the possibility to recover function through targeted repair and neurorehabilitation as long as motoneuron numbers are adequate. Future studies should investigate the possibility that the operation of this network depends on few crucial cells, whose identification might

become strategically important to diagnose spinal damage and to monitor recovery progress and prospects.

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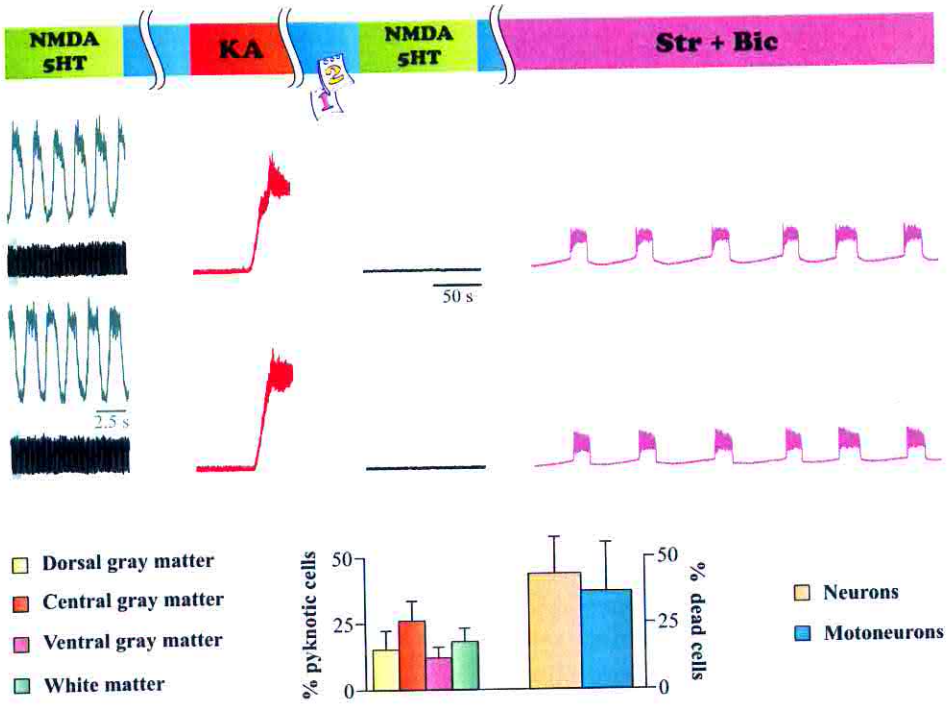
Figure 1. Kainate induced damage to lumbar locomotor networks. The top panel shows the scheme for the experimental protocol that includes control tests for fictive locomotion evoked by NMDA and 5HT, 1 h application of kainate, and subsequent wash with retesting of fictive locomotion the day after. In control condition, NMDA (5 μM) and 5HT (10 μM) elicit alternating oscillations recorded from L2 and L5 lumbar roots of the same side. The traces are also shown at faster time base to reveal the oscillation pattern. Kainate (1 mM) produces strong depolarization of motor pools which is followed by inability to evoke fictive locomotion the day after, although disinhibited bursting (caused by 1 μM strychnine and 10 μM bicuculline administration) is observed. Histograms indicate extent of cell death (left; estimated as % of pyknotic nuclei) in four regions of the lumbar spinal cord 24 h after kainate application, or number of dead premotoneurons and motoneurons (right). Further details in[25].

Figure 2. PM induced damage to lumbar locomotor networks. The top panel shows the scheme for the experimental protocol that includes control tests for fictive locomotion evoked by NMDA and 5HT, 1 h application of PM, and subsequent wash with retesting of fictive locomotion the day after. In control condition, NMDA (5 μM) and 5HT (10 μM) elicit alternating oscillations recorded from L2 and L5 lumbar roots of the same side. The traces are also shown at faster time base to reveal the oscillation pattern. PM depolarizes motor pools. After 1 h washout, fictive locomotion can be evoked by NMDA and 5HT even if cycle amplitude and periodicity have deteriorated: this pattern is detected also the day after together with disinhibited bursting (caused by 1 μM strychnine and 10 μM bicuculline administration). Histograms indicate extent of cell death (left; estimated as % of pyknotic nuclei) in four regions of the lumbar spinal cord 24 h after kainate application, or number of dead premotoneurons and motoneurons (right). Further details in[25].

Figure 3. Focal application of toxic solution produces secondary damage to locomotor networks. Top: scheme indicating experimental arrangement whereby transverse barriers restricted the application of kainate plus PM to the T9-L1 area. Twenty four h later, in the lesioned area (top histograms), there was substantial damage to all cell types, especially in the lateral white matter. Conversely, the bottom histograms indicate minimal damage to region topographically below the barrier placement. Bottom panels show the experimental protocol (see also Fig. 1 legend) and corresponding records from lumbar ventral roots outside the area affected by the toxic solution. After induction of fictive locomotion by NMDA and 5HT, application of kainate plus PM elicits strong depolarization. After 1 h washout, it is not possible to replicate fictive locomotion, although this pattern recovers one day later. Note that 1 Hz pulse trains applied to a

dorsal root below the lesion evoke cumulative depolarization recorded from ventral roots but lacking fictive locomotion-like oscillations.



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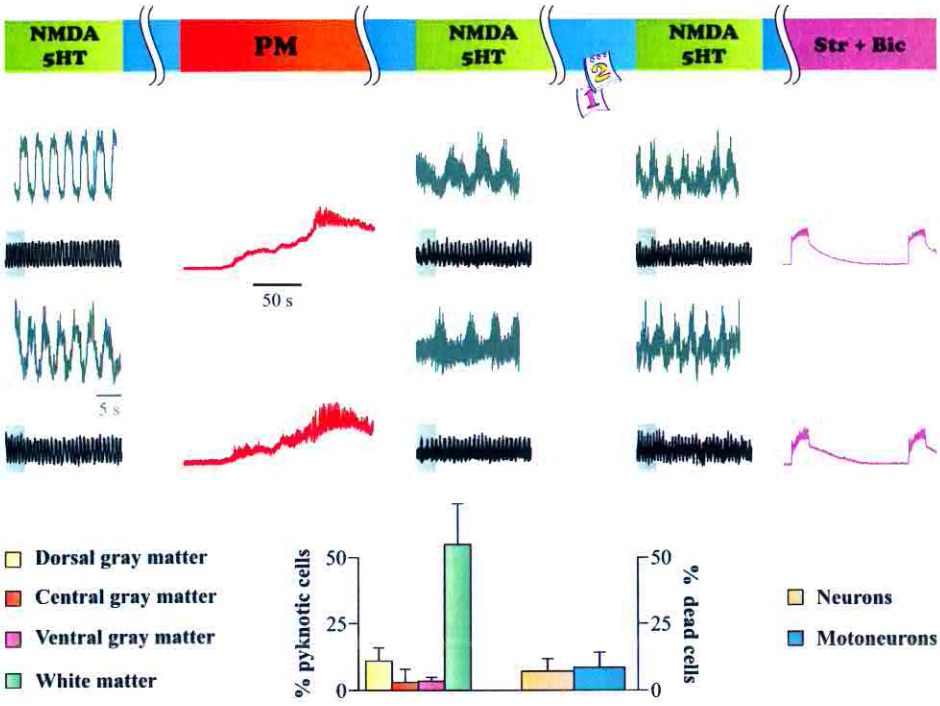

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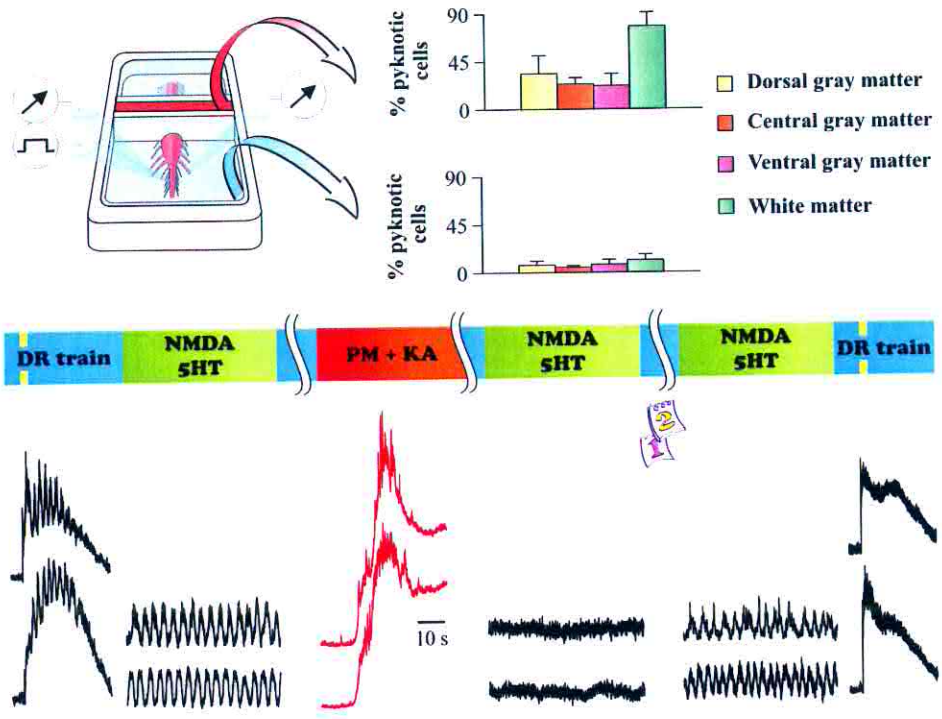

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DISCUSSION

The present PhD project provides new insights into the current understanding of the complex chain of the pathophysiological events occurring early after SCI, and it develops experimental tools to test the suitability of neuroprotective agents. Although a systematic detailed discussion is enclosed in papers, I will summarize the main implications of this research project and compare the effects obtained with different experimental protocols.

1. A novel *in vitro* model of SCI

The principal finding of the first part of the project has been the development of an in vitro model of spinal injury to study the early physiological and histological characteristics of the lesion (Taccola et al., 2008). For this goal, the isolated spinal cord of the neonatal rat has been subjected to different experimental protocols based on either potent excitotoxicity (application of the glutamate agonist kainate) or pathological medium (PM; comprising reactive oxygen species, hypoxia and aglycemia in acid pH conditions) or their combination. The study outcomes have been always fictive locomotion, reflexes and the disinhibited rhythmicity one day after the lesion.

Although the isolated spinal cord preparation, used as our model, has a number of disadvantages to be taken into consideration (which are listed in details on page 32), namely the lack of vascular supply, the limited viability, the mechanical damage due to the dissection and tissue immaturity, it represents a relevant tool to study the operation of CPG in response to spinal injury and the changes in locomotor network activity associated with it. Notwithstanding the fact that spinal injury phenomenon in this model is much simplified and the number of variables is reduced to minimal, such a simplification can be considered advantageous when one is dealing with the intricate cascade of pathophysiological processes, with the scope of understanding the role played by each of these events in the progressive development of the lesion. The other main limitation is related to the use of the neonatal preparation. Unfortunately, adult spinal cord preparations in vitro do not usually show survival beyond a few hours and are usually taken from non-locomotor (sacral) regions (Long et al., 1989). Preliminary tests with a blunt glass micropipette positioned in the spinal cord to simulate mechanical

injury failed to induce a long-lasting suppression of fictive locomotion as preparations spontaneously recovered their activity 24 h later. This observation led us to devise chemically-evoked spinal lesion.

The investigation of the early consequences of a lesion has first required the assessment of the viability of our preparation under control conditions for at least 24 h. Hence, spinal cord preparations have been tested for their main electrophysiological properties shortly after dissection and re-tested 1 day later in standard medium. The isolated spinal preparation, known to survive well in vitro (Kerkut and Bagust, 1995), showed good preservation of locomotor network activity and intrinsic rhythmicity one day after the dissection. What was interesting to observe is that, against an unaltered period of fictive locomotion, disinhibited rhythmicity had been slowed down. We do not know the exact reason for such an alteration; it seems likely that, after 24 h in vitro, lack of descending and afferent inputs led to homeostatic plasticity and neurons adjusted the strength of their excitatory synapses in response to a global activity change (Turrigiano and Nelson, 2004). On the other hand, disinhibited bursting requires a circuitry simpler than fictive locomotion and can be detected even in a single quadrant of spinal cord or organotypic spinal cultures (Bracci et al., 1996 a,b; Ballerini et al., 1999; Darbon et al., 2002). Histological observation of spinal cord preparations kept in standard solution for 24 h was typical of the histology of the rat spinal cord freshly after dissection, as preparations showed well preserved neurons and motoneurons, abundant cell populations in ventral area, comprising neurons and glia throughout the gray and white matter.

Three main lesion protocols were, therefore, investigated: 1. intense neurotoxicity evoked by the powerful glutamate agonist kainate; 2. metabolic perturbation induced by a pathological medium (containing free radicals and hypoxic/aglycemic conditions); 3. co-application of kainate and PM.

2. Excitotoxicity against metabolic perturbation

In analogy with the previous studies demonstrating excitotoxicity of kainate on the rat spinal cord in vivo (Hugon et al., 1989; Magnuson et al., 1999), the first lesion protocol has been directed to explore, with the in vitro preparation, the dynamics of kainate damage, its persistence and preferential localization to different elements of spinal networks. Thus, kainate was applied for 1 h to the isolated spinal cord preparation at 1

mM concentration, which exceeds the one required for maximal depolarization of spinal neurons (Nistri and Constanti, 1979) and approaches the glutamate levels detected after spinal trauma (Liu et al., 1999). The outcome of the transient excitotoxic insult produced by kainate has been the irreversible loss of fictive locomotion, induced either chemically or electrically, while the intrinsic rhythmicity, induced by synaptic inhibition block has persisted, maintaining characteristics of untreated sham preparations. The relative resistance of spinal networks to excitotoxicity, resulting in preservation of disinhibited bursting has been quite unexpected.

The second experimental protocol has been aimed at generating an experimental condition which might mimic the acute clinical lesion when the metabolic insult is temporary and followed by intensive care treatment to correct the metabolic derangement. The metabolic perturbation induced by pathological medium comprised hypoxia, aglycemia, H_2O_2, sodium nitroprussiate, hypo-osmotic, Mg^{2+} free medium and acid medium applied for 1 h. The pathological medium slowed down fictive locomotion and intrinsic bursting: these oscillatory patterns remained throughout without regaining their initial control properties. This phenomenon was associated with polysynaptic reflex depression.

It is interesting to compare the consequences of kainate or PM: kainate always abolished fictive locomotion, while PM induced only a deterioration of locomotor patterns (slow periodicity, small cycle size) observable when locomotor networks were directly activated by NMDA and 5-HT. Electrical pulses, however, failed to produce fictive locomotion.

The principal finding has been the observation that a strong excitotoxic stimulation or a severe metabolic dysfunction evoked distinct changes in spinal network activities in vitro, thus, indicating that different strategies might be necessary to treat the various early components of acute spinal cord lesion.

Histological examination of spinal cord preparations 24 h after kainate has shown large neuronal loss in the central gray matter region, which is believed to contain the key elements for locomotion (Kiehn 2006) and in certain ventral horn areas, with preservation of dorsal horn structure and of white matter throughout. However, motoneuronal loss was limited, as 24 h after kainate only 1/3 of motoneurons were lost. Conversely, after the treatment with PM, the extent of neuronal loss was smaller compared with kainate. Substantial damage mainly affected the white matter surrounding

the ventral gray matter. On average, the total number of neurons and motoneurons after the treatment with PM was close to the one in control.

An intriguing notion arising from the present results is that, should initial excitotoxicity be limited or pharmacologically blocked during the most acute phase of SCI, the metabolic perturbation believed to occur as a consequence of trauma or vascular collapse might leave a network with enough structure for locomotion and its potential deterioration might be prevented once the cellular mechanisms are fully elucidated.

3. Irreversible damage

Since either kainate or metabolic perturbation produced different changes in electrophysiological activity of spinal networks associated with distinct preservation of rhythmic patterns, we have next tested whether, to produce the extensive damage normally observed in vivo following acute SCI, the two experimental paradigms should be combined. This treatment, termed PM+KA, has always lead to full loss of all electrophysiological responses for up to 24 h. Such a finding has been not surprising because of the harsh experimental conditions that mimicked those believed to occur in vivo (Dumont et al., 2001; Hall and Springer, 2004; Norenberg et al., 2004). As our in vitro model suggests distinct roles of excitotoxicity and metabolic dysfunction in the acute injury to locomotor networks, it is thus clear that synergy of action between the powerful excitotoxic agent kainate and deranged metabolism leads to irreversible damage. Morphological examination of the effects of PM+KA on spinal networks 24 h later showed very extensive damage, which included massive neuronal loss throughout the gray matter, large damage to the white matter and strong loss of motoneurons. While the overall number of dead neurons was similar to the one observed after treatment with kainate, motoneuronal loss reached 90%. These data suggest that extensive damage to white matter and motoneurons was likely responsible for the suppression of electrophysiological responses. The present observations are, however, useful to set up a method for producing an irreversible spinal lesion.

4. Magnesium for neuroprotection after the spinal cord injury?

Intravenously administered magnesium has been extensively investigated both preclinically and clinically as a neuroprotective agent for traumatic brain injury (Bareyre et al., 1999, 2000) and stroke (Muir and Lees, 1995; Yang et al., 2000; Chan et al., 2005). The neuroprotective properties of magnesium have also been evaluated in animal models of spinal cord injury by a number of investigators. Magnesium sulfate administration has been shown to improve neurological function (Jellish et al., 2008), decrease membrane damage (Suzer et al., 1999; Kaptanoglu et al., 2003a; Ozdemir et al., 2005), normalize energy balance (Ozdemir et al., 2005), protect axonal function (Suzer et al., 1999), decrease apoptosis (Solaroglu et al., 2005), improve blood-spinal cord barrier function (Kaptanoglu et al., 2003b) and decrease neutrophil infiltration (Gok et al., 2007). While these data provide evidence to support the therapeutic role of magnesium in acute SCI, the neuroprotective effects were only demonstrable with extremely high doses of magnesium sulfate (eg. 600 mg/kg) (Suzer et al., 1999; Kaptanoglu et al., 2003a,b). Such a bolus dose of 600 mg/kg far exceeds the 2-4 gram bolus dose given to patients with emergencies such a pre-eclampsia or cardiac arrest (Gowda and Khan, 2004; Leeman and Fontaine, 2008).

The main finding of the present report is the observation that standard concentrations of extracellular Mg^{2+} unexpectedly enhanced the damage to spinal locomotor networks produced by metabolic perturbation mimicking the biochemical disruption that occurs following an acute spinal injury in vivo. For the first time, these data have shown a detrimental effect of standard Mg^{2+} concentrations that goes against the previously-held assumption that Mg^{2+} should protect against the glutamate receptor contribution to CNS injury (Villmann and Becker, 2007; Ginsberg, 2008). Indeed, magnesium has been tried unsuccessfully in clinical trials for nervous system injury: a large multicenter trial of Mg^{2+} infusion into acute brain lesioned patients has been completed with very unsatisfactory outcome (Maas and Murray, 2007; Temkin et al., 2007). Such clinical results have actually demonstrated that many patients receiving Mg^{2+} infusion fared worse than untreated patients, a result similarly found also for stroke patients (IMAGES, 2004).

The present study has used an in vitro spinal cord model from the neonatal rat to investigate the role of extracellular Mg^{2+} in the lesion evoked by a PM mimicking the

metabolic perturbation occurring in vivo. Hence, we have added 1 mM Mg^{2+} (for 1 h) to the PM solution and have taken, as outcome, locomotor network activity for up to 24 h after the primary insult. Results were clearly different from those formerly obtained when PM was applied in Mg^{2+} free solution (Taccola et al., 2008). PM in 1 mM Mg^{2+} solution largely depressed spinal reflexes and suppressed fictive locomotion (both electrically and chemically induced) on the same and the following day, regardless the increase in stimulus intensity and concentrations of NMDA and 5-HT. Despite the loss of fictive locomotion, the adverse effect detected with PM plus 1 mM Mg^{2+} was not accompanied by a complete destruction of functional network activity because synchronous rhythmicity of spinal networks could always be demonstrated by pharmacological block of synaptic inhibition. Disinhibited bursts readily emerged 24 h after exposure to PM plus 1 mM Mg^{2+}, though at slower period. In summary, application of PM plus 1 mM Mg^{2+} has resulted in large and persistent depression of spinal reflexes and loss of fictive locomotion, indicating a severe impairment of electrophysiological activity of spinal networks. The exact mechanisms responsible for the deleterious action of Mg^{2+} are currently unknown, while they closely resemble the negative outcome of Mg^{2+} i.v. infusion in patients with acute brain trauma or stroke (Ikonomidou and Turski, 2002; IMAGES 2004; Temkin et al., 2007). Changes in intracellular and extracellular Mg^{2+} may develop as a consequence of depolarization and hypoxia that elicit a large increase in Ca^{2+} permeability and depression of Mg^{2+} extrusion pumps (Gotoh et al., 1999; Henrich and Buckler, 2008). Disruption of Mg^{2+}homeostasis may produce profound modifications in the operation of a number of receptor/channels and biochemical pathways. For instance, fluctuations in divalent cations can facilitate the activation of transient receptor potential melastatin related receptor (TRPM) channels with deleterious effects on neuronal survival as suggested with experimental hypoxia protocols (MacDonald and Jackson, 2007; Schlingmann et al., 2007). Furthermore, Mg^{2+} is an essential cofactor for the racemase that converts L-serine into D-serine, the physiological co-agonist of the NMDA receptor (Wolosker et al., 2008). Hence, while a fall in extracellular Mg^{2+} may unplug NMDA channels from their voltage dependent block, a deficit in this divalent cation might also impair the co-activation of the same channels and limit excitotoxicity. In addition, Mg^{2+} can enhance NMDA-mediated currents and reduce desensitization of this receptor by allosterically interacting with the glycine binding site on the NMDA receptor: this interaction may be a key physiological mechanism through which modulation of the NMDA receptor is achieved (Wang and

MacDonald, 1995). Note, however, that NMDA receptor block was insufficient to protect the locomotor networks.

5. Janus-faced nature of magnesium action

We next explored how the functional activity of spinal networks was affected by applying PM with varying Mg^{2+} concentrations. Application of different concentrations of Mg^{2+} ranging from a nominally-Mg^{2+} free medium to one containing 20 mM together with PM have shown a biphasic mode of its action. PM in either Mg^{2+}-free or with larger Mg^{2+} concentrations evoked temporary network depression and enabled fictive locomotion the day after. Conversely, fictive locomotion was absent 24 h after when PM applied with 1 or 2 mM Mg^{2+}. Despite this sustained reduction in network synaptic transmission, the 1 h exposure to PM plus 5 mM Mg^{2+} enabled resumption of fictive locomotion 24 h later. These results suggest that the size of VR reflexes after 24 h from the primary insult was not a reliable predictor of the ability to produce fictive locomotion. Furthermore, 24 h after exposure to PM in 5 mM Mg^{2+} solution, in four out of five preparations there was no recovery in the expression of fictive locomotion by DR stimulus trains. Parallel experiments were performed to find out the effects of changes in extracellular Mg^{2+} on synaptic transmission recorded from VRs in standard Krebs' solution. Application of Mg^{2+} free solution led to a large VR depolarization, accompanied by strong increase in spontaneous network activity, a phenomenon that subsided upon return to standard solution. Application of 5 mM Mg^{2+} conversely reduced spontaneous discharges. In Mg^{2+} free solution the reflex area initially was enhanced and later declined probably because of sustained network depolarization. Recovery was, however, observed within 30 min washout. Application of 5 mM Mg^{2+} moderately depressed VR reflexes, an effect fully reversed after 30 min washout. These results indicate that changes in extracellular Mg^{2+} concentrations were readily translated into altered synaptic transmission within the spinal cord.

Histological analysis indicated extensive white matter versus gray matter damage by PM plus 1 mM Mg^{2+}. However, this phenomenon also occurred with Mg^{2+} free or 5mM Mg^{2+}, suggesting that such damage was not the main process responsible for lack of fictive locomotion. Focusing on the lumbar ventral horn region demonstrated that, in Mg^{2+} free solution plus PM, there was approximately the same number of neurons as in

comparable ventral horn areas of sham preparations: this value fell, on average, by 66% in the sampled area after PM in 1 mM Mg^{2+} solution, while with 5 mM Mg^{2+} the loss of neurons was limited to 37%. Since dorsal and central gray matter areas showed analogous values regardless of the treatment, it is likely that the number of surviving ventral neurons was a crucial factor to allow fictive locomotion. Despite the substantial degree of ventral horn neuronal loss after PM plus 1 mM Mg^{2+}, motoneurons appeared much less sensitive to this insult.

In summary, these observations suggest a number of issues that require future studies. First, the reputedly low Mg^{2+} concentration following CNS injury might be a protective phenomenon to restrict the lesion severity. Second, maintaining extracellular Mg^{2+} at standard concentrations is probably unhelpful for SCI treatment and even dangerous in view of the observed potentiation of the kainate excitotoxicity. Third, raising Mg^{2+} levels to 5 (or more) mM was protective probably via depression of synaptic transmission as demonstrated in the neonatal rat spinal cord (Ault et al., 1980). However, it is unrealistic to consider that such levels of Mg^{2+} should be attained clinically without prejudice for survival.

6. Magnesium and kainate-evoked neurotoxicity

We have investigated if changes in extracellular Mg^{2+} might also affect the excitotoxic action of kainate. Our former study (Taccola et al., 2008) has indicated that irreversible, full damage of spinal networks with total loss of electrical responses is induced by combined application (1 h) of PM (in Mg^{2+} free solution) and kainate (1 mM). We therefore wished to find out if 1 mM Mg^{2+} could actually potentiate the excitotoxic effect of kainate (in combination with PM) on spinal networks when lower concentrations of this glutamate agonist were used. In the present experiments, 1 h administration of PM and kainate always led to irreversible loss of fictive locomotion regardless of the kainate concentration used (50 μM-1 mM) or the doses of extracellular Mg^{2+} . Hence, to evaluate whether the presence of 1 mM Mg^{2+} made the network damage stronger, we applied PM and varying concentrations of kainate with or without this divalent cation and checked for the presence of disinhibited bursting 24 h later. 1 mM Mg^{2+} enabled disinhibited bursting when PM was used together with 50 or 100 μM kainate, although in the latter case this was present in half of the preparations only. Further increases in kainate

concentration were associated with consistent lack of disinhibited bursting. Comparable tests in Mg^{2+} free PM show that all preparations retained disinhibited bursting with addition of 100 μM kainate and a few even with 250 μM. These observations raised the possibility that Mg^{2+} facilitated the excitotoxicity of kainate on network rhythmicity. To test this issue, we investigated the action of kainate alone (1 mM) in Mg^{2+}free solution: this treatment evoked a strong VR depolarization, similar to the one in standard Krebs' solution (Taccola et al., 2008). Mg^{2+} free/kainate solution did not allow subsequent restoration of fictive locomotion, although it enabled disinhibited bursting 24 h later. Burst period and duration were not different from those previously reported for sham preparations or 24 h after kainate in standard Krebs' solution (Taccola et al., 2008). These results suggest that the action of Mg^{2+} was manifested when the experimental protocol induced metabolic perturbation rather than straightforward excitotoxicity. In addition, my own unpublished results have shown that application of different concentrations (ranging from 100 μM to 1 mM) of kainate (for 1 h) in Krebs solution, were always associated with irreversible loss of fictive locomotor patterns.

Our novel finding was that the effects of kainate excitotoxicity were not modulated by extracellular Mg^{2+}. This realization raises interesting implications, namely that the damage produced by excitotoxicity was unrelated to the activity of Mg^{2+} sensitive NMDA channels despite the unavoidably large release of endogenous glutamate. In addition, our data indicate that extracellular Mg^{2+} could modulate spinal lesion mechanisms only when there was concomitant strong metabolic disruption. Although the excitotoxic damage elicited by kainate was insensitive to extracellular Mg^{2+}, 1 mM Mg^{2+} potentiated the effect of combining pathological medium with kainate at low concentrations.

7. Neuroprotection by glutamate antagonists

Efforts to protect central nervous tissue by blocking excitotoxicity with glutamate antagonists or free radical scavengers have yielded disappointing results that have even lead to dismiss the possibility of clinical neuroprotection against trauma or ischemia (Thuret et al., 2006; Faden and Stoica, 2007; Savitz and Fischer, 2007). Previous reports have also shown limited usefulness of glutamate antagonists against long term recovery from spinal injury in vivo, possibly for reasons like the clinical predictiveness/relevance

of the animal models, the adequacy of pharmacological methodology, and poor understanding of the timecourse of the damage development (Faden and Stoica, 2007). Perhaps, limiting acute damage to locomotor networks would require investigation of its early dynamics with a model that can allow on-going monitoring of locomotor activity.

The aim of the present project has been to find out if proof of principle of neuroprotection could be obtained, at least with our in vitro model. Thus, we have investigated the feasibility of neuroprotection of lumbar locomotor networks by conventional glutamate receptor antagonists CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) and APV (D-aminophosphonovalerate) against acute lesions induced by PM plus 1 mM Mg^{2+}. CNQX and APV have been applied in concentrations 10 μM and 50 μM, respectively. The selection of their concentration was based on former reports for the IC_{50} value related to suppression of synaptic transmission in the isolated spinal cord of the rat. Since IC_{50} for CNQX is 1 μM (Long et al., 1990), we applied a ten fold increase in this concentration in order to prevent potential displacement of this antagonist by glutamate (or kainate). As the IC_{50} value for D-APV is 1.4 μM (Evans et al., 1982), we have used it at 50 μM concentration (also in view of the high affinity of glutamate for NMDA receptors; Dingledine et al., 1999). Based on our previous investigation, co-application of 10 μM CNQX and 50 μM APV to the neonatal rat spinal cord resulted in full suppression of locomotor network activity (Beato et al., 1997). Thus, we have tested the following pathological conditions: a. the antagonists were co-applied with the experimental insult to ascertain the contribution of glutamate receptor activation to damage, where it was produced, and to what degree damage prevention might be achieved; b, the antagonists were given after removing the primary insult, in an attempt to mimic the consequences of early treatment of acute spinal injury with intensive care that should start as short as possible after the primary lesion event (Bracken et al., 1990; Pointillart et al., 2000; Hall and Springer, 2004).

The principal finding of the present report is that it has been possible to demonstrate functional neuroprotection against a powerful metabolic insult by applying CNQX and APV either together with or after PM plus 1 mM Mg^{2+}. According to our previous investigation, 1 h application of PM together with 1 mM Mg^{2+} induced irreversible damage to locomotor spinal networks, resulting in suppression of fictive locomotion (Margaryan et al., 2009). Conversely, when PM plus 1 mM Mg^{2+} was applied together with CNQX and APV, fictive locomotion was present already at 1 h washout of this solution, and persisted at 24 h. Although the period of fictive locomotion was not

significantly different from control after the treatment, cycle amplitude was substantially reduced on the same day and remained at low values like the one of sham preparations 24 h later. In half of the preparations tested, fictive locomotor cycles induced by stimulating DR with pulse trains have lead to short-lasting oscillatory cycles. Disinhibited bursting was also fully expressed 24 h after, with faster periodicity than in preparations treated with PM+1mM Mg^{2+}, suggesting improved network function. Reflex amplitude and area were often similar to those normally found for sham preparations in view of their expected decline after 24 h in vitro (Evans 1978; Taccola et al., 2008).

The delayed application of CNQX and APV similarly allowed resumption of fictive locomotor patterns, evoked by NMDA and 5-HT, with periodicity similar to control and halved amplitude. Twenty-four h after, all preparations displayed fictive locomotion with periodicity and cycle amplitude similar to the sham. Disinhibited rhythmicity was also preserved.

Twenty-four h after co-application of PM+1mM Mg^{2+} and glutamate antagonists, damage to the white matter was almost as large (53±3 %) as the one observed with PM+1mM Mg^{2+} alone (Margaryan et al., 2009). Even if the number of pyknotic nuclei in the other areas was very small, pyknotic ventral horn cells were significantly less than after PM+1 mM Mg^{2+} (Margaryan et al., 2009). Also delayed co-application of CNQX and APV significantly reduced the number of white matter pyknotic elements (47±3 %), while the number of pyknotic cells in the other areas remained consistently low. After the delayed administration of CNQX and APV, largest number of surviving neurons was in the ventral horn area (151±46/aoi), a value significantly higher than the corresponding average without glutamate antagonists (105/aoi; Margaryan et al., 2009). Neuronal counts in the dorsal and central areas were, however, similar to those for PM+1mM Mg^{2+} (Margaryan et al., 2009). Likewise, the number of motoneurons (n=13±3/aoi) was similar to the one found after PM+1 mM Mg^{2+} (Margaryan et al., 2009).

In summary, the present data indicate that locomotor network neuroprotection by canonical glutamate antagonists against acute lesions induced by PM has been feasible when introduced during or immediately after the pathological process of spinal injury.

8. NMDA receptor antagonists versus those of AMPA/kainate receptors

We have also tested the relative contribution of NMDA versus AMPA/kainate receptors to protect the locomotor networks from the metabolic damage induced by PM plus 1mM Mg^{2+}. In order to compare the effectiveness of each one of these two antagonists, they were applied separately to the toxic solution. When APV alone was added to PM pus 1 mM Mg^{2+} it could not protect spinal networks as fictive locomotion was always absent both at 1 or 24 h after washout. Disinhibited rhythmicity was poorly expressed with slower periodicity and longer burst duration. Application of CNQX alone together with toxic solution enabled fictive locomotor pattern in 4/5 preparations at 1 h of washout with standard periodicity and amplitude of alternating cycles. Twenty-four h later 3/5 preparations could express fictive locomotion though not on all four VRs, when lower concentrations of NMDA (2 or 3 μM) rather than the standard one (5 μM) were applied. In all five preparations disinhibited bursting was observed with the periodicity and burst duration values similar to those of the sham preparation 24 h later. DR trains of electrical stimuli failed to elicit locomotor-like oscillations either on the same or the day after treatment with PM plus either CNQX or APV. As a result, neither antagonist administered alone could provide efficient degree of protection. CNQX was somewhat better than APV, but the functional outcome was inferior to the result observed with the combination of the two antagonists. The simplest interpretation is that, within the protocol timeframe, damage due to PM comprised a broad excitotoxic component. In fact, similar results were found in chick telencephalic and rat hippocampal neurons, by application of selective antagonists against glutamate excitotoxicity either during the excitotoxic insult (acute treatment) or during the recovery period (posttreatment) (Prehn et al., 1995). In cultures of chick telencephalic neurons, posttreatment with AMPA/kainate receptor antagonists was able to completely protect the cells against glutamate neurotoxicity. On the other hand, blockade of NMDA receptors subsequent to the glutamate exposure produced rather moderate protection. In cultured rat hippocampal neurons, delayed administration of the AMPA/kainate receptor antagonist NBQX up to 4 h after glutamate exposure still produced significant neuroprotection. In contrast, treatment with the NMDA receptor antagonist dizocilpine 4 h after glutamate exposure failed to reduce glutamate-induced damage (Prehn et al., 1995). In animal models of

cerebral ischemia, NMDA receptor antagonists seem to be effective against ischemic injury only when administered before or shortly after the ischemic insult (Meldrum 1990). The AMPA/kainate receptor antagonist NBQX, however, is effective in reducing ischemic damage in rats subjected to global ischemia even when administered several hours after the ischemic insult (Sheardown et al., 1990; Li and Buchan, 1993). Similar results have been also reported from a model of focal cerebral ischemia in rats (Huang et al., 1993).

9. CNXQ and APV against kainate excitotoxicity

According to our former observations (Taccola et al., 2008), 1 h application kainate consistently abolished fictive locomotion and strongly depressed reflex activity. In the present investigation we examined if CNQX and APV might contrast these toxic effects. However, co-application of kainate with CNQX and APV has resulted in less effective neuroprotection. While on the same day, there was no reappearance of fictive locomotion; 24 h later, only two preparations generated fictive locomotor patterns. In the other four preparations, there was only irregular firing from VRs. Morphological examination of preparations displaying fictive locomotion 24 h later and of those, which did not, was done separately. The main difference between these groups in terms of cell pyknosis was a lesser degree of cell loss affecting the dorsal horn area when fictive locomotion was still expressed. When histological analysis was extended to identifying survived cell types, a larger number of neurons were present in the dorsal horn area when fictive locomotion was preserved. Furthermore, a significantly larger number of motoneurons were found in the preparations with fictive locomotion.

When the application of CNQX and APV commenced on the washout of kainate and continued for 2 h, there was actually no recovery in fictive locomotion both with NMDA plus 5-HT and DR stimulus. Nevertheless, it was still possible to record disinhibited bursting.

These observations suggest that co-applied CNQX and APV had limited ability to protect spinal networks as their co-application with kainate functionally protected only 1/3rd of preparations. When their application was delayed, there was total failure. One possible explanation for the restricted neuroprotection and for the ineffective defence from kainate insult when the antagonist application was delayed is the slow pharmacokinetic

properties of CNQX and APV that require a much longer time to block glutamate receptors of the rat spinal cord in vitro (Evans et al., 1982; Long et al., 1990) than kainate to activate them. The most parsimonious explanation is that a substantial component of the kainate evoked lesion occurred rapidly and that more efficient damage inhibition should perhaps be sought in future studies by targeting downstream processes sequential to glutamate receptor overactivity.

Hence, the present investigation provides a model not only for preclinical testing of novel neuroprotective agents, but also to compare whether excitotoxicity or metabolic perturbation (maximized in the presence of 1 mM Mg^{2+}; Margaryan et al., 2009) posed equivalent challenges to neuroprotection.

CONCLUSIONS

The in vitro acute injury model offers novel data potentially interesting to the pathophysiology of spinal injury and its treatment/repair. The present study is the first report of the different sensitivity of spinal reflexes, rhythmicity and fictive locomotion to acute lesion. Hence, further studies will be necessary to clarify the detailed mechanisms responsible for excitotoxicity, metabolic disruption and their combination so that one or more biochemical targets may be identified to test for damage limitation. In addition, more emphasis should perhaps be placed on how to reinstate the function of the locomotor CPG especially when its basic structure remains viable after a lesion such the one due to dysmetabolic insult. The possibility that the operation of this network depends on few crucial cells, whose identification might become strategically important to diagnose spinal damage and to monitor recovery prospects, might also need a detailed investigation

In conclusion, these results raise issues of potential relevance to the management of spinal injuries.

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