Scuola Internazionale Superiore di Studi Avanzati

NEW SPINAL CORD MODELS: CHARACTERIZATION OF EXCITOTOXICITY AND NEUROPROTECTION

Thesis submitted for the degree of "Doctor Philosophiae"

SISSA

Neuroscience Area

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Declaration

The work described in this thesis was carried out at the International School for Advanced Studies (Trieste, Italy), between November 2010 and June 2015.
The candidate has performed the data reported in the present thesis. This data are going to be published in different articles, in preparation. In all cases the candidate performed the data analysis, interpretation and discussion of the results and to the writing of manuscript.
During the first year of PhD the candidate worked with Dr. Taccola in the Spinal Lab in the Istituto Medicina Fisica e Riabilitazione di Udine. The results of that work have been published in 2012 in an article.

CONTENTS

LIST OF ABBREVIATION

5-HT: Serotonin

ADO: Adenosine

ADP: Adenosine diphosphate

ALS: Amiotrophic Lateral Sclerosis

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ATP: Adenosine triphosphate

ASIA: American Spinal Injury Association

BSA: Bovine Serum Albumine

cAMP: cyclic adenosine monophosphate

CPG: Central Pattern Generator

CPPG: (*RS*)-α-Cyclopropyl-4-phosphonophenylglycine

CNS: Central Nervous System

CV: Coefficient of Variation

DAPI: 4',6-diamidino-2-phenylindole

DIV: Days *in vitro*

DMEM: Dulbecco's Modified Eagle Medium

DNA: Deoxyribonucleic acid

EPO: Erythropoietin

FBS: Fetal Bovine Serum

GABA: γ-Aminobutyric acid

GluR2: Ionotropic Glutamate Recetptor subunit 2

IL-β: Interleukin beta

KA: Kainate

L-AP4: L-2-amino-4-phosphonobutyric acid

LDH: Lactate Dehydrogenase

mGluR: Metabotropic Glutamate Receptor

MP: Methylprednisolone

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NBQX: (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione)

NeuN: Neuronal Nuclei

NGF: Nerve Growth Factor

NIH: National Institute of Health

NMDA: N-methyl-D-aspartate

NOS: Nitrous oxide

NSCISC: National Spinal Cord Injury Statistical Center

PARP: Poly (ADP) ribose polymerase

PBS: Phosphate-buffered saline

PKC: Protein Kinase C

PLC: Phospholipase C

PreBötC: Pre-Bötzinger Complex

SCI: Spinal Cord Injury

SMI 32: Anti-Neurofilament H (NF-H), Non-Phosphorylated Antibody

TNFα: Tumor necrosis factor alpha

TRH: Thyrotropin-releasing hormone

TRPM: Transient receptor potential ion channels

ABSTRACT:

Group III metabotropic glutamate receptors (mGluR III) are known to decrease glutamate release and to play an important role in controlling pain as documented in neuropathic pain models. Much less is known about their potential neuroprotective effect against excitotoxicity that is considered important for damage onset of spinal cord injury. Using rat spinal cord organotypic slices model, we investigated if mGluR III receptor activation might contrast excitotoxic cell death evoked by kainic acid (0.1 mM) applied for 1h and followed by wash for further 24h. The specific agonist of mGluR III receptors L-(+)-2-amino-4-phosphonobutyric acid (L-AP4; 1 µM) was either co-applied with kainic acid or administered during washout. Cell death was quantified in terms of percentage of pyknotic nuclei, total number of neurons, motoneurons and astrocytes.

Furthermore we developed for future long-term studies an *in vitro* model of Spinal Cord Isolated from newborn rats maintained for 3 days in medium. We characterize this model using both immunohistochemistry and electrophysiological recordings.

CHAPTER 1: INTRODUCTION

1.1 SPINAL CORD INJURY

Spinal Cord Injury (SCI) is an event related to severe damage to spinal cord that leads to loss of sensory and motor function distal to the point of injury (Hulsebosch 2002). SCI can be traumatic or non-traumatic, producing long-term effects, severe lifelong disabilities that are very problematic for the person affected, but also for their family and society (M. E L Van Den Berg et al. 2010). The Annual Incidence of SCI estimated in 22 new cases every millions of population and there are ~2.5 million people affected by SCI (Rossignol et al. 2007). The average age of the patients is 31.7 years and the ratio between male and female is four to one (John W. McDonald and Sadowsky 2002). Life expectancy in spinal cord injured people increased from World War II (3 months) to now (25 to 30 years) (Hulsebosch 2002). One or more of the following symptoms may characterize SCI: paralysis, loss of the possibility to feel cold, heat and touch, hyper-reflexia or spasm, pain, loss of bladder control, loss of sexual functions. The spinal cord is divided in several segments; starting from the upper to the lower, they are cervical, thoracic, lumbar and sacral. Every segment connects to a specific part of the body (e.g. cervical are related to respiratory movements) consequently, depending on which segment is injured different muscles, organs and sensation are involved. Paralysis can be tetraplegia (or quadriplegia), when the level of injury is above the first thoracic vertebra (T1), or paraplegia, when the level of injury is below the T1 (Figoni 1984; Harkey et al. 2003). Significant improvements have been made on repair and recovery of function and in the early medical and surgical management. The major therapeutic goal during the rehabilitation of patients is to regain a far-reaching autonomy, which involves compensation of the disturbed or missing vegetative and sensorymotor functions. Clinically, spinal cord injury leads to a complete loss of motor, sensory and vegetative functions underneath the point of injury. In this phase of the spinal shock, the vasomotor and visceral motor innervation is distrupted. This results not only in tachycardiac/bradicardiac arrhythmia but also in hypotensive and hyperextensive episodes/dysregulation (so called autonomic dysreflexia). In addition atony of the efferent urine ducts, the stomach and the intestine along with paralytic ileus symptoms, endocrine disruptions like hyperglycemia and derailment of the electrolyte metabolism and malfunction of the body temperature control occur (Gerner 1992; Teasell et al. 2000). Upon the decline of the spinal shock, about 4-6 weeks after the

initial accident, pathological reflexes and usually also spasticity develop due to the absence of supraspinal control (Ditunno et al. 2004). Looking at past research, there are a lot of different pathways that have been explored to describe the pathophysiological development of secondary damage in SCI and the intrinsic regenerative response. Different studies focused on neuronal and glia protection using different pharmacological approaches, from methylprednisolone to cyclooxygenase (Hurlbert 2001; Hurley et al. 2002; Schwab et al. 2004; Short et al. 2000), then the recently discovered EPO and riluzole (Celik et al. 2002; Cifra et al. 2012; Gorio et al. 2002). Another field is related to neurorestoration with promotion of axonal conduction (Nashmi and Fehlings 2001), remyelination (Bunge 2001; J W McDonald 1999; J W. McDonald and Howard 2002), regeneration/plasticity, elimination of the inhibitor factors(Schwab et al. 2005). Further groups focused on the environment near the lesion site studying the role of scar inhibition (Grimpe and Silver 2002; De Winter et al. 2002), the blocking of the inhibitory axonal signal integration into the axon (Dergham et al. 2002; Fournier et al. 2003) and in the end the stimulation of axons via growth factors such as neurotrophins (J W. McDonald and Howard 2002). A lot of these studies started from previous work on brain injury and are only the first approach on the pathophysiology of the Spinal Cord Injury (SCI). The time has come to move further by investigating the pathophysiology of the SCI that at the present day remains largely unknown and there are no effective treatments to restore completely motor performance after SCI (Rossignol et al. 2007; Rowland et al. 2008).

1.2 EPIDEMIOLOGY OF SCI.

1.2.1 Complete and incomplete SCI

SCI can be complete or incomplete from a clinical point of view (Harkey et al. 2003). The clinical scale established by American Spinal Injury Association (ASIA) divides persons affected by SCI in five clusters. From A to E due to severity of neurological loss evaluated by verifying the remaining movement abilities of the person injured:

A) Complete. No sensory or motor function is preserved in the sacral segments S4-5. This will result in complete paraplegia or tetraplegia.
B) Sensory Incomplete. Sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-5 and no motor function is preserved more than three levels below the motor level on either side of the body.

C) Motor Incomplete. Motor function is preserved below the neurological level, and more than half of key muscle functions below the neurological level of injury have a muscle grade less than 3.
D) Motor Incomplete. Motor function is preserved below the neurological level, and at least half of key muscle functions below the neurological level of injury have a muscle grade more than 3.
E) Normal. Sensory and motor functions are normal, in all segments.
(http://www.asia-spinalinjury.org/elearning/ISNCSCI_ASIA_ISCOS_low.pdf).

1.2.2 Traumatic SCI

The National Spinal Cord Injury Statistical Center (NSCISC) report for 2014 underlines that in the United States the three leading causes of spinal cord injury before 2013 were traumatic (figure 1.1). Vehicular accidents, including cars and motorcycles, ranked as the leading cause of SCI (38%), even if there was a steady decrease in this percentage of SCI causes from 46.9% (1990-1994) to 38%(2013). Falls ranked second (30%) followed by acts of violence, primarily gunshot wounds (14%). A decrease in the percentage of SCI due to sports-related activities from 14.4 to 9% occurred during the last twenty years. There has been a significant increase in SCI during the last decade as consequence of falls, probably due to aging of the population (https://www.nscisc.uab.edu/reports.aspx). There is also a progressive increase of traumatic spinal cord diseases among older adults from 79.4 per million in 2007 to 87.7 by the end of 2009 (Selvarajah et al. 2013). For studying traumatic SCI injuries in the last years a lot of animal models were developed and used. For SCI research, it is essential to establish an ideal animal model of injury. Ideal models should meet the following conditions (Akhtar, et al. 2008): (1) simulate damage that is similar to clinical SCI; (2) control over conditions, reproducibility, stability; (3) involve a simple technique that is easy to study; (4) the equipment used to make a model is straightforward and quick to produce. All these principles are still valid for other types of models such as *in vitro* models. Differences in injury exist between experimental and clinical SCI. In both experimental and clinical SCI, contusion and compression are two of the most common injury types. However, in experimental animals, these injuries are frequently induced dorsally and in the thoracic spine, whereas most clinical injuries occur anteriorly and in the cervical region (Akhtar et al. 2008). According to the National Spinal Cord Injury Statistical Center, in 2005, 51% of SCI cases in the U.S. occurred in the cervical region (Akhtar et al.

2008). Most SCI in humans affects the anterior spinal artery that supplies three quarters of cord tissue, in contrast to the dorsal arteries affected in experimental SCI (de la Torre 1981).

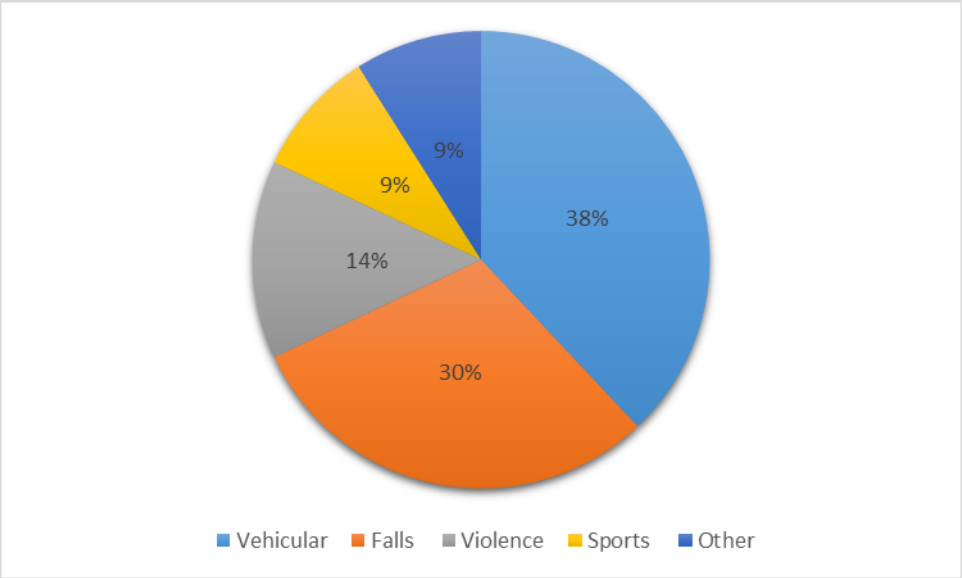

Figure 1.1. Modified from NSCISC 2014 Facts

1.2.3 Non-traumatic SCI

In parallel with a general decrease in the incidence of trauma-related SCI, another trend has been observed, namely that non-traumatic SCI cases are increasing in percentage of the total SCI affected persons reaching a total amount to 30-50% of spinal cord disorders (Nair et al. 2005). Non-traumatic SCI can constitute a very important risk factor during rehabilitation from thrombosis, spasticity or wound infections (McKinley et al. 2002) because it may be caused by non-traumatic events due to vertebral stenosis, tumors, vascular ischemia, inflammatory conditions or subsequent to abdominal aorta surgery (Van den Berg et al. 2010; Bianchetti et al. 2013; Nair et al. 2005). In general, non-traumatic lesions are incomplete with severe clinical symptoms including paralysis and sensory dysfunction (Van den Berg et al. 2010; Nair et al. 2005). Tumor compression, tissue degeneration or vascular problems that can occur during aging increase the risk of non-traumatic SCI in elderly people in contrast to traumatic SCI that is less common during the late phase of life (Van Den Berg et al. 2010). Recently our lab developed an *in vitro* model of damage using a particular type of pathological medium and keeping isolated newborn spinal cord *in vitro* for 24 hours (G. Taccola et al. 2008), mimicking post-ischemia like environment. We also further analyzed the role of magnesium in this particular medium (Bianchetti et al. 2013) unrevelling the role

of this ionic imbalance in ischemic conditions characterized by low oxygen level (Bianchetti et al. 2013; Margaryan et al. 2009; Taccola et al. 2008).

1.3 PHASES OF SCI

Understanding the pathophysiological processes occurring after acute SCI is the first step for developing new neuroprotective strategies based on a pharmacology approach. No matter of its origin, all the different types of traumatic and non traumatic SCI follow similar pathways of evolution (Figure 1.2) that has been divided in three main phases: the acute and immediate (or primary), the secondary and the chronic injury processes (Hulsebosch 2002; Tator 1995).

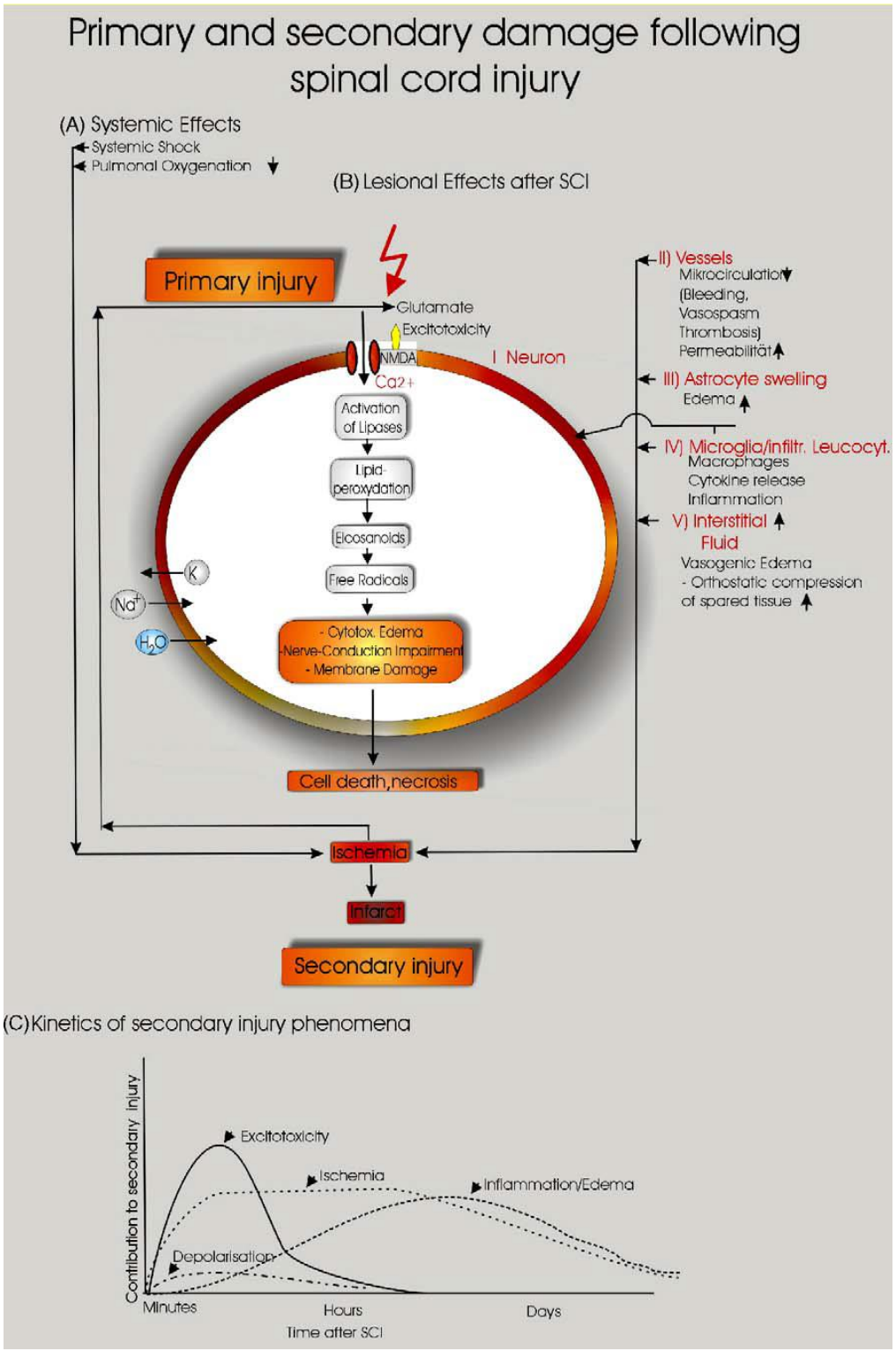

Figure 1.2: Pathophysiology of the postlesional secondary damage. A spinal cord injury expands itself during the first weeks posttrauma due to both (A) systemic and (B,I–V) local effects (modified from Tator, 1995). The cascade of secondary damage is presented in the flow diagram within the schematic neuron. (C) The timeprogression after spinal cord injury: upon the start of the undirected depolarization (loss of membrane integrity) glutamate is released (astrocytes, neurons), this causesthe activation of the neighboring neurons (metabolic stress) and moreover the continuation of the formation of free radicals (Schwab et al. 2006).

1.3.1 The Immediate or primary injury phase (1-2 h)

The first events of traumatic SCI are compressive-contusive-type injuries due to fracture or dislocation of the spinal column and include shearing, laceration, and/or acute stretching. After this mechanical damage, over a matter of a few minutes, the injured neurons respond with abnormal firing of action potentials and significant electrolytic unbalance of the levels of Na^+, Ca^{2+}, K^+. Whenever this condition persists for up to 24 hours, spinal cord networks become severely damaged (Hulsebosch 2002; Rowland et al. 2008). In parallel with this networks impairment, other events occur like parenchymal hemorrhages in the white and grey matter (Kakulas 2004; Quencer et al. 1986). During this initial process there is also an upregulation of proinflammatory cytokines TNFα and IL-β and activation of microglia (Marcello et al. 2013; Pineau and Lacroix 2007). During the following 24 hours, the depolarization induces a massive release of neurotransmitters, including glutamate that is involved in excitotoxicity (Lipton and Rosenberg 1994); this can be one step in the disease process when decreasing the concentration of excitotoxic compounds may limit or arrest the extension of the damage (Norenberg et al. 2004).

1.3.2 The Secondary injury phase (2h to 2 weeks)

The main actors in secondary injury phase are cell death due to ischemia, the electrolytic shift and the edema from the acute primary phase. The whole event is subdivided in two further moments, the early acute and the sub-acute stage (Hulsebosch 2002; Rowland et al. 2008). Within the first two hours, the increase in extracellular concentration of glutamate and other excitatory amino acids such as aspartate causes high level of neuronal deaths via activation of multiple pathways. Thus, this is another timepoint when pharmacology treatments targeting the general biochemical damage can help to constrain the amplification of the lesion (Hulsebosch 2002; Kakulas 2004; Lipton and Rosenberg 1994; Rowland et al. 2008; Tator and Koyanagi 1997). During the subacute stage, that continues for two weeks after the injury, there is a delayed astrocytic response: the periphery of the lesion becomes hypertrophic and proliferative with a strong increase in glial fibrillary acid protein expression. The lesion grows in size from the initial core of cell death with cells at risk of dying in the periphery of the lesion site (Herrmann et al. 2008; Hulsebosch 2002; Pineau and Lacroix 2007).

1.3.3 Glial Scar formation and Chronic phase (>6 months)

After the secondary injury phase, the delayed astrocytic response leads to the formation of a scar that block any possible regenerative axonal sprouting, which is, therefore, insufficient for recovery from severe SCI (Hill et al. 2001) (Figure 1.3 for examples). The beginning of the scar formation consists of Wallerian anterograde degeneration of axons, and demyelination near the lesion site (Norenberg et al. 2004). Subsequently, the astroglial scar replaces the destroyed myelinated axons, while opposing neurite outgrowth (Bovolenta et al. 1993; Norenberg et al. 2004). The final stage is the so-called myelomalacia, a pathological condition of softening of the spinal cord with potential evolution into the formation of intramedullary cysts.

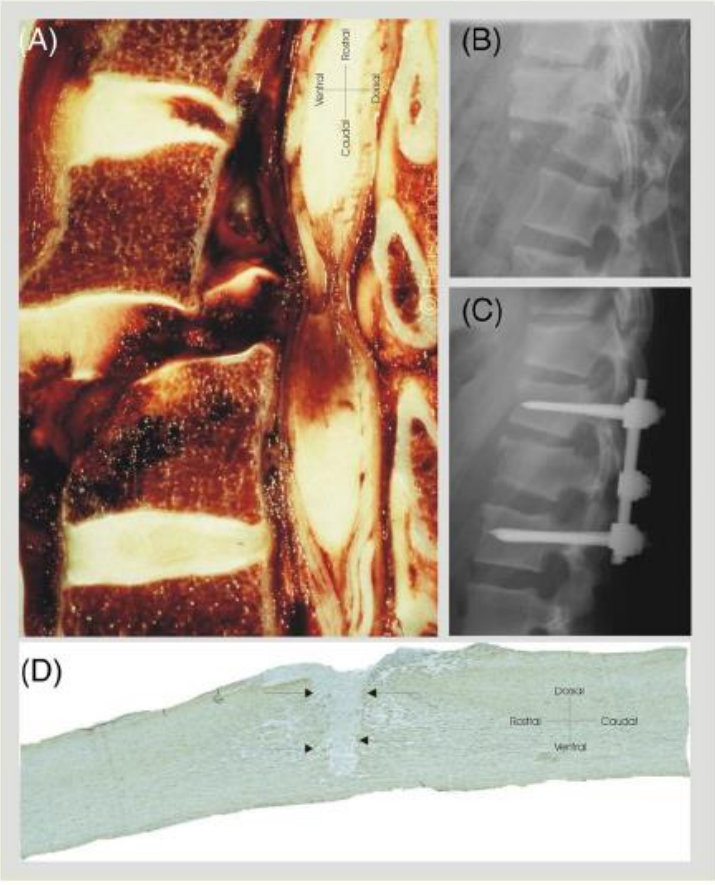

Figure 1.3: Contusion of the spinal cord subsequent to a luxation fracture of the spinal column. (A) Frozen section. Patient died acute of another cause (with kind permission of Dr. v. Rauschning). (B) 35 years old patient with an acute fracture/luxation at T12/L1, contusion of the spinal cord leading to complete paraplegia after a car accident. (C) Repositioning and stabilization of the fracture on the day of the accident after laminectomy using an internal fixation and full persistence of the complete paraplegia. (D) 14 days after an overhemisection-transection injury: a mature scar in an experimental model is clearly detectable (arrows). The scar is, besides myelin, an important barrier for the regeneration of sprouting axons. (From Schwab et al. 2006)

1.4 EXCITOTOXICITY AS A MOLECULAR MECHANISM UNDERLYING SECONDARY DAMAGE AFTER SCI

Death of CNS neurons during acute injury occurs as a result of a complex combination of excitotoxicity, necrosis, apoptosis, edema and inflammatory reactions (Aarts and Tymianski 2004). One process contributing to the propagation of delayed cell death after an acute SCI is excitotoxicity. There is a strong correlation between the loss of ionic homeostasis and excitotoxicity during the acute phase of SCI. Excitotoxicity is a process of overactivation of excitatory amino acid receptors causing neuronal cell death (Park et al. 2004; Szydlowska and Tymianski 2010). The principal excitatory neurotransmitter in the central nervous system (CNS) is glutamate released from vesicles in the presynaptic terminals into the synaptic cleft. Excitotoxicity involves glutamate postsynaptic receptors, in particular ionotropic receptors such those sensitive to NMDA, AMPA or kainate (Aarts and Tymianski 2003; Kumar et al. 1991) and other Ca^{2+} permeable receptors such as TRPM family (Aarts and Tymianski 2003; Kaneko et al. 2006; Schmitz and Perraud 2005). Ionotropic receptors activation increases Na^{+}, K^{+} and Ca^{2+} permeability causing an overload in the network activity of the system and an imbalance in Ca^{2+} compartmentalization triggering cell death pathways relying on calcium-dependent enzymes. (Szydlowska and Tymianski 2010). Tymianski et al. (1993) suggested that Ca^{2+} influx via NMDA channels is more toxic than the one entering via other sources, proposing a sort of “source specificity” (Arundine and Tymianski 2003; Mattson 2000). The imbalance in calcium homeostasis can also occur due to its release from the endoplasmic reticulum or mitochondria due to physical damage during acute injury. The increase of cytoplasmic calcium concentration can trigger downstream neurotoxic cascades including inhibition in energy supply from ATP and activation of enzymes such as proteases, protein kinases, nitric oxide synthase (NOS), calcineurin and endonucleases (Mattson 2000; Szydlowska and Tymianski 2010). All this metabolic stress contributes to glutamate-induced neuronal death occurring during the secondary damage phase (Thayer and Wang 1995). Drugs designed to block the entry of calcium into neurons have failed to produce a positive outcome in clinical trials, as these treatments often elicited side effects such as reduced level of consciousness, hallucinations, hypertension and, in the worst cases, death (Davis et al. 2000; Lee et al. 2000; Szydlowska and Tymianski 2010). Motoneurons are a quite fragile type of cell due to the low levels of glutamate necessary for their activation, and this fragility expose this type of cell to the damaging effects elicited by

high glutamate concentration. AMPA receptors on motoneurons often lack the GluR2 subunit rendering them more permeable to Ca^{2+} (Van Damme et al. 2002). Furthermore, Ca^{2+} binding proteins (e.g. parvalbumin and calbindin) are poorly expressed in the majority of motor neurons limiting their calcium buffering capacity (Ince et al. 1993). Excitotoxicity affects glial cells too, in which the increase in extracellular glutamate concentration leads to a late-response with microglia activation and release of pro-apoptotic factors (Araque et al. 2000), and a decrease in the activity of transporters (Li and Stys 2001). Excitotoxicity is not only involved in SCI, but it is also proposed to underlie a variety of neurological diseases such as stroke, traumatic brain injury, multiple sclerosis, Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Parkinson's disease, and Huntington's disease (Singleton and Povlishock 2004).

1.5 NEUROPROTECTION IN SPINAL CORD

1.5.1 Exogenous compounds for neuroprotection

Although advances in pharmacotherapy for the purpose of limiting neuronal injury and promoting regeneration after spinal cord injury (SCI) have been achieved, only methylprednisolone (MP), administered within the first few hours at high concentration, is used in accordance with USA guidelines (Peter Vellman et al. 2003). MP, which attenuates the peroxidation of membrane lipids and post-traumatic inflammation, has been reported to improve neurobehavioural outcomes in preclinical studies (Braughler and Hall 1984). The administration of MP, however, is not without important side effects; therefore, its use in treating SCI is controversial (Nash et al. 2002; Shen et al. 2005). Another drug is interleukin 10 (IL-10) that allegedly shows therapeutic potential related to its trophic effects on spinal cord neurons, downregulation of the apoptotic factors Bax and caspase 3, and upregulation of anti-apoptotic factors Bcl-2 and Bcl-xl, and neuroprotection against excitotoxicity through the PI3K-AKT pathway. Although it is unlikely that IL-10 alone would improve the condition of an acute SCI patient, its use in conjunction with other treatment models could potentially be valuable, since treatment may necessitate manipulation of spinal cord tissue, leading to harmful secondary inflammation. Nonetheless, published data on IL-10 use in a chronic SCI model are few and discontinued at the present time (Thompson et al. 2013). Recently another compound, namely erythropoietin (EPO) has been described as a glia- and neuro-protective drug (Goldman and Nedergaard 2002; Gorio et al. 2002). In addition to its anti-inflammatory role (Agnello et al. 2002), EPO is claimed to possess protective characteristics,

e.g., through activation of the anti-apoptotic Janus-kinase-2 pathway. Furthermore, it allegedly normalizes the autoregulation of vessel tone and suppresses the release of nitric oxide. Unfortunately, by interfering with blood flow, EPO causes an augmented haematocrit value and an increased aggregation of thrombocytes (Erbayraktar et al. 2003; Schwab et al. 2006).

While other compounds have been studied before such as anaesthetic agents (including Xenon gas, with anti-excitotoxic properties), ormones such as 17-estradiol, progesterone, thyrotropin-releasing hormone (TRH) and its analogues, clinical trials with these substances are few and with frustrating results (Onose et al. 2009).

More recently our lab has focused on the role of other pharmacological compounds, such as riluzole or methoxyflurane, showing neuroprotection effects against excitotoxicity in an in vitro model using kainate as a glutamate analogue for eliciting excitotoxic damage (Mazzone and Nistri 2011; Shabbir et al. 2015). Both these compounds act during the first phases of secondary injury damage by preserving components of the neuronal network activity and by preventing excitotoxicity within a short timeframe. Unfortunately it has been reported that methoxyflurane administered for long time to man can release fluoride with toxic effects on peripheral organs like the kidney (E D Kharasch et al. 1995; E D Kharasch et al. 2006). Although with fewer side effects, riluzole seems to provide incomplete neuroprotection to the spinal cord network after injury (G L Mazzone and Nistri 2011). Other strategies and combined application of several agents seem to be necessary: future studies should address this issue.

1.5.2 Pharmacology and neuroprotection

Activated microglia releases glutamate and neurotoxic pro-inflammatory molecules such as cytokines and cytotoxic factors, thus amplifying excitotoxic damage and exacerbating neurodegeneration (Barger and Basile 2001; Barger et al. 2007; Cunningham et al. 2005; Parker et al. 2002). In addition, prolonged activation of microglia will prevent these cells from carrying out their supportive role to neurons such as release of growth factors (Benoit et al. 2008). Blocking microglial activation has been demonstrated to be neuroprotective in a mouse model of Parkinson’s disease (Wu et al. 2002). In general altering microglial activation in neurodegenerative processes is likely to have multiple beneficial consequences on the progression of pathologies (Williams and Dexter 2014). Different models of SCI have indicated that glutamate receptor antagonists may be useful therapeutic strategies in terms of improved behavioural outcome and neuroprotection (Hulsebosch 2002). The

noncompetitive NMDA ion channel blocker MK-801 (dizocilpine, Merck) and NBQX, a soluble AMPA receptor antagonist, have both demonstrated significant improvements in the contusion model of SCI in outcome measures of improved behaviour and neuroprotection (Faden and Simon 1988; Gorgulu et al. 2000; Li and Stys 2001; Nesic et al. 2001; Wada and Shikaki 1999; Wrathall et al. 1996). In the case of NBQX, the neuroprotection occurs principally in the oligodendrocyte population (Rosenberg, Teng, and Wrathall 1999). Other studies showed that the NMDA antagonists gacyclidine and agmantine, which inhibits nitric oxide synthase too, have demonstrated neuroprotective effects (Yu et al. 2000). Recently the group I metabotropic glutamate antagonists showed neuroprotective effects on a model of contusion SCI (Mills et al. 2002).

1.6 SPINAL CORD METABOTROPIC GLUTAMATE RECEPTORS

After injury there is a short window characterized by large amount of glutamate release at the injury site (McAdoo et al., 1999). This event leads to the activation of glutamate receptors, which are divided into two major types: ionotropic receptors (iGluRs) further divided into 3 groups, NMDA, AMPA and KA receptors; and metabotropic receptors (mGluRs).

The mGluRs are divided in three groups based on sequence homology (table 1), transduction mechanism and pharmacology. Group I mGluRs (mGluR1/5) stimulate phospholipase C (PLC), activate protein kinase C (PKC), and increase Ca^{2+} release from internal storage sites. Both group II (mGluR2/3) and group III (mGluR 4/6-8) are coupled to inhibition of adenylyl cyclase. All the mGluRs are G-protein coupled and their activation affects multiple intracellular signalling pathways having long-lasting effects (Mills et al., 2002).

Family	Receptors	Mechanism	Function	Agonists & Activators	Antagonists
Group I	mGluR 1	G_q ↑Na^+, ↑K^+, ↓glutamate	Increase NMDA receptor acitivty and risk of excitotoxicity	3,5-dihydroxyphenylglycine	
	mGluR 5	G_q ↑Na^+, ↑K^+, ↓glutamate			
Group II	mGluR 2	G_i/G_0	Decrese NMDA receptor acitivty and risk of excitotoxicity	• Eglumegad • Biphenylindanone A • DCG-IV	• APICA • EGLU • Ly-341,495
	mGluR 3	G_i/G_0			
Group III	mGluR 4	G_i/G_0	Decrese NMDA receptor acitivty and risk of excitotoxicity	L-AP4	
	mGluR 6	G_i/G_0			
	mGluR 7	G_i/G_0			
	mGluR 8	G_i/G_0			

Table 1.1

This table lists different families of mGluRs, their main effects, and their agonist and antagonist and their location in the synaptic cleft (Niswender and Conn 2010).

The laminar distribution of mGluRs in the dorsal horn is well characterized (Alvarez et al. 2000; Jia et al. 1999; C D Mills et al. 2001). mGluR1 is found primarily in deeper laminae (III-V), whereas mGluR5 is found in all laminae of the dorsal horn (I-V), but mainly in lamina II. Localization of mGluR2/3 is found predominately in II, with decreasing expression through laminae III and IV.

In the CNS, group I mGluRs are found at both presynaptic and postsynaptic sites and their activation can increase transmitter release (J. P. Pin and Bockaert 1995). Group II and III instead are primarily presynaptic (Liu et al. 1998; Lujan et al. 1996; Neki et al. 1996; Ohishi et al. 1995; Petralia et al. 1996; Shigemoto et al. 1997) and can inhibit neurotransmission (Baskys and Malenka 1991; Forsythe and Clements 1990; Gereau and Conn 1995; Macek et al. 1996; Trombley and Westbrook 1992; Vignes et al. 1995) (Figure 1.4).

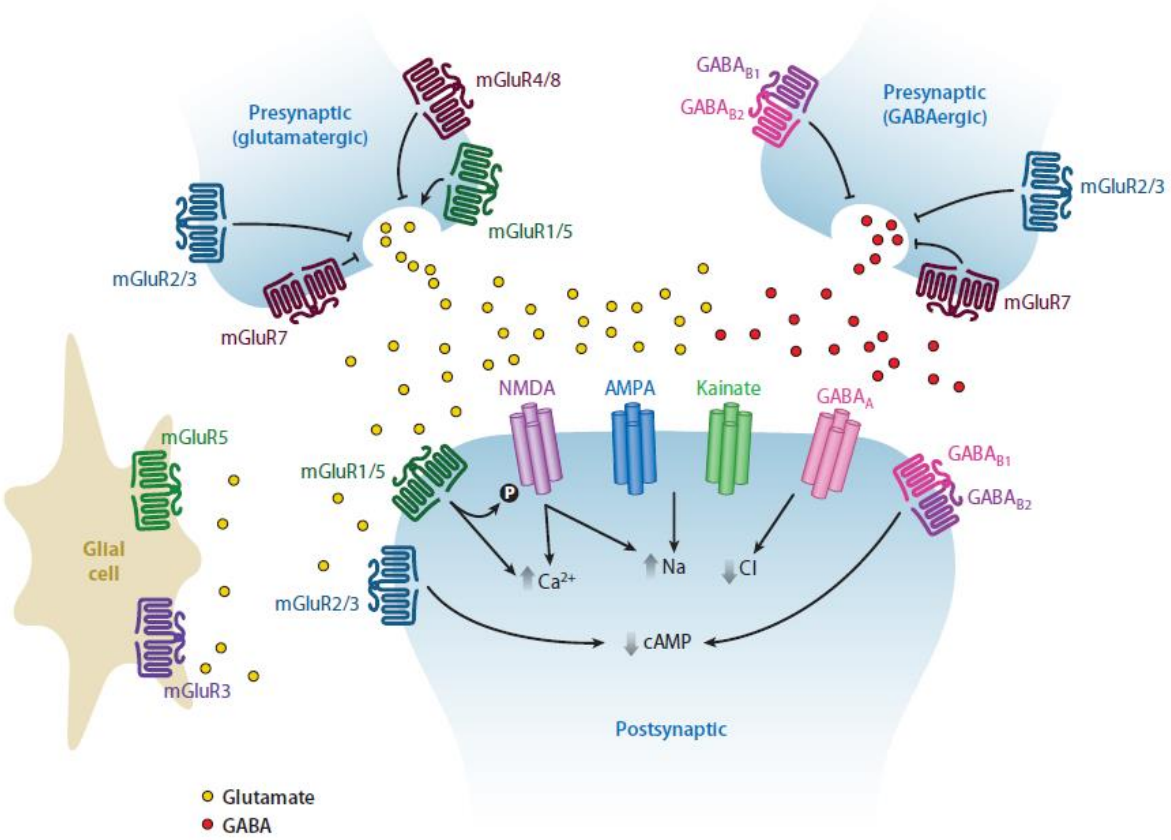

Figure 1.4. Schematic overview of mGluRs at the synapse. In general, group I (green) are localized postsynaptically, and group II (blue) and III (red) receptors are localized in presynaptic locations, (Figure modified from Niswender and Conn, 2010)

A large amount of work has demonstrated that mGluRs are key components in nociceptive processing. Activation of spinal group II mGluRs leads to depression of neuronal hyper-responsiveness following inflammation (Stanfa and Dickenson 1998) and reduces nociceptive behaviour to noxious mechanical stimuli (Dolan and Nolan 2000). Activation of spinal group III mGluRs decrease nociceptive responses and reverse central sensitization (Fisher and Coderre 1996; Neugebauer et al. 2000). Furthermore, activation of group II and III mGluRs may confer neuroprotection by reducing Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Gerber et al. 2000; J.-P. P. Pin and Duvoisin 1995; Takahashi et al. 1996). The typical orthosteric agonist of group III mGluRs is L-AP4, which is highly selective for group III mGluRs relative to other mGluRs or ionotropic glutamate receptors (Schoepp et al. 1999). L-AP4 has submicromolar to low micromolar potencies at mGluRs 4, 6 and 8, but submillimolar to millimolar potency at mGluR7 (Schoepp et al. 1999). Each of the group III mGluRs has been genetically deleted in mice, who display several interesting phenotypes.

Of the groups related to the spinal cord mGluRs 4, 7 and 8, it is known that mGluR4 is predominantly presynaptic (Corti et al. 2002) as mice lacking this receptor show impairment in cerebellar synaptic plasticity and in learning certain complex motor tasks (Pekhletski et al. 1996). These animals also show impaired abilities in spatial memory performance (Gerlai et al. 1998). mGluR4 has also been shown to modulate GABA(A) receptor-mediate seizure activity (Snead et al. 2000), and mGluR4$^{-/-}$ mice lack motor stimulatory effects induced by ethanol (Blednov et al. 2004). mGluR7 exhibits a wide distribution throughout the entire brain, has low affinity for glutamate and is highly localized to active zones of synapses (Kinoshita et al. 1998; Shigemoto et al. 1997). In previous studies it has been proposed that mGluR7 operates like a low pass filter during neurotransmission acting as a brake for overstimulation, as it becomes active only when the glutamate concentration becomes too high (Niswender and Conn 2010). Supporting this hypothesis, mGluR7 knockout mice are prone to manifest seizure and epilepsy, and also have problems in learning (Sansig et al. 2001). mGluR7 function appears to be very important in mediating learning dependent on amygdala activation: moreover, these mice display disorders such as anxiety and depression (both related to amygdala and limbic system) (Callaerts-Vegh et al. 2006; Masugi et al. 1999). Finally, mGluR8 is expressed at lower levels than mGluR4 and mGluR7, but it has wide distribution throughout the CNS. It is localized predominantly presynaptically, although it has been identified at some post-synaptic locations and in the periphery (Lavreysen and Dautzenberg 2008). The mGLuR8 gene is large, spanning exceptionally 1000 kilobases of genomic DNA in the same region. Mutations cause two human disorders, i.e. the Smith-Lemli-Optiz syndrome and retinis pigmentosa (Scherer et al. 1997). mGLUR8 knockout mice show enhanced anxiety and weight gain compared to controls, suggesting a role for mGluR8 in controlling anxiety disorders and network excitability (Linden et al. 2002).

1.7 ADENOSINE RECEPTORS IN SPINAL CORD

Adenosine (ADO) has been shown to be involved in multiple pathological processes like pain, stroke, spinal cord injury and Parkinson's disease (Dunwiddie and Masino 2001; Fredholm et al. 2011; Greene and Haas 1991; Latini and Pedata 2008; Lopes et al. 2011; Paterniti et al. 2011; Sperlágh and Vizi 2011). Former studies demonstrate that ADO activates A_1, A_{2A}, A_{2B}, and A_3 receptor and that inhibition mediated by A_1 receptors is the most common and until now the well-known action on neurons in different brain areas (Porkka-Heiskanen and Kalinchuk 2011; Schmitt et al. 2012). ADO roles are currently

investigated in neuron-glia interactions during sleep deprivation (Porkka-Heiskanen and Kalinchuk, 2011; Schmitt et al., 2012). The latter studies and the above reports state that extracellular ADO rises in proximity of excited neurons to modulate their activity. Mutual interactions between ADO and neuronal activities have been studied thoroughly for the spinal central pattern generator (CPG) controlling swimming movements in tadpoles (Brown and Dale 2000; Brown and Dale 2002; Dale and Gilday 1996; Dale 1998). In this system, rhythmic CPG activity leads to release of ATP into spinal cord networks where it is then degraded enzymatically to ADO which subsequently terminates bouts of swimming. The blocking effect by 8-phenyltheophylline on this ADO action suggests an involvement of A_1 receptors (Dale and Gilday 1996). Focal injection of ATP into newborn mouse brainstem slices does not accelerate pre-Bötzinger complex (preBötC) inspiratory bursting that is normally mediated by P2 receptors because of the rapid degradation of this typically excitatory neuromodulator to ADO that activates A_1 receptors (Zwicker et al. 2011). Furthermore, a modest slowing of inspiratory-related rhythm is seen upon ADO injection into the mouse preBötC slices (Ruangkittisakul and Ballanyi 2010). All these results suggest that the inspiratory CPG in the newborn rat is insensitive to ADO in contrast to its slight depressing effects on the mouse preBötC. As a further example for species differences of ADO effects on CPGs, ADO blocks bursting in rat cervical cord networks caused by inhibition of $GABA_A$ receptors or glycine receptors (Brockhaus and Ballanyi 2000), but it does not affect the “disinhibited” rhythm in the mouse lumbar cord (Witts, Panetta, and Miles 2012).

1.8 SPINAL CORD MODEL *IN VITRO*

In addition to *in vivo* animal models of SCI (Onifer et al. 2007), there have been numerous reports to develop new *in vitro* models that can lead to useful data and discoveries, and to understand mechanisms of SCI pathophysiology. Examples of *in vitro* models are cell cultures, organotypic cultures or isolated spinal cord preparations. *In vitro* models have certain important advantages, for example simplification of the complexity of *in vivo* pathophysiology allows studying certain processes without interference from blood pressure or anesthesia. Primary cultures from spinal cord tissue have been studied for reproducing *in vitro* excitotoxicity (Van Den Bosch et al. 2000; Taylor et al. 2007) and secondary damage due to lack of oxygen (Kaushal and Schlichter 2008). Despite these advantages, these culture models present many disadvantages like the presence of artificial culturing media, random assembly of synaptic contacts, and lack of endogenous substances that may be

fundamental for axon growth and regeneration (Abu-Rub et al. 2010; Silani et al. 2000). Other models are based on organotypic spinal cultures (Guzman-Lenis, Navarro, and Casas 2009), *in vitro* spinal slices (Zhang et al. 2010) and even an incision model (Que et al. 2011). The principal advantage of the organotypic model is the preservation of the basic cytoarchitecture of the tissue with the dorsal/ventral orientation of the spinal segments. Furthermore, this model enables long-term studies stretching over weeks during which changes in network properties can be explored in relation to changes in the local environment (Sibilla and Ballerini 2009). However, these cultures cannot generate locomotor rhythms that require a minimum of three intact spinal segments with undamaged connections. The same limitation applies to acute slices of the spinal cord that can be used for performing functional studies and network pharmacology, though lacking the locomotor circuits.

1.8.1 Neonatal rat isolated spinal cord preparation

Our group has recently developed a novel model of *in vitro* SCI, in order to investigate the rapid events during early secondary damage (G L Mazzone et al. 2010; G. Taccola et al. 2008). It is a model based on the neonatal rat isolated spinal cord, in which we can mimic both non traumatic and incomplete SCI (Kuzhandaivel et al. 2010; Kuzhandaivel et al. 2010; Taccola et al. 2008; Taccola et al. 2010) with the aim of mimicking, under experimental conditions, the acute clinical setting occurring *in vivo.* When *a*pplying kainic acid for one hour, excitotoxic damage is evoked due to large release of endogenous glutamate plus direct activation of glutamate receptors by this agonist. After this application, the solution containing kainate is washed out using standard Kreb's solution for 24 hours. Although this model lacks the immune system responses and vascular supply, it permits monitoring over a period of 24 hours after the kainic acid treatment, the functional responses of the neuronal networks and the number, type and topography of damaged or dead cells. This type of preparation also shows a type of locomotor-like activity (termed fictive locomotion) that can be used as a biomarker for correct performance of the spinal cord networks. This is a very important point due to the relationship between the extent of loss of electrophysiological function and the extent of damage (Mladinic and Nistri 2013; Nistri et al. 2010).

1.8.2 Organotypic slices from rat embryos

Another model recently developed in our laboratory for testing excitotoxicity induced by the glutamate agonist kainate is the organotypic spinal culture that mimics the early pathological processes occurring during the secondary damage caused by kainic acid (Mazzone et al., 2010). With this model, kainate toxicity is primarily directed to neurons rather than glia, and it develops slowly through the hyperactivation of the intracellular enzyme poly(ADP)ribose polymerase-1 (PARP-1) that triggers cell energy failure and DNA damage (a Kuzhandaivel et al. 2010; Graciela L Mazzone and Nistri 2011a; Nasrabady et al. 2011). Our laboratory has evaluated the effects of riluzole on the excitotoxic glutamate release, the release of lactate dehydrogenase (LDH) (used as a marker of cell damage; Hori et al. 2001), metabolic activity of organotypic cultures (with the MTT test; (G L Mazzone et al. 2010)), and number of surviving neurons.

CHAPTER 2: AIMS OF THE STUDY

2.1 Role of mGluR III Group in Excitotoxic Neuroprotection

Our previous studies demonstrated that in the rat isolated spinal cord or organotypic cultures, kainate induces a large neuronal depolarization followed by neuronal death (G L Mazzone et al. 2010; Graciela L Mazzone and Nistri 2011a; G. Taccola et al. 2008). The depolarizing action of kainate is associated with a strong release of endogenous glutamate that peaks in less than 25 minutes in Ca^{2+} free medium(G L Mazzone and Nistri 2011b). The present study follows similar protocols (G L Mazzone and Nistri 2011; Shabbir et al. 2015) to investigate the role of L-AP4, a mGluR III group agonist (Thomsen 1997), in modulating the effects of excitotoxicity induced by kainate on organotypic spinal cord cultures. Group III metabotropic glutamate receptors (mGluR III) are known to decrease glutamate release and to play an important role in controlling pain as documented in neuropathic pain models. Much less is known about their potential neuroprotective effect against excitotoxicity that is considered important for damage onset of spinal cord injury (Pizzi et al. 2000). Using the rat spinal cord organotypic slices model, we wished to investigate if mGluR III receptor activation might contrast excitotoxic cell death evoked by kainic acid (0.1 mM). Furthermore, for better understanding the events that occur during excitotoxicity and L-AP4 effects, we applied CPPG, an mGluR III group antagonist (Niswender and Conn 2010) to find out if block of endogenous activation of mGluR III group by glutamate release leads to an increase of excitotoxic cell death.

2.2 Characterization and developing of a 3 days *in vitro* isolated spinal cord.

Increasing the survival of the rat spinal cord in vitro for more than 1 day is a very interesting challenge. Obtaining this type of new model can lead to the discovery of new information related to the late-onset events that occur in the SCI. Thus, we wished to try to keep an isolated neonatal rat spinal cord *in vitro* for at least 3 days after the laminectomy. For understanding if the spinal cord *in vitro* is an effective model, we evaluated the extent of cell death after the 3 days and the electrophysiological properties such as the ability to generate fictive locomotion (Taccola et al., 2008) elicited by NMDA and serotonin (5-HT).

CHAPTER 3: MATERIALS AND METHODS

3.1 Animal Procedures

3.1.1 Organotypic Slice of rat embryonal spinal cord

Pregnant Wistar rats, at day 13 of gestation, were used for producing embryonic organotypic slice cultures of spinal cord in accordance with previously published procedures (Avossa et al. 2003; L Ballerini et al. 1999; Laura Ballerini and Galante 1998; Gähwiler and Capogna 1997; G L Mazzone and Nistri 2011; G L Mazzone et al. 2010). The fetuses were delivered by caeserean section from anaesthetized rats (10,5% chloral hydrate, 0,4 ml/100 g i.m.) subsequently killed by an intracardiac injection (2 ml) of chloral hydrate. This procedure is in accordance with the regulation of Italian Animal Welfare Act and is in accordance with the National Institute of Health (NIH) guidelines. Fetuses were decapitated and their backs, isolated form their limbs and viscera, were cut into 275 µm thick transverse slices from which the spinal cord was punched out and fixed on a glass coverslip with reconstituted chicken plasma coagulated by one drop of thrombin (200 U/ml). Coverslip were inserted into plastic tubes with 1 ml of medium contained 82% Dulbecco's Modified Eagle's Medium, 8% sterile water for tissue culture, 10% fetal bovine serum (FBS; Invitrogen, Italy), osmolarity 300 mOsm, pH 7.35. For each dissection, 30-40 slices were prepared from thoracic as well as the lumbar segments, and kept in culture for 22 days *in vitro* (DIV) before use. The tubes were kept in a roller drum rotating (120x*g*/h) at 36.5 °C. Dulbecco's Modified Eagle's medium with high glucose (DME/HIGH), penicillin, and streptomycin (purchased from Euroclone, Devon, UK). Fetal calf serum was obtained from Invitrogen, (Carlsbad, CA, USA). Nerve growth factor (NGF) was from Alomone Laboratories (Jerusalem, Israel), chicken plasma from Rockland (Gilbertsville, PA, Usa), and thrombin from Merck, (Darmstadt, Germany).

3.1.2 Newborn rat spinal cord preparation

In accordance with the NIH guidelines and Italian act Decreto Legislativo 27/01/1992 n.116 (implementing the European Community directives n.86/609 and 93/88), 0-2 day old Wistar

rats were anesthetized with urethane. All efforts were aimed at reducing the number of animals used for the present project and at minimizing their suffering. Subsequently, the spinal cord was isolated by laminectomy in Kreb's solution containing (in mM): 113 NaCl, 4.5 KCl, 1 $MgCl_2 \cdot 7H_2O$, 2 $CaCl_2$, 1 NaH_2PO_4, 25 $NaHCO_3$, 11 D-glucose. The solution was gassed with 95% O_2-5% CO_2 to establish a pH of 7.4 at room temperature. For electrophysiological recording, one spinal cord was positioned in an acrylic chamber with a volume of 5 ml that was perfused with Kreb's solution at a flow rate of 5 or 7.5 ml/min. All details about laboratory procedures have been previously published (M Beato et al. 1999; Beato et al. 1997; Bracci et al. 1996a, 1996b) and the experimental setup has been fully reported (Margaryan et al. 2009; G. Taccola et al. 2008; Taccola and Nistri 2006). Drugs were dissolved in Krebs solution and bath applied at the concentration indicated in the text.

3.1.3 Newborn Isolated 3 days *in vitro* spinal cord

After isolation, the neonatal spinal cord was put in a 50 ml tube with 20 ml of Basal Medium (Life Technologies), Nerve Growth Factor 7S 30mg/ml (Sigma Aldrich), Insulin 10 µg/ml (Sigma Aldrich), Amphotericin B (Fungizone) 250mg/ml (Sigma Aldrich), Gentamycin 100 µg/ml (Sigma Aldrich). The medium was oxygenated and keep sterile for all the 3 days.

3.2 Staining and Immunohystochemistry procedures

3.2.1 Immunofluorescence of organotypic slices cultures

Slices were fixed in 4% paraformaldehyde for 1 hour at room temperature and stored in phosphate buffer saline (PBS) until use. Cultures were processed for immunofluorescence analysis by immersion for 10 minutes in trypsin solution (0.05% in sterile water) at 37°C. Slices were then blocked with 3% fetal calf serum (FCS), 3% bovine serum albumin (BSA), 0,3% Triton in PBS (blocking solution) for 1 hour at room temperature, followed by overnight incubation at 4°C in a blocking solution containing the antibodies. The following antibodies were employed: NeuN antibody (Millipore) at 1:500 dilution; SMI 32 antibody at 1:1000 dilution, mGluR4 (Santa Cruz Biotech), mGluR7 (Novus Biologicals), mGluR8 (Millipore) at 1:1000 dilution. The primary antibody was visualized using corresponding secondary fluorescent antibody (Alexa Fluor 488, at 1:500 dilution, Invitrogen, Carlsbad, CA, USA). To

visualize cell nuclei, slices were incubated in 1 µg/ml solution of DAPI for 20 minutes and mounted using DAKO mounting medium (Dako, DK-2600 Glostrup, Denmark).

3.2.2 Quantification of dead cells in organotypic spinal cord cultures

DAPI staining results were analysed using a Zeiss Axioskop2 microscope. The identification and quantification of dead or dying cells in the organotypic cultures was performed as previously shown (G. Taccola et al. 2008), using DAPI nuclear staining and “eCELLence” software. Three different regions of interest (r.o.i.), namely dorsal, central, and ventral, were analysed in each slice (see scheme in figure). The average percent values of nuclei showing condensed chromatin (normalized to the total number of nuclei) were compared between different r.o.i. for controls or treatments and expressed as mean±SD (using at least three different cell culture series for each experimental group).

3.2.3 Quantification of NeuN positive cells in organotypic spinal cord cultures

NeuN positive cells were analysed using a Confocal (Nikon) microscope, equipped with Ar/ArKr (at 488 nm) laser whereby a stack of 25-30 images (20x magnification) were counted with “eCELLence” software using the same intensity threshold (ranging from 0 to 1 where 1 refers to the maximum intensity of the image) and cell diameter parameters for all experiments. The total number of NeuN positive cells was obtained for each experimental condition as the total number of positive cells in all stacks. Multiple entries of the same object were considered as single entities by the “eCELLence” software algorithm.

3.2.4 Quantification of SMI 32 positive cells in Organotypic Spinal Cord Cultures

SMI 32 positive cells were analysed with Zeiss Axioscope Microscopy and “eCELLence” software for counting. We evaluate the total number of nuclei positive to SMI 32 immunostaining in the ventral region of interest near the fissure.

3.2.5 Quantification of mGluR signal intensity

Quantification of Immunofluorescence signals for mGluR 4,7,8 subunits (gray level intensity expressed in arbitrary units, AU) was performed with Meta-Vue imagine software (Molecular devices, Sunnyvale, CA USA) using the densitometry function to calculate mean signal intensity for regions of interest (ventral, central and dorsal) reactive to mGluRs antibodies. The values are mean ± SD (at least 9 different cultures with at least one sample from each culture).

3.2.6 Fluorescence immunohistochemistry procedure in neonatal isolated spinal cord

The free-floating immunofluorescence protocol was used as previously described (Mladinic and Nistri 2013; G. Taccola et al. 2008). The primary antibodies were visualized using appropriate secondary fluorescent Alexa Fuor 488 or 594 antibodies (1:500 dilution, Invitrogen Carlsbad, CA, USA). Sections were stained in 1 µg/ml solution of 4,6-diaminio-2-phenylindole (DAPI) for 20 minutes to visualize cell nuclei and mounted on Superfrost Plus (Menzel-Glazer, Braunschweig, Germany) slides. The immunostaining signal was analysed by Zeiss Axioskop2 microscope (Oberkochen, Germany) using 1µM z sectioning.

3.2.7 Statistical Analysis

Data were collected from at least three independent experiments, and are expressed as mean ± standard error of the mean (SEM), where *n* indicates the number of independent experiments, as indicated in the respective figure legend. Statistical analysis was performed using the SigmaStat (SigmaStat 3.1, SystatSoftware, Chicago, IL, USA): after normality test values were analysed with one-way ANOVA for multiple comparisons (with Turkey-Kramer post-hoc test). For non-parametric values, Kruskal-Wallis one-way analysis of variance and the student-Newman-Keuls method test were used for multiple comparison. The accepted level of significance was $p<0.05$.

3.3 Electrophysiological recording and stimulation

Rhytmic motor Central Pattern Generator (CPG) activity was recorded with suction electrode form the second and fifth ventral lumbar roots (L2, L5). L2 roots contain axons from motoneurons which innervate mainly hindlimb flexor muscles, while L5 roots contain axons that drive primarily hindlimb extensor muscles. Locomotor-associated CPG activity alternates rostrocaudally between L2 and L5 and bilaterally between ipsilateral and contralateral L2 or L5 roots (Clarac et al. 2004; Kiehn 2006; Taccola and Nistri 2006). Rhythmic bursting with these characteristics was induced by combined bath application of serotonin (5-HT; 10 μM; Sigma-Aldrich, Milan) and N-methyl-D-aspartate (NMDA; 3-5 μM; Ascent Scientific, Bristol, UK) and such chemically evoked fictive locomotion was typically stable for time period of more than 3 hours (Clarac et al. 2004; Kiehn 2006; G. Taccola et al. 2008). Single electrical pulses were applied to dorsal root L5 for eliciting a dorsal root/ventral root potential comprising a polysynaptic dorsal root reflex (Marchetti et al. 2001; G. Taccola et al. 2008). Tight-fitting suction electrodes filled with control solution were applied to the distal cut end of L2 and L5 ventral roots for DC-coupled extracellular population recordings via a high input impedance, low noise DC amplifier with a gain of x1000. The signals were bandpass-filtered 0.1-10 kHz and fed at sampling rate of 5 kHz via an Axon Digital Interface (Molecular Device LLC, Sunnyvale, CA, USA) into a personal computer (G Taccola and Nistri 2006). Burst amplitude was calculated at the highest point of the activity bout. Values are given in percentage of control because the absolute amplitude of extracellular signals depends on various parameters such as suction electrode resistance and tightness of the seal between electrode and spinal root. Rhythmic discharges were characterized on the basis of their period that is defined as the time between the onset of two subsequent cycles of oscillatory activity. For averaging period values from different preparations, data from each spinal cord were calculated as the mean of at least 20 burst cycles. The regularity of bursting was determined in terms of the coefficient of variation (CV; given by the standard deviation [S.D.] $mean^{-1}$) of the period. Single burst duration was calculated between the onset of the event and the start of the rapid phase of its decay. Data were quantified as means ± S.D. Parametric data were analysed with t-test, paired t-test for two groups, or one way ANOVA followed by a post-hoc analysis for multiple groups. Non parametric data were evaluated with Rank Sum test, Signed Rank Sum test for two groups,

or Kruskal-Wallis one way ANOVA test followed by a post-hoc analysis for multiple groups. The accepted level of significance was $p<0.05$.

3.4 Organotypic Spinal Cord Slices treatments

As depicted in figure 3.1 the organotypic slices of embryonal spinal cord were subjected to different treatments. In the first treatment (Fig. 3.1A) kainate at the concentration of 100 µM was applied for 1 hour and L-AP4 at the concentration of 1 µM was either co-applied or applied only in the kainate 24 h washout. In the second set of experiments kainate at the concentration of 50 µM was applied for 1 hour and L-AP4 and CPPG both at the concentration of 1 µM were applied only in the 24 hour washout. In the last set of experiments, we applied kainate at the concentration of 100 µM and then L-AP4 1µM and adenosine (ADO) at 100 µM either in co-application or during washout only.

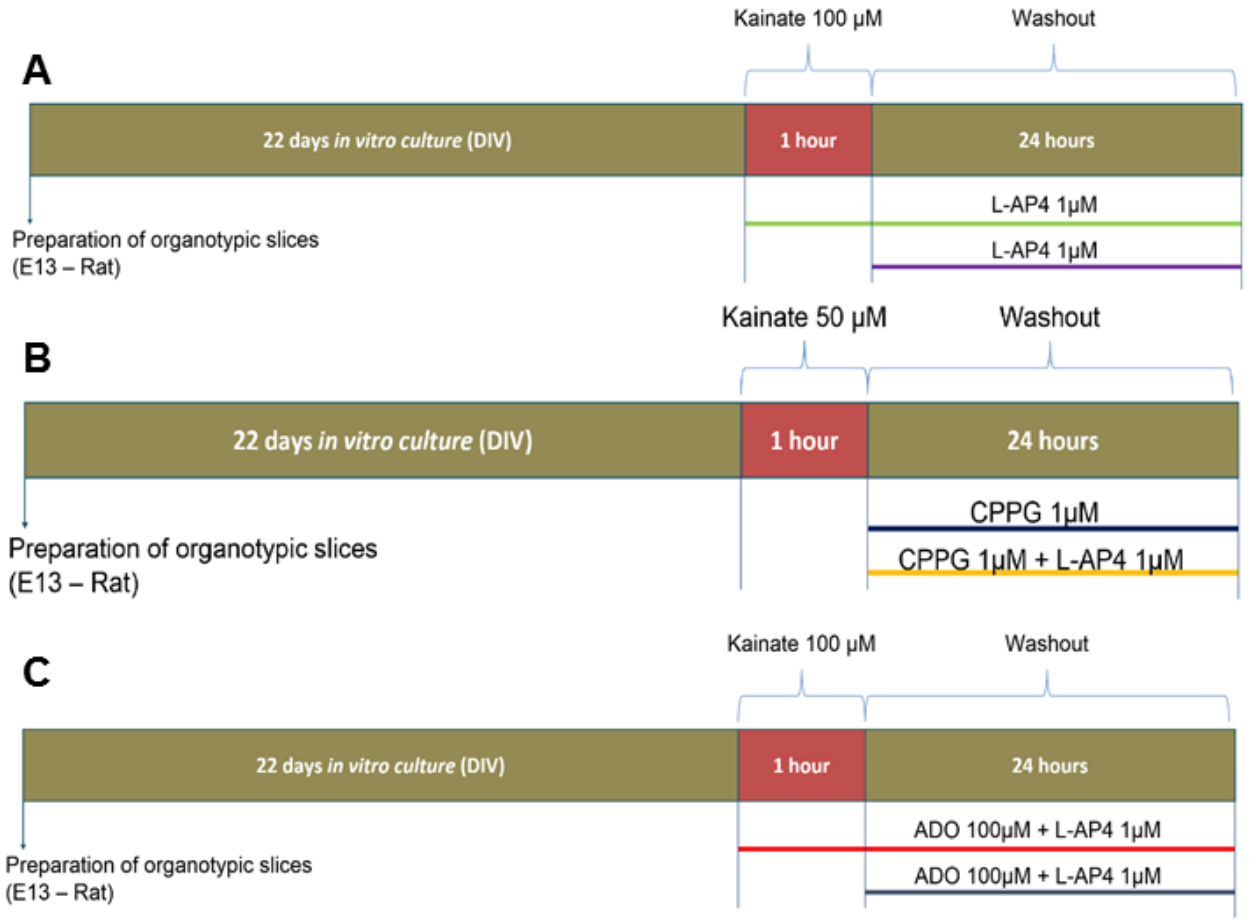

Figure 3.1 Different treatments for organotypic spinal cord cultures. The figure illustrates the three different types of treatment for evaluating the impact of protection by modulation of mGluR III group receptors and

adenosine receptors. Figure 3.1A show the treatment with the application of only L-AP4 after kainate. Figure 3.1B shows the treatment with application of CPPG or co-application of CPPG and L-AP4 during the kainate washout. Figure 3.1C show the coapplication of adenosine (ADO) and L-AP4 during the treatment with kainate or during washout.

CHAPTER 4: RESULTS

4.1 Dead cells quantification after kainate 100μM and L-AP4 1μM application

We identified dead cells (using DAPI) characterized by condensed chromatin (example of pyknotic nuclei is indicated by black arrow in figure 4.1A). Cell death was quantified in terms of percentage of pyknotic nuclei. Percentage of pyknotic nuclei treated with kainate at 100μM concentration is statistically different ($p=0.033$) in all the three regions (ventral, central and dorsal). L-AP4 co-applied with kainic acid decreased pyknosis from 39±1 to 14±3 %, and from 16±4 to 2±1 % in the dorsal and ventral regions, respectively, and from 31±3 to 7±2% in central area. Delayed L-AP4 application was less effective, yet still significant with pyknosis decreasing to 32±2% in dorsal, 7±2% in ventral regions and 23±2 % in central region (Figure 4.1). There is no deleterious effect using only L-AP4 at 1 μM for 24 hours concentration (Figure 4.1C). This results suggests that kainate evokes excitotoxic damage, an effect counteracted by the activation of mGluR III group receptors.

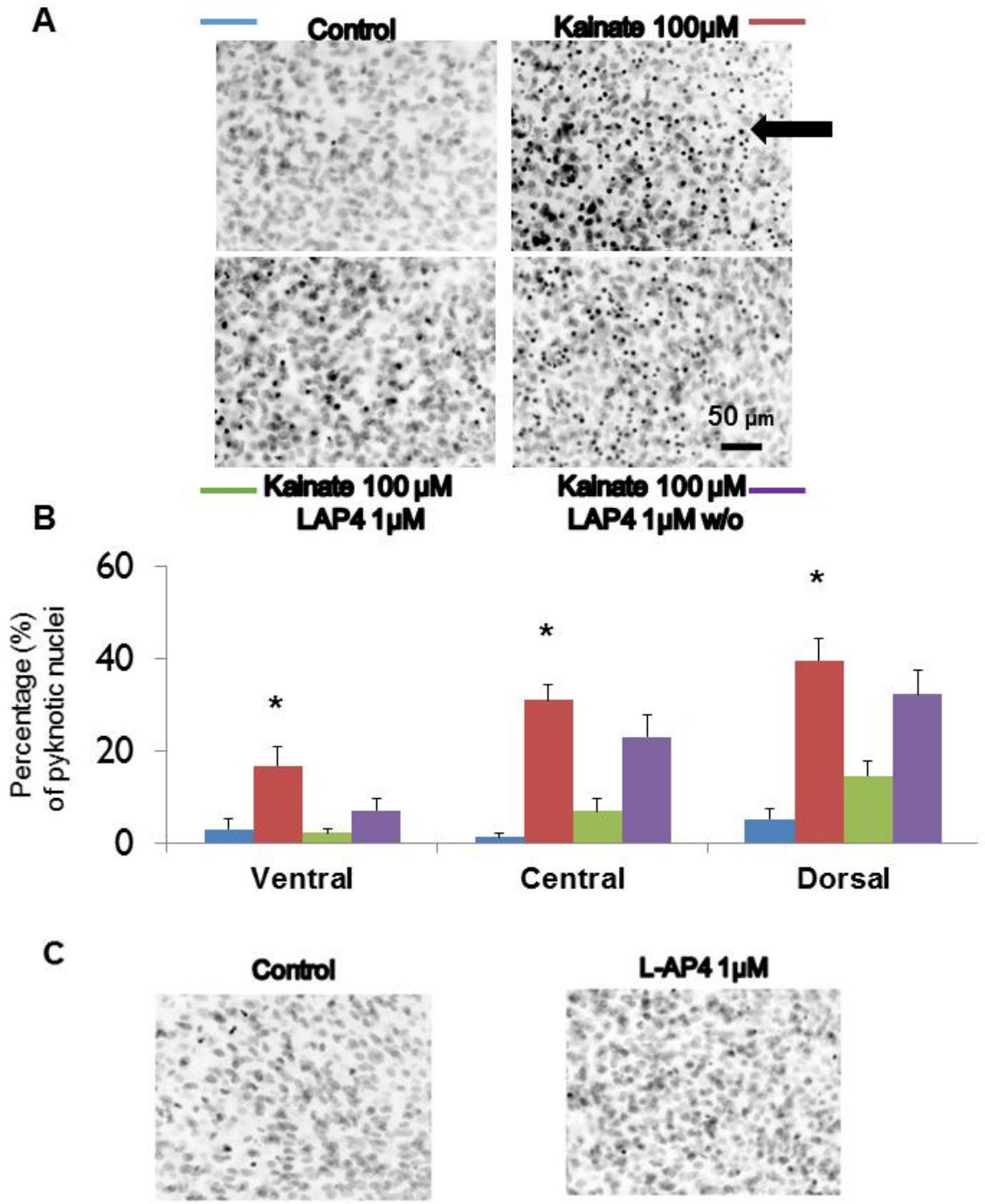

Figure 4.1 Cell death analysis of organotypic cultures 24 hours after application of kainate in the presence of L-AP4 by using the experimental protocol for early excitotoxicity shown in Fig.3.2. (A) Examples of DAPI-stained cells in the central region in control condition, or after kainate for 1 hour at 100 µM and wash out (w/o) with medium, treated with coapplication of kainate 100 µM and L-AP4 1 µM for 1 hour and then washed out with medium and L-AP4 or with the application of L-AP4 only in the washout medium. (B) Histograms show average percent of pyknotic cells in ventral, central and dorsal region in presence of kainate (100 µM) for 1 hour with or without L-AP4 (1 µM). In Blue control condition, in red kainate 100 µM only, in green with co-application of L-AP4 1µM, in purple application of L-AP4 only in the washout (w/o) after 1 hour application of kainate. (C) Comparison of example of central region in control condition and treated with L-AP4 for 24 hours.

4.2 NeuN positive cells quantification after kainate (100µM) and L-AP4 1µM application

To further confirm the action of L-AP4 on kainate induced excitotoxicity, we analyzed the NeuN staining (example of central part of organotypic slices in figure 4.2A) counting the total number of NeuN positive cells. A statistical increase (p=0.039) in total number of NeuN positive residual cells is present in both treatments. For example, in the ventral region from 193±27 with the application of only kainate at the concentration of 100 µM to 245±34 in the co-applied treatment of both kainate 100 µM and L-AP4 1µM. Organotypic slices treated with L-AP4 1µM only during kainate wash-out show improved number of NeuN positive cells too. In the ventral region, values increase from 193±27 to 238±5 with p=0.041 (Figure 4.2). This result shows that the counteracting effect of L-AP4 involves blocking excitotoxic damage to neurons.

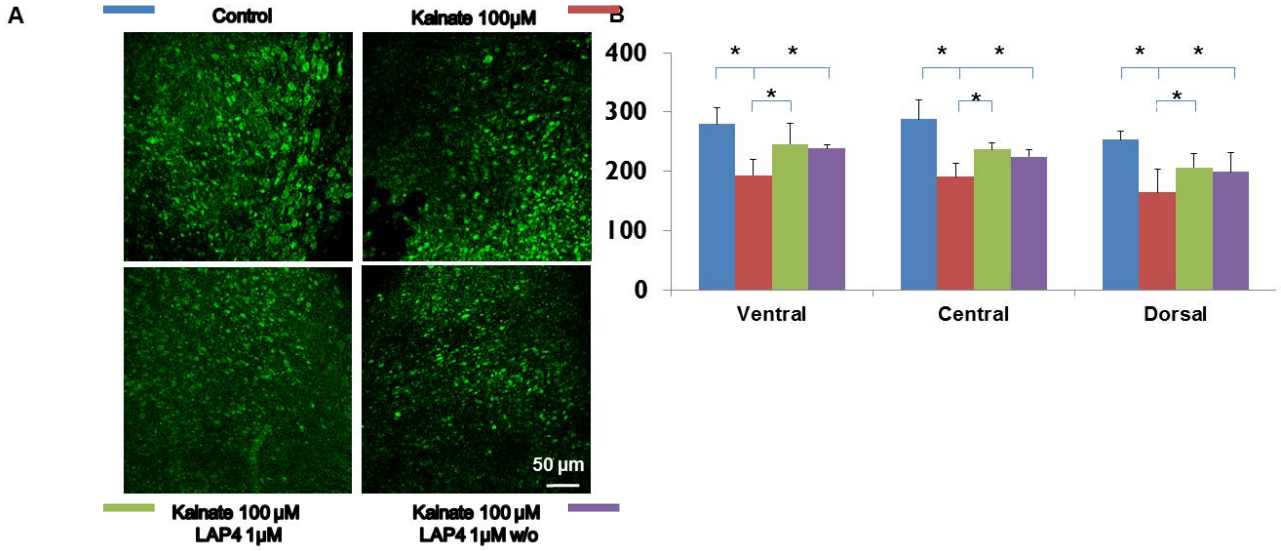

Figure 4.2 Neuronal loss evoked by kainate 24 hours later can be counteracted by L-AP4. (A) Example of central region with NeuN positive neurons 24 hours in complete medium (control), after 1 hour application of kainate (100 µM), or kainate and L-AP4 in co-application or only in the washout (w/o) (lower images, respectively left and right). (B) Histograms showing average number of NeuN positive cell in the three regions analyzed as described above. * P< 0.05 vs kainate (n=8). In Blue control condition, in red kainate 100 µM only, in green with co-application of L-AP4 1µM, in purple application of L-AP4 only in the washout after 1 hour application of kainate.

4.3 SMI 32 positive cells quantification after kainate 100 µM and L-AP4 application

We assessed if the group of neuronal cells protected by excitotoxic damage comprise motoneurons too. We focus on motoneurons because these are the major group of cells that are damaged by excitotoxic events during secondary damage (Van Damme et al. 2002; Ince et al. 1993). We use SMI 32 antibody (G L Mazzone and Nistri 2011; G L Mazzone et al. 2010; G. Taccola et al. 2008) to identify large diameters (>25 µm) cells in the ventral region. Figure 4.3B depicts a strong fall in motoneuron number induced by kainate (100µM) with a significant protection by subsequent application of mGluR III group antagonist L-AP4 either in co-application or only during the washout. The result suggests that mGluR III activation is capable of stopping motoneuron death due to excitotoxic damage.

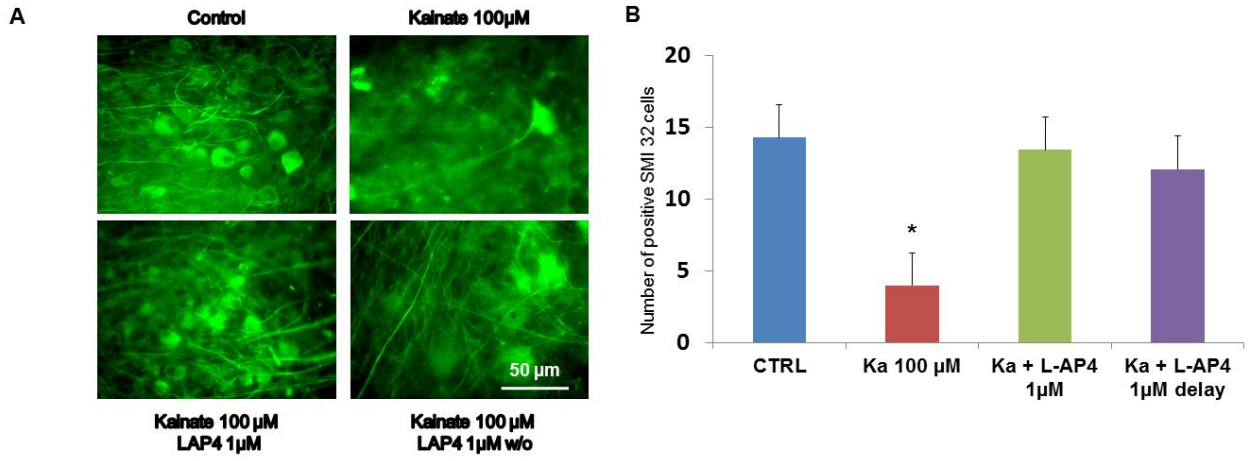

Figure 4.3 Kainate-induced damage of motoneurons in organotypic slice cultures is prevented by L-AP4 applied even after 1 hour of treatment with kainate. (A) Example of motoneuron staining (with SMI 32 antibody) in control, or 24 hours after kainate (100 µM), or kainate co-applied or followed by L-AP4. (B) Histograms show number of motoneurons from 8 experiments with 3 slices; * P<0.05 vs control.

4.4 Cell death evaluation after treatment with CPPG, kainate and L-AP4

Focusing on the neuroprotection effect of L-AP4, we further wished to evaluate the role of mGluR III group receptors in excitotoxic events following kainate treatment. For investigating these events we used a lower concentration of Kainate (50 µM) to prevent maximum damage level at one hour. Previous studies from our laboratory have shown that the release of glutamate is slower following 50 µM kainate even if the final damage is as strong as after

100 µM (G L Mazzone and Nistri 2011). Figure 4.4 shows that in presence of CPPG during the washout after kainate at 50 µM there is more excitotoxic damage compared with the damage in kainate alone (e.g. in ventral region: from 9.6±1 to 35±3 %). Co-application of L-AP4 1 µM with CPPG 1 µM in the washout leads to levels statistically different ($p<0.05$) from both kainate 50 µM and kainate plus CPPG 1 µM treated slices. There is no effect using only CPPG at 1 µM for 24 hours in terms of cell death (Figure 4.4C). The result suggests that decreasing the activity of mGluR III receptors leads to an increase in glutamate release leading to a massive cell death. The co-application of both mGluR III group agonist and antagonist decreases the cell death probably due to the different affinity of the two compounds with a smaller counteracting effect of L-AP4.

4.5 NeuN positive cells quantification after kainate 50µM and CPPG and L-AP4 1µM application

To better investigate the role of CPPG in kainate induced excitotoxicity, we analyzed the NeuN staining (example of central part of organotypic slices in figure 4.2A). A statistical decrease ($p<0.05$) in total number of NeuN positive cells is observed in all regions but not incremented by CPPG treatment. For example, in the ventral region from 194±23 with the application of kainate at the concentration of 50 µM, treatment with kainate 50 µM and CPPG 1µM during washout left the same damage (190±34). Organotypic slices treated with L-AP4 1µM co-applied with CPPG 1µM show an apparent increase in the total number of NeuN positive cells without reaching control values (Figure 4.5).

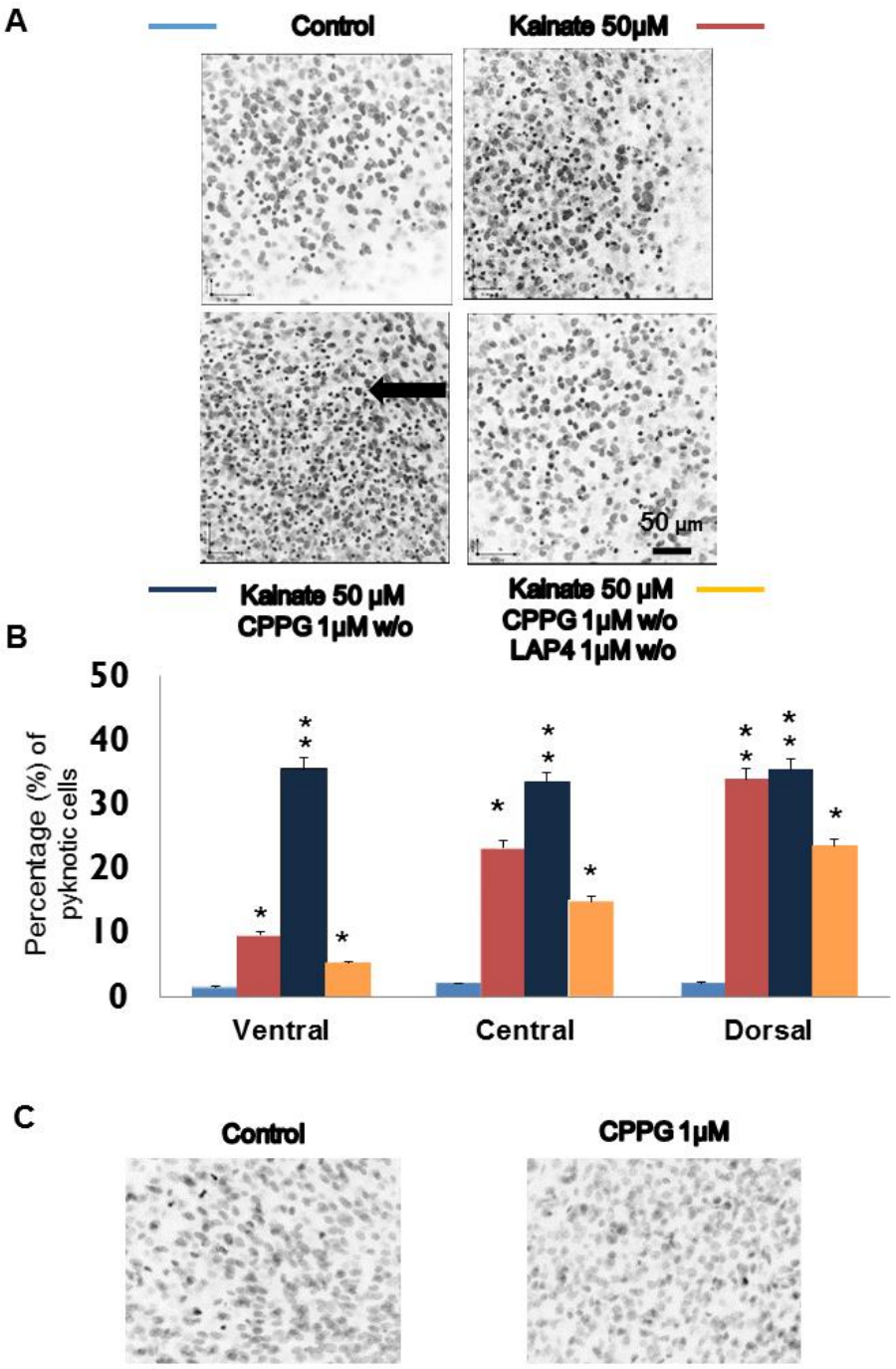

Figure 4.4 Characterization of organotypic cultures 24 hours after application of kainate and CPPG. (A) Examples of DAPI-stained cells in the central region in control condition, after kainate (50 µM) for 1 hour with or without CPPG and co-application with L-AP4, CPPG and KA 50 µM. (B) Histograms show average percent of pyknotic cells in ventral, central and dorsal regions in presence of kainate (50 µM) for 1 hour with or without CPPG (1 µM) and coapplication of the three compounds. In Blue control condition, in red Kainate 100 µM only, in green with co-application of L-AP4 1µM, in purple application of L-AP4 only in the washout after 1 hour application of Kainate. In light blue control condition, in red Kainate 50 µM only, in heavy blue with co-application of CPPG 1µM, in orange combined application of L-AP4 1µM, Kainate 50 µM and CPPG 1µM. (C) example of central region in control condition and with the application of only CPPG for 24 hours.

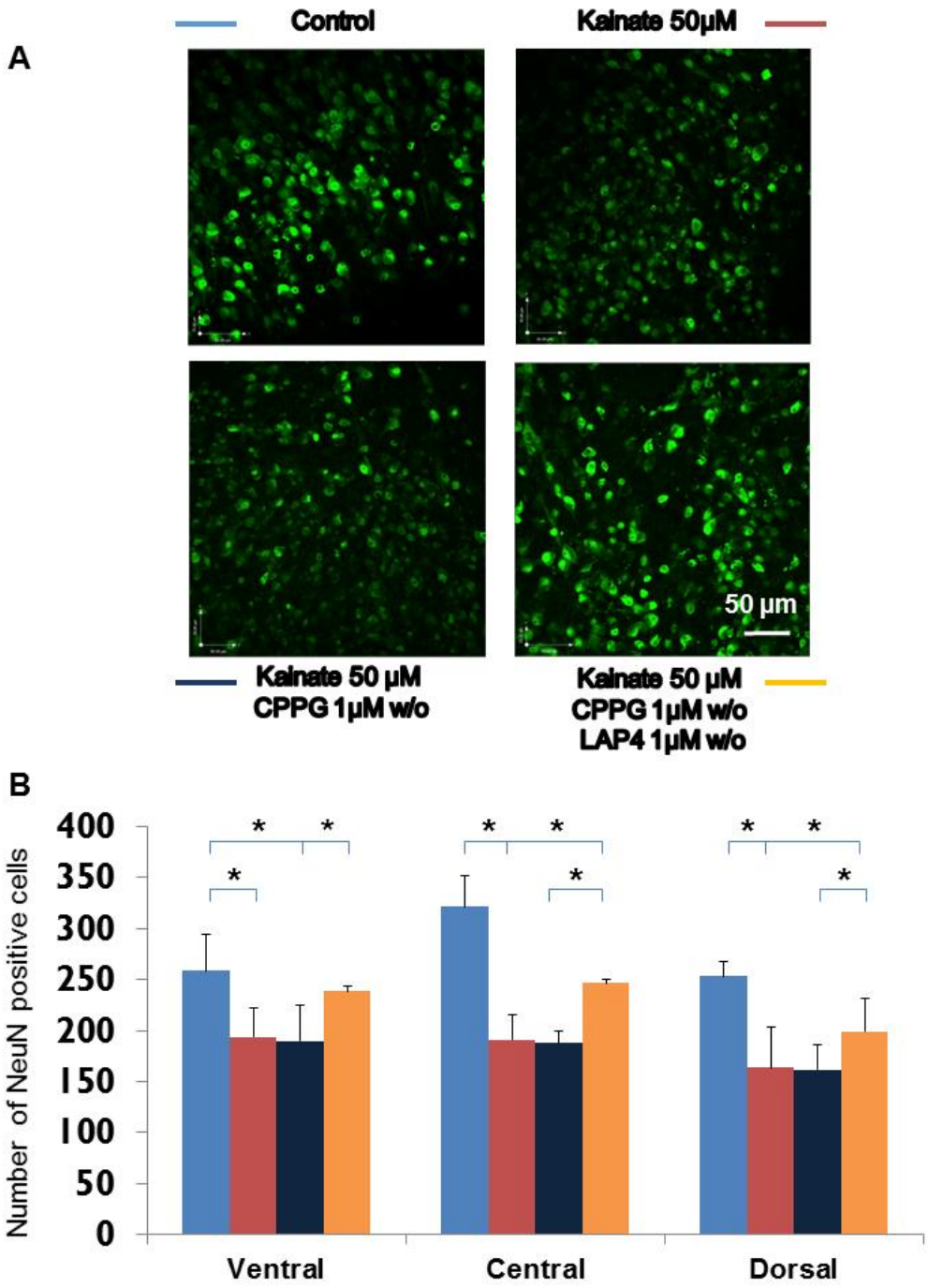

Figure 4.5 Neuronal loss evoked by kainate and CPPG. (A) Example of central region with NeuN positive neurons after 24 hours in complete medium (control), after 1 hour application of kainate (50 µM) and CPPG (1 µM), or kainate, CPG and L-AP4 in co-application during the kainate washout (lower images, respectively left and right). (B) Histograms showing average number of NeuN positive cells in the three regions analyzed after the protocol described above. * P< 0.05 (n=8). In light blue control condition, in red kainate 50 µM only, in dark blue with co-application of CPPG 1µM, in orange application of L-AP4, CPPG only in the washout phase after 1 hour application of Kainate.

4.6 SMI 32 positive cell quantification after kainate 50 µM and CPPG application

Using the same protocol of SMI 32 antibody staining for identifying motoneurons, we further evaluated the impact of CPPG-kainate treatment on these cells. Figure 4.6B depicts a strong fall in motoneuron number induced by kainate (50µM) and CPPG (1 µM) in the washout similar to the effect by kainate alone. The coapplication of L-AP4 alongside with CPPG in the washout phase results in a partial increase in the number of motoneurons without fully counteracting the kainate excitotoxic treatment. These data suggest that without fast, strong activation of mGluR III group receptors the degree of excitotoxicity is too high for their survival.

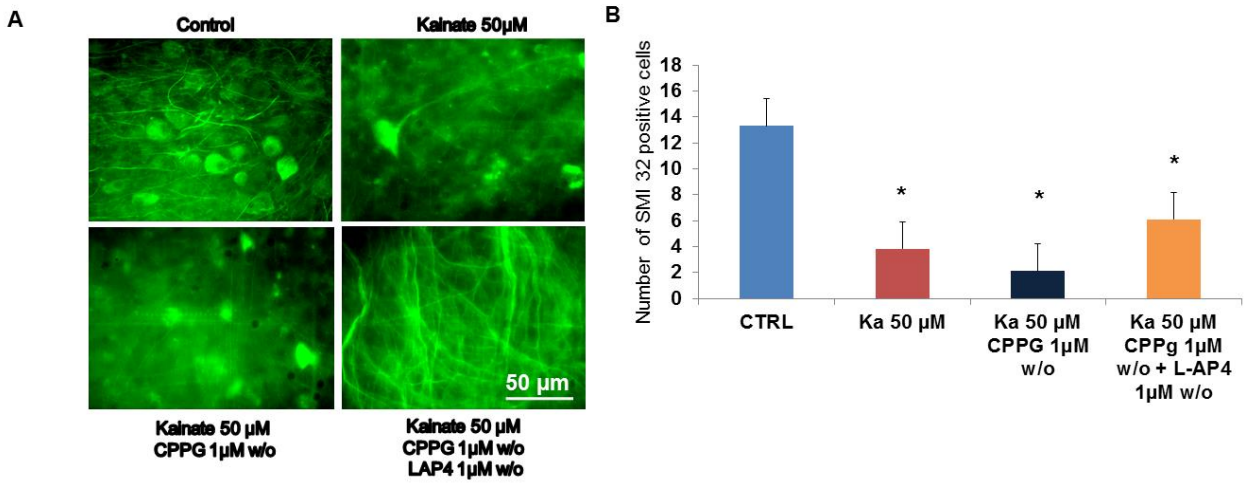

Figure 4.6 CPPG effect on motoneurons. (A) Example of motoneuron staining (with SMI 32 antibody) in control, or 24 hours after kainate (50 µM), or kainate followed by CPPG in washout or co-application of CPPG and L-AP4 during washout. (B) Histograms showing numbers of motoneurons from 8 experiments with 3 slices; * P<0.05 vs control.

4.7 Characterization mGluR receptor subunits

For a better characterization of the events following excitotoxic damage in the spinal cord organotypic slices we performed immunostaining against three types of mGluR III group subunits that are reported in the spinal cord (Niswender and Conn 2010). We analyzed the total intensity of the fluorescence signals of mGluR 4 antibody, mGluR 7 antibody and

mGluR 8 antibody to evaluate if there are differences in the localizations of these receptors in our model and within the different treatments.

4.7.1 mGluR 8 characterization

Figure 4.7 shows that the intensity of the signal of the mGluR8 antibody is no different in control condition between the three regions of interest. In the organotypic slices treated with kainate at 100 µM concentration, there is a decrease in the values of the signal ($p<0.01$). L-AP4 co-application counteracts this fall in fluorescence intensity (Figure 4.7C). When slices are treated with L-AP4 only during kainate washout, a stronger fluorescence signal than after kainate alone is observed, although this value does not reach the control level.

4.7.2 mGluR 4 characterization

We next proceeded to characterize the signal intensity of mGluR 4 type subunit. Figure 4.8 shows that also in this case there is no difference between the three regions of the organotypic spinal cord slice. The fluorescence levels after the different treatments appear to be broadly comparable with the pattern observed with the mGluR8 one. Notably, there is a large decrease of the signal intensity following kainate 100 µM, good preservation with kainate and L-AP4 coapplication, and a smaller decrease in the slices treated with L-AP4 only during the kainate washout.

4.7.3 mGluR 7 characterization

Finally, the immunohistochemistry signal of mGluR 7 units is comparable with the two reported above: thus, there is a strong decrease following kainate 100 µM, a good protection level with either co-application of L-AP4 and kainate or delayed application of L-AP4 in the three regions of interest.

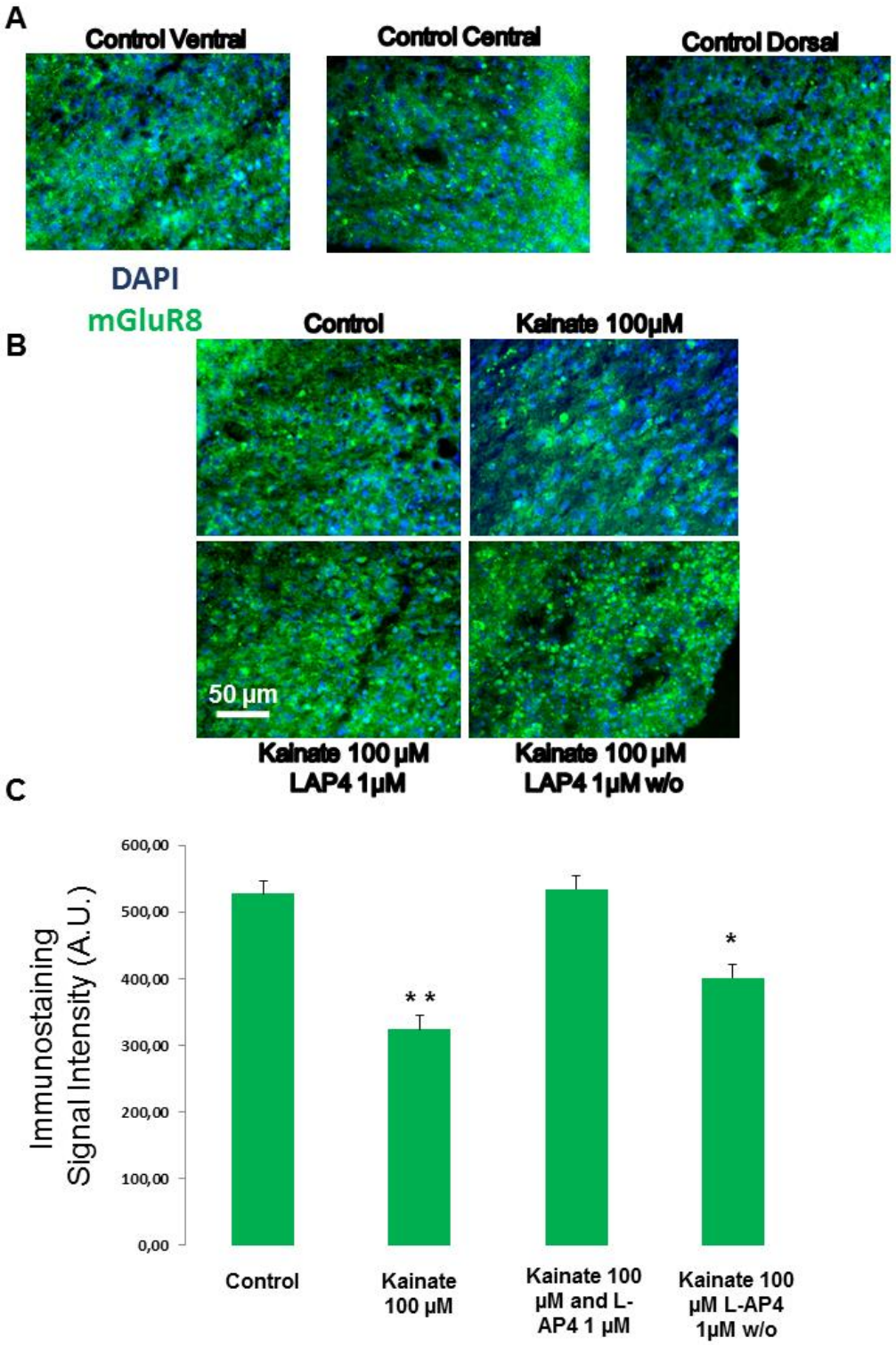

Figure 4.7 Signal Intensity of mGluR8 immunocytochemistry. Panel A shows an example of ventral, central and dorsal tissue. There are no differences in immunostaining signal intensity among these three regions. Panel B shows an example of the central region of the organotypic slice immunohistochemistry under various treatments. C shows histograms depicting the decrease in arbitrary units (A.U.) of the signal between control and treatments (**p<0.01, *p<0.05 n=11). w/o=washout of kainate.

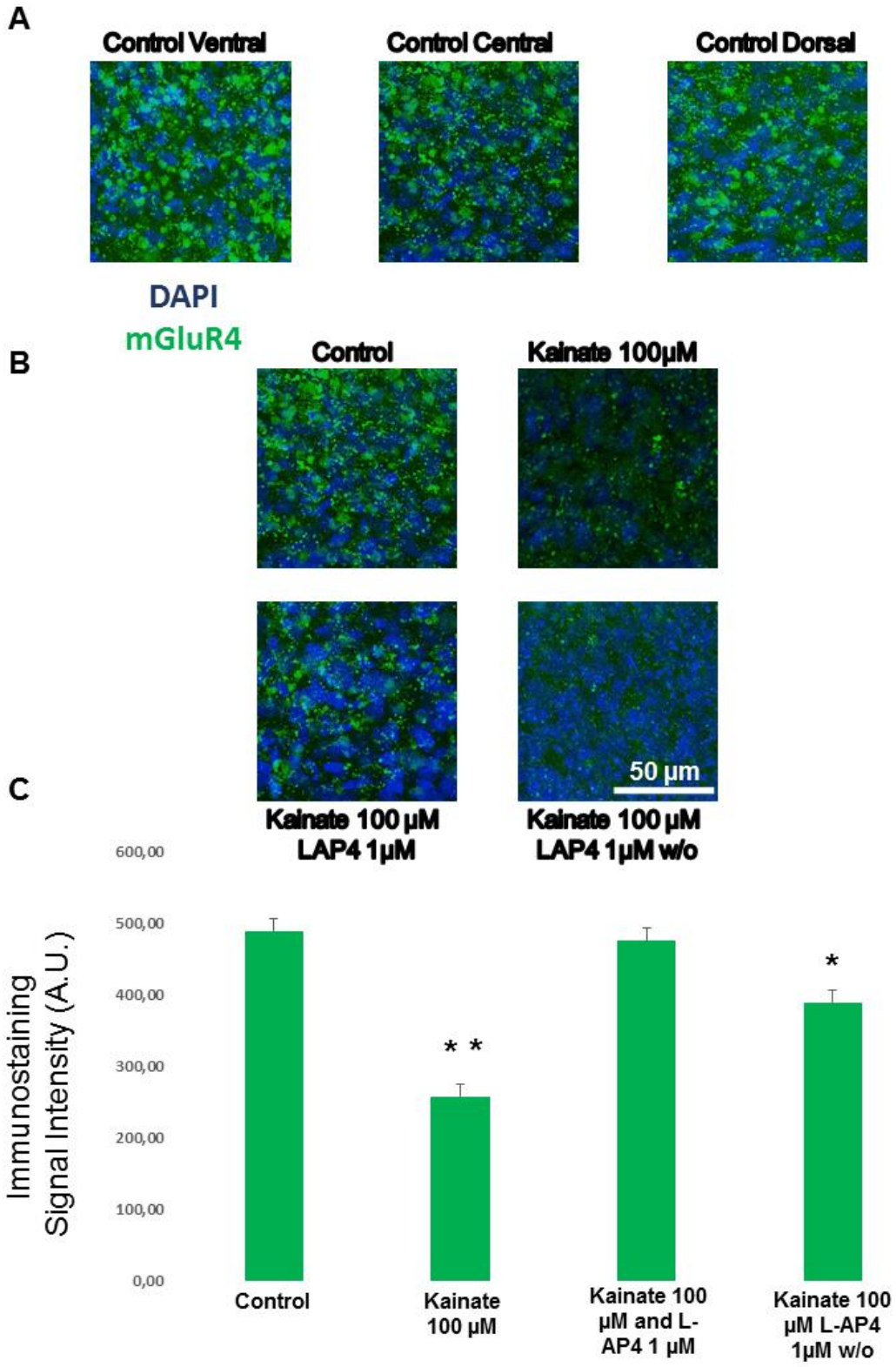

Figure 4.8 Signal Intensity of mGluR 4 immunohistochemistry. Panel A shows no signal difference among the three regions (ventral, central, dorsal, respectively). Panel B shows an example of the central region of organotypic slices under various treatments. Panel C shows histograms indicating the decrease in arbitrary units (A.U.) of the signal between control and treatments (**p<0.01, *p<0.05 n=9). w/o = washout of kainate.

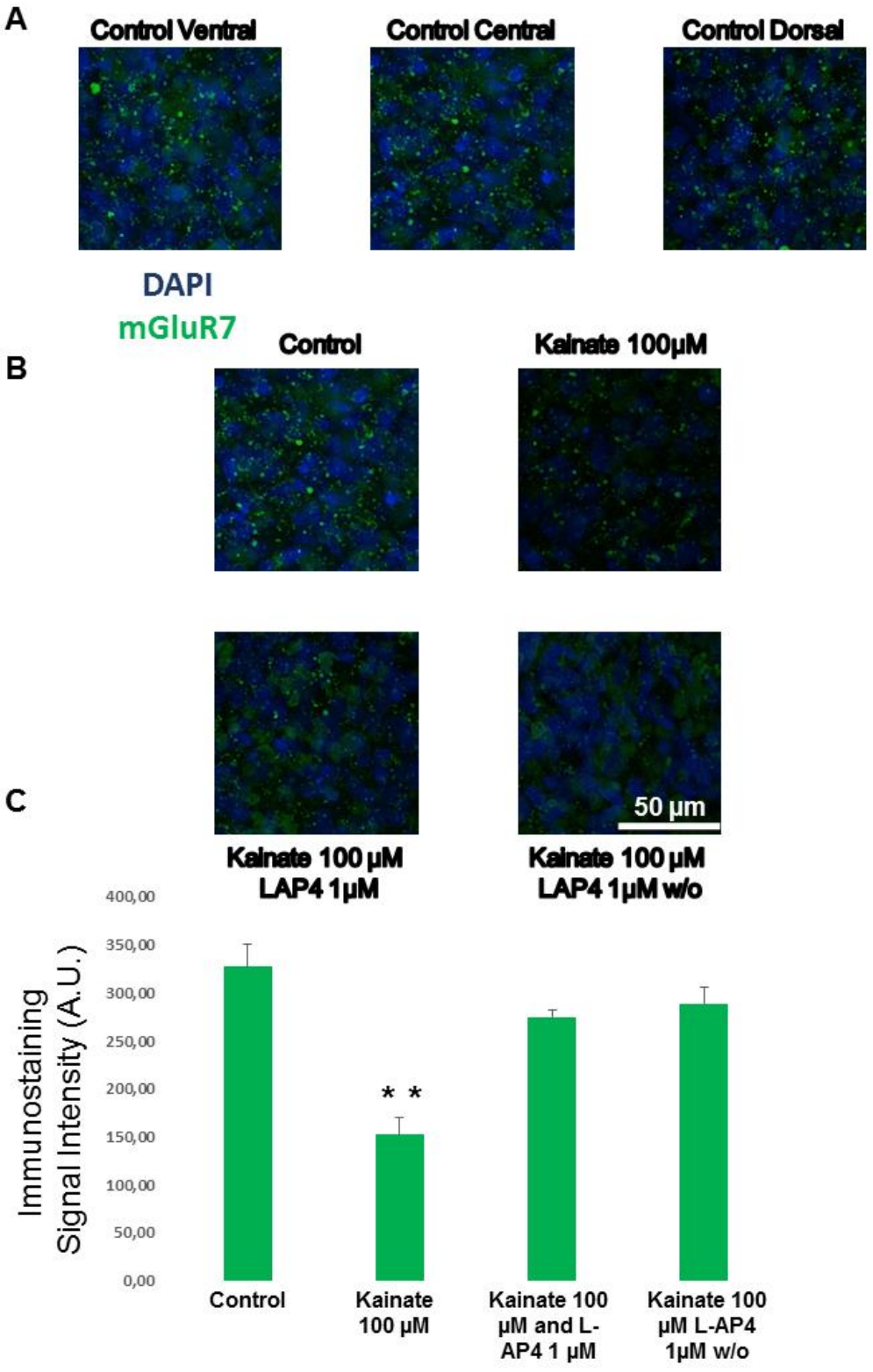

Figure 4.9 Signal Intensity of mGluR 7 immunohistochemistry. Panel A shows is no signal difference among the three regions (ventral, central, dorsal, respectively). Panel B shows an example of the central region of the organotypic slice immunohistochemistry under various treatments. Panel C shows histograms indicating the decrease in arbitrary units (A.U.) of the signal between control and treatments (**p<0.01, *p<0.05 n=11). w/o=washout of kainate.

4.8 Dead cells quantification after kainate 100μM and L-AP4 1μM and adenosine 100 μM application

My previous work (see attached reprint) has demonstrated that adenosine inhibits the excitatory currents recorded from spinal cord neurons. On the basis of these results we used adenosine co-applied with L-AP4 to explore a potential increase in cell survival after kainate, in particular in central and dorsal regions in which L-AP4 seems to be less effective. We applied adenosine at 100 μM concentration together with L-AP4 at 1 μM concentration or only during the kainate washout. The preliminary results of nuclear staining with DAPI and immunohistochemistry with NeuN and SMI 32 (as done before for the other treatments) show no significant improvement in comparison with L-AP4 alone ($p>0.05$; $n=3$).

4.9 Spinal Cord 3 days *in vitro* histology characterization

To understand if it is viable to keep a neonatal isolated spinal cord *in vitro* after dissection for more than 1 day, we analyzed the newborn rat isolated spinal cord maintained for 3 days in vitro. We performed a DAPI staining comparing data with the freshly fixed tissue (dissected and fixed) to find out the degree of cell death. Results in Figure 4.10 show no difference between the two spinal cord specimens, suggesting that is possible to keep the isolated spinal cord in vitro for this length of time. We next analyzed if neurons (labeled with NeuN antibody), and motoneurons (labeled with SMI 32 antibody) remain viable after 3 days *in vitro*. The results in panel B and C of Figure 4.10 demonstrate that there is no difference in the number of neurons and motoneurons, thus confirming the observations obtained using DAPI staining.

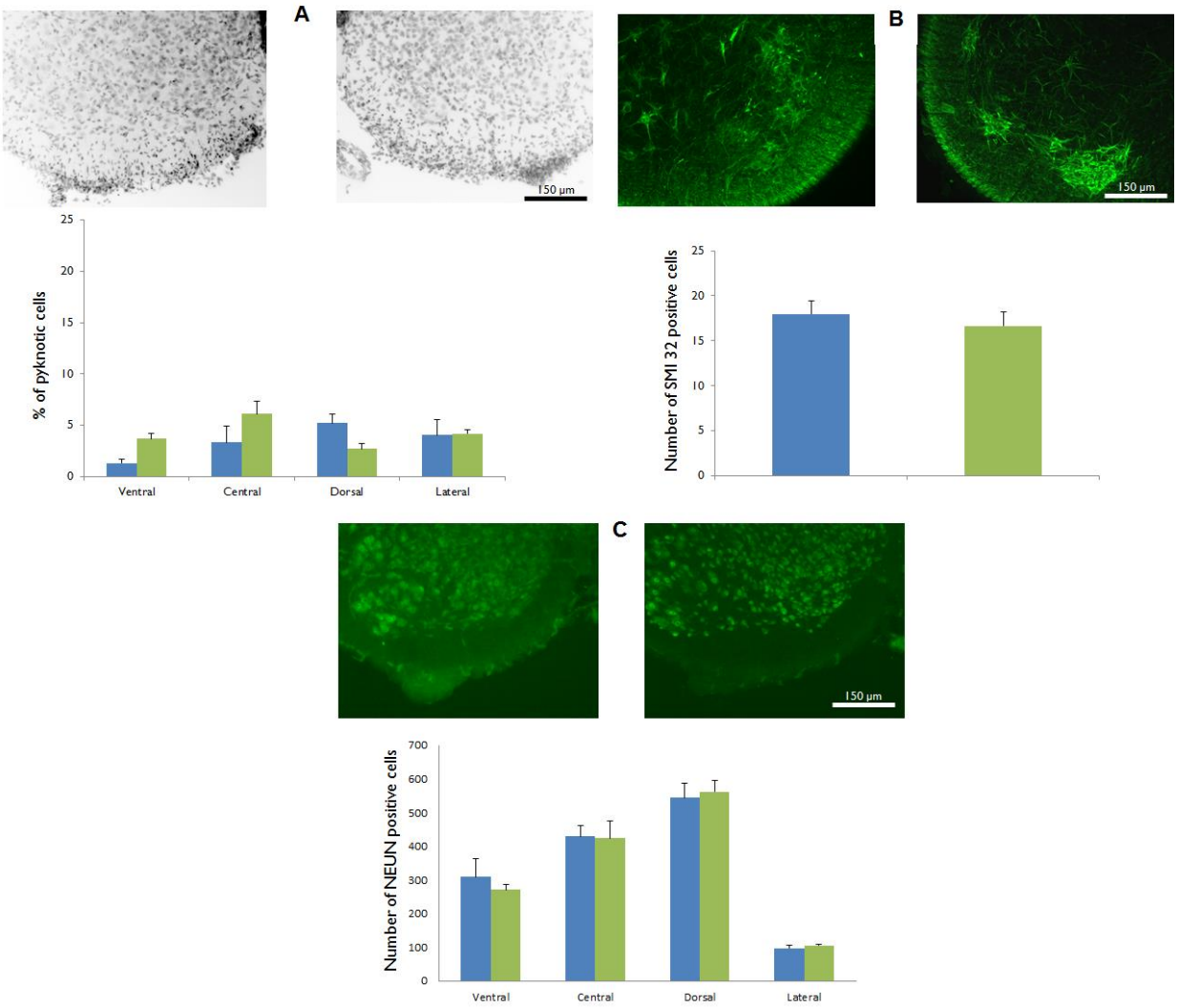

Figure 4.10 Histological comparison between fresh fixed and 3 days *in vitro* neonatal isolated spinal cords. (A) Example of ventral horn of isolated spinal cord, on the left the fresh fixed tissue, on the right the 3 days *in vitro* spinal cord: the histograms show that there is no difference between the two conditions concerning the percentage of pyknotic nuclei in various regions of interest ($p>0.05$ $n=12$). (B) Immunohistochemistry results for SMI 32 positive cells (motoneurons) showing no statistical difference between the two conditions ($p>0.05$ $n=12$). (C) Immunohistochemistry data for NeuN positive cells (neuronal nuclei) indicating no difference between fresh fixed and 3 days *in vitro* spinal cords ($p>0.05$ $n=12$).

4.10 Electrophysiological recording from Spinal Cords kept 3 days *in vitro*

To support our histological results, we performed electrophysiological recording with NMDA (3-5 µM) and 5-HT (10 µM) to elicit fictive locomotion both in P0-P3 fresh isolated neonatal spinal cord and in 3 days *in vitro* isolated spinal cord. In line with histology results as depicted

in figure 4.11, there is no statistical difference in the average rhythmicity between the two conditions. Analysis of data from 8 spinal cords shows s rhythm with a mean cycle for P0-P3 freshly isolated preparations of 5.48 ± 0.83 s and a period CV of 0.18±0.04, while for 3 days *in vitro* isolated spinal cords a mean cycle of 6.11 ±1.56 s and a period CV of 0.0.16±0.03 (p>0.05) is present.

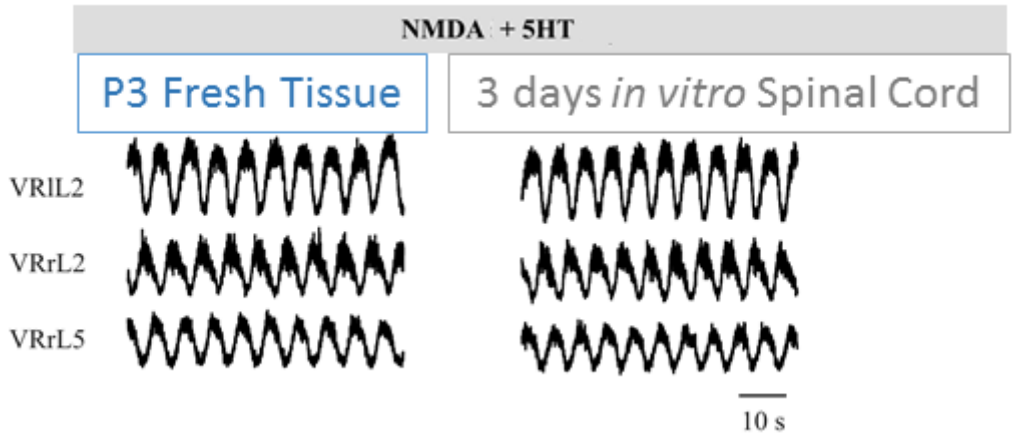

Figure 4.11 Fictive locomotion of P3 fresh isolated spinal cord and 3 days *in vitro* isolated spinal cord. The figure shows the absence of significant differences between the two conditions. Two examples of fictive locomotion lasting 60 seconds elicited by bath application of NMDA and serotonin (5-HT) are shown.

4.11 A_1 adenosine receptor modulation of chemically and electrically evoked lumbar locomotor network activity in isolated newborn rat spinal cords.

See article attached. Taccola G, Olivieri D, D'Angelo G, Blackburn P, Secchia L, Ballanyi K.; Neuroscience 2012

CHAPTER 5: DISCUSSION

5.1. L-AP4 neuroprotection against kainate induced excitotoxicity

In previous experiments of our laboratory conducted by Taccola et al. (2004), L-AP4 was able to block strongly and reversibly synaptic transmission evoked by dorsal root stimulation and thus decreased spinal reflexes. The principal finding of this thesis is the novel demonstration that L-AP4, either co-applied with kainate or after washout, on a model of spinal cord injury is able to counteract excitotoxicity by reducing cell losses and the number of pyknotic nuclei. In particular, neuroprotecting motoneurons in the ventral part of the spinal cord organotypic cultures was an important observation, in connection with the high vulnerability of this cell type (Van Damme et al. 2002). These effects suggested that L-AP4 was able not only to act as a spinal analgesic drug (Fisher et al. 2002; Mills et al. 2002b; Onaka et al. 1996), but also to have an effect on the levels of glutamate release and subsequent activation of postsynaptic pathways that lead to excitotoxicity. We can suggest that the activation of mGluR III group is similar to the one demonstrated in brain by Martin et al. (2007) modulating glutamate release by inhibiting P/Q-type Ca^{2+} channels and decreasing cAMP. Using both L-AP4 as an agonist and CPPG as an antagonist of the mGluR III group on organotypic spinal cord cultures, we suggest that these effects are related to the mGluR III group activation that are located in the pre-synaptic cleft (Niswender and Conn 2010). The decrease of the glutamate released in the synaptic cleft has a very important impact on the postsynaptic ionotropic glutamate receptor activation and contrasting kainate (0.1-0.5 mM) that strongly and rapidly increases extracellular levels of glutamate, a phenomenon that does not disappear with washout as demonstrated by Mazzone and Nistri, (2011b). Moreover, kainate binding to specific receptors leads to an increase in intracellular calcium levels leading to cell death phenomena in spinal cord (A. Kuzhandaivel, Nistri, and Mladinic 2010; A. Kuzhandaivel et al. 2011; G L Mazzone et al. 2010): The mGluR III group activation seems to reduce the total increase of excitation probably through various different patterns. Deactivation of excitotoxic pathways is not only related to glutamate release, a recent work done by McMullan et al. (2010) shows that metabotropic glutamate receptors can inihibit microglial glutamate release, so probably L-AP4 neuroprotection is related, at least in part, to a similar cascade in our model too. . Furthermore, we investigated the role of mGluR III group antagonist CPPG that has been demonstrated to increase the damage related to kainate excitotoxicity by a strong deactivation of mGluR III group receptors. These findings underline the entanglement

between mGluR III group receptors and the modulation of the release of glutamate. The high neuroprotection of motoneurons in the ventral area can suggest that different regions of the spinal cord differ for mechanisms underlying cell death becauset theh mGluR III group agonist L-AP4 seems to have a somewhat different extent of neuroprotection. Pizzi et al (1999) underlined neuroprotection by L-AP4 in motoneurons. suggesting that these neurons can be much more sensitive to this fine tuning by the mGluR III group receptor. Our model demonstrates that the neuroprotection by L-AP4 can be potentially helpful for future spinal cord injury treatments. Furthermore, the effect of CPPG suggests that during the excitotoxic damage exerted by kainate there is an activation of mGluR III group receptor, but this activation is not enough to protect the cells from the harmful events related to kainate application. These events are presumably related to increased intracellular calcium and ionic imbalance (A. Kuzhandaivel et al. 2011; G L Mazzone and Nistri 2011). These important effects are elicited by a relatively small concentration of L-AP4 (1 µM) that has been demonstrated have no toxic effect if used alone. All these results agree with previous data reported by Pizzi et al., (1999) and Tomiyama et al., (2001) comparing this secondary damage due to excitotoxicity to the neuronal degeneration typical of ALS These data, open further possibilities to study the relationship between SCI, traumatic or not, and other neurodegenerative diseases related of of the spinal cord. The present work not only is consistent with these observations but it also indicates that Ca^{2+} dependent and independent release mechanisms activated by kainate can be largely counteracted by the activation of mGluR III group receptors. Moreover, we can suggest that glutamate accumulating extracellularly actually has the potential to moderate its own action via activation of mGluR receptors, this can once more be demonstrated by the increasing of cell death due to the CPPG presence. The different levels of neuroprotection in the different regions of interest seem not to be related to different type of localization of the different type of mGluR III subtypes. This result can be explained in two ways, the first is related to the type of analysis used, suggesting that a qualitative analysis such as the fluorescence signal intensity cannot detect small differences among the three subtypes of receptor; the other reason can be related to the specificity of the organotypic spinal cord cultures. The spinal cord cultures are characterized by a high number of neurons compared to other cell populations such as microglial cells or astrocytes: this high number of neurons might amplify the size of responses to treatments, but on the other hand it can mask relatively small differences like the metabotropic glutamate receptor distribution. The preliminary results using adenosine in co-application with L-AP4 suggest that the neuroprotection with mGluR

III group agonist probably reached a maximum level and cannot be increased by combined treatments with other substances. Moreover, considering that adenosine is able to shut down the rhythmic bursting at 2 mM concentration (G Taccola et al. 2012), probably an increase in the concentration of adenosine can induce multiple effects. These pharmacological results should be evaluated in future experiments using different concentrations of both adenosine and L-AP4 on organotypic spinal cord cultures or other types of spinal cord models.

5.2. Neonatal spinal cord Isolated for 3 days *in vitro*

Having a good model for studying long-term events after spinal cord injury is very important for the future of research (Akhtar et al. 2008). A model must follow 4 principles to be considered a good model: 1) simulate damage that is similar to clinical SCI; (2) control over conditions, reproducibility, stability; (3) involve a simple technique that is easy to study; (4) the equipment used to make a model is straightforward and quick to produce. Our results show that it is possible to maintain for at least 3 days in vitro an isolated spinal cord from neonatal rat and to possess a model that displays all 4 principles. Our model can be used to simulate damage (in particular excitotoxic damage) with high control over the experimental conditions. Having a significant number of samples allows a demonstration of a high reproducibility and stability of the model, and the whole process is very simple and easy to manage because it starts from previous models that are already used in spinal cord injury research such as the newborn isolated spinal cord. All these results can lead to new perspectives in the study of the role of late onset events after injury and this new model shows that in an oxygenated medium the isolated spinal cord suffers no damage and no events of necrosis and cell death after the dissection. Another important keypoint in the development of a good spinal cord model is the presence of electrical activity. Our model shows that not only electrical activity is maintained but also we are able to elicit the same pattern of responses using the same concentration of NMDA and 5-HT with fictive locomotion rhythm equal to the one elicited in freshly dissected newborn spinal cord. Having this model will help to analyze what happen in the late onset events at the central patter generator level, responsible of the fictive locomotion. The previous model has been important for understanding the basics of the spinal cord networks with or without injuries: we hope that another type of model can lead to other studies focusing on molecular biology

and electrophysiology of late-onset events. These new results can verify some of the findings that have been discovered withthe organotypic spinal cord cultures and give us a better understanding of the complex organization of the spinal cord tissue and networks. In future experiments we can analyze the role of mRNAs that can be translated after the initial injuries or other events related to changes in electrical activity during the first days of spinal cord injuries.

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