Scuola Internazionale Superiore di Studi Avanzati – Trieste

Study of the neuroprotection mechanisms in a model of spinal cord injury *in vitro*.

Thesis submitted for the degree of "Doctor Philosophiae"

Academic year 2013 – 2014

Candidate
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Supervisor
Prof. Andrea Nistri

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International School for Advance Studies (ISAS)

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Dedicated to my Beloved Father

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Declaration

The data reported in the present thesis have been published or currently under review in listed articals herewith. The candidate has performed all the electrophysiological experiments including isolated spinal cord preparations, data acquisition, and data analysis. In addition, the candidate has also performed some immunohistochemistry experiments, in all cases, including spinal cord isolation and slice preparation, immunohistology and data analysis. In all the reports, candidate has contributed to interpretation of data, its discussion and manuscript writing:

- **Ayisha Shabbir**, Elena Bianchetti, Andrea Nistri (2015). The volatile anesthetic methoxyflurane protects motoneurons against excitotoxicity in an *in vitro* model of rat spinal cord injury. Neuroscience *285*, 269 – 280.

Ayisha Shabbir, Elena Bianchetti, Renato Cargonja, Miranda Mladinic, Kristina Pilipović and Andrea Nistri (2014). Motoneuron survival after excitotoxic stress is related to HSP70 expression in a rat spinal cord injury model *in vitro*. Manuscript under revision.

Abstract

Excitotoxicity is the major contributor to the pathophysiological damage after acute spinal cord injury which is often incomplete, yet it produces paralysis with uncertain outcome for gait recovery despite early intensive care support. Neuroprotecting the spinal cord during the early phase of injury is an important goal to determine a favourable outcome to suppress delayed pathological events that extend the primary damage and amplify the loss of motor function often with irreversible consequences. While intensive care and neurosurgical intervention remain mainstay treatments, effective neuroprotection requires further focused experimental studies under controlled conditions.

To better understand the pathophysiological mechanism of spinal lesion an *in vitro* model of rat spinal cord has been developed by our laboratory whereby injury is mimicked by a moderate excitotoxic insult. Such an injury suppresses the locomotor networks together with partial loss of motoneuronal population. The present thesis explores if the volatile general anesthetic methoxyflurane can protect spinal locomotor networks from kainate induced excitotoxicity and whether motoneuronal survival after excitotoxicity relies on cell expression of heat shock protein 70 (HSP70), a cytosolic neuroprotective protein binding and sequestering metabolic distress-generated proteins.

The protocols involved 1 h excitotoxic stimulation on day 1 followed by electrophysiological and immunohistochemical testing after 24 h. A time-limited (1 h), single administration of methoxyfluorane together with kainate (or with 30 min or 60 min delay), prevented any depression of spinal reflexes, loss of motoneuron

excitability, and histological damage. Methoxyfluorane per se temporarily decreased synaptic transmission and motoneuron excitability. These effects were readily reversible on washout. When methoxyfluorane was applied with or after kainate, spinal locomotor activity recorded as alternating electrical discharges from lumbar motor pools was fully preserved after 24 h. Furthermore to test the second hypothesis, the motoneurons were investigated for their expression of apoptosis inducing factor (AIF; a known biomarker of cell death) which became preferentially localized to the nucleus in pyknotic cells after excitotoxicity. The surviving motoneurons showed strong expression of HSP70 with no nuclear AIF. The sham preparations did not show any AIF nuclear translocation whereas the preparations treated with kainate (100 µM) were the most affected. VER155008, a pharmacological inhibitor of HSP70, per se induced neurotoxicity comparable to that of kainate. Electrophysiological recording indicated depression of motoneuron field potential with strong decrease in excitability and impaired synaptic transmission following kainate or VER155008. Their combined application elicited more intense neurotoxicity. Interestingly, motoneurons in the spinal cord (24 h *in vitro*) showed large expression of HSP70 compared to freshly dissected tissue, suggesting that HSP70 up-regulation was critical for spinal cord preparation survival *in vitro*.

These data suggest that a volatile general anesthetic could provide strong electrophysiological and histological neuroprotection that enabled retention of locomotor network activity even one day after the excitotoxic challenge. Our study also showed that HSP70 is important for motoneuronal survival. It is hypothesized that the benefits of early neurosurgery for acute SCI might be enhanced if, in addition to injury decompression and stabilization, the protective role of general anesthesia is maximized. Another potential future strategy to neuroprotect motoneurons could be the upregulation of HSP70 activity by either using its pharmacological enhancers or by inducing its over-expression.

Introduction

1. Spinal Cord

The spinal cord is part of central nervous system and extends caudally. It is protected by vertebral bones. All the motor and sensory communication (except that of cranial nerves) between brain and the body travels through nerve fibers present in white matter tract of spinal cord. The processing of ascending and descending stimulus is processed by grey matter of spinal cord and is also involved in automated motor patterns such as walking.

The role of spinal cord has been realized and expressed by the Persian physician and philosopher Avicenna (AD 980 - 1037) for at least a millennium ago:

> "If all the nerves exited directly from the brain, the brain would be bigger. For example, nerves innervating the hands and feet would travel a longer distance and, thus, would be more prone to injury; they would also become less able to innervate the big muscles of the thigh and the calf. Therefore, God created the spinal cord below the brain. The spinal cord is like a channel coming out of a fountain in the way that nerves emerge from both sides and go down, thus putting the organs closer to the brain. That is why God placed the spinal cord into a hard bony channel called the spine to protect it from injury."
> (Naderi et al., 2003)

2. Spinal cord injury

Spinal cord injury (SCI) disrupts normal motor, sensory and autonomic functions. Although the majority of new cases of spinal cord injury (SCI) are associated with an incomplete lesion, severe locomotor deficit usually ensues with resultant paralysis and uncertain recovery (van den Berg et al., 2010; McDonald and Sadowsky, 2002; Nair et al., 2005). The other symptoms of SCI include loss of sensation (heat, cold and touch), loss of bowel and bladder control, hypereflexia or spasms, pain, respiratory difficulties and changes in sexual behavior. When the level of injury is above the first thoracic vertebra, it is referred to as tetraplegia or quadriplegia and such injury affects arms, hands, trunk, legs and pelvic organs. Paraplegia injury (injury occurring below first thoracic nerve) affects all or parts of trunk, pelvic organs and legs.

2.1. Epidemiology

Annual incidence of acute traumatic SCI is 15 – 40/million worldwide (Rowland et al., 2008; Sekhon and Fehlings, 2001). SCI is associated with severe physical, psychological, social and economic impact on patients and their families. The estimated lifetime cost of a young SCI patient (25 year old), with high cervical tetraplegia, is approximately US $3 million (Priebe et al., 2007). The major contributor to SCI are vehicular accidents with 50 % of cases followed by falls and work related accidents (30%) (Rowland et al., 2008; Singh et al., 2014).

In addition to traumatic SCIs, the non-traumatic SCIs are on rise and are as important (van den Berg et al., 2010). Non-traumatic SCIs account for 30 – 50% of spinal cord disorders (Nair et al., 2005) and are usually incomplete (van den Berg et al., 2010; McKinley et al., 1999). The major causes of non-traumatic SCI are vertebral stenosis (54%; McKinley et al., 1999), spinal tumors (26%; Nair et al., 2005) and other diseases (vascular, infective, genetic, inflammatory, metabolic and degenerative).

2.2. Classification

As early as 2500 BC, Imhoteo (Egyptian physician) classified spinal cord injury as – "an ailment not to be treated" in Edwin Smith surgical papyrus (Hughes, 1988). But despite being a serious and hopeless injury in the past, the quest to find a cure to SCI has not been dampened, resulting in its proper classification. As of today, clinicians refer SCI as complete or incomplete. The first scale to grade the neurological loss was devised at the Stokes Manville hospital before World War II and was widely circulated by Frankel in 1970's. The American Spinal Injury Association (ASIA) impairment scale follows Frankel scale with some important improvements and is clearer.

American Spinal Injury Association (ASIA) impairment scale

A = Complete: No motor or sensory function is preserved in the sacral segments S4-S5.

B = Incomplete: Sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-S5.

C = Incomplete: Motor function is preserved below the neurological level, and more than half of the key muscles below the neurological level have a muscle grade less than 3.

D = Incomplete: Motor function is preserved below the neurological level, and at least half of key muscles below the neurological level have a muscle grade of 3 or more.

E = Normal: Motor and sensory function are normal.

2.3. Pathophysiology

The understanding of pathophysiological processes that accompany SCI is essential for the development of neuroprotective strategies. The typical consequences of spinal cord injury in human are severe below injury, due to axonal disruption or demyelination. The schematic representation in Figure 1 shows damage to the

corticospinal tract (major descending pathway from the brain to the spinal cord) by injury that deprives motoneurons and interneurons of inputs (Thomas et al., 2014). This alters the excitation and inhibition balance of signals to motoneurons which results in recruitment of fewer motoneurons. Some motor units get weak, while some are paralysed and motoneuron death will initiate with denervation. The motor unit properties differ with different type of muscles like those for hand or limb (Thomas et al., 2014). Muscle function also depends upon the site of injury, namely how close or far away from the lesion motor pools are.

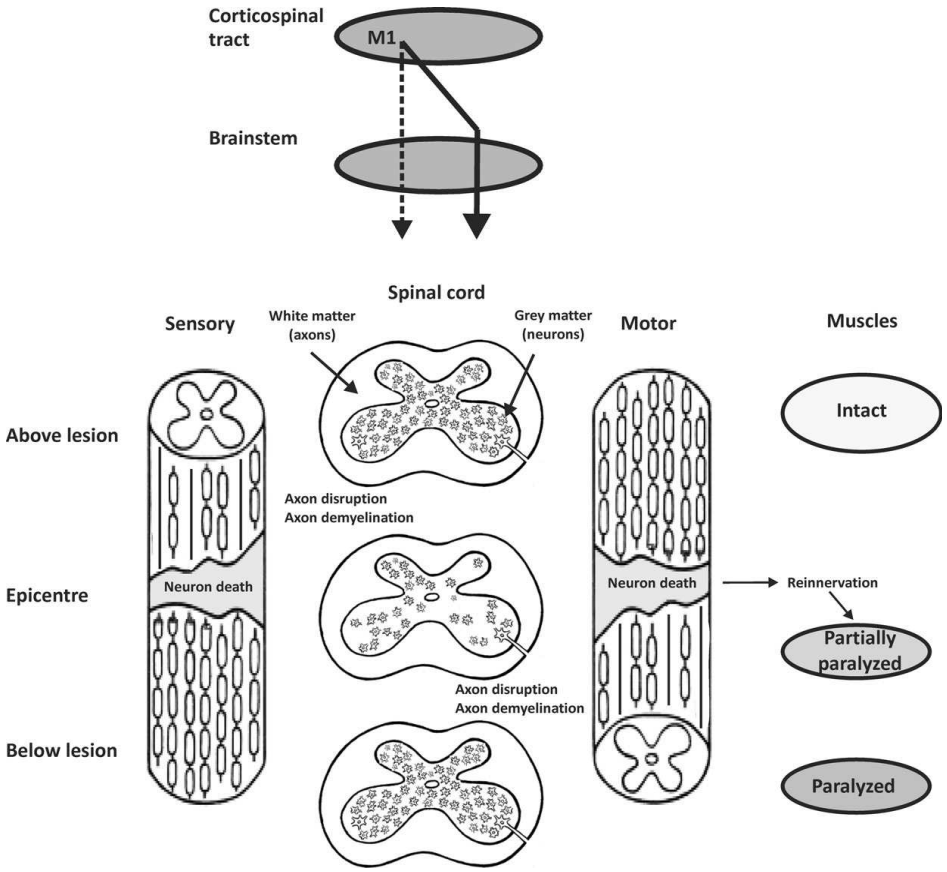

Figure 1. *Schematic of typical spinal cord injury consequences in human. The descending inputs from corticospinal tracts cross the midline (solid arrow) to reach spinal neurons but some descend via ipsilateral pathways (dashed arrow) (Thomas et al., 2014).*

All these changes in the spinal cord after injury are part of a major process that can be better described in two major phases, namely primary and secondary. The primary injury results in traumatic severing of axons, hemorrhage, early necrosis, and activation of microglia. Primary injury is accompanied with “spinal shock” which can be defined as loss of sensation and motor paralysis, although reflexes gradually recover later (Hiersemenzel et al., 2000). The extension of primary lesion over the first few hours and days to surrounding spinal areas represents the secondary injury phase (Park et al., 2004; Rowland et al., 2008; Sekhon and Fehlings, 2001). It involves vascular dysfunction, edema, ischemia/hypoxia, excitotoxicity, free radical production, inflammation and delayed cell death (Dumont et al., 2001; Hall and Springer, 2004; Kuzhandaivel et al., 2011). These processes are explained in detail below.

2.3.1. Primary Injury

The injury resulting from a displacement of the vertebral column is known as compressive-contusive injury and is the most commonly observed among acute SCIs. It can last up to 2 h and exerts force directly on spinal cord causing disruption of axons, damage to blood vessels and cell membranes. The primary lesions rarely transect or fully disrupt the anatomical continuity of spinal cord. The damage to central grey matter is mostly due to the initial necrosis because of the cell membrane disruption and ischemia. The up-regulation of pro-inflammatory cytokines, like TNFα, IL-6 and IL-1β, activates the microglia (Pineau and Lacroix, 2007). The number of spared axons, coursing through the lesion site, is of high therapeutic importance (Rowland et al., 2008). Even as little as 5% of surviving axons is useful to preserve some neurological function in animal models (Fehlings and Tator, 1995; Kakulas, 2004). The concentration of extracellular glutamate can also reach excitotoxic levels within minutes after injury (Wrathall et al., 1996) as a result of cell lysis from mechanical impact and synaptic/non-synaptic release to the epicenter of injury.

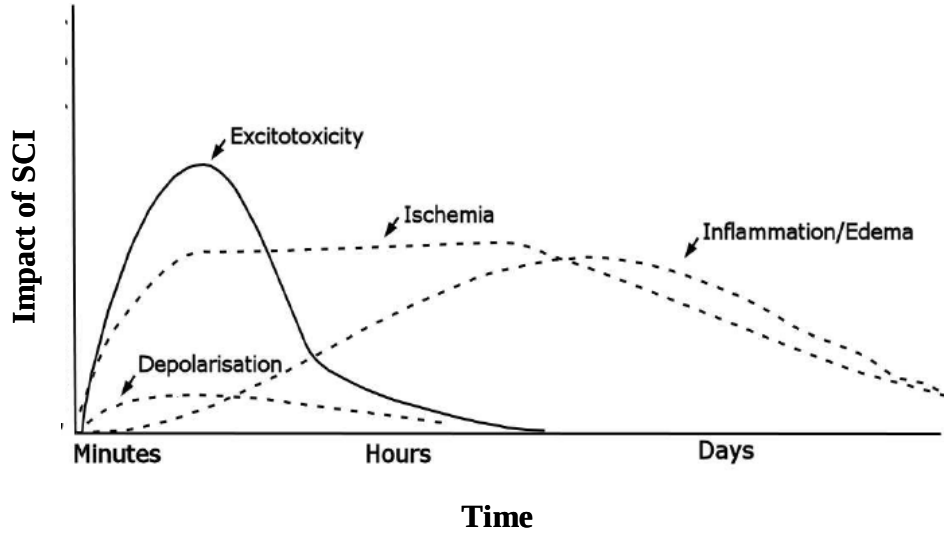

Figure 2. *Time course of spinal cord injury impact. Excitotoxicity starts within minutes after injury and peaks in hours to damage neurons followed by ischemia and inflammation. These events contribute to secondary phase of injury which ultimately leads to cell death (modified from Dirnagl et al., 1999).*

2.3.2. Secondary Injury

The secondary injury begins minutes after primary injury and can last from several months to years (Park et al., 2004).

Acute phase (2 h – 2 weeks) begins shortly after the time of injury. The microvascular damage results in ischemia causing hypoxia and metabolic dysfunction at the epicenter of injury and adjacent areas (Rowland et al., 2008). This deprivation leads to cell death through mitochondrial dysfunction as neurons are highly sensitive to oxygen and nutritional deficit (Kuzhandaivel et al., 2011). The release of excitatory neurotransmitters, glutamate and aspartate, causes cytotoxicity (Park et al., 2004) because the rise in glutamate results in hyperactivation of glutamate receptors which might lead to neuronal cell death (Lipton and Rosenberg, 1994). The hyperactivity of

glutamate causes Na^+ influx resulting in edema (Choi, 1987; Hall and Springer, 2004) and triggers cell death cascades (Kuzhandaivel et al., 2011). See Figure 3.

Subcute phase may last from 1 to 2 months post injury. The invasion of inflammatory cells due to breakdown of the blood brain barrier also contributes to secondary damage (Popovich et al., 1997). The inflammatory response includes invasion of neutrophiles and cytokines. These cytokines mediate the generation of free radicals which will also lead to cell death. This inflammatory response is also thought to be neuroprotective as it promotes tissue sparing and functional recovery (Hohlfeld et al., 2007; Rapalino et al., 1998).

Chronic Stage (> 6 months) is a virtually life-long phase for spinal cord injury patients. The glial scar is formed following spinal cord injury by activation of astrocytes around the lesion together with invading meningeal fibroblasts, microglia, vascular endothelial cells and oligodendrocyte precursor cells (Chung et al., 2014; Fawcett and Asher, 1999). This glial scar can turn into cysts (with contribution to local ischemia, inflammation, and hemorrhage; Josephson et al., 2001; Williams et al., 1981) and keeps expanding from days to weeks after injury, thus making injury worse. The loss of oligodendrocytes leads to demyelination of spared axons and contributes to injury progression.

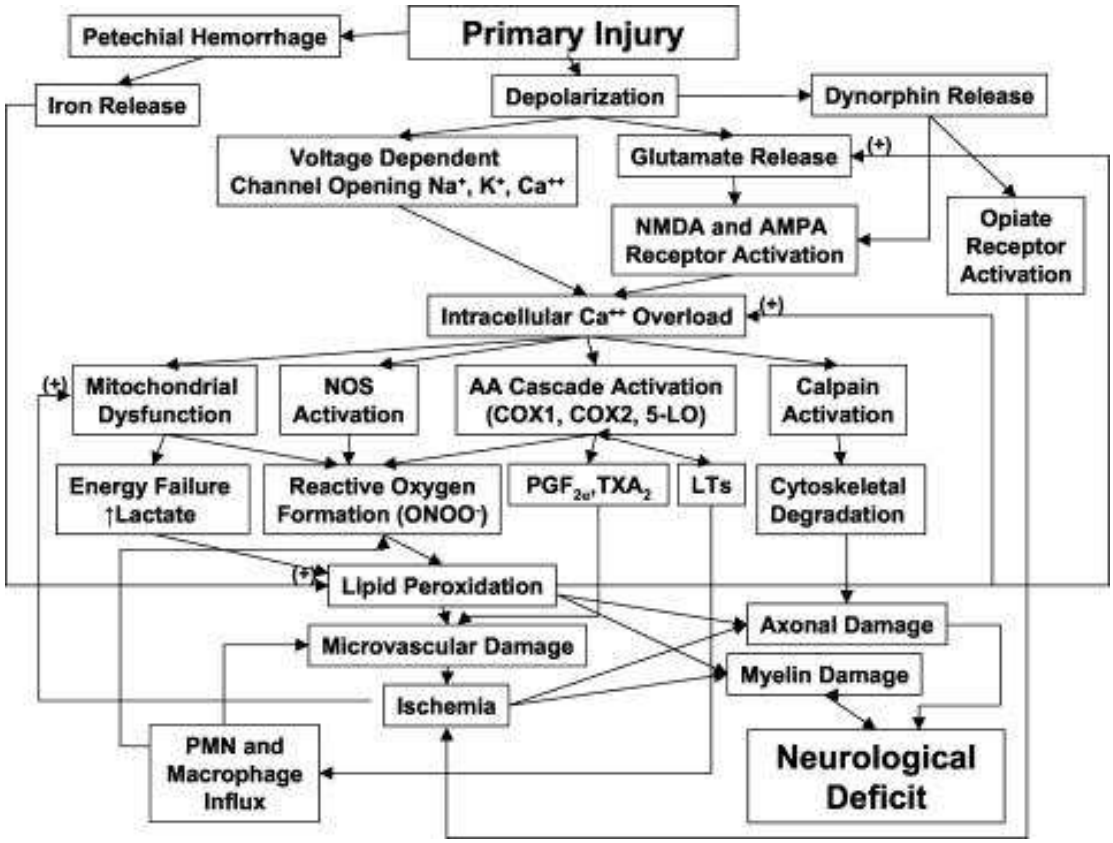

Figure 3. *Pathophysiology of secondary spinal cord injury (Hall and Springer, 2004). 5-LO = 5-lipoxygenase; TXA_2 = thromboxane A_2; $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$; LTs = leukotrienes; $ONOO^-$ = peroxynitrite anion; PMN = polymorphonuclear leukocyte.*

2.3.3. Excitotoxicity

The major excitatory neurotransmitter in central nervous system is glutamate. It acts through metabotropic and ionotropic receptors. Hence, excitotoxicity refers to a neurotoxic state caused by hyperactivation and prolonged exposure to glutamate resulting in neuronal cell death (Park et al., 2004). It has been shown that the concentration of glutamate by induced experimental spinal injury reaches neurotoxic levels when administered to an uninjured spinal cord (Li et al., 1995). The excessive activation of glutamate receptors like NMDA, AMPA and kainate leads to influx of Na^+ and Ca^{2+}. This influx triggers a chain of biochemical processes like uncoupling of mitochondrial electron transfer from ATP synthase, activation and hyperactivity of calpains, Ca^{2+} dependant proteases, protein kinases, phospholipases and nucleases (Szydlowska and Tymianski, 2010; Wang et al., 2005). These events result in mitochondrial dysfunction and eventually cell death. AMPA and kainate receptors are also permeable to Ca^{2+} and are heteromers consisting of combination of 4 subunits, GluR1-4. The permeability of Ca^{2+} through these channels is controlled by GluR2 subunit. The expression of GluR2 actually reduces Ca^{2+} permeability (Bleakman and Lodge, 1998). The decrease in this subunit's expression is directly related to enhanced permeability of Ca^{2+} through AMPA receptors, making neurons highly vulnerable to kainate induced excitotoxicity (Friedman, 1998; Pellegrini-Giampietro et al., 1997). Furthermore, reduction in desensitization of AMPA receptors due to high glutamate concentrations causes functional abnormalities (Li and Stys, 2000).The GluR2 subunits are absent from AMPA receptors on oligodendrocyte, making them more permeable to Ca^{2+}: the importance of AMPA receptors for oligodendrocytes is demonstrated by the strong white matter lesion in certain spinal cord injury models (Agrawal and Fehlings, 1997; Li and Stys, 2000). Application of non-NMDA glutamate receptor antagonists helps recovery from white matter spinal cord injury. Conversely, NMDA antagonists are ineffective, indicating different mechanisms of glutamate mediated damage between white and grey matter (Agrawal and Fehlings, 1997; Li and Stys, 2000; Park et al., 2004). Excitotoxicity is proposed

to be the cause of cell death is many traumatic and non-traumatic neuronal diseases like spinal cord injury, brain injury, stroke and neurodegenerative diseases (multiple sclerosis, Parkinson's disease, Alzheimer's disease, Amyotrophic Lateral Sclerosis (ALS) and Huntington's disease).

3. Cell Death Pathways

Cell death after spinal cord injury involves complex heterogeneous processes that follow biochemical cascades which are not yet fully understood (Kroemer et al., 2009). The nomenclature committee on cell death (NCCD) has proposed definitions for distinct cell death pathways in 2012. I will be discussing 3 of these pathways in my thesis, namely necrosis, apoptosis and parthanatos.

3.1. Apoptosis

Apoptosis is a normal process of cell elimination during development of white and grey matter of spinal cord. During the secondary spinal cord injury phase, apoptosis becomes more prominent than in the early phase of injury. Apoptosis can be triggered by cytokines, inflammation, free radicals and excitotoxicity. Emery et al. (1998) reported the existence of apoptosis after traumatic spinal cord injury in man. Apoptosis can be observed in various cell types after SCI: neurons, oligodendrocytes, microglia and astrocytes (Dumont et al., 2001). Morphological indicators of apoptosis include rounding of the cell, formation of blebs, chromatin condensation, nuclear fragmentation, increased intracellular Ca^{2+} , endoplasmic reticulum dysfunction and budding of apoptotic bodies (Galluzzi et al., 2012).

Extrinsic pathway – this kind of apoptosis is receptor dependent and is evoked by the extracellular signal generated by tumor necrosis factor (TNF). It is mediated by Fas ligand (FasL) and Fas receptor and/or inducible nitric oxide synthase produced by macrophages. FasL binds to Fas receptor (Leskovar et al., 2000; Sakurai et al., 1998)

and induces a programmed sequence of caspase activation which involves caspase-8 (inducer caspase) and caspase-3 and caspase-6 (effector caspases) (Eldadah and Faden, 2000). The activation of effector caspases results in cell death. The inducible nitric oxide synthase triggers caspase-3 cell death pathway. Extrinsic pathway in neurons after SCI is triggered by higher concentration of Ca^{2+} which induces mitochondrial damage, release of cytochrome c and caspase activation. The cytochrome c binds with apoptosis activating factor-1 to activate caspase-9 (inducer) and activates the effector caspases (caspase-3 and 6) to cause cell death (Budd et al., 2000; Kuida, 2000).

Intrinsic pathway - The signaling cascade for intrinsic apoptotic trigger is diverse, but all these signals are linked to mitochondrion-centered control mechanisms. Apoptosis, when initiated from inner mitochondrial membrane, develops via the permeability transition pore complex (PTPC). It starts on the outer membrane of mitochondria due to the formation of pores by the activity of BAK and BAX (BCL-2 protein family) (Galluzzi et al., 2012). The oligomerization of BAX and BAK induces mitochondrial dysfunction and changes the mitochondrial membrane potential, with production of reactive oxygen species (ROS), formation of membrane pores leading to loss of mitochondrial constituents including cytochrome-c, apoptosis inducing factor (AIF) and endonuclease G (EndoG) (Galluzzi et al., 2012; Gross et al., 1999). The release of cytochrome-c into the cytosol activates the adaptor apoptotic protease activating factor-1 (Apaf-1) which facilitates the formation of the apoptosome (a caspase activating complex made up of cytochrome c, Apaf-1 and dATP) (Liu et al., 1996). The apoptosome in turn triggers the caspase cascade involving caspase 9 and caspase 3 (Figure 4). AIF translocates to nucleus causing DNA fragmentation. The intrinsic apoptosis can be a caspase dependent or independent phase, whose relative contribution to cell death execution can be estimated by use of caspase inhibitors which may provide short term cytoprotection.

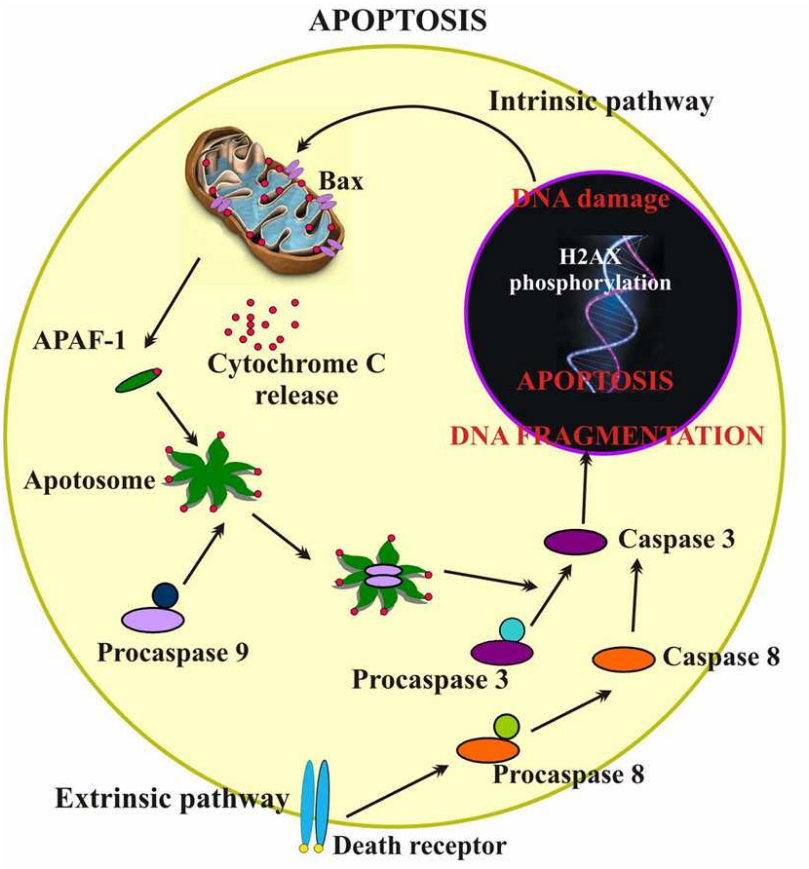

Figure 4. *Schematic representation demonstrating intrinsic and extrinsic pathways of apoptosis (Kuzhandaivel et al., 2011).*

3.2. Necrosis

Necrosis was traditionally considered as accidental cell death with no morphological traits of programmed cell death like apoptosis or autophagy. Nowaday, necrosis is believed to be programmed or regulated. Various circumstances can result in programmed necrosis including DNA damage, excitotoxicity, and activation of death receptors (Galluzzi et al., 2012). The immediate physical spinal cord injury causes necrosis at the injury site resulting in early death of cells. because cells swell, and an energy crisis arises due to mitochondrial dysfunction which leads to cytoplasmic membrane lysis (Cohen, 1993; Lu et al., 2000). Necrosis can be further characterized depending on the specificity of signaling modules. The RIP1- and/or RIP3-dependent

necrosis is regulated by RIP1 (receptor-activating protein 1) and/or RIP3 (Degterev et al., 2008; He et al., 2009) activation through the tumor necrosis factor receptor-1 (TNFR1) and it produces the disintegration of mitochondria, lysosomes and plasma membranes. The RIF1 dependent activation can be inhibited by using an antagonist, neurostatin-1 (Degterev et al., 2005, 2008; Wang et al., 2007).

3.3. Parthanatos

The term parthanatos comes from the Greek word Thanatos, meaning death. It was a process first described by David and colleagues in 2009. It is a caspase-independent cell death pathway. Parthanatos starts by the production of poly-ADP-ribose (PAR) which is produced by poly-ADP-ribose polymerase-1 (PARP1) (David et al., 2009). The production of PARP1 is linked to the DNA fragmentation. PARP1 has four distinct sites; 1) two zinc zippers containing DNA-binding domain, 2) caspase cleavage site with nuclear localization signal (NLS), 3) an automodification domain for protein-protein interactions with BRCT motif, and 4) a catalytic site at C-terminal with NAD^+-fold (Amé et al., 2004). PARP1 acts to promote cell death in the case of massive DNA damage. This can occur due to oxidative stress resulting in overproduction of nitric oxide (NO) which interacts with superoxide anion to produce peroxy-nitrite and causes DNA damage that activates PARP1 (Dawson et al., 1991; Xia et al., 1996). PARP-1 synthesizes a polymer of ADP-ribose, PAR, linked by glycosidic bonds. PAR synthesis was first described by Chambon et al. (Chambon et al., 1963). ADP-ribose is formed by hydrolysis of NAD^+ and releases nicotinamide. The PAR polymer might be linear or branched, but it is heterogeneous in length and branching (one branch every 20-50 ADP-ribose residues) (D'Amours et al., 1999). The more complex and heavier (high molecular weight) PAR polymer is, more toxic it becomes (David et al., 2009). PAR, when it binds to its target protein, changes protein activity by functioning as site specific covalent modification, a protein binding matrix or a steric block (Kim et al., 2005). The over activation of PARP1

depletes NAD^+ pools resulting in energy depletion (ATP) which results in cell death (Chiarugi, 2005; D'Amours et al., 1999). The over activation of PARP1 also facilitates the release of apoptosis inducing factor (AIF). AIF has high affinity for PAR through PAR binding sites. The release of AIF from mitochondria is triggered when PAR translocates to the cytosol from the nucleus (Andrabi et al., 2006; Wang et al., 2011). AIF then translocates to nucleus causing DNA fragmentation. However, the precise mechanism of induction of AIF by PAR is still poorly understood.

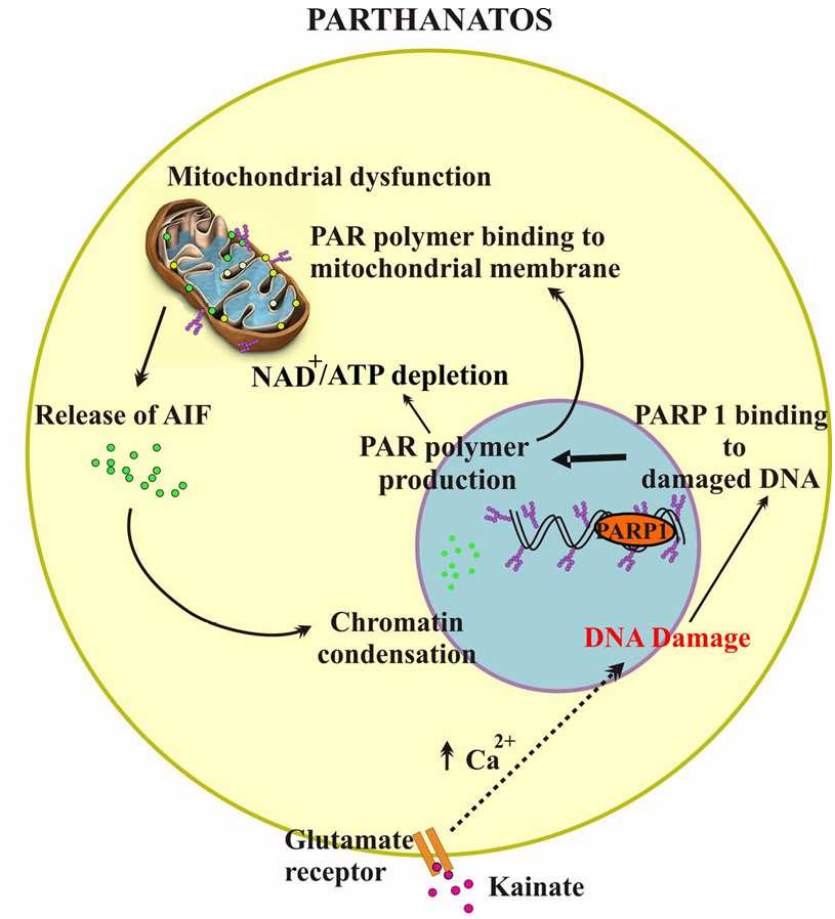

Figure 5. *Schematic representation demonstrating parthanatos pathway mediated by PARP1 (Kuzhandaivel et al., 2011).*

Several studies with different models of spinal cord injury have identified the presence of PARP1 dependent cell death, parthanatos. Our lab has also demonstrated the presence of parthanatos in neuronal cells of grey and white matter after excitotoxicity (Bianchetti et al., 2013; Kuzhandaivel et al., 2010a, 2011; Nasrabady et al., 2011a). After ischemia and traumatic brain injury (TBI), oxidative stress is found to be the major cause of parthanatos (David et al., 2009). The pharmacological inhibition of PARP1 has resulted in neuronal protection in experimental models of stroke, ischemia, neurotrauma, excitotoxicity and Parkinson's disease (Mandir et al., 1999, 2000; Szabó and Dawson, 1998; Whalen et al., 1999). The schematic representation of the parthanatos process is shown in Figure 5.

4. Heat Shock Protein (HSP)

The heat shock proteins are highly conserved among all the animals and even plants. These chaperones perform very crucial processes in an animal's life like maintaining cellular homeostasis. The expression of HSPs increases markedly under stressful conditions including neurodegenerative diseases (Brown, 2007). The classification of HSPs is based on their molecular weight, homology and function. The 70 kDa heat shock protein (HSP70) family is an ATP-dependent molecular chaperone (Figure 6).

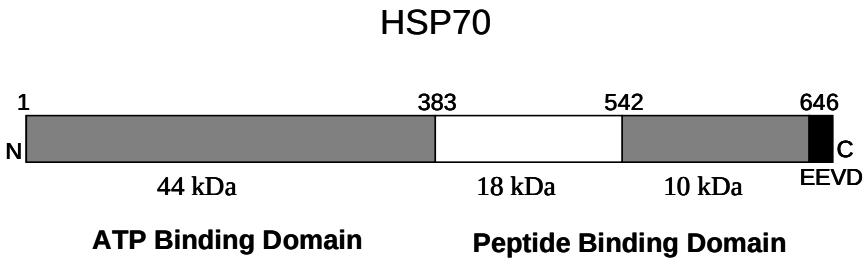

Figure 6. *Diagrammatic structure of HSP70. EEVD is the highly conserved sequence at the carboxy terminal.*

HSP70 assists in the process of correct folding newly synthesized polypeptides, the assembly of multi-protein complexes, and the transport of proteins across cellular membranes under normal conditions (Boorstein et al., 1994; Kroemer, 2001; Mayer, 2013). The synthesis of HSP70 enhances the ability of cells to cope with increased concentrations of unfolded or denatured proteins under various stress conditions (Giffard et al., 2004). Neurons are particularly vulnerable to protein misfolding and to intracellular accumulation of insoluble protein aggregates (Kroemer, 2001). The increased expression of HSP70 has neuroprotective effects in various animal models of neurodegenerative diseases (Brown, 2007) and this effect is dependent on its ability to bind AIF and to inhibit programmed cell death (Garrido et al., 2001; Sevrioukova, 2011). Spinal motoneurons show high levels of HSP70 in various neurodegenerative disease models (Brown, 2007) and after ischemia of the rat spinal cord (Carmel et al., 2004; Cizkova et al., 2004).

HSP70 interacts with AIF to prevent cell death (Giffard and Yenari, 2004; Gurbuxani et al., 2003; Ravagnan et al., 2001; Ruchalski et al., 2006). Upon release of AIF from damaged mitochondria, HSP70 captures it and prevents its nuclear import. The peptide binding domain of HSP70 is responsible for such an interaction. HSP70 also interacts with the apoptotic protease-activating factor (Apaf-1). In particular, when cytochrome-c is released by mitochondria in response to stress, it oligomerizes with Apaf-1 to recruit procaspase-9 to form the apoptosome (Mosser and Morimoto, 2004): binding of the HSP70 ATP binding-domain to Afap-1 prevents this process. The apoptosome formation can be inhibited *in vitro* by addition of purified HSP70, prior to the addition of cytochrome-c to the cytosolic extracts (Saleh et al., 2000). Furthermore, HSP70 can exert its anti-apoptotic effects upstream of caspase activation by blocking the caspase-3 activation. The processing of procaspase-3 is required for caspase 3 activation but it has been shown that over-expression of HSP70 can inhibit it (Buzzard et al., 1998; Mosser et al., 1997). HSP70 also prevents necrotic cell death by interfering with reactive oxygen species (ROS) (Giffard and Yenari, 2004). The Figure 7 summarizes the potential intrinsic targets of HSP70.

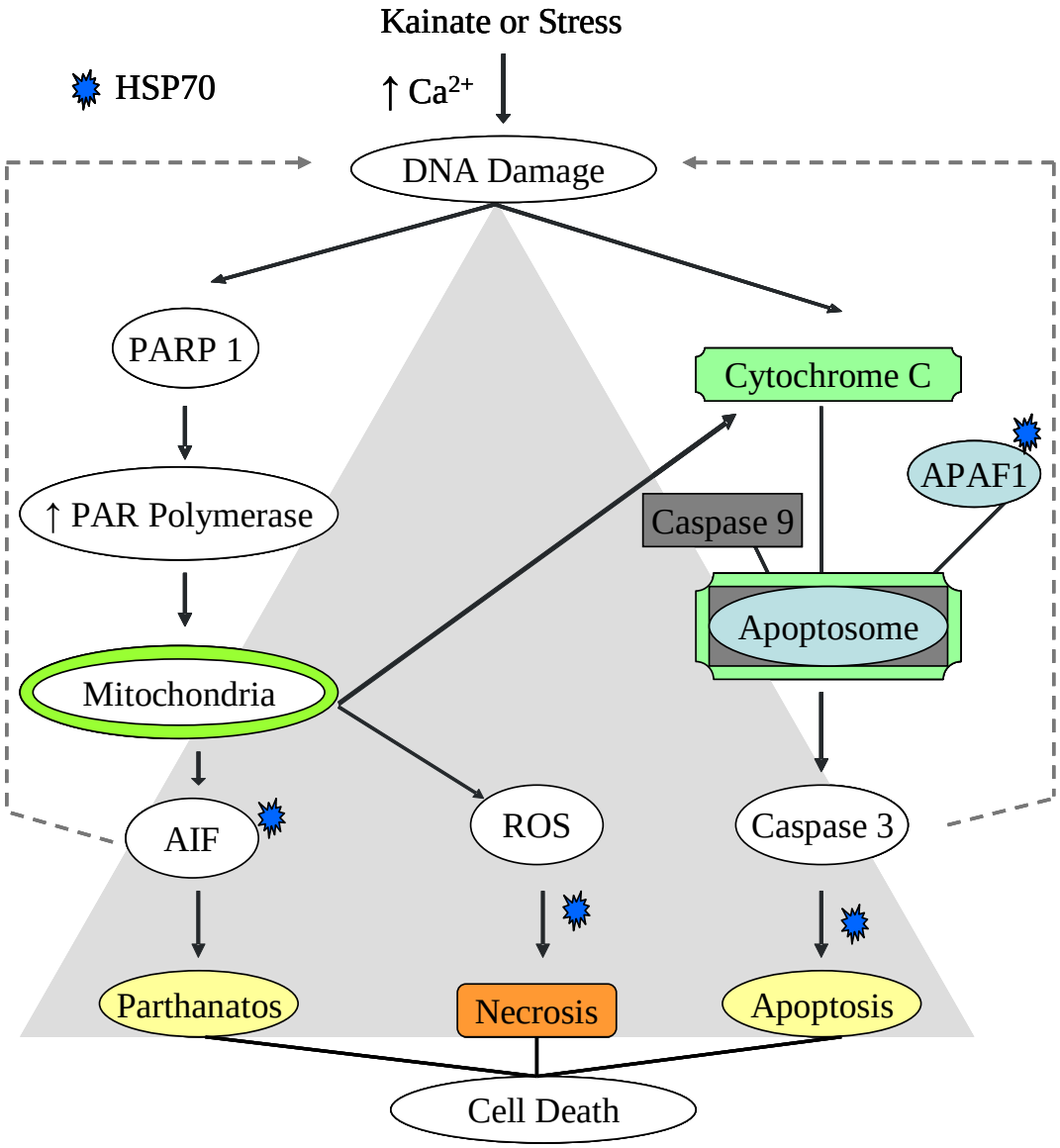

Figure 7. *Influence of HSP70 in suppression of apoptosis, necrosis and parthanatos. The dotted grey lines indicate the cyclic nature of the pathways, resulting in DNA damage (chromatin condensation, DNA fragmentation), in absence of HSP70. This DNA damage results in cell death (indicated by grey triangle).*

5. General Volatile Anesthetics

In recent years it has been established that the spinal cord is the primary site responsible for the immobilization by most anesthetics. It has also been shown that the isolated spinal cord preparation is the most suitable to study their action on motoneurons (Sonner et al., 2003). The clinically used general anesthetics are found to be neuroprotective in rodent model of brain ischemia (Burchell et al., 2013; Mackensen et al., 1999; Warner et al., 1995). One possibility for neuroprotection by anesthetics is inhibition of glutamate receptor mediated excitotoxicity. Volatile anesthetics are known to reduce glutamate release (Patel et al., 1995), glutamate mediated calcium influx (Bickler et al., 1994) acting through NMDA receptors (Yang and Zorumski, 1991) in the brain. Nevertheless, the mechanism by which these anesthetics operate is not completely understood.

Earlier it was hypothesized that the action of general volatile anesthetics was based on nonspecific interaction with the cell membrane bilayer (Elliott et al., 1992; Franks and Lieb, 1994). This lead to the idea of exploring membrane bound proteins, particularly ion channels. The inhibitory GABA and glycine receptor channels were identified as targets of anesthetics (Daniels and Smith, 1993; Mihic et al., 1997). More recently, focus has been directed to background K^+ channels that cause membrane hyperpolarization and increase neuronal input conductance (Sirois et al., 2000). In particular, the functional role of TASK channels (subgroup of background K^+ channels) has been explored in relation to the anesthetics action on rat motoneurons where they are abundantly expressed (Lazarenko et al., 2010). These TASK channels are pH sensitive and generate weakly rectifying K^+ currents after exposure to volatile general anesthetics (Duprat et al., 1997). There are several reasons to believe that these channels play an important role in motoneuron function as well as in other neuronal populations (Bayliss et al., 2003; Lesage and Lazdunski, 2000). A prominent K^+ current in motoneurons cannot be explained by known presence of inward rectifying Kir2.2 and Kir2.4 channels (Karschin et al., 1996; Prüss et al., 2005). Among these TASK channels, TASK-1 and TASK-3 are most commonly expressed in the CNS (Karschin et al., 2001; Talley et al., 2001) where

TASK-3 channels are implicated in mitochondrial dysfunction (Bittner et al., 2010). The mechanism of spinal cord injury pathophysiology involves mitochondrial dysfunction, a process in which TASK-3 channels are implicated (Bittner et al., 2010). Marnic et al. have found that the motoneurons in the ventral horn of cervical spinal cord highly expressed somatodendritic TASK-3 channels, Figure 8 (Marinc et al., 2012). The functional role of TASK-1 and TASK-3 channels has also been studied with respect to anesthetics in conditional knock-out mice in which it has been found that higher doses of anesthetics are required to elicit immobilization in mice lacking TASK-1 and TASK-3 channels (Lazarenko et al., 2010). These data suggest that TASK channels on motoneurons are molecular substrates to general anesthetics actions.

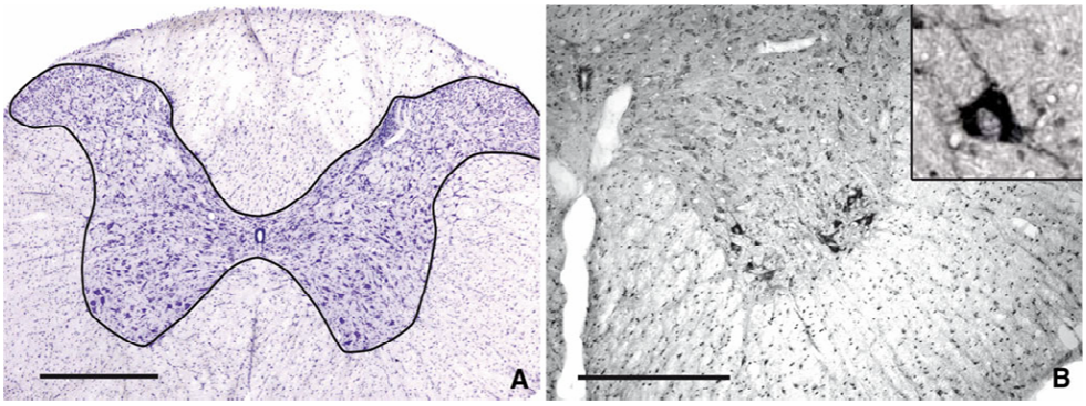

Figure 8. *Localization of TASK-3 channels in spinal cord motor neurons. (A) Cresyl violet staining of a spinal cord section from the cervical region. (B) Immunocytochemistry with the anti-TASK-3 antibody detected strong TASK-3 expression in motor neurons of the anterior horn. The channel protein can be found in somata and dendrites (see inset). Bar represent 1 mm in (A), and 500 µm in (B) (Marinc et al., 2012).*

These considerations suggest the desirability of investigating the influence of a general anesthetic on the mechanisms of spinal injury. For this purpose, the physical and chemical properties of the volatile anesthetic methoxyflurane (Andersen and Andersen, 1961) makes it a suitable candidate for further exploration. Methoxyflurane possesses a number of effects, such as activation of the calcium dependent ATPase in the sarcoplasmic reticulum via increased fluidity of lipid bilayer (Elliott et al., 1992).

GABA and glycine receptors are both ligand-gated ion channels. The GABA receptors are heteromeric complexes formed by assemblies of different glycoprotein subunits, whereas glycine can be heteromeric (complexes of alpha subunits) or homomeric (complexes of alpha and beta subunits). Methoxyflurane also binds to GABA receptors to produce the depressant effects (Moody et al., 1988) via GABA mediated chloride channels. It was shown that GABA antagonist Ro15-4513 blocked the effect of methoxyflurane in mice whereas GABA inverse agonists had no effect on methoxyflurane induced sleep time in mice (Moody and Skolnick, 1988). The effects of volatile general anesthetics on glycine receptors were found to be sensitive to ether, alkane and alcohol but not to other anesthetics like etomidate, propofol, barbiturates, alpha-chloralose and steroidal anesthetics in various animal models and cell lines (Koltchine et al., 1996; Krasowski and Harrison, 2000; Mascia et al., 1996; Pistis et al., 1997; Prince and Simmonds, 1992). Glutamate is an excitatory neurotransmitter and its postsynaptic actions are mediated by a variety of receptors. The NMDA glutamate receptor opens a non-selective cation channel characterized by high Ca^{2+} permeability (Seeburg, 1993) and glycine is an NMDA receptor co-agonist (Kaplita and Ferkany, 1990). Several studies suggest the interaction between NMDA receptor and volatile general anesthetics. The potency of volatile anesthetics is shown to be increased by the NMDA receptor antagonist MK-801 in rat brain (Foster et al., 1987). Isoflurane blocked NMDA mediated currents by decreasing the frequency of channel opening and mean channel opening time in rat cultured hippocampal neurons (Yang and Zorumski, 1991). In another study performed with radioligand binding assay (membrane cells were obtained from rat cerebral cortex), the effects of volatile general anesthetics on NMDA receptors showed antagonist (MK-801) binding to be

markedly inhibited by volatile general anesthetics in non-competitive manner: glycine reversed most of the inhibition (Martin et al., 1995).

6. Therapeutic Approaches - Past, Present and Future

Progressive tissue degeneration initiates in the secondary phase of spinal cord injury, but, at the same time, it also prompts a number of neuroprotective and regenerative responses. Regenerating axons can be observed within the first 24 h after injury (Kerschensteiner et al., 2005), a process is known as abortive sprouting (Hill et al., 2001; Schwab and Bartholdi, 1996) because it does not lead to any major improvement on its own. Spontaneous remyelination can also occur in Schwann cells and oligodendrocytes after axonal demyelination (Gilson and Blakemore, 2002). Spontaneous behavioral improvement can be observed perhaps due to functional plasticity and local circuit formation or adaptation, a phenomenon typically reported in rodents and humans after incomplete spinal cord injury (Bareyre et al., 2004; Edgerton et al., 2004; Frigon and Rossignol, 2006). Neurorehabilitation can also play an important role in recovering locomotor network after injury (Baptiste and Fehlings, 2007). Here I will be discussing three major therapeutic approaches to the acute phase of spinal cord injury.

6.1. Neuroprotection

The minimization of secondary damage after injury has been attempted many times with the help of various pharmacological substances (Kwon et al., 2011; Schwab et al., 2006), often adapting strategies employed for brain stroke.

Lipid Peroxidase Inhibition - The most studied substance in this regard is methylprednisolone (MP) as, in high doses, it was used to inhibit post traumatic lipid peroxidation (Braughler and Hall, 1981). MP is thought to reduce the inflammation, formation of a cytotoxic edema, the release of glutamate and formation of free

radicals. However, certain side effects, (like facilitated infection, delayed wound healing, diabetic complications and septic shock) have been reported for the high dose of MP in humans, making its clinical use still controversial.

Calcium Channel Blocker - Nimodipine is an L-type calcium channel blocker. Since excitotoxic glutamate release is calcium dependent, this drug is thought to be neuroprotective against subarachnoid hemorrhage (Fehlings et al., 1989).

Anti-inflammatory Agents – Because the inflammatory response after spinal cord injury has profound effects on the spinal cord, anti-inflammatory agents have been tested as neuroprotective. In particular, COX2 inhibitors and the antioxidant resveratrol have been used as anti-inflammatory substances. Studies have shown neuroprotection after reseveratrol in an experimental stroke model (Baur and Sinclair, 2006).

Erythropoietin - Another, rather new, neuroprotective substance is erythropoietin, an endogenous hormone normally responsible for increased red blood production via maturation of reticulocytes. It has been shown to increase spared tissue and decreased the cavity volume size at injury site in rats along with some neuronal regeneration (Huang et al., 2009). The recovery of hindlimb motor function after contusive spinal cord injury has been observed in rats by acute administration of recombinant human erythropoietin and it was suggested that the locomotor recovery was the result of erythropoietin mediated sparing of spinal cord sensory and motor pathways (Cerri et al., 2012).

Neurotrophic Factors - Neurotrophic factors like neurotrophin 3 and 4 (NT-3 and NT-4), brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) have all shown therapeutic potential for spinal cord injury (Blesch et al., 2002; Schnell et al., 1994). However, they are not sufficient on their own because they cannot help injured nerve fibres to overcome the axon growth inhibitory environment created after spinal cord injury. They rather seem to be more useful in combination

with other treatment strategies to contribute to functional recovery (Fouad et al., 2005; von Meyenburg et al., 1998).

Inhibitory-factor Blockers – In recent years, new approaches to surmount the negative action of myelin derived inhibitors that impede regrowth have shown interesting preclinical results after spinal cord injury. Schnell and Schwab have reported the first attempt to promote regeneration with a monoclonal antibody against NogoA (Schnell and Schwab, 1990). Recently, clinical trials with the humanized NogoA antibody have been started (Buchli et al., 2007). This substance act on the GTPase Rho which inhibits regeneration (He and Koprivica, 2004): its antagonists (Cadotte and Fehlings, 2011) may be neuroprotective by increasing regeneration and functional recovery after spinal cord injury (Dergham et al., 2002).

Sodium Channel Blocker - Excitotoxicity is the result of glutamate hyperactivity and use of glutamate inhibitors/antagonists might lead to some neuroprotection. CNQX and APV, when coapplied with kainate, have produced partial protection of locomotor networks in an *in vitro* rat model of spinal cord injury as NMDA and 5HT have elicited fictive locomotion in 1/3 of preparation (Margaryan et al., 2010). However, only few oscillatory cycles have been observed after a train of DR pulses with some recovery of cumulative depolarization (Margaryan et al., 2010). It is suggested that the slow pharmacokinetics of CNQX and APV require a long time to block glutamate receptor of rat spinal cord (Long et al., 1990) that is significantly later of the glutamate receptor hyperactivity. Another such substance is riluzole, that is a sodium channel blocker already approved for symptomatic use in patients with amyotrophic lateral sclerosis (ALS) (Kitzman, 2009; Schwartz and Fehlings, 2001; Springer et al., 1997). Clinical trials with riluzole are underway for spinal neuroprotection after acute traumatic spinal cord injury (Wilson and Fehlings, 2014). Riluzole apparently exerts its neuroprotective effects by blocking voltage sensitive sodium channels. In addition, riluzole antagonizes presynaptic calcium dependent glutamate release. When riluzole is used in combination with methylprednisolone, it is claimed to reduce oxidative stress by improving mitochondrial function and

enhancing glutamate uptake (Mu et al., 2000). Our laboratory has reported minimal neuroprotective effects of riluzole on locomotor networks after excitotoxic spinal cord injury (Sámano et al., 2012) with more encouraging results on spinal organotypic cultures (Mazzone and Nistri, 2011a).

PARP1 Inhibitors - The role of PARP1 in excitotoxicity induced cell death has already been discussed. In recent years, our laboratory has been exploring the possibilities to improve neuroprotection by using PARP1 inhibitors (Kuzhandaivel et al., 2010a; Nasrabady et al., 2011a, 2011b, 2012). The isolated neonatal spinal cord has shown decreased number of pyknotic cells after use of pharmacological inhibitor of PARP1 by PHE (6-5(H)-phenanthiridinone) following kainate induced excitotoxicity (Kuzhandaivel et al., 2010a). Another potent PARP1 inhibitor is PJ34 (2-(dimethylamino)-N-(5,6-dihydro-6-oxophenanthridin-2yl)acetamide). It exerts significant neuroprotection against acute stroke even if given as late as 10 minutes prior to reperfusion time (Abdelkarim et al., 2001). Studies in our laboratory have also shown PJ34 to be moderately neuroprotective in spinal cord injury models with some recovery of locomotor network activities (Mazzone and Nistri, 2011b; Nasrabady et al., 2011b, 2012).

Heat Shock Proteins – The increased expression of HSP70 has neuroprotective effects in various animal models of neurodegenerative diseases (Brown, 2007) and this effect is dependent on its ability to bind AIF and to inhibit programmed cell death (Garrido et al., 2001; Sevrioukova, 2011). Spinal motoneurons show high levels of HSP70 in various disease models (Brown, 2007; Carmel et al., 2004; Cizkova et al., 2004). HSP70 has been shown to neuroprotect motoneurons against ALS degeneration (Gifondorwa et al., 2007) and oxidative stress (Robinson et al., 2008) in a mouse model. The use of enhancers for HSP70 like celestrol has proved to be neuroprotective in differentiated human neurons (Chow and Brown, 2007). HSP70 has also been tested on knockout (KO) mice and the decreased level of functional recovery was observed in KO when compared to wild type mice after spinal cord injury (Kim et al., 2010). The HSP70 inhibitor VER155008 (5'-*O*-[(4-cyanophenyl)

methyl]-8- [[(3,4-dichlorophenyl) methyl]amino]-adenosine) (William-son et al., 2009) is known to antagonize the neuroprotective action of HSP70 after mild traumatic brain injury (Saykally et al., 2012). These data indicate that exploring the role of HSP70 for spinal cord injury treatments can be fruitful.

General Anesthesia with gas anesthetics - Volatile anesthetics depress reflex activity by suppressing excitability of spinal motoneurons in rats (Collins et al., 1995). Furthermore, volatile anesthetics are known to be neuroprotective against excitotoxicity in the brain (Kudo et al., 2001; Popovic et al., 2000; Ren et al., 2014) and spinal cord (Nout et al., 2012). Anesthetics like isoflurane and urethane have less deleterious effects (compared to propofol, morphine, pentobarbital and alpha-chloralose) on outcomes of induced injury when used either during the lesion procedure or postoperatively in a brain injury models (Cochrane et al., 1988; O'Connor et al., 2003; Statler et al., 2000, 2006a, 2006b). For spinal cord injury models, halothane has resulted is better neurological outcomes compared to other anesthetics like ketamine and pentobarbital (Grissom et al., 1994; Ho and Waite, 2002; Leal et al., 2007; Salzman et al., 1990), indicating neuroprotective effects of general volatile anesthetics during neurosurgery. Likewise, when isoflurane was administered during a study of rat spinal cord injury, it facilitated the recovery of locomotor function much better than pentobarbital (Nout et al., 2012). These studies suggest that the choice of general anesthetic has an impact on the outcome of experiments and can be a good neuroprotective strategy.

6.2. Neurorepair

After spinal cord injury, cell transplantations have been investigated to replace lost tissue components, remyelinate denuded axons, provide guidance structures and express growth factors. The cell types tested for this purpose were embryonic stem cells (McDonald et al., 1999), Schwann cells (Kuhlengel et al., 1990), olfactory ensheathing cells (Li et al., 1997) and marrow stromal cells (Chopp et al., 2000; Hofstetter et al., 2002). Embryonic stem cells can allegedly differentiate into

oligodendrocytes, astrocytes and neurons once transplanted into the injured spinal cord (Harper et al., 2004; Kerr et al., 2003; McDonald et al., 1999). Nevertheless, this is a controversial issue and the challenge in cell transplantation remains survival, differentiation and integration (Enzmann et al., 2006).

The most studied cell type for transplantation after spinal cord injury is the Schwann cell. These cells can produce a cocktail of axonal growth factors like NGF, NT-3, GDNF, BDNF and FGF (Oudega and Xu, 2006). In addition to these growth factors, they express axon guidance cell adhesion molecules on their surfaces (Daniloff et al., 1989). After spinal cord injury, Schwann cells are reported to reduce the size of spinal cysts, remyelinate axons and enhance function recovery (Baron-Van Evercooren et al., 1992; Xu et al., 1995).

The other cell types that can be transplanted are olfactory nerve cells as they have the ability to regenerate throughout life. The olfactory ensheathing cells have been reported to facilitate axonal growth and functional recovery when transplanted into the injured medulla oblongata or spinal cord (Li et al., 1997; Ramón-Cueto and Nieto-Sampedro, 1994; Ramón-Cueto et al., 1998).

The central nervous system axons can grow over long distances as long as the right environment is provided. Certain bridging strategies like peripheral nerve bridge transplantation have been shown to partially restore function in the completely transected spinal cord (Cheng et al., 1996; Tsai et al., 2005). All together combination of nerve grafts with other transplants (Schwann cells and olfactory ensheathing cells; Fouad et al., 2005) or substances (chondroitinase) have been described as more effective in recovering functionality.

A new, interesting method for neurorepair is the use of nanoparticals/tubes for targeted drug delivery to spinal lesion sites. Studies on nanodrug delivery after spinal cord injury are not well characterized owing to the problem of maintaining high concentration of active drug within spinal cord to achieve neuroprotection. Methylprednisolone has been administered using nanoparticles of poly-lactic-co-glycolic acid applied topically over the injured spinal cord and it has seemingly resulted in regeneration and functional recovery even after 7 days of primary insult (Chvatal et al., 2008). In another study, titanium nanowires were used to deliver drugs

in order to study the drug effects on pathophysiology and functionality after spinal cord injury in rats: this approach showed good neuroprotection only when drugs were delivered within 30 minutes from injury (Tian et al., 2012). A lot of research is still necessary to clarify this subject.

6.3. Neurorehabilitation and Plasticity

Neurorehabilitation has been an established treatment after spinal cord injury. For instance, complications like urinary tract infections can be minimized with physical activity. Neurorehabilitation is also helpful in improving cardiovascular function and patients wellbeing (Curtis et al., 1986; Hicks et al., 2003, 2005). One procedure is body weight support during treadmill training that can improve function in both rodents and humans (Lünenburger et al., 2006; Rossignol, 2000). The rationale stems from the realization that local spinal cord circuits, even in the absence of supraspinal inputs, can be modulated to produce movement patterns (Grillner, 2002). While spontaneous recovery after spinal cord injury can be accounted for by plasticity of remaining fibers (Wolpaw and Tennissen, 2001), the spinal cord can learn specific motor tasks with improved performance through training (Edgerton et al., 2004). Stepping and standing are therefore facilitated (Edgerton et al., 2001; De Leon et al., 1999). Other rehabilitation procedures like running wheels, environmental enrichment and robotic devices have been used in rodent models (Engesser-Cesar et al., 2005; Lankhorst et al., 2001). Better understanding of mechanism for adaptation and plasticity after spinal cord injury is needed to devise new strategies. Another form of therapy that can activate neural components after incomplete spinal cord injury is electrical current stimulation (Grahn et al., 2014). The use of electrical stimulation to test the function of nervous system was first introduced by an Italian physician and scientist Luigi Galvani (1792) in frog legs (Parent, 2004). This kind of treatment can cause muscles to contract which can help restoration of motor function (Jackson and Zimmermann, 2012).

7. Spinal Cord Injury Models

The spinal cord injury models have evolved over the past decades and have contributed to a better understanding of the injury. Rats are the most commonly used animals as they are inexpensive, readily available and have similar functional and morphological characteristics of injury when compare to humans. The injury models can be classified as contusion, compression, distraction, dislocation, transaction or chemical. A transient acute injury of the spinal cord can be induced by contusion methods such as weight drop (Scheff et al., 2003), electromagnetic impactor (Stokes, 1992) and air gun device (Marcol et al., 2012). Compression models usually include contusion-compression processes in the sense that they involve an acute impact followed by prolonged compression of the spinal cord. However, compression is distinct from mere contusion because it implies lasting pressure analogous to the one of spinal fractures. Compression models include clips (Rivlin and Tator, 1978), calibrated forceps (Blight, 1991), and balloon compression (Tarlov et al., 1953) as well as spinal cord strapping (da Costa et al., 2008). The aim of the distraction model is to stimulate the tension forces present in the cord after spinal cord injury. Distraction method involves the stretching of spinal cord (Silberstein and McLean, 1994). The most sophisticated modeling of this method is known as Harrington distraction (Dabney et al., 2004) and involves laminectomy at the intended injury location. This method has not been validated in humans yet. Dislocation models mimic the column shift observed after traumatic human spinal cord injury (Fiford et al., 2004). It was developed on rats for vertebral displacement. The other model is transection that could be partial or complete (Spillmann et al., 1997). Transection is used to study neuronal regeneration after spinal cord injury.

Chemical models are used where secondary injury mechanism/pathways are to be studied after spinal cord injury. The ischemia followed by spinal cord injury can be induced by injections or direct application of photosensitive dyes (Watson et al., 1986). Oxidative stress is produced by reagents like superoxide, hydroxyl radical and peroxynitrate (Bao and Liu, 2002). Zymosan and phospholipase A2 can be used to mimic inflammatory response after spinal cord injury (Liu et al., 2006). Twenty % of

chronic spinal cord injury cases are accompanied by syringomyelia (fluid filled cavity formation) which can be recreated by use of kaolin or quisqualic acid injections into the spinal cord (Lee et al., 2005). Excitotoxic injury can be produced by glutamate, aspartate, NMDA or kainate (Liu et al., 1999; Taccola et al., 2008).

7.1. Our Laboratory *In Vitro* Neonatal Spinal Cord Injury Model

Cell lines or primary cultures can be used to study cell death and neuroprotection mechanisms for preclinical screening, but these models do not allow testing network activity/function like locomotor-like activity. The *in vivo* models pose some serious concerns as the use of anesthetics administered before and during the lesion procedure can attenuate the severity of damage, there is usually lack of intensive care for experimental animals after injury, and the difficulty in inducing a non-traumatic lesion without affecting the general metabolic state of animal. To overcome these problems, our laboratory has recently developed a novel model of acute spinal cord injury *in vitro* based on the neonatal rat isolated spinal cord (Taccola et al., 2008). This method is equally helpful in studying non-traumatic and incomplete spinal cord injury (Kuzhandaivel et al., 2010a, 2010b; Nasrabady et al., 2011b; Taccola et al., 2008). This model allows exploring the pathophysiology of spinal cord injury over the 24 hours by monitoring the changes in locomotor function and relating them to molecular and cellular alterations within spinal networks (Nistri, 2012). We have used this model to induce excitotoxic damage by use of kainate (Mazzone et al., 2010; Nasrabady et al., 2011a; Shabbir et al., 2014) and ischemic injury by application of pathological medium characterized by oxygen-glucose deprivation (Bianchetti et al., 2013; Margaryan et al., 2009). The basic protocol starts with 1 hour application of kainate/pathological medium followed by 24 hour washout in kreb's solution at room temperature. Sham preparations are kept for 24 hour without any toxic medium application. Although this model has some obvious limitations like lack of vascular supply, absence of an immune system and the rat neonatal age, it has the great advantage of correlating the outcome of injury after 24 hours by monitoring locomotor like activity (fictive locomotion) in relation to number, type and

topography of damaged or dead cells. It also allows the preservation of the tissue for further immunological and molecular studies as the extent of loss of electrophysiological function depends on the extent of neuronal damage (Nistri et al., 2010).

8. Central Pattern Generator (CPG)

The ability to move depends on the locomotor rhythmic behavior of that specie such as flying, swimming or walking (Büschges et al., 2011). The inherent complexity of the central nervous system (CNS) in mammals has led to the studies involving vertebrate models with relatively fewer neurons like the lamprey (Grillner et al., 1998) and the zebra fish (Kyriakatos et al., 2011). Vertebrates share many similarities in locomotor behavior and controlling system (Grillner, 1985; Grillner et al., 1977) because the same centers initiate locomotor activity in lampreys as in primates (through the activity of lower brainstem reticulospinal neurons). This system activates localized spinal neuronal networks which generate the timing and pattern of complex, rhythmic, coordinated muscle activities (Goulding, 2009). Grillner et al. (1998) described the controlling system of locomotion that is prevented by a tonic inhibitory system induced by basal ganglia (Figure 9). The descending excitatory inputs can therefore stimulate the local circuits to produce rhythmic alternating motor commands to limb muscles without the need for peripheral inputs. Thus, this system is termed locomotor central pattern generator (CPG). The activity of CPG network is directly proportional to the speed of locomotion. In addition to descending signals from reticulospinal projection neurons, the CPG can be activated by pharmacological excitatory substances or by patterned sensory inputs under experimental conditions (Kjaerulff et al., 1994; Marchetti and Nistri, 2001; Marchetti et al., 2001; Taccola and Nistri, 2006).

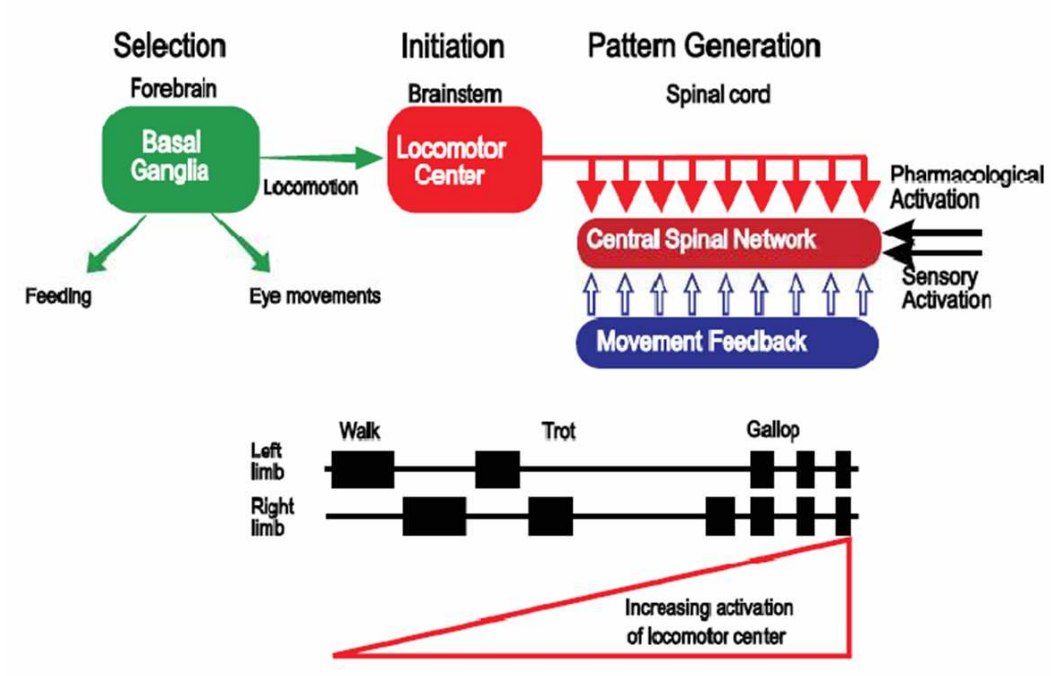

Figure 9. *Schematic representation of locomotion general controlling system in vertebrates (Grillner et al., 1998).*

More recently a two-level structure of the CPG at spinal level has been proposed, comprising a rhythm generator (RG) that distributes regular rhythmic commands to a pattern formation circuit responsible for the correct motoneuron activation in terms of flexor and extensor discharges on bioth sides of the body (McCrea and Rybak, 2008).

8.1. Localization of CPG Network

The rostrocaudal extent of rhythmogenic capacity of the hindlimb locomotor CPG was proposed first by Grillner and Zangger (Grillner and Zangger, 1979). They demonstrated that alternating rhythmic activity could be evoked in ankle flexors and extensors, when the caudal lumbar cord (L6–S1 segments) was isolated from the rest of the spinal cord by using transverse sectioning. They suggested that the

rhythmogenic capacity of the CPG controlling hindlimb locomotion to be distributed throughout the lumbar enlargement (L3–S1 in cats). Similar results were obtained when other laboratories studied spontaneous or drug induced rhythmic activity before and after transverse trans-sectioning at different spinal levels (Bracci et al., 1996; Christie and Whelan, 2005; Kjaerulff and Kiehn, 1996; Kudo and Yamada, 1987). L1–L3 in rodents and L3–L5 in cats (rostral lumbar segments) have a greater ability to generate rhythmic motor output in isolation than caudal segments (L4–L6 and L6–S1, respectively). These studies therefore suggest that the CPG is distributed along the lumbar cord but its excitability has a rostrocaudal gradient. The reason can be the larger proportion of intraspinal inputs reaching more rostral segments (Berkowitz, 2004) and/or the differential distribution of neuronal receptors for neuromodulatory substances in the rostral or caudal cord (Christie and Whelan, 2005). Thus, it has been suggested that spinal interneurons directly involved in producing rhythmic activity are limited to the T13 and L2 (Kiehn, 2006). Transverse distribution of CPG in spinal cord was studied by activity-labeling studies (Dai et al., 2005; Kjaerulff et al., 1994) and multi-electrode recording (Tresch and Kiehn, 1999). It was reported that rhythmogenic CPG neurons are located ventrally (laminae VII, VIII, and X). It has also been confirmed with dorsal horn ablation studies, demonstrating the presence of fictive locomotion (chemically induced by NMDA and 5-HT) by recording from VRs (Taccola and Nistri, 2006).

8.2. Elements of CPG Network

The same side of the body needs both excitatory and inhibitory networks for the alternation between flexor and extensor motoneuron pools (Kiehn, 2006). The model that has been suggested for mammalian locomotor CPG has a rhythm generating layer (flexor-extensor) and a pattern generating layer (left-right side) (Kriellaars et al., 1994; Lafreniere-Roula and McCrea, 2005). This model suggests that the pattern-generating neurons are connected monosynaptically to motor neurons, whereas the neurons in the rhythm generating layer have two or more synapses upstream from motor neurons and project directly to pattern-generating neurons. The origin of

rhythm generation in lamprey CPG is the result of ipsilateral excitatory glutamatergic interneurons (Grillner, 2003). These interconnected excitatory neurons seem to work as burst generating units. They provide a rhythmic, excitatory glutamatergic synaptic drive to motor neurons and other ipsilateral inhibitory and left-right coordinating CPG neurons in each segment. It has been proposed that excitatory CPG neurons might have some intrinsic pacemaker-like properties due to the presence of rhythmic activity after blocking of inhibitory system (Smith et al., 2000).

The other two elements of the network are flexor-extensor coordinating circuits and left-right coordinating circuits. The flexor-extensor coordination involves ipsilateral inhibitory networks since flexors and extensors are activated synchronously in the presence of synaptic inhibition block (Beato and Nistri, 1999; Cowley and Schmidt, 1997).

The right-left coordinating circuitries have been studied mostly in mammals to show that these complex circuitries consist of intrasegmental (binding motor synergies along the spinal cord) and intersegmental (coordinate homonymous muscle activity at segmental level) commissural interneurons (CINs). The CINs are both inhibitory and excitatory. Thus, the functioning of CPG network in proposed to operate by contribution of excitatory neurons responsible for rhythm generation and glycinergic CINs, that are directly involved in left-right alterations (Kiehn, 2006, 2011).

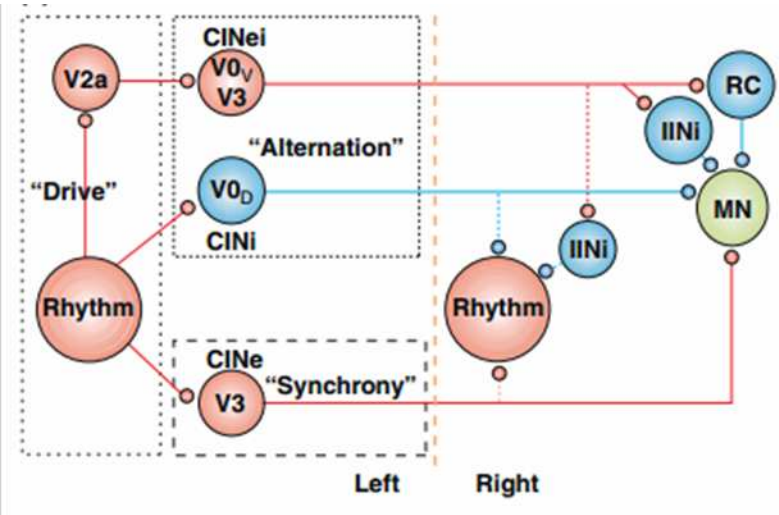

Figure 10. *Organization of left–right coordinating CPG circuits in rodents. The core of the left–right system in rodents is composed of commissural interneurons (CINs) acting directly via inhibitory CINs (CINi), or indirectly, via excitatory CINs (CINei) on contralateral motor neurons (MN). This system is involved in left–right alternation. Left–right synchrony is obtained via a single excitatory system (CINe) acting directly on motor neurons. To obtain left–right coordination during locomotion, these crossed connections should also connect to the rhythm-generating core (indicated with dotted lines) on the other side of the cord and/or corresponding commissural interneurons. A single neuron in the diagram represents a group of neurons. Inhibitory interneurons are blue. Excitatory interneurons are red, and motor neurons green. Dotted line indicates the midline. Abbreviations: IINi: ipsilaterally projecting inhibitory interneuron. RC: Renshaw Cell. MN: motor neuron (Kiehn, 2011).*

Multiple neuronal subtypes are involved in the different aspects of left-right coordination. The interneurons play important role in the operation of the neuronal circuitry. Ventral progenitor cells are the ones to give rise to motoneurons and 4 classes of interneurons (V0 – V3) (Rabe et al., 2009; Zhang et al., 2014). Among them, V0 and V3 give rise to CINs that are studied to understand the genetic basis of locomotor coordination. V0 neurons are mostly inhibitory (Lanuza et al., 2004) whereas V3 are predominantly excitatory (Bernhardt et al., 2013). V2 interneurons comprise two subpopulations; one expressing Chx10 and Lhx3 and the other expressing Gata2 and Gata3 (Karunaratne et al., 2002; Smith et al., 2002). The ablation of Chx10 and Lhx3 results in high variability of cycle period and amplitude

of locomotor bursts (Crone et al., 2008) during fictive locomotion. The other important factor is correct axonal guidance during the formation of the functional neuronal circuitry. There are four families of axonal guides with their receptors which have been identified in recent years, namely ephrin-Eph (Imondi et al., 2000), Slits-Robo (Brose and Tessier-Lavigne, 2000), semaphorins (neuropilin and plexin co-receptors) (Castellani and Rougon, 2002) and netrins with DCC, Unc-5/RCN and neogenin receptors (Barallobre et al., 2005; Tessier-Lavigne and Goodman, 1996).

8.3 Fictive Locomotion

The term fictive swimming in lamprey spinal cord was introduced by Grillner and colleagues (Grillner et al., 2001) and the corresponding mechanism is the mammalian isolated spinal cord is known as fictive locomotion (FL). The fictive locomotion is product of CPG networks and is produced by rhythmic alternating oscillations between left-right and flexor-extensor motor pools. In an isolated spinal cord preparation, fictive locomotion can be elicited chemically and/or electrically. The chemically induced FL can be achieved by application of certain drugs like NMDA and serotonin (5HT). These pharmacological agents raise spinal network excitability to activate a sufficient number of CPG neurons (Christie and Whelan, 2005; Grillner et al., 2001; Kjaerulff and Kiehn, 1996). Electrical stimulation can be applied by train of stimuli to a single dorsal root (DR) which can be recorded from left- right L2 ventral roots (VRs; flexor motor pool commanding hindlimb) and left-right L5 VRs (extensor motor pool commands to hindlimb) (Nasrabady et al., 2011b; Nistri et al., 2010; Taccola et al., 2004, 2008). Similar activity can be observed when electrical stimulation is applied *in vivo* animals and to spinal cord injury people. In humans with complete spinal cord injury, epidural stimulation at the level of T11 – L1 elicits locomotor-like EMG activity (Dimitrijevic et al., 1998).

9. Field Potential

Motoneurons are an essential component of locomotor network activity and many of them can survive after spinal cord injury. While studies conducted on motoneurons at single cell level provide important information on cell properties, the response of more than one motoneuron is required when investigating the functional outcome of any neuroprotective therapy. Field potential (FP) recordings can show the collective behaviour of the motoneuronal population in the ventral motor area. Further, the advantage of FP recording is that they can more easily be obtained than intracellular or whole cell recording and are much more stable for long term investigations. Recording evoked field potentials is a widely applied technique *in vitro* (Bear et al., 1992; Fulton and Walton, 1986; Zhou and Poon, 2000) and *in vivo* (Berger and Averill, 1983).

The extracellular potentials induced by electromotive force of excitable membranes are called field potentials (FPs). The potential (and current) fields at any point in the extracellular medium are given by the sum of signals resulting from each active current component entering (source) and leaving (sink) the recorded site. As a result, the net balance of all the currents that flow into a small extracellular local volume represents the field potential (Zhou and Poon, 2000). Any excitable membrane (spine, dendrite, soma, axon or axon terminal) and any type of transmembrane current can contribute to the field potentials that are the expression of all the ionic processes ranging from fast action potentials to the slow fluctuations in glia. The amplitude of the field potential is inversely proportional to the distance of the recording electrode from the current source. The distance also leads to inclusion of other interfering signals. The field potentials can come from multiple sources such as synaptic activity (fast sodium action potential), calcium spikes, and intrinsic currents (Buzsáki et al., 2012). Many extracellular currents must overlap to give a measurable signal as synaptic activities are slow events (Elul, 1971; Logothetis and Wandell, 2004). The excitatory currents are mediated by neurotransmitters acting on NMDA and AMPA

receptors, whereas GABA (and glycine) receptors mediate inhibitory currents. In general, inhibitory currents contribute little to field potentials (Bartos et al., 2007).

In the present study, they provide the average strength of the motoneuronal pool activation in the ventral horn of the spinal cord. The evoked field potentials are stimulus intensity dependent, their amplitude increases with increasing stimulus as shown in Figure 10. At high stimulus voltage, the response gets saturated and an artifact can over-shadow the potential.

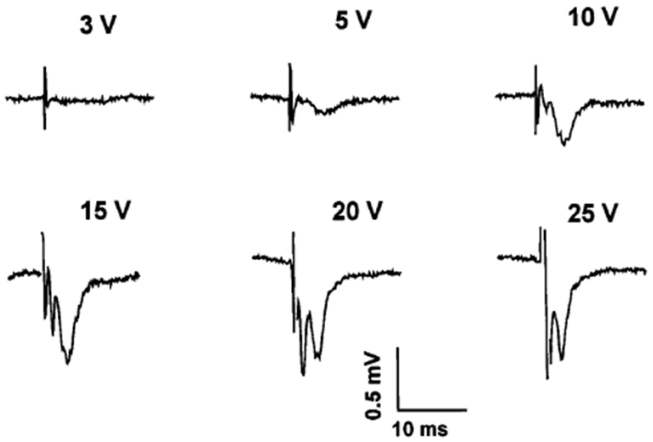

Figure 10. *Effects of stimulus intensity on field potential (Zhou and Poon, 2000).*

Aims of the Study

During the early phase of spinal cord injury, neuroprotective interventions can be extremely important to determine the injury outcome by preventing delayed pathological events. Thus, in relation to this issue, two main questions were investigated in the present project. First, can a volatile general anesthetic protect the spinal cord? This enquiry is prompted by the observation that volatile general anesthetics are neuroprotective in many injury models of the CNS probably because these agents inhibit the excitotoxicity induced glutamate release. Second, since a number of motoneurons survive the first insult, what endogenous mechanisms are used by such cells to withstand damage, and do they remain functional? Using the *in vitro* model of neonatal spinal cord injury, the aims of the present study are:

- Potential neuroprotective effect of the volatile general anesthetics **Methoxyflurane** on;

1. Motoneurons survival and their functional outcomes.
2. Electrophysiological properties of CPG network activity.

I further enquired what intrinsic mechanism might be involved in motoneuron survival after spinal cord injury with the goal to understand:

- The role of **heat shock protein 70 (HSP70)** in spinal cell survival by studying:

1. Expression of HSP70 and AIF in motoneurons after excitotoxicity.
2. Pharmacological inhibition of HSP70 by VER155008 and its consequences on cell survival and electrophysiological properties.

Materials, methods and results

Section I

The volatile anesthetic methoxyflurane protects motoneurons against excitotoxicity in an *in vitro* model of rat spinal cord injury.

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THE VOLATILE ANESTHETIC METHOXYFLURANE PROTECTS MOTONEURONS AGAINST EXCITOTOXICITY IN AN *IN VITRO* MODEL OF RAT SPINAL CORD INJURY

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Abstract—Neuroprotection of the spinal cord during the early phase of injury is an important goal to determine a favorable outcome by prevention of delayed pathological events, including excitotoxicity, which otherwise extend the primary damage and amplify the often irreversible loss of motor function. While intensive care and neurosurgical intervention are important treatments, effective neuroprotection requires further experimental studies focused to target vulnerable neurons, particularly motoneurons. The present investigation examined whether the volatile general anesthetic methoxyflurane might protect spinal locomotor networks from kainate-evoked excitotoxicity using an *in vitro* rat spinal cord preparation as a model. The protocols involved 1 h excitotoxic stimulation on day 1 followed by electrophysiological and immunohistochemical testing on day 2. A single administration of methoxyflurane applied together with kainate (1 h), or 30 or even 60 min later prevented any depression of spinal reflexes, loss of motoneuron excitability, and histological damage. Methoxyflurane per se temporarily decreased synaptic transmission and motoneuron excitability, effects readily reversible on washout. Spinal locomotor activity recorded as alternating electrical discharges from lumbar motor pools was fully preserved on the second day after application of methoxyflurane together with (or after) kainate. These data suggest that a volatile general anesthetic could provide strong electrophysiological and histological neuroprotection that enabled expression of locomotor network activity 1 day after the excitotoxic challenge. It is hypothesized that the benefits of early neurosurgery for acute spinal cord injury (SCI) might be enhanced if, in addition to injury decompression and stabilization, the protective role of general anesthesia is exploited.
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Key words: spinal locomotor network, fictive locomotion, motoneurons, spinal reflex.

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Abbreviations: 5HT, 5-hydroxytryptamine; ChAT, choline acetyltransferase; CPG, central pattern generator; CV, coefficient of variance; DAPI, 4′,6-diamidino-2-phenylindole; DR, dorsal root; DR-VRPs, dorsal root ventral root potentials; FL, fictive locomotion; FP, field potential; KA, kainic acid (kainate); MF, methoxyflurane; NMDA, *N*-methyl-D-aspartate; ROI, region of interest; VR, ventral root.

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INTRODUCTION

Although the majority of new cases of spinal cord injury (SCI) are associated with an incomplete lesion, severe locomotor deficit usually ensues with resultant paralysis and uncertain recovery (McDonald and Sadowsky, 2002; Nair et al., 2005; van den Berg et al., 2010). One essential factor for this negative outcome is the extension of the primary lesion over the first few hours and days to surrounding spinal areas (secondary lesion) (Sekhon and Fehlings, 2001; Park et al., 2004; Rowland et al., 2008). Hence, it is important to protect, with various strategies (Boulenguez and Vinay, 2009), initially-spared nervous tissue from further damage which, according to experimental preclinical data, might be already complete within the first 24 h (Mazzone and Nistri, 2014). One major mechanism for lesion progression is excitotoxicity whereby sustained activation of glutamate receptors leads to neuronal degeneration via a delayed cell death process termed parthanatos (Mandir et al., 2000; David et al., 2009; Kuzhandaivel et al., 2010). The realization of the existence of a time lag between primary injury and cell loss should prompt attempts to neuroprotect the lesioned spinal cord (Baptiste and Fehlings, 2008). Investigating these issues demands animal models, though it is difficult to relate the severity of an acute injury to later outcome because *in vivo* animal studies do not readily allow repeated time-related measurements of pathophysiological mechanisms.

In the attempt to circumvent these difficulties, we have developed an *in vitro* model of SCI based on transient (1 h) application of the potent glutamate analogue kainate to mimic a clinical scenario (Taccola et al., 2008; Kuzhandaivel et al., 2011; Nasrabady et al., 2011a) in which intensive care treatment is applied as soon as possible to provide life support, correct metabolic imbalance and, perhaps, restrict the early component of excitotoxic stress (Baptiste and Fehlings, 2008; Munce et al., 2013). One important characteristic of this model is the possibility to analyze the functional activity of locomotor networks by recording their cyclic oscillatory discharges (alternating between flexor and extensor motor pools), and relate them to the topography and extent of the induced lesion. Notwithstanding the intrinsic limitations of this model (e.g. lack of blood supply), several features appear to be reminiscent of the clinical scenario like the limited extent of cell damage which primarily concerns neurons (rather than glia), and motoneurons in particular that are very vulnerable to this type of injury. Furthermore,

there is very high sensitivity of locomotor networks to damage as their electrical activity is suppressed even when substantial cell numbers remain apparently unscathed. These properties may render this model as a simple predictive tool for further testing the *in vivo* effectiveness of novel devices (Nistri, 2012). Our former studies of SCI model neuroprotection have so far obtained limited success (Nasrabady et al., 2011a, 2012) in line with disappointing clinical data (Baptiste and Fehlings, 2008) and should stimulate further work in this field.

The present investigation explored the usefulness of a volatile general anesthetic like methoxyflurane as a protective agent against experimental SCI. Volatile anesthetics are known to be neuroprotective against excitotoxicity in the brain (Popovic et al., 2000; Kudo et al., 2001; Ren et al., 2014) and spinal cord (Nout et al., 2012) although no data are currently available for their efficacy to preserve locomotor function. Methoxyflurane is a convenient drug for such studies because of its physical properties (Andersen and Andersen, 1961; Seto et al., 1992) and its pharmacological activity on synaptic and firing properties has been previously reported (De Jong et al., 1968; Richens, 1969). Hence, we tested the ability of methoxyflurane to contrast the histological and functional damage evoked by kainate. We employed two protocols of administration, namely the anesthetic was started after the application of kainate, or applied together with kainate as a test for proof of principle for the effectiveness of this treatment.

EXPERIMENTAL PROCEDURES

Spinal cord preparations

Thoraco-lumbar spinal cord preparations were isolated from urethane anesthetized (0.2 ml of i.p. of a 10%, w/v solution) neonatal Wistar rats of 0–2 day postnatal age according to the guidelines of NIH and with the approval of the local ethical committee. The experimental set up was as described previously (Taccola and Nistri, 2006a). The isolated spinal cords were superfused with Kreb's solution (in mM: 113 NaCl, 4.5 KCl, 1 $MgCl_2 \cdot 7H_2O$, 2 $CaCl_2$, 1 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose, gassed with 95% O_2, 5% CO_2, pH 7.4 at room temperature at rate of 7.5 mL/min). In accordance with the three Rs objective, all efforts were made to minimize the number of animals used for the experiments and their suffering. The experiments were performed in accordance with the ethical guidelines for the care and use of laboratory animals of National Institutes of Health (NIH) and the Italian act D. L. 27/1/92 n. 116 (implementing the European Community directives n. 86/609 and 93/88). All experimental protocols were approved by the ethical committee of the International School for Advanced Studies.

Electrophysiological recordings

Spinal reflexes and motoneuron field potential (FP). Dorsal root-ventral root potentials (DR-VRPs) were evoked by stimulating a single dorsal root (DR) through a suction electrode with 0.5–10 V stimuli (0.1–0.2 ms duration) and recorded from the homolateral ventral root (VR) by using glass suction electrodes (Taccola and Nistri, 2006a) filled with Kreb's solution as previously reported (Ostroumov et al., 2011). Usually five synaptic responses were averaged for further analysis.

Recent investigations have demonstrated that spinal motoneurons are most vulnerable to excitotoxicity (Mazzone et al., 2010) and sensitive to membrane potential changes evoked by gas anesthetics (Marinc et al., 2012). Thus, to monitor their functional responses after excitotoxic stimulation and methoxyflurane application, motoneuron field potentials (FP) due to synchronous motoneuron firing (Fulton and Walton, 1986) were recorded with an extracellular pipette located in the lumbar ventral horn region of sagitally hemisected (24 h after dissection) spinal cords continuously superfused with Kreb's solution (Ostroumov et al., 2011). Lumbar motoneurons were approached blindly through the medial cut surface of spinal cords and identified (in L3–L5 segments) on the basis of their antidromic response to stimulation (2 Hz) of the corresponding VR. After establishing the stimulus threshold value by

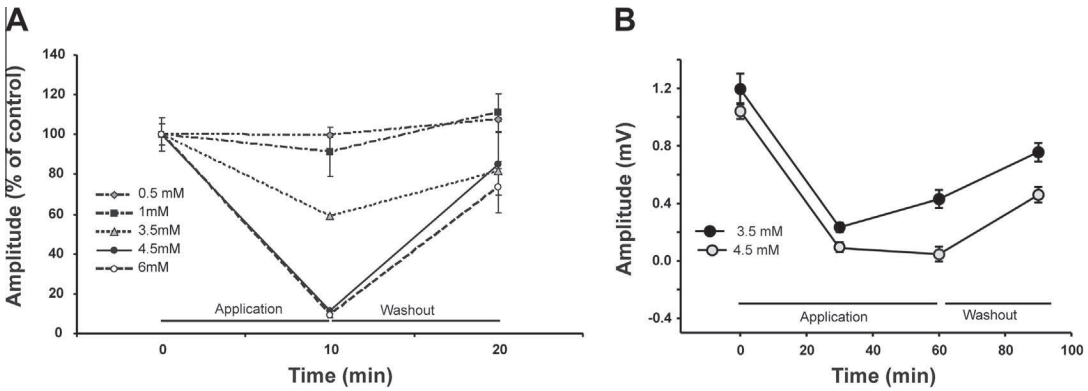

Fig. 1. Changes in DR-VRP amplitude after methoxyflurane treatments *in vitro*. (A) DR-VRP amplitude vs time following application of various concentrations of methoxyflurane. Strong DR-VRP reduction appeared with concentration larger than 1 mM and became very strong at 4.5–6 mM with recovery on washout. Data are from three experiments for each anesthetic concentration. (B) Acute effect of methoxyflurane (MF; 4.5 mM) applied for 1 h at 3.5 mM or 4.5 mM concentration ($n = 3$ for both): complete depression of polysynaptic reflex response was apparent with gradual recovery. Data are represented as mean ± SEM.

straddling the pulse intensity, data were normally collected using as standard stimulus strength a pulse of 5 V (0.1 ms). For analysis, an average of at least 10 sweeps was used for each FP recording. Responses were acquired and analyzed with pClamp software (version 9.2; Molecular Devices, Sunnyvale, CA, USA).

Fictive locomotion (FL) recording.
Extracellular recordings were obtained from left L2 VR (flexor motor signals), left and right L5 VRs (extensor motor signals; Taccola and Nistri, 2006b) in DC-coupled mode. To elicit FL via DR stimulation, electrical stimuli were applied to a single ipsilateral homosegmental DR with a train (30 stimuli at 2 Hz) of pulses (0.1 ms duration). Typical fictive locomotor cycles emerged on top of the cumulative depolarization induced by stimulation strength $\geqslant 2\times$ threshold (threshold taken as the minimum intensity to elicit a detectable response in the homolateral VR; Taccola et al., 2004; Nasrabady et al., 2011a,b). Chemically induced FL was elicited by co-application of *N*-methyl-D-aspartate (NMDA; 2.5–4 μM) (Tocris Bioscience, Bristol, UK) and 5-hydroxytryptamine (5-HT; 10 μM) (Sigma, Milan, Italy) (Kiehn and Kjaerulff, 1998; Taccola et al., 2004; Nasrabady et al., 2011a,b). Continuous generation of at least 20 cycles alternating between homosegmental and left–right $\geqslant$ 3 L VRs was set as criterion for the presence of FL. These rhythmic discharges were analyzed for their period value (time between the onset of two cycles of oscillatory activity), coefficient of variation (CV), and amplitude using at least 20 continuous oscillatory cycles.

Experimental lesion and neuron-protection protocols

The excitotoxic insult mimicking spinal lesion relies on the *in vitro* application of kainate (Taccola et al., 2008) for 1 h in standard Krebs's solution. Kainate was applied at the threshold concentration of 50 μM which is known to abolish locomotor network activity (Mazzone et al., 2010; Nasrabady et al., 2011b).

Along with the excitotoxic lesion by kainate, we applied three protocols for neuroprotection by using the volatile anesthetic agent methoxyflurane (Medical development international Ltd., ABN 14106340667, Victoria, 3171, Australia). Methoxyflurane was applied through perfusion along with Kreb's solution in an airtight container at room temperature (20 °C). The drug inlet tube was positioned in close proximity of the spinal cord preparation to allow changing the bath solution in about 1 min. It is noteworthy that, among the series of halogenated volatile anesthetics, methoxyflurane shows good water solubility and stability (Eger and Eger, 1985; Seto et al., 1992), and it has high affinity for lipids (Elliott et al., 1992; Soares et al., 2012). Before application, vigorous mixing for at least 3 min was performed to ensure its full solubility. Various concentrations (0.5–6 mM) were tested for the acute effect of methoxyflurane on the amplitude of DR-VRPs as exemplified in Fig. 1A for 10 min application and Fig. 1B for 60 min application of the anesthetic. Thus, the concentration of 4.5 mM was chosen as it fully depressed the DR-VRPs in a reversible fashion (even after 60 min application) and accorded with previous findings (Richens, 1969). The first neuroprotection protocol implied co-application of kainate (50 μM) with methoxyflurane (4.5 mM) for 1 h. In the second protocol, kainate (50 μM) was applied alone for first 30 min followed immediately by 30 min co-application with methoxyflurane (4.5 mM) and later by methoxyflurane (4.5 mM) alone for 30 min. The third protocol comprised 1 h application of kainate (50 μM) alone, washout and subsequent application of methoxyflurane (4.5 mM; 1 h) alone. All these treatments were followed by 24 h continuous washout with Kreb's solution at room temperature. Electrophysiological and immunohistochemical data were compared among

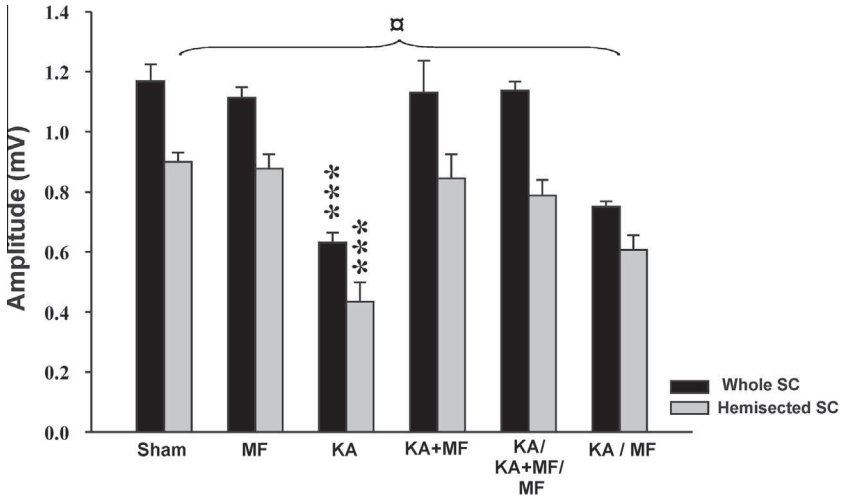

Fig. 2. Changes in DR-VRP amplitude after pharmacological treatments *in vitro*. DR-VRPs generated by electric stimulation ($\geqslant 2\times$ threshold) in whole spinal cord and hemisected spinal cord in sham and treated groups after 24 h *in vitro*. Note significant preservation of DR-VRPs after MF co-applied with kainate (KA + MF), with 30 min delay (KA/KA + MF/MF) or kainate (60 min) followed by methoxyflurane (60 min; KA/MF). $^{***}P \leqslant 0.001$ by one-way ANOVA on rank's (Dunn's test), when KA was compared to sham and all treated groups. $^{¤}P < 0.001$ between sham and KA/MF (KA applied alone for 60 min which was followed by washout with MF alone for 60 min) for hemisected spinal cords; n = 5, 4, 7, 7, 4 for whole SC and 7, 3, 7, 7, 4 for hemisected SC, respectively. Data are represented as mean ± SEM.

Table 1. Effect of treatments on VR polarization level *in vitro*

Experimental group	VR polarization change (mV) Mean ± SEM, *n*
KA (60 min) (50 μM)	2.52 ± 0.36, 4
MF (60 min) (4.5 mM)	−1.67 ± 0.26***, 4
KA + MF (60 min)	1.64 ± 0.26$^{\$}$, 5
KA (30 min)/KA + MF (30 min)/MF (30 min)	0.76 ± 0.24$^{**,\$}$, 5
KA (60 min)/MF (60 min)	0.90 ± 0.29$^{**,\$}$,4

The data refer to the results obtained with the three protocols of neuroprotection (see 'Experimental procedures' section). KA, kainate (50 μM); MF, methoxyflurane (4.5 mM); KA + MF, coapplication for 60 min; KA/KA + MF/MF: 30 min delayed application of MF continuing for further 30 min; KA/MF: application of MF delayed 1 h after kainate. One-way ANOVA (Bonferroni *t*-test) was performed, $^{***}P < 0.001$ and $^{**}P < 0.01$ when compared with KA alone and $^{\$}P < 0.001$ when compared to MF alone.

distinct groups that included, after 24 h *in vitro*, sham (untreated), methoxyflurane-treated, kainate-treated, and kainate plus methoxyflurane-treated with concomitant or staggered application as indicated above.

Immunofluorescence procedure

Full details of the immunohistochemical procedures have recently been published (Cifra et al., 2012). In brief, after electrophysiological analysis, paraformaldehyde-fixed spinal cords were cryoprotected with 30% sucrose and sectioned (35 μm) with a sliding microtome. Immunostaining was performed on T13 to L5 segments, in accordance with our former studies (Cifra et al., 2012; Bianchetti et al., 2013). After incubation in blocking solution (5% donkey goat serum, 5% bovine serum albumin, 0.3% Triton-X 100) for 3 h at room temperature, the primary antibody for ChAT (choline acetyltransferase, goat polyclonal, Chemicon, Millipore, AB144P, used 1:50) was incubated at 4 °C for 2 days to identify motoneurons visualized as large cells (soma diameter > 20 μm) in the ventral horn. For neuronal identification, the NeuN primary antibody (mouse monoclonal, Chemicon, Millipore, MAB377, used 1:50) was added one day later. Primary antibodies were visualized using secondary donkey anti-mouse Alexa fluor 488 or donkey anti-goat 594 antibodies (1:500, Invitrogen, Carlsbad, CA, USA). Sections were finally stained with 4′,6-diamidino-2-phenylindole (DAPI) for 20 min and analyzed as detailed below. All antibodies have been previously used and validated for immunostaining with our preparations (Taccola et al., 2008; Cifra et al., 2012; Bianchetti et al., 2013).

Cell death analysis

The method has been recently reported in detail (Bianchetti et al., 2013). Briefly, using a Zeiss Axioskop2 microscope (Oberkochen, Germany) and Metavue

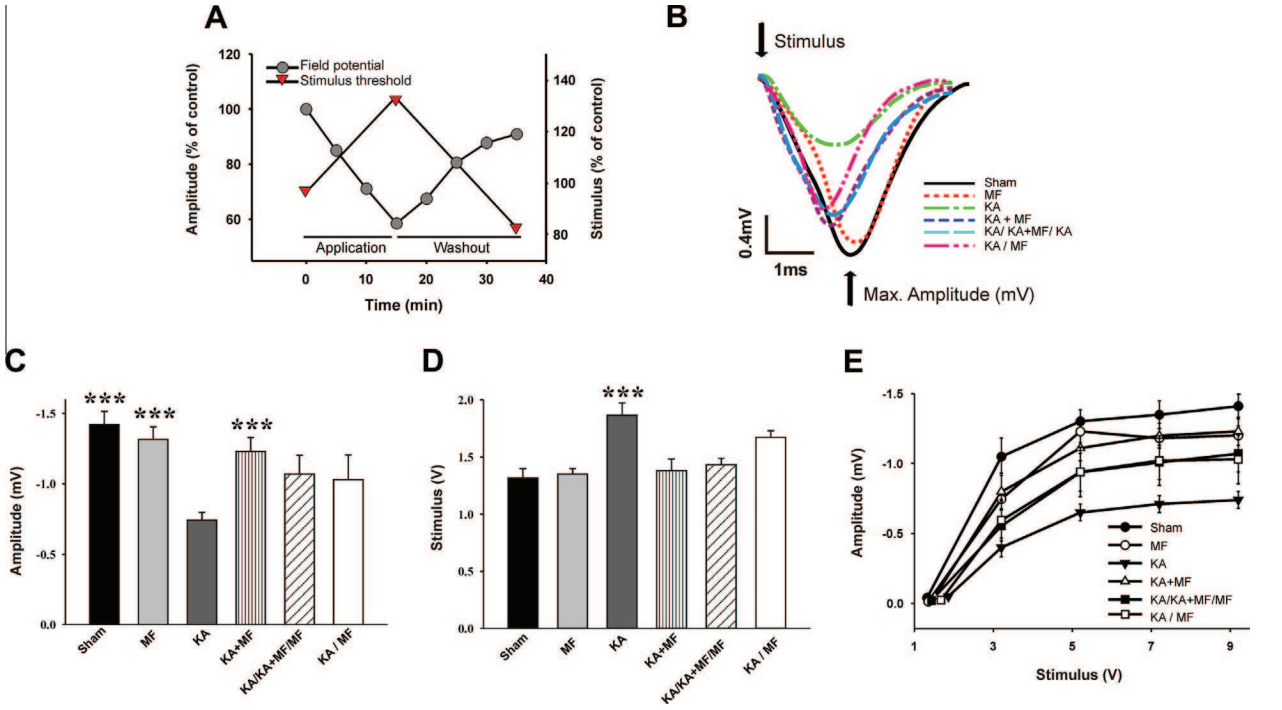

Fig. 3. Changes in antidromic field potentials (FPs) recorded from lumbar VRs. (A) Methoxyflurane (MF; 4.5 mM) reduced the maximum field amplitude (circles) to half within 15 min from the start of its application and also raised the stimulus threshold (inverted triangles), followed by quick recovery on washout. Error bars within data points ($n = 3$). (B) Examples of antidromic FPs superimposed to aid comparison. The arrows point to the record trace where the amplitude value of the FP was recorded. See Fig. 2 legend for abbreviations of protocols. (C and D) Histograms of FP amplitude and stimulus threshold compared among sham and treated groups after 24 h *in vitro*: the largest depression of motoneuron FP and stimulus threshold was found after KA (kainate; 50 μM), an effect strongly prevented by all neuroprotective protocols (KA + MF, KA/KA + MF/MF and KA/MF). (E) input/output curves of antidromic FPs recorded under incrementing stimulus intensity show preventive effect of MF for all stimulus strengths. One-way ANOVA (post hoc Bonferroni *t*-test) was performed, $^{***}P \leqslant 0.001$ when compared to KA. For panels C and E $n = 9, 5, 7, 10, 5, 7$; $n = 15, 7, 14, 11, 11, 7$ for stimulus threshold histograms, respectively. Data are represented as mean ± SEM.

software (Metamorph suite supplied by Molecular Devices, Sunnyvale, CA, USA) to acquire images, cell counting was performed after DAPI staining or NeuN positivity (for neurons). Data were quantified with 'eCELLence' software (Glance Vision Tech., Trieste, Italy). For each histological cross section of the spinal cord, three different areas were investigated as previously reported in detail (Mazzone et al., 2010; Cifra et al., 2012; Bianchetti et al., 2013): dorsal grey matter (Rexed laminae I–IV), central grey matter (Rexed laminae V–VII and X), and ventral grey matter (Rexed laminae VIII–IX). In each area, a 460 × 185 μm region of interest (ROI) was analyzed. For each experimental group, three spinal cords were used and, for each spinal cord, three different sections from T13 to L5 were examined. Pyknosis was readily observed as a change in nuclear morphology resulting from chromatin condensation. Motoneurons were counted in the ventral horn ROI from T13 to L5.

Data analysis

Statistical analysis was done using SigmaStat 3.5 (Systat Software, Chicago, IL, USA). The data were analyzed with two-tailed Student's *t*-test or with analysis of variance (ANOVA; Bonferroni, Tukey, Dunn's) as appropriate. Respective tests are stated in figure captions along with their *P*-values. The significance level was $P \leqslant 0.05$. The numerical data were expressed as mean ± SEM. For electrophysiological experiments *n* refers to number of preparations, while for immunohistochemical experiments *n* indicates the number of tissue sections.

RESULTS

Effect of methoxyflurane on kainate-evoked depression of synaptic reflexes

In the light of results illustrated in Fig. 1A, B, the present study explored how 4.5 mM methoxyflurane (1 h) affected the DR-VRP amplitude recorded from whole or hemisected spinal cord preparations. Fig. 2 summarizes data obtained with the various lesion protocols, namely transient excitotoxic stimulation (50 μM kainate for 1 h; KA) followed by washout for the subsequent 24 h and retesting of DR-VRPs. This approach was also investigated when methoxyflurane was co-applied with kainate for 1 h (KA + MF), or co-applied in the second half of the kainate application and followed by a further 30 min administration (KA/KA + MF/MF), or applied for 1 h after kainate had been administered for 1 h and then washed out (KA/MF). Finally, data were collected also for methoxyflurane application per se (MF). For sake of

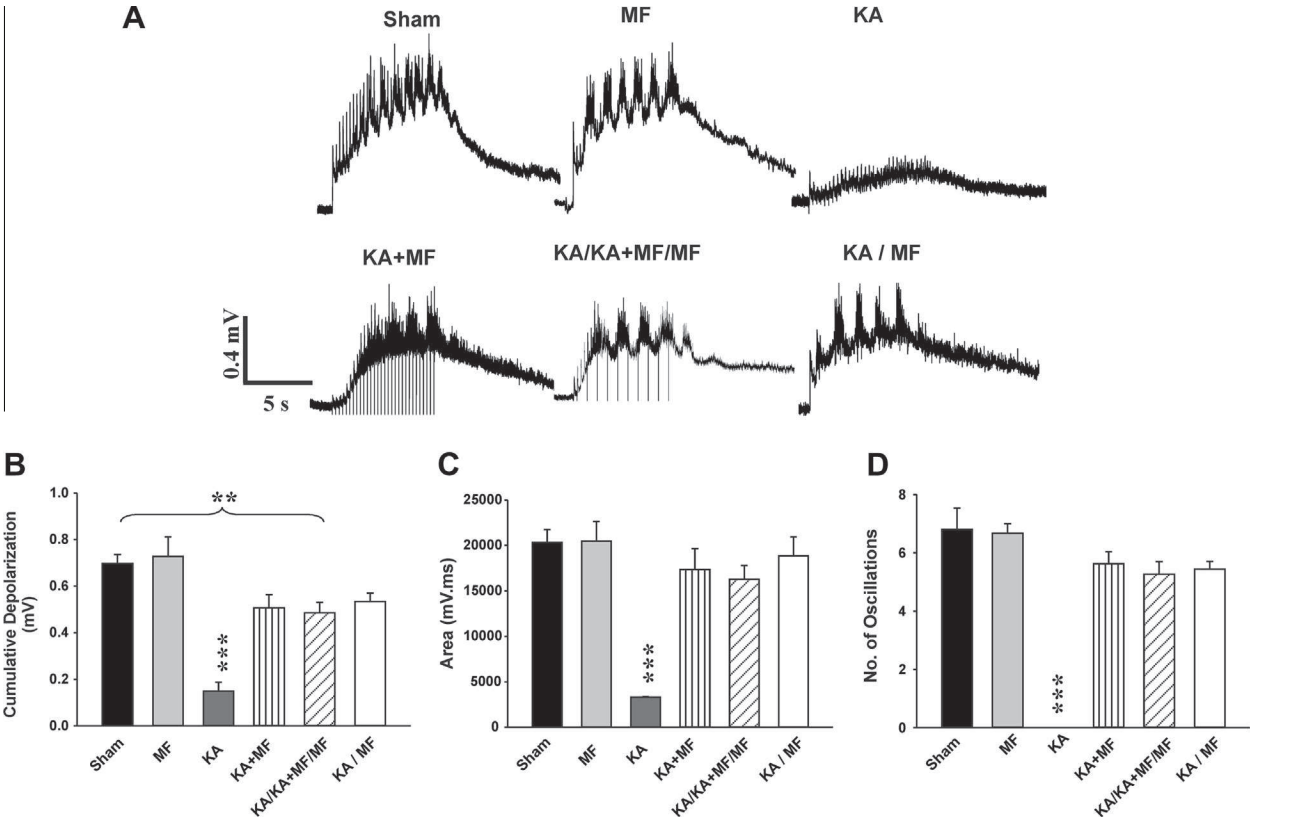

Fig. 4. Neuroprotective effect of methoxyflurane (MF; 4.5 mM) on cumulative depolarization and fictive locomotion (FL) induced by DR electrical stimuli after 24 h *in vitro*. (A) Examples of MF effects alone or applied with or after KA (50 μM): preservation of oscillations superimposed on cumulative depolarization is observed. See Fig. 2 legend for abbreviations of protocols. (B) Histograms of cumulative depolarization amplitude that was significantly decreased when compared to sham values after KA and preserved when MF was applied with the other protocols. (C and D) shows the preservation of area and oscillations in all three neuroprotective (KA + MF, KA/KA + MF/MF and KA/MF) protocols and MF alone has no toxic effect on preparations whereas KA has decreased both values significantly. One-way ANOVA with Bonferroni *t*-test was performed, $^{***}P \leqslant 0.001$ (KA compared to sham and all treated groups), $^{**}P = 0.01$ (sham vs KA/KA + MF/MF), $n = 5, 3, 3, 8, 6, 5$ respectively. Data are represented as mean ± SEM.

comparison, we also show results from whole or hemisected spinal cord preparations because the latter was more suitable to record motoneuron FPs in view of the ease of electrode tracking without crossing the pial surface and potential injury damage. Previous experiments have indicated that the spinal network activity remains fully viable in the rat hemisected preparation (Bracci et al., 1996; Ostroumov et al., 2007, 2011).

While kainate alone largely depressed DR-VRPs recorded one day later in accordance with previous data (Mazzone et al., 2010), this effect of kainate was fully prevented when methoxyflurane was co-applied even when the anesthetic was started with a 30 min time lag or after kainate washout, indicating effective neuroprotection of polysynaptic transmission. In the rat spinal cord, kainate is known to induce a large VR depolarization (Taccola et al., 2008; Nasrabady et al., 2011b). Table 1 summarizes the data of VR polarization changes recorded using the various experimental protocols. Thus, the average VR depolarization recorded 60 min from the start of 50 μM kainate application was 2.52 ± 0.36 mV (n = 4) vs pre-drug baseline, whereas the value recorded 60 min after application of methoxyflurane alone was −1.67 ± 0.26 mV (n = 4), demonstrating opposite effects on VR polarization. When kainate was applied together with methoxyflurane, or the anesthetic was applied later (30 or 60 min), a significantly smaller amplitude of the VR depolarization was observed. This finding shows that the spinal network depolarization evoked by KA was largely reduced by MF.

Methoxyflurane contrasted depression of motoneuron firing by kainate

Our former report (Mazzone et al., 2010) has indicated that spinal motoneurons are highly vulnerable to kainate-induced excitotoxicity and that substantial motoneuron loss (even after moderate kainate concentrations) is a crucial process to impair spinal network function. We, thus, investigated whether methoxyflurane could protect motoneuron function monitored as synchronous firing of one segmental motoneuron population in the face of kainate challenge. First, we explored how methoxyflurane could affect motoneuron spiking by measuring the maximum FP amplitude and threshold recorded from lumbar VRs (Fig. 3A). Methoxyflurane (4.5 mM) nearly halved the response magnitude within 15 min and largely raised the firing threshold with rapid recovery on washout. When tests were extended to preparations after 24 h *in vitro* from the drug administration protocols, as exemplified in Fig. 3B (average antidromic FPs are superimposed to aid comparison), differing degrees of FP reduction were observed. Hence, the largest depression of the motoneuron FP was detected 24 h after kainate (50 μM) with strong prevention when methoxyflurane was administered together with (or staggered after) kainate. The anesthetic alone induced insignificant decrease in the motoneuron FP. These data are quantified in Fig. 3C, D. Fig. 3E depicts input/output plots for antidromic FPs recorded under various protocols, indicating that methoxyflurane could prevent the depressant action by kainate regardless of the number of activated motoneurons that varied in relation to the stimulus intensity.

Fictive locomotion protection by methoxyflurane

Former studies have indicated that even a moderate excitotoxic lesion induces a clear dissociation between limited histological damage and complete loss of FL despite preservation of reflex activity (Mazzone et al., 2010; Kuzhandaivel et al., 2011). In this scenario the vulnerability of motoneurons is thought to play an important role. The present study investigated whether methoxyflurane could prevent the disappearance of

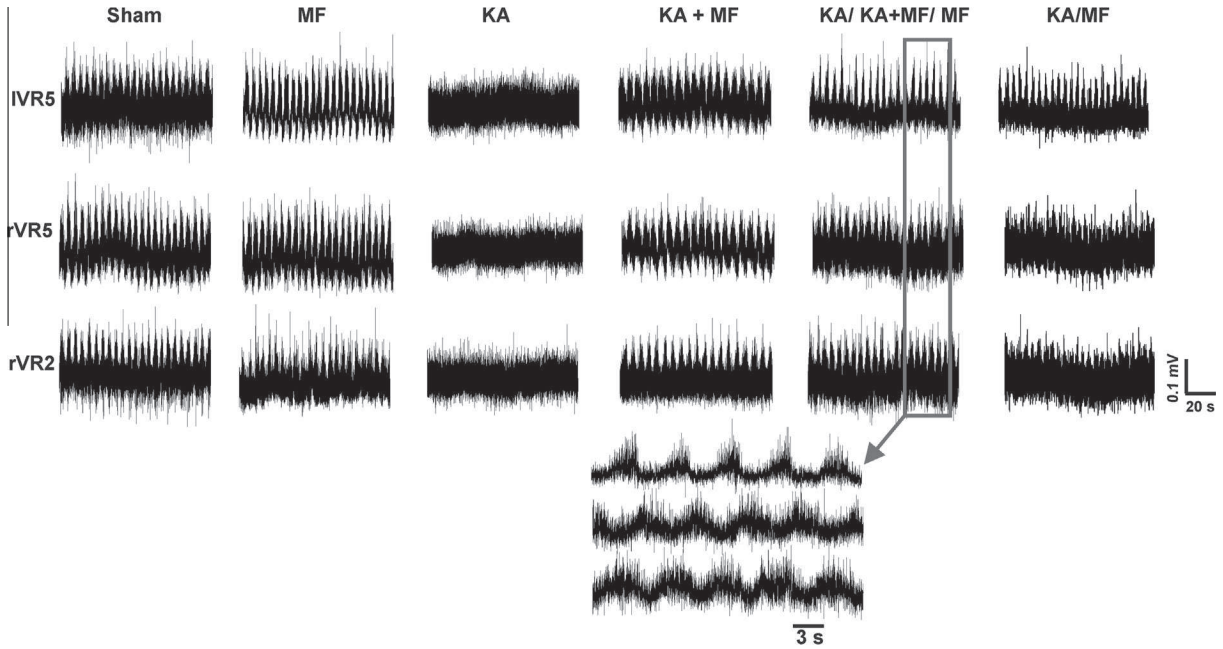

Fig. 5. Effect of methoxyflurane (MF; 4.5 mM) on fictive locomotion (FL) induced by chemical stimuli (2.5–4 μM NMDA and 10 μM 5HT) after 24 h *in vitro*. VR records show alternating cyclic oscillations in control conditions, or after MF applied alone or with the excitotoxic insult (kainate (KA); 50 μM). See Fig. 2 legend for abbreviations of protocols. The higher gain inset indicates alternating cycles between lumbar VRs.

locomotor network activity evoked by kainate. To this end, we induced FL with either repeated DR stimuli or co-application of NMDA and 5HT. Fig. 4A shows that one day after 1 h application of 50 μM kainate, the amplitude of cumulative depolarization was decreased and oscillations typical of locomotor networks (Marchetti et al., 2001) were abolished. When methoxyflurane was co-applied with kainate, oscillations were preserved (Fig. 4A, D) even though the cumulative depolarization amplitude was decreased vs sham values (Fig. 4B), while the total depolarization area was preserved (Fig. 4C).

Likewise, sustained locomotor cycle activity elicited by NMDA and 5HT was suppressed 24 h after kainate, yet retained when kainate had been applied together with methoxyflurane as exemplified in Fig. 5 in which the higher gain inset indicates alternating cycles between lumbar VRs. Average data are summarized in Fig. 6A–C that indicates good preservation of cycle amplitude and period (and its CV value) by methoxyflurane, notwithstanding kainate application.

Histological characteristics of spinal cord preparations following excitotoxic protocols

Fig. 7A shows percent values of pyknotic nuclei (markers of cell death) under various experimental conditions. As reported earlier (Nasrabady et al., 2011b), pyknosis evoked by kainate was the highest in the dorsal horn area and was significantly prevented in all three ROIs when this agent was applied together with methoxyflurane. Likewise, neuronal numbers, even in the most sensitive ROI, were fully preserved (Fig. 7B) as exemplified in Fig. 7C in which neurons were identified with NeuN staining (green) and cell nuclei with DAPI (blue).

As motoneurons are a small population of cells in the spinal network (Cifra et al., 2012), their lowered number may strongly impact network activity. Analysis of motoneuron numbers identified on the basis of their ChAT immunoreactivity observed in sections from T13 to L5 segments shows that they were substantially reduced 24 h after kainate application (Fig. 8A, B), a phenomenon fully prevented by co-application of methoxyflurane either together or at the end of kainate administration.

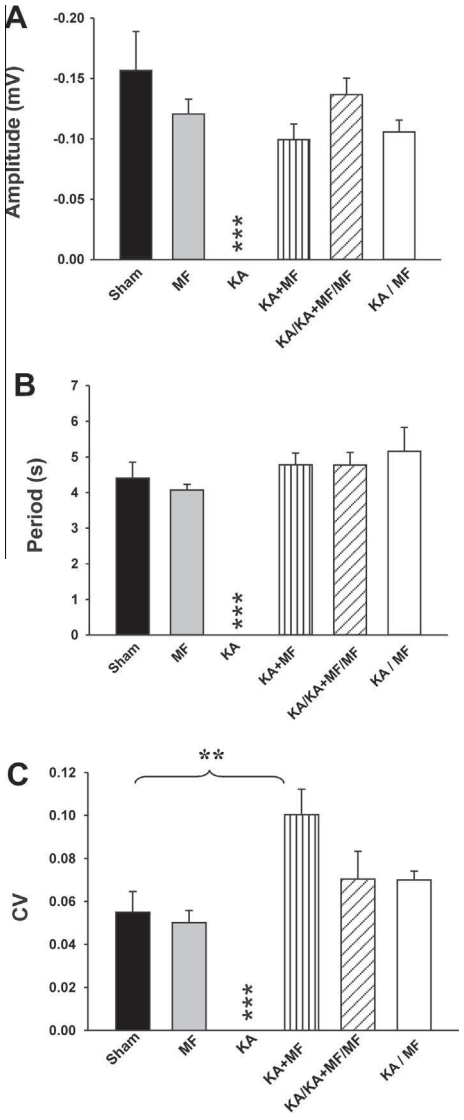

Fig. 6. Chemically induced fictive locomotion (FL) in *in vitro* spinal cord preparations after 24 h. See Fig. 2 legend for abbreviations of protocols. (A–C) FL was completely absent in KA treated SC, whereas in all the other groups cycle amplitude and period (and its CV, coefficient of variance) were well preserved, indicating good neuroprotection of locomotor networks. One-way ANOVA (Bonferroni *t*-test) was performed, $^{***}P \leqslant 0.001$ (KA compared to sham and all treated groups), $^{**}P = 0.01$, $n = 6, 3, 3, 8, 6, 4$ respectively. Data are represented as mean ± SEM.

DISCUSSION

The principal result of the present study is the demonstration that the volatile anesthetic MF was effective to protect spinal locomotor networks from excitotoxicity. This observation included electrophysiological as well as histological protection that was afforded even to motoneurons that are known to be very vulnerable to this type of injury.

Choice of methoxyflurane as a neuroprotective agent

In vitro studies, like the present one, are best suited to investigating drugs which can be applied via the bathing solution (Kreb's medium). Thus, among the wide series of halogenated volatile anesthetics, methoxyflurane appeared to be a convenient agent because of its chemical and physical properties (Andersen and Andersen, 1961; Eger and Eger, 1985; Seto et al., 1992; Soares et al., 2012). In general, when their administration is prolonged, volatile anesthetics may produce toxic effects including apoptosis in the nervous tissue (Sanders et al., 2008; Zhao et al., 2011). Furthermore, methoxyflurane administered to man can release fluoride with toxic effects on peripheral organs like the kidney (Kharasch et al., 1995, 2006), making this drug suitable only for short term use. For these considerations, the present investigation employed 1 h as the maximum duration of methoxyflurane application (either together with kainate or delayed after the start of kainate administration). This protocol is supported by the recent finding that

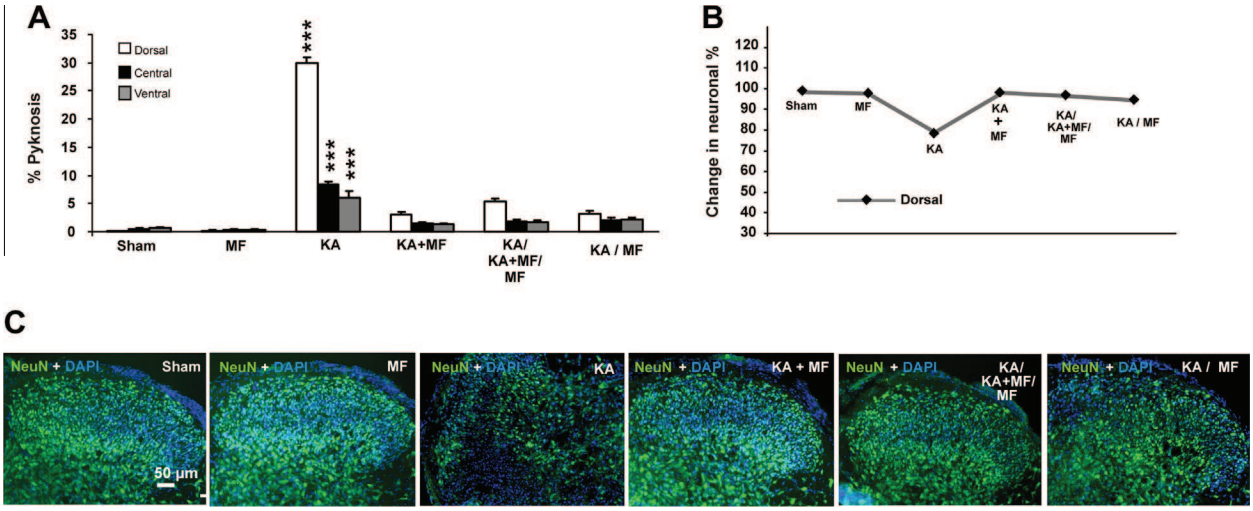

Fig. 7. Histological changes in the rat spinal cord *in vitro* following excitotoxic protocols. See Fig. 2 legend for abbreviations of protocols. (A) Histograms show percentage of pyknotic cells in the different grey matter ROIs (dorsal, central and ventral horn). Note statistically significant decrease in pyknotic cell number in all ROIs from samples treated with methoxyflurane (MF; 4.5 mM) after the KA (kainate; 50 µM) insult versus KA treated samples. (B) Decrease in dorsal horn neuronal number after KA damage, while good preservation was observed in all groups treated with MF. Data are the average taken from nine sections from three rats. (C) Examples of dorsal horn neuronal staining from all protocols as this region is normally showing the highest neurotoxic action by kainate. $^{***}P \leqslant 0.001$ when data are compared with KA alone (Mann–Whitney test). Data are represented as mean ± SEM.

a short application of a volatile anesthetic can be highly neuroprotective against rat brain ischemia (Ren et al., 2014). In accordance with these data, the present study never observed any neurotoxic action by 1 h application of methoxyflurane in terms of electrophysiological or histological parameters.

The concentration of methoxyflurane (4.5 mM) for the majority of the current tests was empirically selected on the basis of its rapid and reversible depression of electrophysiological responses. The anesthetic concentration of methoxyflurane expressed as 1 MAC (minimum alveolar concentration) in man is 0.16% (Quastel and Saint, 1986; Elliott et al., 1992). Previous reports indicate that clinically relevant concentrations do not suppress neuronal activity completely (MacIver et al., 1989; Elliott et al., 1992; Vahle-Hinz and Detsch, 2002). In the present study the *in vitro* system was devoid of circulatory support and required a concentration comparable to the human plasma free concentration of methoxyflurane (low mM range) to provide general anesthesia (Eger and Eger, 1985; Soares et al., 2012). It is interesting that, in addition to its anesthetic properties, methoxyflurane is a validated agent to produce analgesia after acute trauma (Coffey et al., 2014).

Methoxyflurane induced neuroprotection

Former studies have shown kainate to be an effective drug to induce delayed excitotoxicity which was the strongest in the dorsal horn (where most neurons (Cifra et al., 2012) and kainate-sensitive glutamate receptors (Agrawal and Evans, 1986; Wang et al., 2005; Pinheiro and Mulle, 2006) are found) and on motoneurons that, 24 h later, were more than halved in numbers. It is noteworthy that the present protocols demonstrated a relatively slow onset of synaptic or motoneuron depression by methoxyflurane, and partial inhibition of the neuronal depolarization evoked by kainate in analogy with the recent report that a general anesthetic antagonizes kainate-mediated depolarization in the frog spinal cord *in vitro* (Daló and Hackman, 2013). Thus, efficient prevention of neurotoxicity could be detected even with slowly developing pharmacological responses evoked by methoxyflurane. This finding supported the protocol of methoxyflurane application with a 30 or even 60 min delay after the start of kainate administration: in either case strong neuroprotection was always observed. Motoneuron field potentials were uniformly protected (in terms of amplitude and stimulus threshold) in line with the full preservation of the motoneuron numbers measured immunohistochemically.

A simple interpretation of these data is that administering methoxyflurane effectively stopped the slow emergence of the non-apoptotic cell death process typically triggered by excitotoxicity (Kuzhandaivel et al., 2011) and peaking within the first 1–2 h.

The *in vitro* spinal cord preparation is intrinsically unsuitable for long term investigations into the locomotor network function because of poor survival after 24 h. However, organotypic slice cultures of the rat spinal cord are amenable to long term studies stretching over a few weeks and have shown that the neuronal damage caused by excitotoxicity is complete within the first 24 h from the lesion (Mazzone et al., 2013).

It is useful to recall that the current data were obtained with an *in vitro* preparation which, while being a useful model for SCI preclinical studies (Kuzhandaivel et al., 2011), clearly differs from the clinical setting of human SCI. Nevertheless, tissue immaturity of the neonatal rat spinal cord may impart certain properties similar to those of the excitotoxic process of the human neonatal brain (Hagberg, 1992) whose neurotoxicity is significantly inhibited by the gas anesthetic isofurane (Burchell et al., 2013).

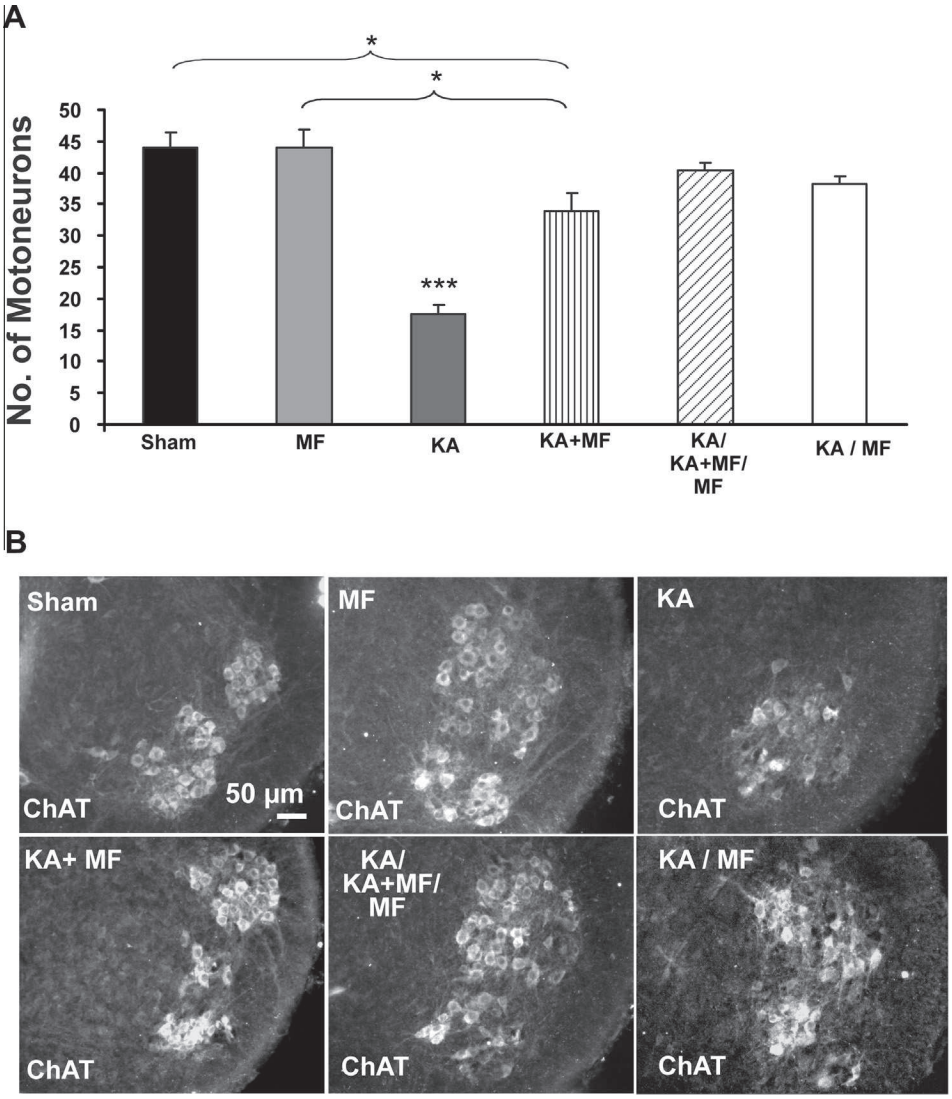

Fig. 8. Motoneuron number change after excitotoxic KA (kainate; 50 µM) treatment *in vitro*. (A) Histograms show the number of motoneurons counted in ventral ROI. See Fig. 2 legend for abbreviations of protocols. The number of motoneurons statistically decreased after KA excitotoxicity, while it was preserved in all groups after application of MF (4.5 mM). Examples of motoneuron ChAT staining are shown in (B) from each protocol. Data are the average taken from nine sections from three rats. One-way ANOVA (Bonferroni *t*-test) was used, $^{***}P \leqslant 0.001$; $^{*}P = 0.02$ (sham vs KA + MF); $^{*}P = 0.03$ (MF vs KA + MF). Data are mean ± SEM.

Preservation of locomotor network function

Previous studies have shown how fictive locomotion is readily lost after an excitotoxic stimulation that may leave spinal reflexes relatively preserved (Taccola et al., 2008; Mazzone et al., 2010; Nasrabady et al., 2011b). In particular, fictive locomotion induced by a train of DR pulses is the most prone to neurotoxicity so as it is difficult to protect it even with the mechanism-targeted drug N-(6-Oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino) acetamide hydrochloride (PJ-34) (Nasrabady et al., 2011b). It was, therefore, important to observe that, despite some reduction in the cumulative depolarization elicited by DR pulse trains, oscillatory VR discharges were preserved. This observation was also supported by demonstrating that chemically evoked fictive locomotion was also preserved 24 h later when methoxyflurane had been applied together with or after kainate. The unchanged periodicity of the locomotor rhythm when compared to sham preparations suggests that, within the organization of the central pattern generator for locomotion (central pattern generator (CPG); Kiehn, 2006; Brownstone and Wilson, 2008), the ''clock'' oscillatory function (Taccola and Nistri, 2006a; McCrea and Rybak, 2008) had been well protected. Hence, not only motoneurons received a beneficial treatment, but also oscillatory interneurons and premotoneurons retained their full functional activity. To the best of our knowledge, a relatively brief application of methoxyflurane has so far been the most effective neuroprotective tool in our experimental model.

Clinical neurophysiological monitoring of SCI patients at early time after the acute SCI have shown that irregular electromyographic discharges can be recorded from various leg muscles and are related to pathological processes like spasticity (McKay et al., 2011). Although

there is, so far, no evidence for long term expression of real locomotion in the rat once the spinal cord has been protected with methoxyflurane against kainate toxicity, it seems likely that the alternating VR rhythmicity found one day after the lesion was indeed the expression of the locomotor CPG because similar patterns are observed in man when there is locomotor recovery from SCI (McKay et al., 2012).

Clarification of the mechanisms underlying this action by methoxyflurane will require future experiments. It is likely that neuroprotection was due to combinatorial effects such as depression of synaptic transmission, reduced axonal firing and decreased intrinsic neuronal excitability as these are common actions exerted by gas general anesthetics (De Jong et al., 1968; Collins et al., 1995; Vahle-Hinz and Detsch, 2002). It is, however, noteworthy that simple reduction of glutamatergic transmission and voltage-activated Na^+ conductances by riluzole (Cifra et al., 2013) is inadequate for early neuroprotection against excitotoxicity in the rat spinal cord (Sámano et al., 2012), although clinical data indicate that twice-daily administration of riluzole for 2 weeks induces a favourable outcome in the case of spinal cervical injury (Grossman et al., 2014). Neuroprotective effects of riluzole have also been reported for experimental ischemic damage to the rat spinal cord (Wu et al., 2014).

It is tempting to suggest that, like related anesthetics, methoxyflurane activated background K^+ conductances such as TASK1 (Sirois et al., 2000; Lazarenko et al., 2010) strongly expressed in spinal motoneurons (Marinc et al., 2012). This phenomenon would be consistent with the detected VR hyperpolarization and is likely to constrain neuronal excitation and downsize related intracellular Ca^{2+} overload that, during excitotoxicity, develops slowly (Szydlowska and Tymianski, 2010) to trigger the development of cell damage in the spinal cord (Kuzhandaivel et al., 2011). This hypothesis requires further work especially when specific tools to activate or block such channels become available. Future experiments will also be necessary to find out if the neuroprotective action by methoxyflurane might be observed with other commonly used anesthetics like propofol: this is an interesting issue since it may help to clarify if spinal network neuroprotection is a common feature of general anesthesia or specific to the mechanism of action of methoxyflurane. The relative contribution by propofol or gas anesthetics to neuroprotection and the related mechanisms have recently been discussed (Zhou et al., 2012).

CONCLUSIONS

Present guidelines for treating acute spinal injury recommend intensive care to correct homeostatic imbalance, and to maintain life supporting functions (Donovan, 1994; Fehlings et al., 2010). Early neurosurgical intervention is also proposed to stabilize the lesion and to relieve tissue compression. SCI patients subjected to early neurosurgical intervention are reported to have a better outcome and the same is proposed for preclinical animal studies (Cadotte and Fehlings, 2011; Furlan et al., 2011, 2013). It is interesting that complex neurosurgery requires general anesthesia, a procedure probably contributing to damage limitation if applied as soon as possible. We suggest that the present study is the first one to validate, under strictly controlled experimental conditions, the usefulness of a volatile anesthetic to protect spinal neurons and to ensure that spinal locomotor network function remains intact despite a potent neurotoxic challenge.

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Materials, methods and results

Section II

Motoneuron survival after excitotoxic stress is related to HSP70 expression in a rat spinal cord injury model *in vitro*.

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Excitotoxicity-induced motoneuron cell death is related to insufficient HSP70 expression in a rat spinal cord injury model in vitro

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Running Title: HSP70 and cell death in damaged spinal cord.

Abstract

Acute spinal cord injury is often anatomically incomplete, yet it produces paralysis with uncertain outcome for gait recovery despite early intensive care. In an in vitro model of the rat spinal cord in which injury was mimicked with moderate excitotoxic insult, we have observed how this paradigm readily suppressed locomotor network activity together with motoneuron death, arising slowly via a parthanatos-like mechanism and always limited in number. We wondered whether survival of motoneurons was related to their expression of HSP70, a cytosolic neuroprotective protein binding and sequestering metabolic distress-generated proteins. In the present experiments excitotoxicity evoked by kainate (1 h application) induced delayed (24 h), moderate loss of motoneurons that became pyknotic with nuclear expression of the apoptosis inducing factor (AIF), a known biomarker of cell death. Surviving cells showed strong expression of HSP70 with no nuclear AIF. The HSP70 pharmacological inhibitor VER155008 per se induced neurotoxicity comparable to the action of kainate in extent and AIF nuclear translocation. Electrophysiological recording indicated depression of motoneuron field potential with strongly decreased excitability and impaired synaptic transmission following kainate or the HSP70 blocker. Their combined application elicited more intense neurotoxicity. Interestingly, motoneurons in the spinal cord (24 h in vitro) showed large expression of HSP70 in comparison with freshly dissected tissue, suggesting that HSP70 upregulation was critical for survival in vitro. Our data indicate that HSP70 was one important

contributor to motoneuron survival from acute injury, outlining a potential future strategy to neuroprotect these cells by enhancing the activity of this protein.

Keywords

Heat shock proteins; parthanatos; excitotoxicity; AIF; spinal networks

Abbreviations

KA, kainic acid (kainate); HSP70, heat shock protein 70; VER155008, 5'-*O*-[(4-cyanophenyl)methyl]-8-[[(3,4-dichlorophenyl) methyl]amino]-adenosine; AIF, apoptosis inducing factor; DR, dorsal root; VR, ventral root; DR-VRPs, dorsal root ventral root potentials; FP, field potential; ChAT, choline acetyltransferase; DAPI, 4',6-diamidino-2-phenylindole; AU, arbitrary unit; NADH, nicotinamide adenine dinucleotide; ATP, adenosine triphosphate; DMSO, dimethyl sulfoxide.

Introduction

Excitotoxicity is one of the hallmarks of acute spinal cord injury (SCI) and develops rapidly[1] because of increased levels of extracellular glutamate.[2,3] We have recently set up an in vitro model of SCI caused by the glutamate analogue kainate[4–6] and observed that the extent of neuronal damage is typically self-limited in close analogy to most clinical cases where the primary lesion is often anatomically incomplete.[7–9] Among spinal neurons, motoneurons are highly vulnerable to excitotoxicity that triggers a non-apoptotic cell death mechanism (parthanatos[2,10,11]). It is, however, noteworthy that a substantial number of motoneurons survive. We enquired what mechanisms might be responsible for tilting the fine balance between cell survival and death, and focused our study on Heat-Shock Protein 70 (HSP70) that has been reported to block both apoptosis and parthanatos by binding to protease activating factor-1 (Apaf-1) thereby preventing the constitution of the apoptosome, or by binding to apoptosis inducing factor (AIF), preventing the AIF-mediated chromatin condensation.[12–15]

HSP70 is an ATP-dependent molecular chaperone that, under normal conditions, assists the folding of newly synthesized polypeptides, the assembly of multi-protein complexes, and the transport of proteins across cellular membranes.[16,17] Under various stress conditions, the synthesis of HSP70 enhances the ability of cells to cope with increased concentrations of unfolded or denatured proteins.[18] Neurons are particularly vulnerable to protein misfolding and to intracellular accumulation of insoluble protein aggregates since they cannot dilute their level by cell division.[16] The increased expression of HSP70 has neuroprotective effects in various animal models of neurodegenerative diseases[19] and this effect is dependent on its ability to bind AIF and to inhibit programmed cell death.[20,21]

Spinal motoneurons show high levels of HSP70 in various neurodegerative disease models[19] and after ischemia of the rat spinal cord.[22,23]

The aim of present study was to investigate the spinal cell expression of HSP70 following kainate-mediated excitotoxicity and after pharmacological block of HSP70 to unveil its potential role in preventing cell death in rat spinal cord in vitro and in preserving electrophysiological activity. To this end, we used the HSP70 inhibitor VER155008 (5'-*O*-[(4-cyanophenyl)methyl]-8-[[(3,4-dichlorophenyl) methyl]amino]-adenosine)[24] that was initially introduced as a sensitizing agent to chemotherapy against cancer cells relying on HSP70 for their survival.[25–27] VER15508 is known to antagonize the neuroprotective action of HSP70 after mild traumatic brain injury.[28]

Results

Excitotoxicity-evoked cell death of motoneurons

Previous reports have shown that transient application of kainate (≥50 μM concentration) induces delayed cell death of spinal neurons, especially of motoneurons even though a significant number of them survives.[2,4] In the present study, we first validated the extent of motoneuron death observed 24 h after the application (1 h) of 50 or 100 μM kainate (as exemplified in Figure 1a and b): the number of large ventral horn cells immunopositive to ChAT (thereby identified as motoneurons) was approximately 2/3rd of the value in sham preparations kept for 24 h in vitro. In keeping with previous observations, the global number of dead motoneurons was not dependent on the kainate concentration once the neurotoxic threshold had been passed.[4] A comparable degree of motoneuron loss was obtained when we applied for 24 h VER155008 (50 μM; see

supplemental Figure S1 for biological tests to validate this concentration). This observation suggested that effective HSP70 activity was important to ensure survival in in vitro conditions. Thus, it seemed interesting to explore if HSP70 played a major role in survival against excitotoxicity.

HSP70 expression in control or excitotoxic conditions

We, therefore, investigated motoneuron immunoreactivity for HSP70 that might be a mechanism for neuroprotection,[29,30] and for AIF taken as an index of cell death.[2,31] Figure 2a shows examples of double immunoreactivity of motoneurons (24 h in vitro) and expressing HSP70 (green), and/or AIF (red), while cell nuclei were visualized with DAPI (blue). In sham conditions (24 h in vitro), most motoneurons were immunopositive for HSP70 and negative for AIF (Figure 2a1). Following 50 μM kainate application, motoneurons with AIF positivity as well as motoneurons expressing HSP70 were clearly visible 24 h later (Figure 2a2); a group co-expressing HSP70 and cytoplasmic AIF was also present. After 100 μM kainate application, the AIF expression was stronger and fewer motoneurons were HSP70 positive (Figure 2a3). Clear AIF immunoreactivity was also observed 24 h following the application of 50 μM VER155008 with a few motoneurons stained for both AIF and HSP70 (Figure 2a4). This observation is consistent with the reportedly distinct sites for VER155008 and AIF binding on HSP70.[12,13,15,32]

The histograms of Figure 2b quantify the percent value of motoneurons (24 h in vitro) expressing nuclear AIF under the various experimental protocols. The highest value was observed following 100 μM kainate application, whereas sham preparations were essentially negative.

We next enquired how HSP70 was expressed in single motoneurons after administration of kainate or VER155008. Figure 2c shows the average fluorescence intensity (AU) for single cell HSP70 expression. Twentyfour h after 50 or 100 μM kainate, the signal intensity distinguished two motoneuron groups. One showing HSP70 positivity (with no AIF nuclear expression) observed in sham preparations as well as in the majority of kainate or VER155008 treated spinal cords (see shaded bars). Conversely, the second group (absent in sham preparations) showed weaker HSP70 positivity (together with AIF nuclear translocation; open bars) in kainate or VER155008 treated spinal cords.

Contrasting expression of AIF and HSP70 in motoneurons

To further analyze the distribution of these two markers within single motoneurons, we used line scan confocal microscopy of cells in sham conditions or after 50 μM kainate (Figure 3). In this example from a sham preparation, the z-stack analysis was run at the site (pink circle) of crossing of the two pink lines to generate stack images as indicated at the bottom and right margin of Figure 3a. Figure 3b shows the motoneuron sample cell with DAPI-stained nucleus and poor AIF expression used for the line scan (yellow) across its soma. The graphs of Figure 3b1 show the distribution between cytoplasmic and nuclear compartment of DAPI (blue), AIF (red) and HSP70 (green). It is noteworthy that the AIF signal was very low throughout the line scan, while HSP70 was found predominantly outside the nucleus. Figure 3c shows that, on the contrary, following 50 μM kainate treatment (1 h), two patterns of AIF expression were detected. In the cell labeled d1 in Figure 3d, AIF was clearly expressed in the cytoplasm with a signal spike just appearing in the juxta-membrane region of the nucleus, while HSP70 was uniformly

expressed at low level. The cell labeled as d2 (Figure 3d) showed strong AIF expression in all three compartment and poor HSP70 expression. In Figure 3d1-d2, both cells displayed, after kainate treatment, strong signal for DAPI (blue line) perhaps indicative of developing chromatin condensation concurrent with cell death, which is a hallmark of kainate-evoked excitotoxicity.[33,34] These data suggest that AIF nuclear translocation was associated with poor HSP70 expression.

Functional changes in motoneuron activity after kainate application and HSP70 inhibition

Previous studies have shown irreversible electrophysiological deficit of motoneurons following excitotoxicity.[4,35] In the present report, we first investigated whether the damage evoked by 50 or 100 μM kainate was intensified by the application of VER155008 (50 μM). To isolate motoneuron electrical activity, we recorded the motoneuron field potential (FP) evoked by antidromic stimulation of a single VR as exemplified in Figure 4a. This response represents the synchronous spike generation by the motoneurons contained in the lumbar segment of the stimulated VR. Kainate or VER155008 similarly depressed the field potential, a phenomenon synergized when the two drugs were sequentially applied (1 plus 24 h). Not only the amplitude but also the excitability of motoneurons was significantly depressed (Figure 4b and c). In particular, it was necessary to treble the stimulus strength to evoke the field potential after kainate plus VER155008 application (Figure 4c and d). The depression of field amplitude was uniformly observed for various stimulus intensities suggesting that functionally distinct classes of motoneuron with differential excitability were all similarly depressed (Figure

4d). Qualitatively similar data were obtained with 100 μM kainate, although the field amplitude depression was larger and the effect of combining kainate with VER155008 even more depressant (Figure 4a-d).

We next enquired whether a concentration of kainate (10 μM), which is sub-threshold for excitotoxicity,[4] could synergize with the administration of VER155008 to damage motoneurons. Figure 5a-d shows that, while the functional depression of the field potential by 10 μM kainate was modest, subsequent application of VER155008 elicited a strong decrease in field amplitude and stimulus threshold, clearly larger than that produced by VER155008 alone.

We also explored whether synaptic transmission which is known to be impaired after kainate application[33] was depressed by VER155008. Table 1 summarizes the data pertaining to polysynaptic reflex amplitude (DR-VRP) induced by single DR stimulation applied to the same lumbar segment from which the field potential was recorded. Thus, kainate had minimal effect at 10 μM, and a strong reduction at 50-100 μM. VER155008 alone halved the reflex amplitude and synergized the depression evoked by kainate. It is noteworthy that VER155008 applied after 100 μM kainate led to almost complete disappearance of reflex activity, indicating extensive functional damage to the spinal networks beyond the motoneuron neurotoxicity.

These data indicated that efficient HSP70 activity was probably important to contrast the excitotoxic damage elicited by kainate, and that it was also important for the survival of sham preparation for 24 h in vitro, in view of the deleterious effects produced by VER155008 per se. This latter issue was explored by comparing HSP70 expression in intact spinal cord tissue fixed immediately after dissection versus preparations kept in

vitro for 24 h. Figure 6a shows that, in a freshly fixed preparation, immunohistochemical expression of HSP70 in motoneurons was rather low by comparison with the one seen after 24 h in vitro. Figure 6b indicates that the HSP70 signal was significantly stronger 24 h in vitro versus freshly fixed preparations.

Discussion

The principal finding of the present study is that, in an excitotoxicity model of SCI in vitro, strong expression of HSP70 was an important contributor to limit motoneuron cell death. When HSP70 was pharmacologically inhibited, a significantly larger histological and functional damage emerged. These results suggest that, in future, exploiting the neuroprotective properties of HSP70 might be a helpful strategy to improve motoneuron survival after acute SCI.

The in vitro model of SCI

Our previous studies have indicated that the neonatal rat spinal cord is an advantageous model to investigate basic mechanisms of cell death, especially those concerning the secondary lesion that amplify the severity of the initial insult.[4,5,33] The limited degree of tissue damage is not directly related to the electrophysiological dysfunction because the locomotor circuitry is highly sensitive to these protocols with ensuing arrest of its activity. This preparation is, of course, immature, yet it recapitulates most of the characteristics of the in vivo SCI including the delayed onset of secondary damage, the incomplete nature of the histological loss, and the principal cell death mechanisms (parthanatos) also detected in vivo.[36,37] It is also worth noting that prenatal and neonatal

SCI is not uncommon in humans,[38–40] thereby making the present model relevant also to the understanding of neonatal SCI pathophysiology.

In vitro and clinical cases of acute SCI are characterized by the gradual onset of secondary damage, implying that there is an ongoing contrast between injury-progression mechanisms and intrinsic neuroprotective processes. In the current study, we focused on the role of HSP70 because its expression starts to rise after injury with a minimum delay of 30 min, and it reaches a maximum at 24 h in close time correlation with our experimental protocols.[22,23,25]

HSP70 as a gatekeeper of motoneuron survival pathways

Previous reports have shown that, after excitotoxic injury, spinal motoneurons die mainly through a process of programed cell death termed parthanatos, although a substantial number of these cells can survive the insult.[2] The present study supports the notion that their survival is in relation to HSP70 expression whereby high levels of HSP70 protect motoneurons from parthanatos by preventing nuclear translocation of AIF.

Programmed cell death pathways such as apoptosis or parthanatos are major physiological process responsible for the demise of damaged cells and are involved in the pathophysiology of SCI.[2,5,35,41–44] One critical step in the parthanatos cell death process consists in the permeabilization of mitochondrial membranes, leading to the release of proteins such as AIF[10] that is a flavin adenine dinucleotide-containing, NADH-dependent oxidoreductase suggested to play a crucial role in the maintenance of mitochondrial morphology and energy metabolism.[21] Following insult, AIF undergoes proteolysis and

translocates to the nucleus, where it triggers chromatin condensation and large-scale DNA degradation in a caspase-independent manner.[16,21]

In the presence of high level of cytoplasmic HSP70, released AIF is bound and sequestered by this protein, thereby reducing its ability to migrate to the nucleus with irreparable cell damage.[12,14] It is worth noting that the pharmacological inhibitor of HSP70, VER155008, binds to a distinct site that corresponds to the protein activation domain by ATP.[32]

Our data showed that, in sham conditions, the majority of motoneurons were moderately immuno-positive for HSP70 and negative for nuclear AIF. Indeed, maintenance of the spinal cord in vitro was associated with a clear rise in HSP70 expression that was almost absent in freshly-dissected preparations. This observation suggests that the in vitro condition caused a certain stress for motoneurons probably compensated at least in part by HSP70 expression. With an excitotoxic stimulus, metabolic distress is expected to be enhanced so that HSP70 expression may become an even more important player in contrasting cell damage (via AIF release). Our confocal analysis confirmed that 24 h after kainate, motoneurons with strong nuclear AIF immunostaining lacked expression of HSP70, whereas motoneurons with strong cytoplasmic expression of HSP70 did not show nuclear AIF. With a larger kainate concentration, the number of motoneurons with AIF translocation was increased, together with the smaller number of motoneurons expressing a significant HSP70.

We currently do not know the precise mechanisms that allowed certain motoneurons to survive excitotoxicity, yet we noted that those that did, could express higher level of HSP70. This result was further supported by the observation that inhibiting HSP70 was

per se sufficient to evoke as much damage as kainate, and that the combined application of kainate and VER155008 was the most severe insult for motoneuron firing. Nevertheless, because a number of motoneurons survived in vitro after VER155008 administration, we suspect that cell survival processes additional to HSP70 became important to ensure cell viability. Future studies are necessary for their identification.

Histological survival and electrophysiological activity

Even if, 24 h after kainate application, the loss of the motoneuron population was about 1/3rd, it is possible that further delayed neurotoxicity might emerge later (beyond the survival of the in vitro preparation). This hypothesis might be applicable to those motoneurons already expressing AIF (24 h) prior to pyknosis and perhaps already showing functional deficit. This view is consistent with the observation of the discrepancy between histological loss (limited) and electrophysiological deficit (very strong) at 24 h. By recording directly the motoneuron field potential, we could find an intense reduction in the response amplitude (implying a smaller number of electrically excitable cells) with broadening of the field (indicative of scattered cell firing), plus clearly diminished excitability that required much stronger electrical pulses to elicit motoneuron firing (suggesting alterations in the motoneuron intrinsic properties). The latter finding was obtained after kainate or VER155008 application or their combined administration: hence, even surviving motoneurons likely suffered from a functional deficit. Likewise, premotoneuron networks were also largely affected by such treatments with widespread depression of polysynaptic reflexes. It is important to note that shortly after an acute SCI, network excitability is drastically depressed in man, a condition

known as “spinal shock” from which recovery is uncertain.[45] This situation could be mimicked in our in vitro model, indicating that histology and electrophysiology did not go in parallel as indicators of spinal damage. Because the inhibitor of HSP70 was so effective in depressing synaptic transmission and antidromic motoneuron firing, it is tempting to speculate that ongoing network activity in vitro demands strong expression and function of HSP70. In conclusion, the present data suggest that motoneuron survival is supported by their HSP70 expression in a pathophysiological model of SCI, and that HSP70 is even an important contributor to ensure full motoneuron functionality of in vitro preparations.

Material & Methods

Animal care

In accordance with the three Rs objective, all efforts were made to minimize the number of animals used for the experiments and their suffering. The experiments were performed in accordance with the ethical guidelines for the care and use of laboratory animals of National Institutes of Health (NIH) guidelines and the Italian act D. L. 27/1/92 n. 116 (implementing the European Community directives n.86/609 and 93/88). All experimental protocols were approved by the ethical committee of the International School for Advanced Studies.

Spinal cord preparations

As reported earlier,[6] neonatal Wistar rats (0-2 days) were used to prepare thoraco-lumber spinal cord preparations removed under anesthesia with urethane (i.p. 0.2 ml; 10% w/v

solution). Kreb's solution (in mM: 113 NaCl, 4.5 KCl, 1 $MgCl_27H_2O$, 2 $CaCl_2$, 1 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose, gassed with 95% O_2, 5% CO_2, pH 7.4 at room temperature) was used to superfuse (7.5mL/min) isolated spinal cords subsequently maintained in vitro for up to 24 h.

Electrophysiological recordings

One lumbar dorsal root (DR) was stimulated (0.5-10 V stimuli of 0.1 – 0.2 ms duration) through a suction electrode to evoke dorsal root-ventral root potentials (DR-VRPs) which were recorded from the homolateral ventral root (VR) by using a glass suction electrode[6] filled with Kreb's solution. Analysis was done by averaging 5 responses at 30-60 s interval. Field potentials (FPs) due to synchronous firing of motoneurons[46] were recorded with an extracellular glass pipette in the lumbar ventral horn region after 24 h in vitro.[47] After the stimulus threshold values were established, responses were collected at varying stimulus strength (ranging from 3 to 9 V; 0.1 ms duration). The average of 10 sweeps for each field potential was used for further data analysis. The data were acquired and analyzed with pClamp software (version 9.2; Molecular Devices, Sunnyvale, CA, USA).

Experimental lesion and chemical treatments

To mimic a spinal lesion,[33] kainic acid (kainate; Tocris Bioscience, Bristol, UK) was used to produce excitotoxic injury in vitro. Kainate was applied for 1 h at the concentration of 10, 50 or 100 μM, where 50 μM is the threshold concentration to abolish locomotor network activity.[4,5] After 1 h treatment with kainate, preparations were kept in Kreb's for 24 h at room temperature and subjected to further electrophysiological and histochemical

analysis. The HSP70 antagonist VER155008 (Tocris Bioscience, Bristol, UK) was used to block the activity of HSP70 completely.[26] The VER155008 concentration was selected on the basis of experiments described below and applied for 24 h.

Functional tests for HSP70 inhibition

Initial experiments were run to validate that the VER155008 concentration (50 μM) reported in the literature[26,48,49] was effective to inhibit the action of HSP70 in functional tests using cell lines constitutively expressing HSP70.[48,49] Thus, preliminary experiments were performed on SH-SY5Y cells and HEK 293T cells to check cell survival 24 h after VER155008 application. For both SH-SY5Y and HEK 293T cells, cell death was evaluated and quantified using Trypan Blue staining (Sigma-Aldrich, T8154) at the concentration of 0.4%, taking pyknosis of an index of cell death.

On HEK 293T cells obtained from the SISSA in-house bank, we checked cell death and HSP70 expression following VER155008 application for 24 h. SH-SY5Y cells were cultured in Ham's F12:EMEM (1:1) medium supplemented with 15% fetal bovine serum, 1% Non Essential Amino Acids (NEAA) and antibiotics.[50] HEK cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and antibiotics.[51] Once confluent, the cells plated in 35 mm Petri dishes were divided into three experimental groups: one treated with VER155008 (50 μM) dissolved in DMSO (Sigma-Aldrich, D4540) and applied via the culture medium, one control group in standard culture medium, and one treated with DMSO alone (0.1 % v/v). After 24 h, cells were fixed in 4% paraformaldehyde for 20 min at room temperature followed by washing. SH-SY5Y cell images were acquired using a LEICA 6000 microscope in phase

contrast mode (x40 lens). Supplemental Figure 1a shows examples of SH-SY5Y pyknosis observed after VER15508 application: after 24 h the dead cells were 9 %, a value clearly larger than the effect due to DMSO alone.

Immunocytochemistry was performed on HEK 293T cells: after incubation in blocking solution (5% normal goat serum, 5% bovine serum albumin, 0.3% Triton-X 100) for 1 h at room temperature, the primary antibody for HSP70 (Abcam, ab6535, 1:200) was incubated at 4 ºC overnight. This antibody has been previously validated for immunohistochemistry.[52–55] The primary antibodies were visualized using secondary anti-mouse Alexa Fluor 488 (Invitrogen, CA, USA, 1:500). Specimens were finally stained with DAPI for 20 min and images were acquired using a LEICA 6000 microscope (x20 lens). Supplemental Figure 1b shows examples of HSP70 expression by HEK cells after 24 h in vitro. When cells were treated with VER155008, substantial pyknosis was observed together with loss of HSP70 expression. These results indicated that VER155008 at the concentration of 50 μM was toxic to cells via HSP70 inhibition.

Immunofluorescence procedure on spinal cord preparation

The procedure for staining and identifying motoneurons was reported earlier.[42,56] Briefly, paraformaldehyde-fixed spinal cords were cryoprotected with 30% sucrose and sectioned (35 μm) with a sliding microtome. Immunostaining was performed on sections from T13-L3 segments as they contain the locomotor central pattern generator.[57] After incubation in blocking solution (5% donkey serum, 5% bovine serum albumin, 0.3% Triton-X 100) for 3 h at room temperature, the primary antibody for ChAT (choline acetyltransferase, goat polyclonal, Chemicon, Millipore, AB144P, 1:50) was incubated at 4 °C for 2 days to

identify motoneurons visualized as large cells (soma diameter >20 μm) in the ventral horn. After incubation in blocking solution (10% goat serum, 5% bovine serum albumin, 0.3% Triton-X 100) for 3 h at room temperature, the primary antibody for AIF (Upstate, Millipore, 04-430, 1:50) and for HSP70 (Abcam, ab6535, 1:200) were incubated at 4 °C overnight. Primary antibodies were visualized using secondary donkey anti-goat 594 antibodies or goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 594 (1:500, Invitrogen, CA, USA). Sections were stained with DAPI for 20 min and analyzed as detailed below. ChAT and AIF antibodies have been previously used and validated for immuno-staining with our preparations.[42,56]

Immunofluorescence data quantification

These procedures have been described in detail earlier.[42] In brief, using a Zeiss Axioskop2 microscope (Oberkochen, Germany) and Metavue software (Metamorph suite supplied by Molecular Devices, Sunnyvale, CA, USA) to acquire images, motoneuron numbers were counted on the basis of ChAT positivity in the T13-L5 ventral horn (Rexed laminae VIII–IX). For each experimental group, three spinal cords were analyzed and, for each spinal cord, three different sections from T13 to L5 were examined. Fluorescence intensity (expressed in arbitrary units; AU) was measured using constant setting and threshold values to allow data comparison. Data were quantified with 'eCELLence' (Glance Vision Tech., Trieste, Italy) software.

Confocal images of single motoneurons were acquired with a LEICA microscope (TCSSP2), using 0.5-μm z sectioning as reported by Oh et al., (2006). After reconstructing the cell image, we selected a single central optical section that comprised

the largest nuclear staining with DAPI. Thereafter, we performed a line scan of such an image to verify the distribution of AIF and HSP70 immunofluorescence signals in the nucleus and in the cytoplasm. The number of reconstructed motoneurons showing AIF nuclear translocation was quantified with ImageJ (http://rsb.info.nih.gov/ij).

Data analysis

Statistical analysis was done with SigmaStat 3.5 (Systat Software, Chicago, IL, USA). The normality test (Anova Tukey) was performed first to distinguish parametric from non-parametric data that were then analyzed with either Student's t-test or any other test as appropriate. The significance level was $P \leq 0.05$. For electrophysiological experiments *n* refers to number of preparations, while for immunohistochemical experiments *n* indicates the number of tissue sections.

Conflict of Interest

The authors have no conflict of interest.

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Title and legends to figures

Figure 1 Change in motoneuron number 24 h after excitotoxic injury by kainate (KA; 50μM and 100 μM) and VER155008 (50 μM). (a) Example to motoneuron ChAT immunolabeling are shown for various experimental protocols and histogram (b) shows the number of motoneurons counted in ventral horn, where it is significantly higher in sham when compared to all other groups by Student's t-test. ***$P \leq 0.001$ and data are mean ± SD (n = 9 for each group).

Figure 2 Expression of AIF and HSP70 in motoneurons 24 h after experimental treatment. From left to right a1-4 shows example of staining for AIF (red), HSP70 (green) and DAPI (blue) in a sham prepration, treated with 50 or 100 μM kainate or 50 μM VER155008, respectively. (b) shows histograms of percent change in motoneuron number expressing nuclear AIF (nAIF) as an index of cell death after the same treatment. $P \leq 0.001$, n=9 sections from 3 spinal cord for each group. (c) shows immunofluorescence signal (AU) of single motoneurons expressing HSP70 alone (shaded bars) or together with nuclear AIF (open bars). One way ANOVA (Bonferroni t-test) was performed; ***$P \leq 0.001$ when sham (shaded bar) and kainate (open bar) was compared to all the other groups. $^{\$}P \leq 0.001$ and $^{@}P \leq 0.001$ compared to kainate (KA; 50μM and 100 μM). Data are mean ± SD (n = 9 for each group).

Figure 3 Confocal analysis of DAPI, HSP70 and AIF signal in motoneurons after KA 50μM. (a) example of confocal analysis of a single sham motoneurons. The pink lines are

centered on a motoneuron whose nucleus is outlined (in pink colour). This cell is then subjected to z-stack analysis in the z-plane (right and bottom insets). A line scan across this cell (as exemplified in b) yields the plots depicted in b1 in which the three markers are shown for the cytoplasmic and nuclear compartments. Note strong nuclear distribution of DAPI (blue) versus AIF and HSP70. (c) Similar analysis is used for the sample obtained 24 h after KA (50μM). Two cells are exemplified in d where d1 shows strong cytoplasmic location of AIF with low expression of HSP70 as quantified in the plot for d1. The cell d2 shows more homogeneous distribution of AIF across the cytoplasm and nucleus (with minimal HSP70 expression). Note DAPI signal colocalized with AIF in the nucleus. Scale bar: 30 μm.

Figure 4 Effect of HSP70 antagonist (VER155008; 50μM) and kainate (KA; 50-100μM) on motoneuron field potential (FP) evoked by antidromic activation of a single VR. (a) Representative FP traces of experimental groups superimposed for comparison purpose. Note the significant reduction in field amplitude in the histograms (b) in comparison with sham (n = 10, 9, 9, 5, 10, 5 spinal cords, respectively). The histograms (c) show significant increase in stimulus threshold when VER155008 was applied for 24 h after KA (50 μM and 100 μM) 1 h application. (d) input/output curves of antidromic field potentials for various stimulus intensities; n=14, 12, 13, 5, 11, 5 spinal cords, respectively for data in c and d. One way ANOVA (Bonferroni t-test) was performed; **$P \leq 0.01$, ***$P \leq 0.001$. Data are mean ± SME.

Figure 5 Effect of HSP70 antagonist (VER155008; 50μM) and a concentration of kainate (KA; 10 μM) subthreshold for neurotoxicity on FP evoked by antidromic activation of a single VR. (a) represents the average FPs following various treatments as indicated. In the histograms (b) the amplitude of FPs was significantly decreased when kainate was applied with VER155008, whereas kainate alone has no significant effect on FP (n=8, 7, 9, 7). Stimulus threshold for FP (c) was significantly increased among all experimental groups. (d) shows field amplitude for incrementing stimulus strength; n=14, 10, 10, 8. One way ANOVA (Bonferroni t-test) was performed; *P = 0.03, **P ≤ 0.01, ***P ≤ 0.001. Data are mean ± SME.

Figure 6 Expression of HSP70 in freshly fixed (FF) vs sham (24 h) preparations. (a) expression of HSP70 was relatively high in sham spinal cord when compared to FF. Histograms (b) show fluorescence intensity (AU) significantly lower in FF with ***P ≤ 0.001 when Student's t-test was performed. Data are mean ± SD.

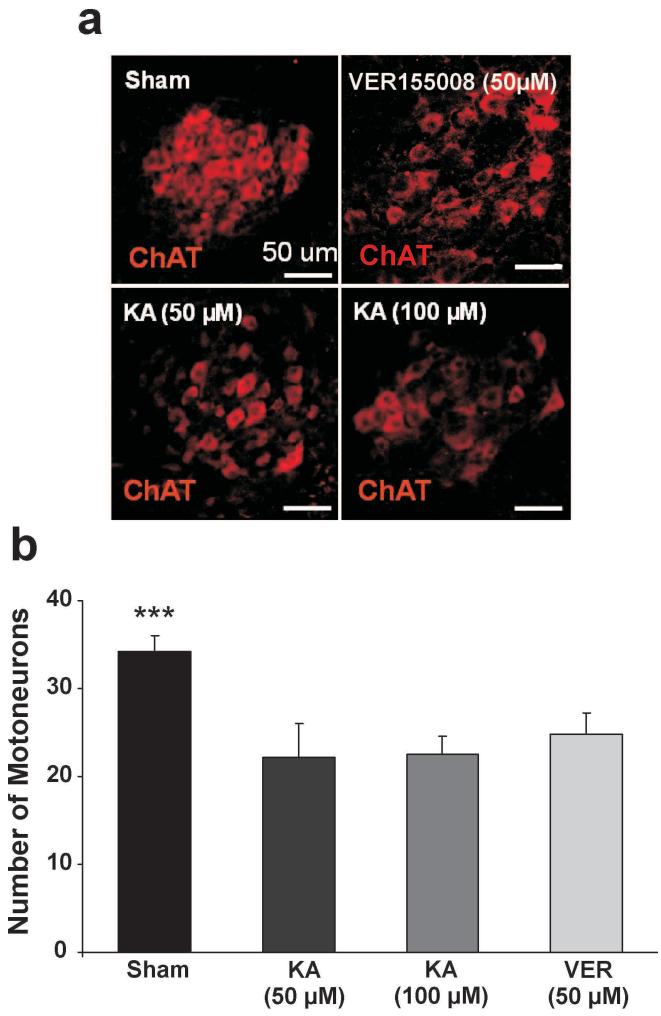
Supplemental Figure S1 Validation of the VER155008 concentration (50 μM) as inhibitor of HSP70, using cell lines constitutively expressing HSP70. (a) Examples of SH-SY5Y pyknosis observed after VER15508 application (phase contrast images). Cell death was quantified in (b) with 9 % pyknosis measured after VER155008 application for 24 h. *P = 0.03 when Mann-Whitney test was performed, n = 4. The effect of the solvent DMSO was negligible. (c) DAPI and HSP70 signals in HEK cells in sham conditions (top row), or following VER155008 application for 24 h (bottom row). Arrows indicate examples of pyknotic nuclei. As shown in the histograms (d), cell death after the

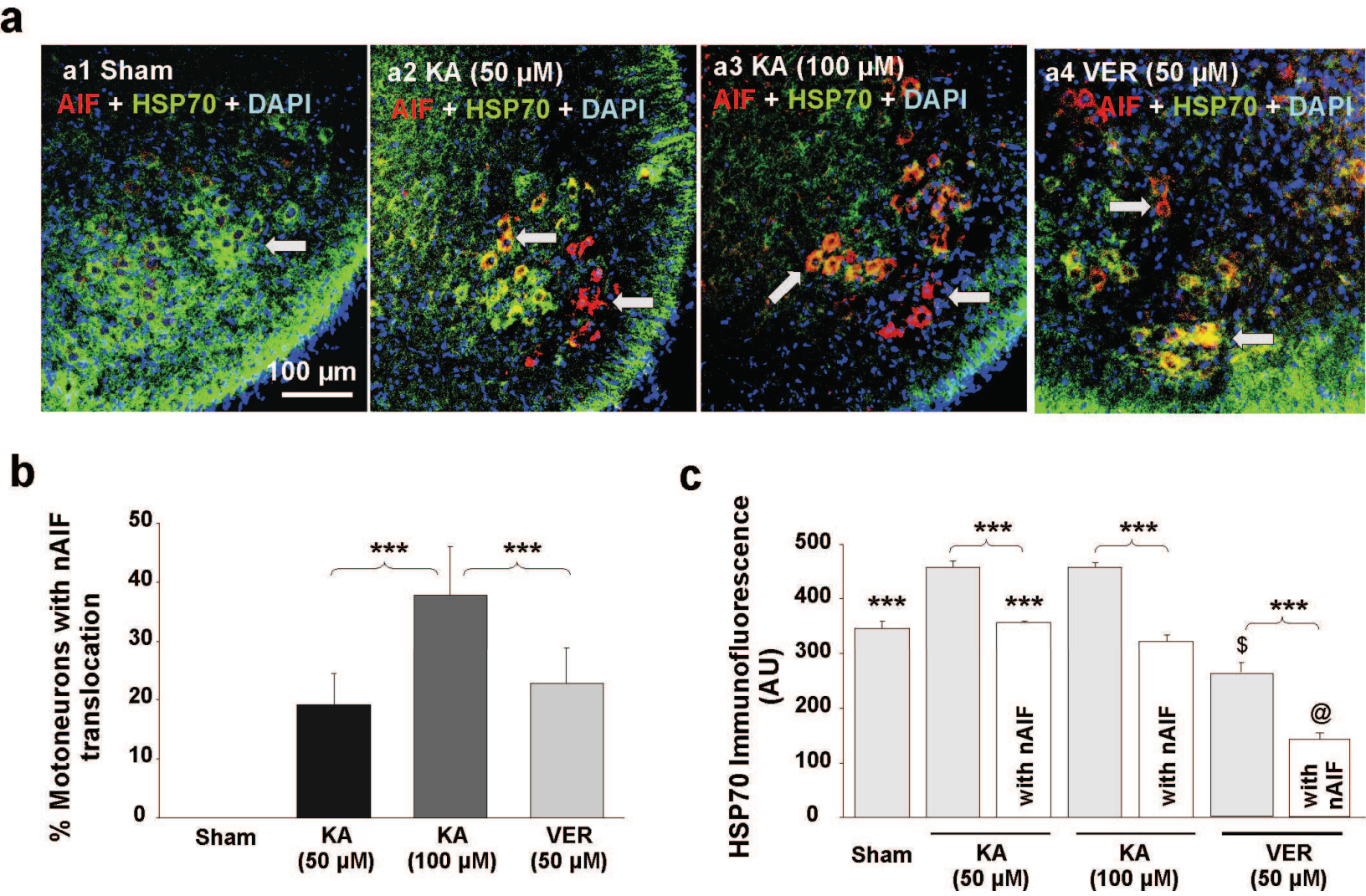
application of VER155008 was 24 % of the total cell number, a value significantly different from sham or DMSO conditions. ***$P \leq 0.001$, *$P = 0.02$ when Student's t-test was performed, $n = 4$. Data are mean ± SD.

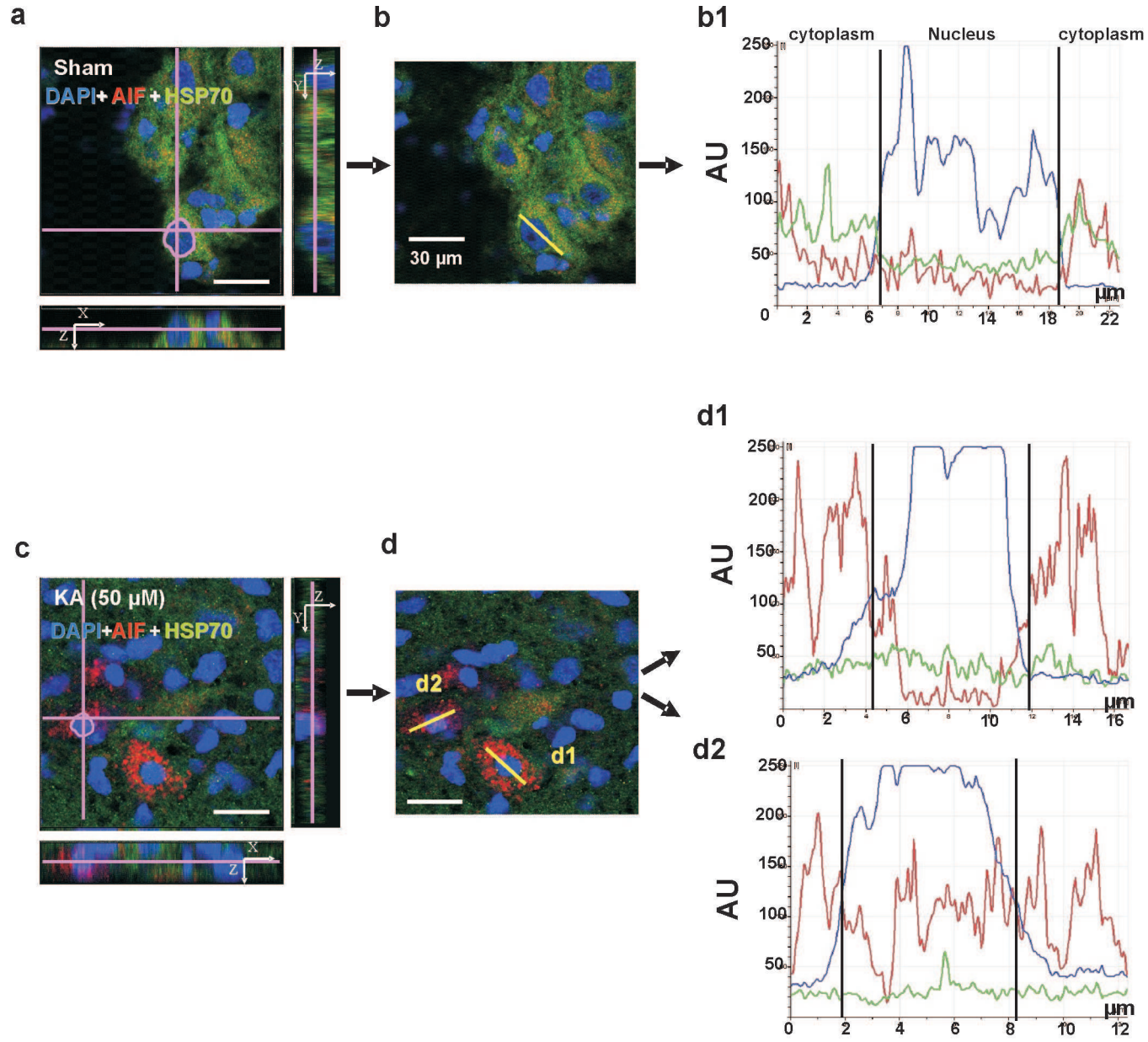
Table 1 Changes in DR-VRP amplitude in sham and pharmacologically treated spinal cord preparations after 24 h *in vitro*.

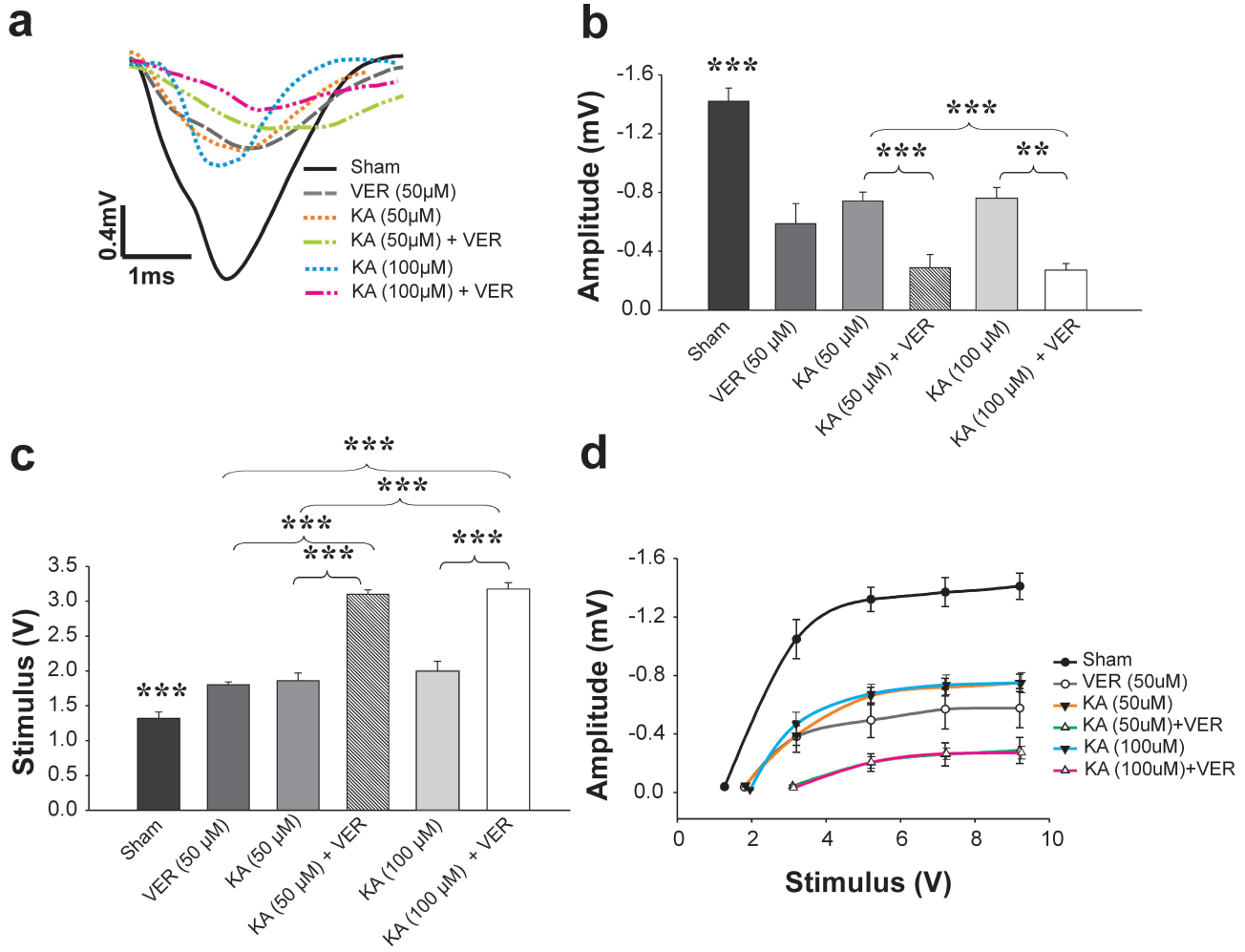
Treatment	**Amplitude (mV) ± SEM**
Sham	0.86 ± 0.02
VER155008 (50 μM)	0.38 ± 0.05***
KA (10 μM)	0.63 ± 0.09
KA (10 μM) + VER	0.17 ± 0.04***, $
KA (50 μM)	0.46 ± 0.06 ***
KA (50 μM) + VER	0.17 ± 0.02 ***, β, $, ^
KA (100 μM)	0.41 ± 0.02 ***
KA (100 μM) + VER	0.08 ± 0.01 ***, β, $, ^, ¤

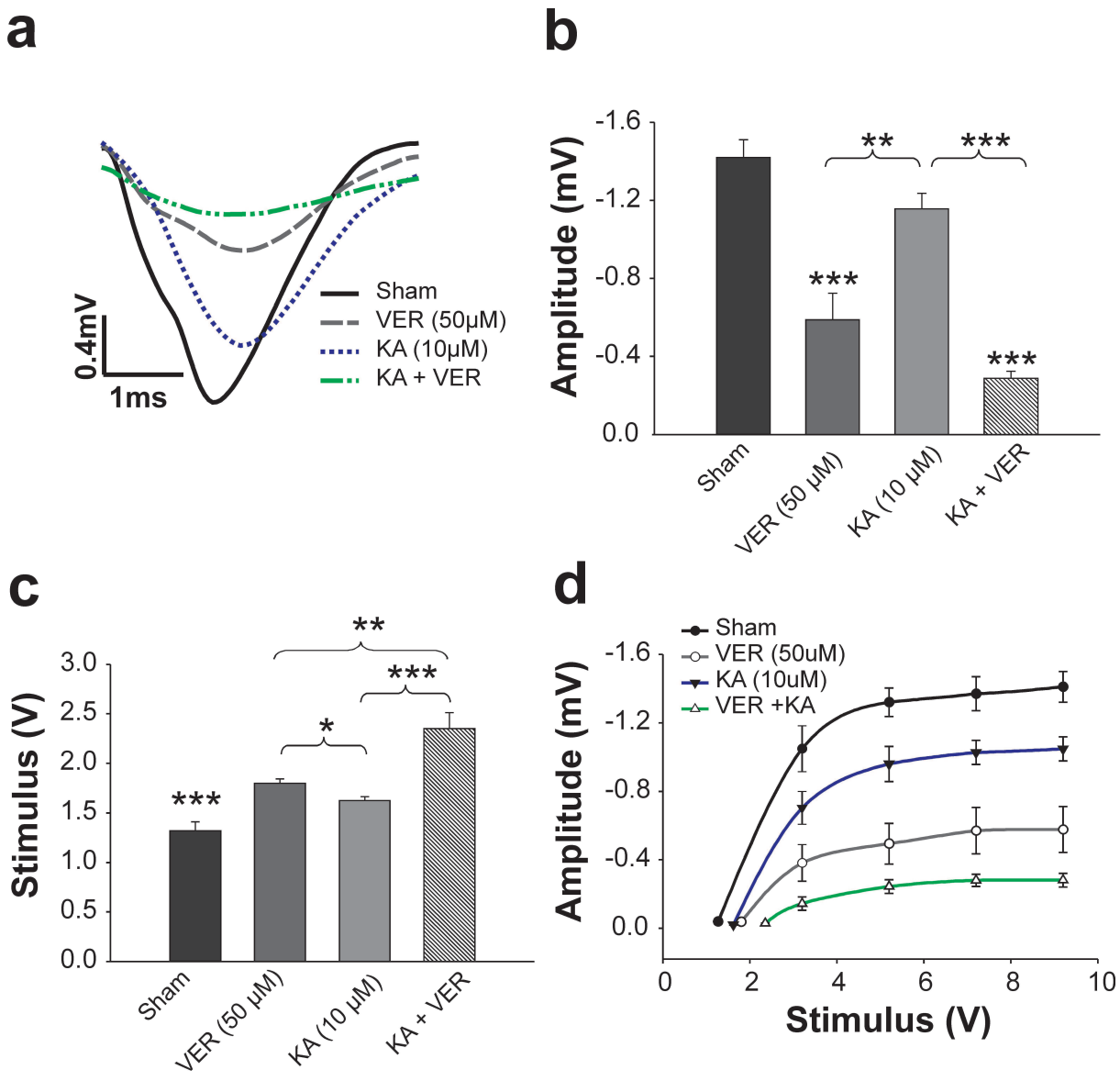
n = 7, 4, 8, 3, 7, 6, 9, and 3 spinal cords, respectively. KA; kainate. One way ANOVA (Bonferroni t-test) was performed; ***P ≤ 0.001 when compared to sham. $^{\beta}$P ≤ 0.002 when compared to VER155008 alone. $^{\$}$P ≤ 0.01 when compared to KA (10 μM). ^P ≤ 0.001 when compared to KA (50 μM) and $^{¤}$P ≤ 0.001 when compared to KA (100 μM). Data are mean ± SEM.


a
Sham
ChAT
50 um
VER155008 (50μM)
ChAT
KA (50 μM)
ChAT
KA (100 μM)
ChAT
b
Number of Motoneurons
40
30
20
10
0
***
Sham
KA
(50 μM)
KA
(100 μM)
VER
(50 μM)


a
a1 Sham
AIF + HSP70 + DAPI
a2 KA (50 μM)
AIF + HSP70 + DAPI
a3 KA (100 μM)
AIF + HSP70 + DAPI
a4 VER (50 μM)
AIF + HSP70 + DAPI
100 μm
b
% Motoneurons with nAIF translocation
0
10
20
30
40
50
***
***
Sham
KA (50 μM)
KA (100 μM)
VER (50 μM)
c
HSP70 Immunofluorescence (AU)
0
100
200
300
400
500
***
***
***
***
***
$
@
with nAIF
with nAIF
with nAIF
Sham
KA (50 μM)
KA (100 μM)
VER (50 μM)


a
Sham
DAPI+AIF+HSP70
b
30 μm
b1
cytoplasm
Nucleus
cytoplasm
AU
μm
c
KA (50 μM)
DAPI+AIF+HSP70
d
d2
d1
d1
AU
μm
d2
AU
μm


a
0.4mV
1ms
Sham
VER (50μM)
KA (50μM)
KA (50μM) + VER
KA (100μM)
KA (100μM) + VER
b
Amplitude (mV)
-1.6
-1.2
-0.8
-0.4
0.0
***
***
***
**
Sham
VER (50 μM)
KA (50 μM)
KA (50 μM) + VER
KA (100 μM)
KA (100 μM) + VER
c
Stimulus (V)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
***
***
***
Sham
VER (50 μM)
KA (50 μM)
KA (50 μM) + VER
KA (100 μM)
KA (100 μM) + VER
d
Amplitude (mV)
-1.6
-1.2
-0.8
-0.4
0.0
0
2
4
6
8
10
Stimulus (V)
Sham
VER (50uM)
KA (50uM)
KA (50uM)+VER
KA (100uM)
KA (100uM)+VER


a
0.4mV
1ms
Sham
VER (50μM)
KA (10μM)
KA + VER
b
Amplitude (mV)
-1.6
-1.2
-0.8
-0.4
0.0
**
***
***
***
Sham
VER (50 μM)
KA (10 μM)
KA + VER
c
Stimulus (V)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
***
*
***
Sham
VER (50 μM)
KA (10 μM)
KA + VER
d
Amplitude (mV)
-1.6
-1.2
-0.8
-0.4
0.0
Sham
VER (50uM)
KA (10uM)
VER +KA
0
2
4
6
8
10
Stimulus (V)

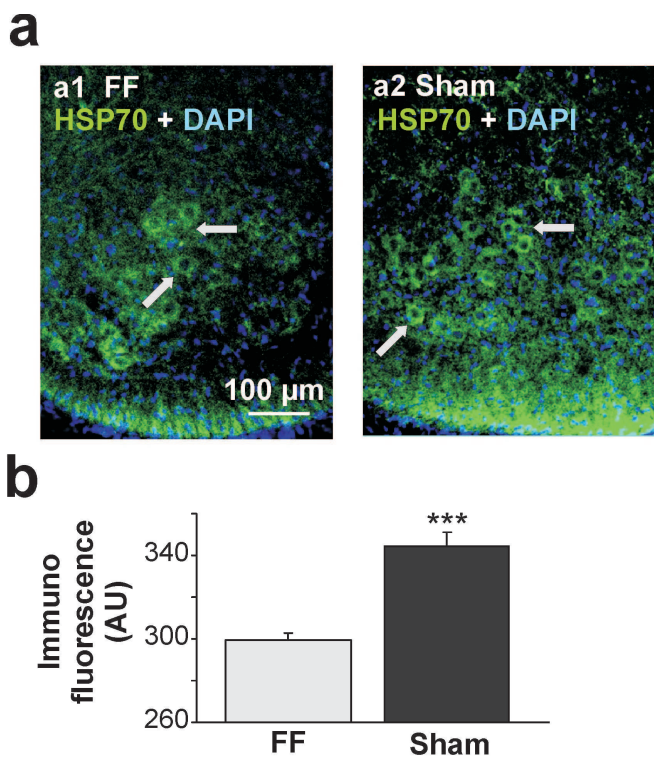

a
a1 FF
HSP70 + DAPI
100 µm
a2 Sham
HSP70 + DAPI
b
Immuno fluorescence (AU)
340
300
260
***
FF
Sham

Table 1 Changes in DR-VRP amplitude in sham and pharmacologically treated spinal cord preparations after 24 h *in vitro*.

Treatment	**Amplitude (mV) ± SEM**
Sham	0.86 ± 0.02
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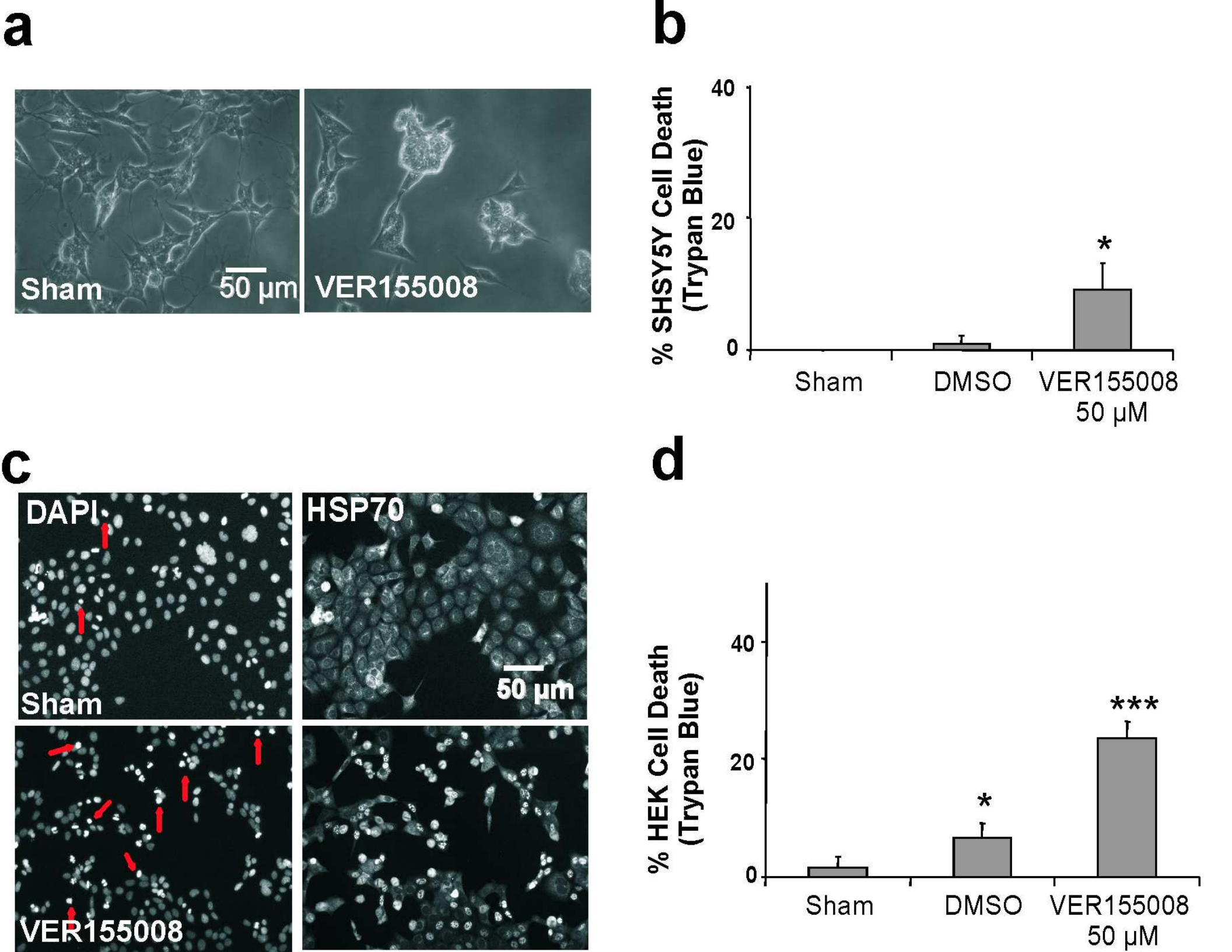
n = 7, 4, 8, 3, 7, 6, 9, and 3 spinal cords, respectively. KA; kainate. One way ANOVA (Bonferroni t-test) was performed; ***P ≤ 0.001 when compared to sham. $^{\beta}$P ≤ 0.002 when compared to VER155008 alone. $^{\$}$P ≤ 0.01 when compared to KA (10 µM). ^P ≤ 0.001 when compared to KA (50 µM) and $^{¤}$P ≤ 0.001 when compared to KA (100 µM). Data are mean ± SEM.


a
Sham
50 µm
VER155008
b
% SHSY5Y Cell Death (Trypan Blue)
40
20
0
*
Sham
DMSO
VER155008 50 µM
c
DAPI
HSP70
Sham
50 µm
VER155008
d
% HEK Cell Death (Trypan Blue)
40
20
0
*
***
Sham
DMSO
VER155008 50 µM

Discussion

The principal findings presented in this thesis demonstrate a few important aspects related to the basic mechanisms underlying the acute phase of spinal cord injury. In an excitotoxicity model of spinal cord injury *in vitro*, the volatile anesthetic methoxyflurane was very effective to protect spinal locomotor networks (Paper I). The neuroprotective mechanism against excitotoxicity, in part, relies on strong expression of heat shock protein 70 (HSP70) in motoneurons (Paper II). The details of our novel studies are reported and explained in attached papers; however, in this section I will briefly discuss the main findings of present thesis.

1. *In vitro* Spinal Cord Injury (SCI) Model

The current data were obtained using an *in vitro* model of SCI based on transient (1 h) application of the potent glutamate analogue kainate to mimic a clinical scenario (Kuzhandaivel et al., 2011; Nasrabady et al., 2011a; Taccola et al., 2008). Although these preparations are immature and have some limitations like tissue immaturity and lack of vascular supply yet they show most of the characteristics of *in vivo* spinal cord injury including the delayed onset of secondary damage, the incomplete nature of the histological loss, and the cell death mechanisms, in particular parthanatos (Genovese and Cuzzocrea, 2008; Kuzhandaivel et al., 2011; Wu et al., 2009). One important characteristic of this model is the possibility to analyze the functional activity of locomotor networks by recording their cyclic oscillatory discharges (alternating between flexor and extensor motor pools), and relate them to the topography and extent of the induced lesion. The locomotor networks are highly sensitive to damage as their electrical activity is suppressed even when substantial cell numbers remain apparently intact. It is also worth noting that prenatal and neonatal SCI is not uncommon in humans (Barnes et al., 2008; Fenger-Gron et al., 2008; Selvarajah et al., 2014), thereby making the present model relevant also to the understanding of neonatal SCI pathophysiology.

2. Methoxyflurane as Neuroprotective Agent

Since general volatile anesthetics tend to exert some neuroprotective effect when used clinically, this notion gave rise to the idea of using this halogenated volatile anesthetic which can be applied through the bath solution (Kreb's medium) in *in vitro* studies like the present one. Methoxyflurane seemed to be the valid candidate because of its chemical and physical properties (Andersen and Andersen, 1961; Eger and Eger, 1985; Seto et al., 1992; Soares et al., 2012). The favorable concentration of methoxyflurane (4.5 mM), that can suppress synaptic transmission fully, was chosen after trying various concentrations between 0.5 – 6 mM. Thus, the concentration of methoxyflurane (4.5 mM) for the majority of the current tests was empirically selected on the basis of its rapid and reversible depression of electrophysiological responses. Methoxyflurane (4.5 mM) was applied for 1 h owing to the fact that prolonged administration of volatile general anesthetics may produce toxic effects including apoptosis of nervous tissue (Sanders et al., 2008; Zhao et al., 2011), whereas short application of such anesthetics can be highly neuroprotective against rat brain ischemia (Ren et al., 2014).

2.1. Effects of Methoxyflurane on Functional Outcomes

Excitotoxicity induced by kainate (50 µM for 1 h) as described in previous reports from our lab (Mazzone et al., 2010; Nasrabady et al., 2012; Taccola et al., 2008) resulted in a functional deficit. It significantly reduced the amplitude of polysynaptic DR-VRPs, increased the FP stimulus threshold and decreased the FP amplitude after 24 h. In the present study, no neurotoxic action of methoxyflurane alone was observed after 1 h application. Methoxyflurane, when applied together with kainate (coapplication and 30 min delayed) or after kainate washout (1 h kainate followed by 1 h methoxyflurane), showed very strong neuroprotection of polysynaptic transmission in whole and hemisected spinal cords by preventing the depressive

effects of kainate (50 µM) after 24 h. Kainate is known to produce large ventral root (VR) depolarization in rat spinal cord (Nasrabady et al., 2011b; Taccola et al., 2008), but when it was applied in combination with methoxyflurane (co-application, 30 min delay and after washout), it significantly reduced the VR depolarization amplitude, measured 60 min from the start of application. This relatively slow onset of synaptic or motoneuron depression by methoxyflurane and partial inhibition of neuronal depolarization evoked by kainate is in analogy with the recent studies by Daló and Hackman (2013) that a general anesthetic antagonizes kainate mediated depolarization in an *in vitro* model of the frog spinal cord.

Spinal motoneurons are the most vulnerable to kainate induced excitotoxicity (Mazzone et al., 2010) and a substantial number of motoneurons are required for proper spinal network function expressed as motor output (Nistri, 2012). We, thus, investigated whether methoxyflurane could protect motoneuron function monitored as synchronous firing of motoneuron population with/after kainate. The direct application of methoxyflurane for only 15 min was sufficient to halve the FP amplitude and largely raised the firing threshold recorded from lumbar VRs, with rapid recovery on washout. After 24 h *in vitro*, when methoxyflurane had been applied with or after kainate, it strongly prevented the depression of motoneuron FP amplitude in comparison with kainate alone. Methoxyflurane also prevented the increase in the stimulus threshold. These findings strongly suggest that methoxyflurane is highly neuroprotective. A simple interpretation of these data is that administering methoxyflurane effectively stopped the slow emergence of the non-apoptotic cell death process typically triggered by excitotoxicity (Kuzhandaivel et al., 2011) and peaking within the first 1-2 h.

2.2. Histological Effects of Methoxyflurane

We have also conducted a detailed histological analysis on the preparations to access methoxyflurane effects on the neuronal population, in particular motoneurons. The pyknosis was significantly higher in kainate treated spinal cords as reported earlier

(Nasrabady et al., 2011b), but it was significantly decreased in all the methoxyflurane treated groups together with or after kainate. The neuronal number (identified by NeuN nuclear staining) was also preserved even in the most vulnerable region (dorsal horn). While motoneurons are just a small population of cells in the spinal network (Cifra et al., 2012), they are essential for motor function. Hence, when we counted their number after ChAT immuno-staining, they were found to be well preserved, therefore supporting efficient network activity in methoxyflurane treated spinal cords (with or after kainate).

2.3. Effect of Methoxyflurane on Locomotor Network Function

Kainate induced excitotoxicity was shown to abolish fictive locomotion (FL) in spinal cord *in vitro* preparations (Nasrabady et al., 2011b; Mazzone et al., 2010; Taccola et al., 2008). It is important to mention that the fictive locomotion induced by the train of DR pulses is the most sensitive to neurotoxicity, as even a mechanism targeted drug like PJ34 has failed to preserve it effectively (Nasrabady et al., 2011b). After 24 h *in vitro* followiing methoxyflurane applied together with or after kainate, except for some reduction in cumulative depolarization, the oscillatory VR discharges and total area of depolarization evoked by DR pulse trains were preserved. A similar result was seen with chemically induced FL by NMDA and 5HT. The preservation of periodicity of locomotor rhythms suggest that the “clock” oscillatory function (McCrea and Rybak, 2008; Taccola and Nistri, 2006) within the organization of the central pattern generator for locomotion (Brownstone and Wilson, 2008; Kiehn, 2006) had been well protected. Thus, not only motoneurons were spared, but also oscillatory interneurons and premotoneurons retained their full functional activity.

2.4. Mechanism of Methoxyflurane Neuroprotection

To better understand the mechanism underlying the neuroprotective effect of methoxyflurane, further experimentation is required. Nevertheless, it is feasible to propose that this neuroprotective effect is result of combinatorial effect of synaptic transmission depression, reduced motoneuronal firing and decreased intrinsic neuronal excitability as these are common actions of volatile general anesthetics (Collins et al., 1995; De Jong et al., 1968; Vahle-Hinz and Detsch, 2002).

Impaired glutamate uptake is a major process contributing to neuronal cell death after excitotoxicity. The activation of metabotropic glutamate receptors are responsible for higher levels of extracellular glutamate and these receptors are highly expressed in rat spinal cord where they regulate synaptic transmission (Marchetti et al., 2003). General volatile anesthetics may neuroprotect by blocking the glutamate receptor mediated neurotoxicity in *in vivo* models of brain ischemia (Popovic et al., 2000). It has also been reported that the volatile general anesthetic isoflurane reduces the size of lesion when maintained for longer time periods after microinjections of NMDA or AMPA into the rat cerebral cortex (Harada et al., 1999; Kimbro et al., 2000). Thus, it can be suggested that general volatile anesthetics like methoxyflurane may produce neuroprotective effects via mechanisms involving the attenuation of glutamate neurotoxicity by either reducing its release (or enhancing its uptake), or antagonizing postsynaptic glutamate receptors.

TASK1 channels are strongly expressed in spinal motoneurons (Marinc et al., 2012). They can constrain neuronal excitation and downsize related intracellular Ca^{2+} overload that, during excitotoxicity, develops slowly (Szydlowska and Tymianski, 2010) to trigger the development of cell damage in the spinal cord (Kuzhandaivel et al., 2011). Methoxyflurane can also protect spinal cord by activating background K^+ channels such as TASK1, in analogy to chemically-related anesthetics (Lazarenko et al., 2010; Sirois et al., 2000). This hypothesis can further be tested once highly selective tools to block or activate these channels will be available.

The spinal cord *in vitro* normally shows spontaneous synaptic activity mediated predominantly by glutamate, GABA and glycine receptors distributed within the local networks impinging on motoneurons. However, pharmacological block of these receptors (while inhibiting ongoing activity) does not hyperpolarize motoneurons (Bracci et al., 1997; Mazzone et al., 2010). This suggests that resting membrane potential is controlled by other factors like background K^+ channels, particularly that of TASK family. Thus, if methoxyflurane had acted mainly on tramsitter-activated channels, motoneuron hyperpolarization would not have been observed. However, methoxyflurane has evoked hyperpolarization which suggests that the principal target of this anesthetic action is K^+ channels rather than ligand gated ion channels.

3. Motoneurons Cell Death Pathways and Potential Intrinsic Role of HSP70

Previous reports have shown that, spinal motoneurons die mainly through a process of programed cell death termed parthanatos (David *et al.* 2009; Kuzhandaivel *et al.* 2011; Mandir *et al.* 2000) after excitotoxic injury, although a substantial number of these cells can survive the insult (Kuzhandaivel et al., 2011). The present study (Paper II) supports the notion that their survival is in relation to HSP70 expression, whereby high levels of HSP70 protect motoneurons from parthanatos by preventing nuclear translocation of AIF (Giffard and Yenari, 2004; Gurbuxani et al., 2003; Ravagnan et al., 2001; Ruchalski et al., 2006). Furthermore, the expression of HSP70 starts to rise after injury with a minimum delay of 30 min, and it reaches a maximum at 24 h in close time correlation with our experimental protocols (Carmel et al., 2004; Cizkova et al., 2004; Hecker and McGarvey, 2011).

Our data indicated that the majority of motoneurons were moderately immuno-positive for HSP70 and negative for nuclear AIF in sham conditions after 24 h. Indeed, maintenance of the spinal cord *in vitro* was associated with a clear rise in HSP70 expression that was almost absent in freshly-dissected preparations. This observation suggests that the *in vitro* condition caused a certain stress for motoneurons probably compensated at least in part by HSP70 expression. With an excitotoxic stimulus, metabolic distress is expected to be enhanced so that HSP70 expression may become an important player in contrasting cell damage (via AIF release).

We studied the distribution of HSP70 and AIF in sham and kainate (50 µM) preparations and our confocal line scan analysis confirmed that 24 h after kainate, motoneurons with strong nuclear AIF immunostaining lacked expression of HSP70, whereas motoneurons with strong cytoplasmic expression of HSP70 did not show nuclear AIF. After kainate treatment, strong DAPI signal was observed in the nucleus of motoneurons which perhaps indicated the development of chromatin condensation as an index of cell death, a hallmark of kainate induced excitotoxicity (Taccola et al.,

2008; Wang et al., 2005). With a larger kainate concentration, the number of motoneurons with AIF translocation was increased, together with the smaller number of motoneurons expressing a HSP70. These data suggested that AIF nuclear translocation was associated with poor HSP70 expression.

3.1. Inhibition of HSP70 by VER155008

VER155008 was used to block the HSP70 activity completely (Massey et al., 2010). The VER155008 concentration (50 µM) reported in the literature (Budina-Kolomets et al., 2014; Jiang et al., 2013; Massey et al., 2010) was validated on the basis of initial experiments performed on cell lines constitutively expressing HSP70 (Budina-Kolomets et al., 2014; Jiang et al., 2013). SH-SY5Y cells and HEK 293T cells were quantified, using Trypan Blue staining, for cell death after 24 h of VER155008 application. The cells which were treated with VER155008 showed substantial pyknosis together with loss of HSP70 expression in comparison with sham and DMSO treated cells. These data indicated that VER155008 (50 µM) was toxic to cells via HSP70 inhibition. For our further experiments VER155008 was used at the standard 50 µM concentration for 24 h *in vitro*.

3.2. Excitotoxicity Induced Cell Death of Motoneurons

In the present study, we validated the extent of motoneuron death observed 24 h after kainate (50 or 100 µM for 1 h) washout. The motoneurons were identified as large ventral horn cells to ChAT immunopositivity and were found to be significantly reduced in kainate treated spinal cords when compared to sham. In keeping with previous observations, the global number of dead motoneurons was not linearly dependent on the kainate concentration once the neurotoxic threshold had been passed (Mazzone et al., 2010). The cords treated with VER155008 only also showed comparable loss of motoneurons suggesting that effective HSP70 activity was important to ensure survival in *in vitro* conditions.

3.3. Expression of HSP70 and AIF after Excitotoxicity and VER155008

Motoneurons were further investigated for their immunoreactivity to HSP70 (that might be a mechanism for neuroprotection; (DeMeester et al., 2001; Welsh et al., 1992), and for AIF expression taken as an index of cell death (Kuzhandaivel et al., 2011; Yu et al., 2006). In sham conditions, most motoneurons were immunopositive for HSP70 but not for AIF, whereas clear AIF immunoreactivity was observed after kainate (50 or 100 µM for 1 h) and VER155008 (50 µM) in the majority of the motoneurons (few motoneurons were co-stained for both AIF and HSP70). This observation is consistent with the reportedly distinct sites for VER155008 and AIF binding on HSP70 (Giffard and Yenari, 2004; Gurbuxani et al., 2003; Ruchalski et al., 2006; Schlecht et al., 2013). When motoneurons were quantified for AIF translocation into the nucleus, the highest value was observed following 100 µM kainate application, whereas sham preparations were essentially negative. The signal intensity analysis for HSP70 distinguished two motoneuron groups 24 h after kainate (50 or 100 µM for 1 h) and VER155008 (50 µM); one with strong HSP70 positivity (with no AIF nuclear expression) observed in the majority of sham preparations as well as in the kainate or VER155008 treated spinal cords, and another one (absent in sham preparations) that showed weaker HSP70 positivity (together with AIF nuclear translocation) in kainate or VER155008 treated spinal cords.

3.4. Functional Deficit after Excitotoxicity and HSP70 Inhibition in Motoneurons

We next investigated the effect of kainate and VER155008 on motoneuron synaptic transmission and their field potentials. Previous studies have shown irreversible electrophysiological deficit of motoneurons following excitotoxicity (Mazzone et al.,

2010; Nasrabady et al., 2011b). In the present study, we recorded the motoneuron field potential (FP) evoked by antidromic stimulation of a single VR to investigate whether the damage evoked by 50 or 100 µM kainate was intensified by the application of VER155008 (50 µM). An intense reduction was observed in the response amplitude (implying a smaller number of electrically excitable cells) with broadening of the field which is indicative of scattered cell firing. The increased stimulus threshold also suggested alterations in the motoneuron intrinsic properties. Hence, our data indicated that even the surviving motoneurons suffered from a functional deficit. We also enquired whether a concentration of kainate (10 µM), which is sub-threshold for excitotoxicity (Mazzone et al., 2010), could synergize with the administration of VER155008 to damage motoneurons. Although, the functional depression of the field potential by 10 µM kainate was modest, subsequent application of VER155008 elicited a strong decrease in field amplitude and increase in stimulus threshold, clearly larger than that produced by VER155008 alone. Likewise, widespread depression of polysynaptic reflexes was observed in all the treated groups except for 10 µM kainate. This deficit was particularly strong in all the instances when VER155008 was co-applied with kainate, indicating extensive functional damage to the spinal networks beyond the motoneuron neurotoxicity. It is suggested that network activity *in vitro* demands strong expression and function of HSP70.

In the present study it was evident that the electrophysiological deficit was much stronger than histological damage when HSP70 was inhibited. This effect might be somewhat similar to the condition of spinal shock (depressed network excitability) which is observed in humans shortly after acute spinal cord injury and is not closely related to the extent of lesioned tissue (Hiersemenzel et al., 2000). Although we currently do not know the precise mechanisms that allowed certain motoneurons to survive excitotoxicity, it is apparent that high levels of HSP70 were usually associated with survival. This result was further supported by the observation that inhibiting HSP70 caused as much damage as kainate alone.

Conclusion and Future Prospective

The novel findings discussed in the present thesis are interesting, yet intriguing. To the best of our knowledge, a relatively brief application of methoxyflurane has so far been the most effective neuroprotective tool in our experimental model. Methoxyflurane has strongly protected spinal neurons as well as locomotor network function against a potent neurotoxic insult. Our lab's work with riluzole has indicated that simple reduction of glutamatergic transmission and voltage activated Na^+ conductances (Cifra et al., 2013) is inadequate for early neuroprotection in the rat spinal cord after excitotoxicity (Sámano et al., 2012). Conversely, neuroprotective effects of riluzole have been reported for experimental ischemic damage to the rat spinal cord (Wu et al., 2014). The shorter application time of methoxyflurane without any neurotoxicity is also an important result since longer exposure (5 – 6 h) to volatile general anesthetic can be eventually neurotoxic (Sanders et al., 2008). We suggest that, in addition to early neurosurgery of acute spinal cord injury, the neuroprotective role of volatile general anesthetics should be exploited to aim for a better recovery. In future more experiments will be necessary to find out if this neuroprotective affect is exclusive to methoxyflurane or is a common mechanism of action for other anesthetics like propofol.

In conclusion to the second part of thesis regarding HSP70 assisted neuroprotection mechanisms, our data suggest that motoneuron survival is supported by their HSP70 expression in a pathophysiological model of SCI. Nevertheless, we suspect that additional processes for cell survival are in action to ensure cell viability owing to the fact that a few motoneurons still survived *in vitro* after strong HSP70 inhibition. Future studies are necessary for the identification of such unknown mechanisms. These findings can be further strengthened by the use of potent HSP70 enhancing agents like celestrol and arimoclomol. Celastrol is known to elevate the cellular expression of HSP70 and recently it has been shown to prevent the onset and progression of damage after traumatic brain injury *in vivo* (Eroglu et al., 2014). Arimoclomol is a drug going through the second phase of clinical trails for the treatment of amyotrophic lateral sclerosis (ALS) (Brown, 2007). The induced overexpression of HSP70 before ischemia in rat brain *in vivo* is demonstrated to be neuroprotective and the process is attributed to anti-apoptotic activity of HSP70 among other mechanisms (Giffard et al., 2004).

The novel findings of this thesis have translational value for the current approaches used to deal with spinal cord injury mechanism and its neuroprotection. It is clear that our results will need direct validation with *in vivo* injury models as well as human clinical cases. Nonetheless, we suggest that early intervention against the secondary damage of spinal cord injury has now more exciting prospects for improving the functional outcome.

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List of Abbreviation

5HT	5-hydroxytryptamine
AIF	apoptosis inducing factor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Apaf-1	apoptotic protease activating factor-1
APV	2-amino-5-phosphonopentanoic acid
ASIA	American Spinal Injury Association
ATP	adenosine triphosphate
AU	arbitrary unit
BDNF	brain-derived neurotrophic factor
BDNF	Brain-derived neurotrophic factor
ChAT	choline acetyltransferase
CINs	commissural interneurons
CNQX	7-nitro-2,3-dioxo-1,4- dihydroquinoxaline-6-carbonitrile
CNS	central nervous system
CPG	central pattern generator
CV	coefficient of variance
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DR	dorsal root
DR-VRPs	dorsal root ventral root potentials
FGF	fibroblast growth factor
FL	fictive locomotion
FP	field potential
GABA	γ-Aminobutyric acid

Gata2 and 3	GATA binding protein 2 and 3
GDNF	Glial cell-derived neurotrophic factor
GluR1-4	glutamate receptors 1-4
GTPase	Guanosine-5'-triphosphatase
HEK 293T	human embryonic kidney 293T cells
HSP70	heat shock protein 70
IL-1β	interleukin 1β
IL-6	interleukin 6
KA	kainic acid (kainate)
KO	knockout
MF	methoxyflurane
MK-801	[5R,10S]-[+]-5-methyl-10,11- dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine
MP	methylprednisolone
NADH	nicotinamide adenine dinucleotide
NGF	nerve growth factor
NLS	nuclear localization signal
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NT-3 and NT-4	neurotrophin 3 and 4
NT-3	Neurotrophin-3
PAR	poly-ADP-ribose
PARP1	poly-ADP-ribose polymerase-1
PHE	6-5(H)-phenanthiridinone
PJ34	N-(6-Oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino) acetamide hydrochloride
PTPC	permeability transition pore complex
RG	rhythm generator
Rho	ρ factor

RIP1	receptor-activating protein 1
RIP3	receptor-activating protein 3
Ro15-4513	Ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-1,4-benzodiazepine-3-carboxylate
ROI	region of interest
ROS	reactive oxygen species
TNFR1	tumor necrosis factor receptor-1
TNFα	tumor necrosis factor α
VER155008	5'-O-[(4-cyanophenyl)methyl]-8-[[(3,4-dichlorophenyl) methyl]amino]-adenosine
VR	ventral root

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