UNIVERSITÀ DEGLI STUDI DI MESSINA

TESI DI DOTTORATO DI RICERCA IN BIOLOGIA APPLICATA

E MEDICINA SPERIMENTALE

CURRICULUM IN MEDICINA SPERIMENTALE

XXIX CICLO

SSD BIO/14

The role of mTOR signaling pathway in Brain and Spinal Cord Injury

Candidata:

DOTT.SSA MARIKA CORDARO

<table>
<tr><td>Correlatore:</td><td>Relatore:</td></tr>
<tr><td>**Ch.mo Prof.**</td><td>**Ch.ma Prof.ssa**</td></tr>
<tr><td>**SALVATORE CUZZOCREA**</td><td>**EMANUELA ESPOSITO**</td></tr>
</table>

Coordinatore:

Ch.mo Prof. SALVATORE CUZZOCREA

ANNO ACCADEMICO 2014-2016

The choice of a young man depends on his inclination,

but also by the good fortune to meet a great teachers.

Rita Levi Montalcini

INTRODUCTION

Central nervous system (CNS) is one of the complex systems in the body that consists of brain and spinal cord. Any disease or traumatic assault may lead to the degeneration of CNS including loss of homeostasis. CNS injuries constitute a major cause of morbidity and mortality includes the life threatening injuries such as traumatic brain injury (TBI) and spinal cord injury (SCI). TBI and SCI are caused by both primary and secondary injuries influencing the cascades of cellular and molecular events, which will cause further damage in the system, and loss of body functions. The consequences of the secondary injury include mitochondrial dysfunction, neurotransmitter accumulation, blood-brain barrier (BBB) and blood spinal cord barrier disruption, apoptosis, excitotoxic damage, initiation of inflammatory, and immune processes which is followed by initial primary mechanical trauma. Secondary injury involves the production of highly reactive species, reactive oxygen species (ROS), reactive nitrogen species (RNS), or free radicals which will cause damage to protein structure, DNA, and cell membrane and leads to oxidative stress which plays a major role in the pathophysiology of CNS injury. The progression of the damage starts from the primary impact on brain or spinal cord and will continue for hours, days, and weeks after the initial mechanical insult which will result in tissue damage (Samantaray *et al.*, 2009; Khalatbary *et al.*, 2010; Bains and Hall, 2012b; Bhalala *et al.*, 2013; Naseem and Parvez, 2014).

Plus the stress response, autophagy is a highly essential cellular response to damage and influences the improvement and progression of post-traumatic disease (Wang *et al.*, 2015).

The term autophagy, from Greek "self-eating" refers to a range of processes, including chaperone-mediated autophagy, microautophagy and macroautophagy, which regulated process of degradation and recycling of cellular constituents, participated in organelle turnover and the bioenergetic management of starvation of spinal cord injury.

The mammalian target of rapamycin (mTOR), a conserved serine/threonine kinase, is the catalytic subunit of two fundamentally distinct complexes: complexes-mTOR complex 1 (mTORC1) and complexes-mTOR complex 2 (mTORC2) that individually plays an essential role in the control of cell proliferation. Both complexes localized to distinctive subcellular sections, thus affecting their initiation and role (Wullschleger *et al.*, 2006; Betz and Hall, 2013). The mTORC1 stimulate protein synthesis by mRNA translation and cell development by entering the G1 phase of the cell cycle, however mTORC2, firstly identified as a regulator of the actin cytoskeleton, has been indicated to phosphorylate members of the AGC kinase

family, including Akt, which is linked to several pathological conditions (Menon and Manning, 2008; Foster and Fingar, 2010; Sparks and Guertin, 2010). They have distinctive downstream targets, different biological functions and importantly, different sensitivity to the drug rapamycin. mTORC1 is pharmacologically inhibited by short-term rapamycin management, whereas mTORC2 is resistant to short-term rapamycin treatment, although long-term treatment can prevent mTORC2 complex assembly (Phung *et al.*, 2006; Sarbassov *et al.*, 2006).

One of the most important mTOR inhibitor studied until today was Rapamycin.

Rapamycin, an inhibitor of the mTOR pathway, can extend lifespan and improve age-related functional decline in mice, thereby providing the first proof of principal that a pharmaceutical agent can slow the aging process in mammals. These outcomes have proven robust in repeated studies; however, their potential translational relevance towards a means to slow aging or prevent age-related disease in otherwise healthy humans remains unclear. Part of the challenge in addressing the potential of rapamycin (or its analogs) as a pro-longevity therapeutic lies in its known clinical risks for adverse side effects. Primary amongst these are metabolic defects that include hyperglycemia, hyperlipidemia, insulin resistance and increased incidence of new-onset type 2 diabetes. In healthy rodents, treatment with rapamycin also causes a relatively rapid, dose-dependent impairment of markers of glucose homeostasis. The natures of the metabolic effects/defects caused by rapamycin remain ambiguous regarding their role in longevity and healthy aging. Fang et al. suggested the effects of rapamycin on metabolism depend on the length of treatment with a detrimental effect on glucose metabolism in the short-term whereas mice treated chronically with rapamycin actually became insulin-sensitive. On the other hand, Blagosklonny has proposed that the presumed metabolic impairments caused by rapamycin may simply be a consequence of its action as a "starvation-mimetic" and, further, may be fundamentally required for its pro-longevity effect (Blagosklonny, 2011; Wilkinson *et al.*, 2012; Fang *et al.*, 2013; Miller *et al.*, 2014).

Considering a lot of Rapamycin-induced side effect, in these years, a number of inhibitors of the PI3K/AKT/mTOR pathway has been identified such as Temsirolimus and KU0063794.

Temsirolimus was the first mTORC1 inhibitor investigated in clinical trials in the late 1990s in patients with cancer. Is an ester derivative of rapamycin and it is a specific inhibitor of mTORC1 that interferes with the synthesis of proteins that regulate proliferation, growth, and

survival of tumor cells. Treatment with temsirolimus leads to cell cycle arrest in the G1 phase and stops tumor angiogenesis by reducing synthesis of VEGF (Duran *et al.*, 2006).

KU0063794 is a second-generation mTOR inhibitor targeting mTORC1 and mTORC2, including p70S6K, 4E-BP1 and Akt. Specifically, inhibits the phosphorylation of S6K1 and 4E-BP1, which are downstream substrates of mTORC1, and it inhibits Akt phosphorylation on Ser473, which is the target of mTORC2 (Garcia-Martinez *et al.*, 2009; Zhang *et al.*, 2013). In a recent study it has been demonstrated that KU0063794 decreasing the viability and growth of renal cell carcinoma cell lines, Caki-1 and 786-O, and showed anti-fibrotic activity in Keloid disease (Syed *et al.*, 2013; Zhang *et al.*, 2013). Previously it has been showed that mTOR plays a key role in modulation of macrophage/microglia activation, reduction of IL-1β and TNFα production, expression of nitric oxide synthase, prevention of apoptosis neuronal loss and demyelination both in the first and second phases of the damage after injury (Kanno *et al.*, 2012). Moreover, it has been demonstrated that rapamycin treatment significantly improved the neurological recovery from SCI and increased the number of surviving neurons at the lesion epicenter (Chen *et al.*, 2013b).

However, the mechanism of autophagy related inflammation after SCI and TBI is still unclear. So, in this regard in this thesis we have evaluated the effect of Ku0063794, as potential treatments for inflammation in SCI and TBI models.

CHAPTER 1: CENTRAL NERVOUS SYSTEM INJURY

1.1 SPINAL CORD INJURY

Spinal cord injury has a significant impact on quality of life, life expectancy and economic burden, with considerable costs associated with primary care and loss of income. In one study, quadriplegics ranked recovery of arm and hand function as a priority, whereas paraplegics rated recovery of sexual function as most important (when measured against recovery of bladder/bowel function, and eradicating autonomic dysreflexia, improving walking movements and trunk stability, regaining normal sensation and eliminating chronic pain)(Anderson, 2004).

The normal architecture of the human spinal cord can be radically disrupted by injury (Bunge *et al.*, 1993; Kakulas, 1999). SCI is heterogeneous in cause and outcome and can result from contusion, compression, penetration or maceration of the spinal cord. SCI leads to the death of cells, including neurons, oligodendrocytes, astrocytes, precursor cells, and any resulting cavities and cysts may interrupt descending and ascending axonal tracts, although circumferential white matter is often spared (Horky *et al.*, 2006). The pathophysiology of SCI comprises both **primary** and **secondary** mechanisms of injury.
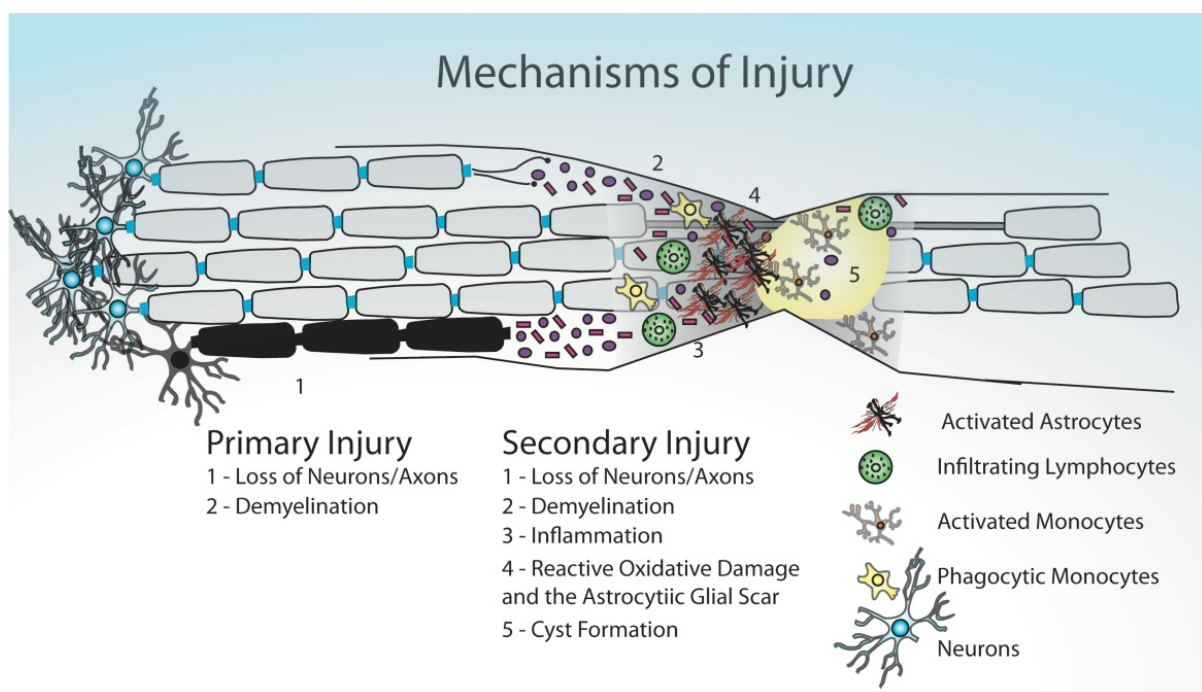
Figure 1 – Primary and secondary injury in SCI.

The "*primary injury*" refers to the forces that impart the primary mechanical insult to the spinal cord, which in its mildest form causes a cord concussion with brief transient neurologic deficits and in its most severe form causes complete and permanent paralysis. After the initial insult to the spinal cord, additional structure and function are lost through active "*secondary damage*" such as apoptosis of oligodendrocytes, loss of myelin, generation of oxidative stress, activation of immune system response and inflammation process (Crowe *et al.*, 1997).

Demyelinated axons are observed up to a decade after human SCI, and the extent to which these axons survive unmyelinated or become remyelinated by central or peripheral myelin is a subject of ongoing investigation (Guest *et al.*, 2005; Totoiu and Keirstead, 2005). Resident and invading inflammatory cells (including neutrophils, microglia, macrophages and T cells) can have a range of destructive and reparative roles. SCI culminates in glial scarring, a multifactorial process that involves reactive astrocytes, glial progenitors, microglia and macrophages fibroblasts and Schwann cells (Bruce *et al.*, 2000; Jones *et al.*, 2002; Jones *et al.*, 2003; Jones *et al.*, 2005). Progressive expansion of the injury across more than one segment (syringomyelia) can also occur over months or years, sometimes proving fatal (Silver and Miller, 2004; Fawcett, 2006).

In contrast to these destructive events, commonly observed pathological features do indicate some spontaneous repair after SCI (Beattie *et al.*, 1997). Whereas there is little or no neurogenesis in the injured spinal cord, proliferation in the ependymal and peri-ependymal canal generates new precursor cells that exclusively differentiate into glial cells (Yamamoto *et al.*, 2001; Azari *et al.*, 2005; Yang *et al.*, 2006). Limited axon sprouting does occur and lesions might even be spanned by trabeculae containing axon sprouts. Sprouting is largely impeded by geometrical and molecular factors, and few axons regenerate over long distances back to their original targets (Beattie *et al.*, 1997; Hill *et al.*, 2001; Pettigrew *et al.*, 2001). However, various forms of cortical, brainstem and spinal plasticity occur that could contribute to limited compensatory recovery (Raineteau and Schwab, 2001; Weidner *et al.*, 2001). After SCI, new spinal circuits can bypass the lesion, including sprouting of injured corticospinal axons onto spared, long descending propriospinal tracts that increase connectivity with lumbar motor neurons (Raineteau *et al.*, 2002; Bareyre *et al.*, 2004). Cortical sensorimotor areas can functionally rearrange and, at the subcortical level, the rubrospinal system can reorganize and compensate for much of the function lost after corticospinal injury (Raineteau and Schwab, 2001; Bareyre *et al.*, 2004; Thuret *et al.*, 2006).

Therefore, although there is some spontaneous repair after CNS injury, it is incomplete. Further recovery of function will require a combination of effective and safe therapeutic interventions

1.1.1 CLASSIFICATION OF SPINAL CORD INJURY

Injuries are classified in general terms of being neurologically "**complete**" or "**incomplete**" based upon the sacral sparing definition (Waters *et al.*, 1991). "**Sacral Sparing**" refers to the presence of sensory or motor function in the most caudal sacral segments as determined by the examination (i.e. preservation of light touch or pin prick sensation at the S4-5 dermatome, DAP or voluntary anal sphincter contraction). A complete injury is defined as the absence of sacral sparing (i.e. sensory and motor function in the lowest sacral segments, S4-5), whereas an incomplete injury is defined as the presence of sacral sparing (i.e. some preservation of sensory and/or motor function at S4-5) (Kirshblum *et al.*, 2011).

The following **ASIA Impairment Scale (AIS)** designation is used in grading the degree of impairment:

- ✓ A = Complete. No sensory or motor function is preserved in the sacral segments S4-S5.
- ✓ B = Sensory incomplete. Sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-S5, AND no motor function is preserved more than three levels below the motor level on either side of the body.
- ✓ C = Motor incomplete. Motor function is preserved below the neurological level, and more than half of key muscle functions below the single neurological level of injury have a muscle grade less than 3 (Grades 0–2).
- ✓ D = Motor incomplete. Motor function is preserved below the neurological level, and at least half (half or more) of key muscle functions below the NLI have a muscle grade >3.
- ✓ E = Normal. If sensation and motor function as tested with the ISNCSCI are graded as normal in all segments, and the patient had prior deficits, then the AIS grade is E. Someone without a SCI does not receive an AIS grade.

Figure 2 Standard neurological classification of spinal cord injury

1.1.2 EPIDEMIOLOGY, INCIDENCE AND CAUSES

SCI epidemiology has been studied extensively over the past 40 years. Studies focused on descriptive epidemiology, including overall incidence rates, age, gender, race, cause of injury, level and completeness of injury (Kraus *et al.*, 1975; Bracken *et al.*, 1981; Griffin *et al.*, 1985). Published reports of SCI incidence in the United States vary from 25 to 59 new cases per million population per year with an average of 40 per million (Acton *et al.*, 1993; Price *et al.*, 1994; Thurman *et al.*, 1994; Johnson *et al.*, 1997; Surkin *et al.*, 1998). This would translate to approximately 12 400 new SCIs in 2010. The incidence of SCI in the rest of the world is much lower than in the United States (Devivo, 2012). There are several possible explanations for this. One is the relative absence in most countries of SCI because of acts of violence. However, there also appear to be fewer SCIs related to motor vehicle crashes in other countries. Possible explanations for this would be a lower average passenger miles of exposure, greater use of seat belts, or safer driving habits and road conditions. Conversely, lower incidence could also result from greater mortality at the site of the accident. Finally, incomplete case ascertainment may have occurred in many of these studies as they are not

typically population-based but rather rely on referrals to specialized centers. No studies have addressed the reasons for international variation in SCI incidence.

SCI incidence rates are lowest for the pediatric age group, highest for persons in their late teens and early twenties, and generally decline consistently thereafter, although some studies suggest a secondary increase in incidence rates among the elderly.(Acton *et al.*, 1993; Price *et al.*, 1994; Hagen *et al.*, 2010)**.** Among persons enrolled in the combined US data set, the mean age at injury has increased from 28.3 years during the 1970s to 37.1 years between 2005 and 2008 (DeVivo and Chen, 2011). These figures mirror the increasing median age of the general US population, which were 30 years in 1980 and 36.9 years in 2010.

The average age at injury is a few years higher in most other countries than in the United States (Devivo, 2012). This is likely because of the lower rate of injuries due to violence that typically occur among younger persons, although other factors such as the average age of the general population and differences in other cause-specific incidence rates also likely have a role in raising the average age at injury in other countries. In 2001, 13% of the US population was >65 years, compared with 18% in Japan and 15% in Europe.
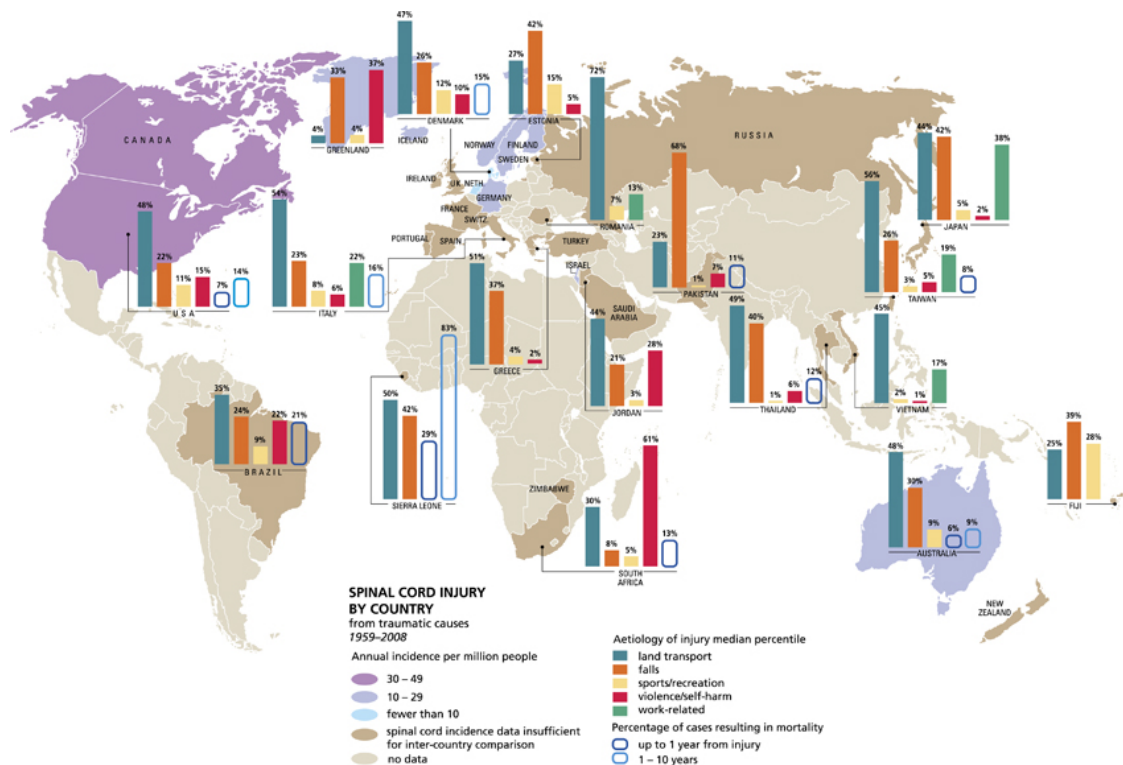
Figure 3 Global mapping of spinal cord injury from traumatic

causes by country 1959–2008

SCI occurs predominantly among men and will continue to do so in the future. SCI annual incidence rates are typically 3 to 4 times higher for men than women (Devivo, 2012).

However, the percentage of new injuries occurring among men in the combined US data set has declined slightly over time from 80.9% during the 1970s to 77.1% since 2000 (Devivo, 2012). A similar trend has occurred in Norway where the incidence rate was 5.3 times higher among males than females between 1952 and 1956, but only 4.2 times higher between 1992 and 2001 (Hagen *et al.*, 2010). This trend toward a slightly increasing percentage of women among new SCIs should continue because injuries among older persons are increasing, and SCIs among the elderly are more evenly split between men and women than SCIs that occur among teenagers and young adults.

In the United States, motor vehicle crashes are the leading cause of SCI (Acton *et al.*, 1993; Price *et al.*, 1994; Devivo, 2012). Although the percentage of SCIs owing to motor vehicle crashes in the combined US data set has fluctuated over time, it is approximately the same today (48.3% since 2000) as it was during the 1970s (47.6%) (Devivo, 2012). Injuries due to acts of violence peaked in the 1990s (21%) but have since declined dramatically (12% since 2000). 18 Overall, sports-related SCIs have declined slightly from 14.2% during the 1970s to 10.0% since 2000 (Devivo, 2012). Injury prevention initiatives have reduced the occurrence of SCIs in many sports, most notable diving, American football and trampolines. However, SCIs from winter sports such as snow skiing have increased.

Falls are the leading cause of SCI among persons aged >60 (Price *et al.*, 1994).Therefore, it is not surprising that the proportion of new SCIs owing to falls has been increasing steadily as injuries among older persons have become more frequent. During the 1970s, falls accounted for 16.2% of new SCIs in the combined US data set compared with 21.8% since 2000 (Devivo, 2012). This trend is likely to continue, with a corresponding decline in sports and violence-related SCIs that do not typically occur among older persons.

1.1.3 PHARMACOTHERAPY

Pharmacotherapy early after SCI is aimed at neuroprotection to minimize the secondary injury. Several trials with different neuroprotective pharmacological agents have been reported in literature. These pharmacological agents block one or more of the mechanisms of secondary injury following trauma to the cord. Unfortunately, till date, no neuroprotective pharmacological agent has been conclusively shown to be clinically effective in human subjects with SCI (Tator, 2006).

1.1.3.1 Methylprednisolone

Animal studies have demonstrated that methylprednisolone (MP) reduces the secondary cord injury following trauma by inhibiting lipid peroxidation, improving spinal blood flow, enhancing the postinjury activity of Na^+/K^+-ATPase, and facilitating the recovery of extracellular calcium ions (Young, 1991; Hall *et al.*, 1992). Three MP trials have been reported from North America (Bracken *et al.*, 1984; Bracken *et al.*, 1990; Bracken *et al.*, 1997). The NASCIS 2 trial (Bracken *et al.*, 1990) suggested clinical efficacy that did not translate into effectiveness. Subsequent study from France (Pointillart *et al.*, 2000) and evidence-based analysis (Hurlbert, 2000; Short *et al.*, 2000; Hurlbert, 2001; Hurlbert, 2006) have not supported the routine use of MP in acute SCI. Despite equivocal effectiveness, MP is widely used in many North American centres, for want of a better alternative. The drug is given within 8 h of injury in a bolus dose of 30 mg/kg followed by a maintenance dose of 5.4 mg/kg/h over next 23 h.

1.1.3.2 GM1 Ganglioside

Animal studies have shown that monosialotetrahexosylganglioside (GM-1) ganglioside enhances the functional recovery of damaged neurons (Ferrari and Greene, 1998). The first randomized control trial, with 34 patients, suggested beneficial effects of GM-1 (Geisler *et al.*, 2001). However, a later adequately powered multicenter study failed to establish the effectiveness of this agent in SCI (Geisler *et al.*, 2001).

1.1.3.3 Thyrotropin Releasing Hormone

Thyrotropin releasing hormone (TRH) is a partial endorphin antagonist. Endorphins are released after SCI and postulated to exacerbate posttraumatic ischemia by reducing spinal blood flow secondary to systemic hypotension (Faden *et al.*, 1981). A smaller study (Pitts *et al.*, 1995) suggested beneficial effects of TRH in SCI. A larger study to evaluate the efficacy has not been carried out.

1.1.3.4 Nimodipine

Nimodipine is a Ca^{2+} channel blocker. It can potentially minimize the injury following SCI by counteracting vasospam, ischemia, and infarction that contribute to secondary damage(Guha

et al., 1987; Pointillart *et al.*, 1993). A single center randomized trial that compared four treatment arms viz. nimodipine, MP, MP and nimodipine, and placebo failed to demonstrate any significant benefits of nimodipine alone or in combination with MP (Pointillart *et al.*, 2000).

1.1.3.5 Gacyclidine

Gacyclidine is an N-methyl-D-aspartate antagonist. It blocks the toxic effects of glutamate, which is released following SCI. A multicenter RCT failed to establish efficacy of Gacyclidine at 1 year following SCI (Tator, 2006).

1.1.3.6 Minocycline

Minocycline is a tetracycline derivative (a bacteriostatic antibiotic) that is currently in common clinical use for the treatment of acne and chronic periodontitis. Animal experiments have shown minocycline has a neuroprotective effect following SCI. It reduces axonal loss at the site of injury, decreases oligodendrocyte apoptosis, and prevents activation of microglia and macrophages (Lee *et al.*, 2003; Wells *et al.*, 2003; Stirling *et al.*, 2004). A pilot study to evaluate minocycline in patients with acute spinal cord injuries has been initiated in Calgary, Alberta, Canada (Kwon *et al.*, 2005).

1.1.3.7 Estrogen

Early treatment with the estrogen 17 β-estradiol has been shown to have a neuroprotective effect following experimental SCI in rats (Yune *et al.*, 2004; Sribnick *et al.*, 2006). 17β-Estradiol reduces apoptosis in the penumbra around the zone of necrosis, thereby minimizing the secondary damage.

1.2 TRAUMATIC BRAIN INJURY

Traumatic brain injury is a nondegenerative, noncongenital insult to the brain from an external mechanical force, possibly leading to permanent or temporary impairment of cognitive, physical, and psychosocial functions, with an associated diminished or altered state of consciousness (Topal *et al.*, 2008). TBI can manifest clinically from concussion to coma and death. Injuries are divided into 2 subcategories: *primary injury*, which occurs at the moment of trauma, and *secondary injury*, which occurs immediately after trauma and produces effects that may continue for a long time. Changes to the cerebral environment involve a complex interplay between cellular and molecular processes, in which glutamate-driven excitotoxic effects, oxidative stress, inflammation, ion imbalance, and metabolic disarray are major components. These pathways induce progressive neuronal loss through necrosis and apoptosis (Morganti-Kossmann *et al.*, 2007; Kalia *et al.*, 2008). Also, important are the intracellular changes that are determined by the excessive influx of calcium, which affects mitochondrial integrity, depleting cells of an essential source of energy. The metabolic disarray caused by accumulation of lactate results in cytotoxic swelling of cells, which, together with the increased permeability of the cerebral vasculature, leads to brain oedema, elevated intracranial pressure, and reduced cerebral perfusion(Morganti-Kossmann *et al.*, 2007; Kalia *et al.*, 2008; Bains and Hall, 2012a).
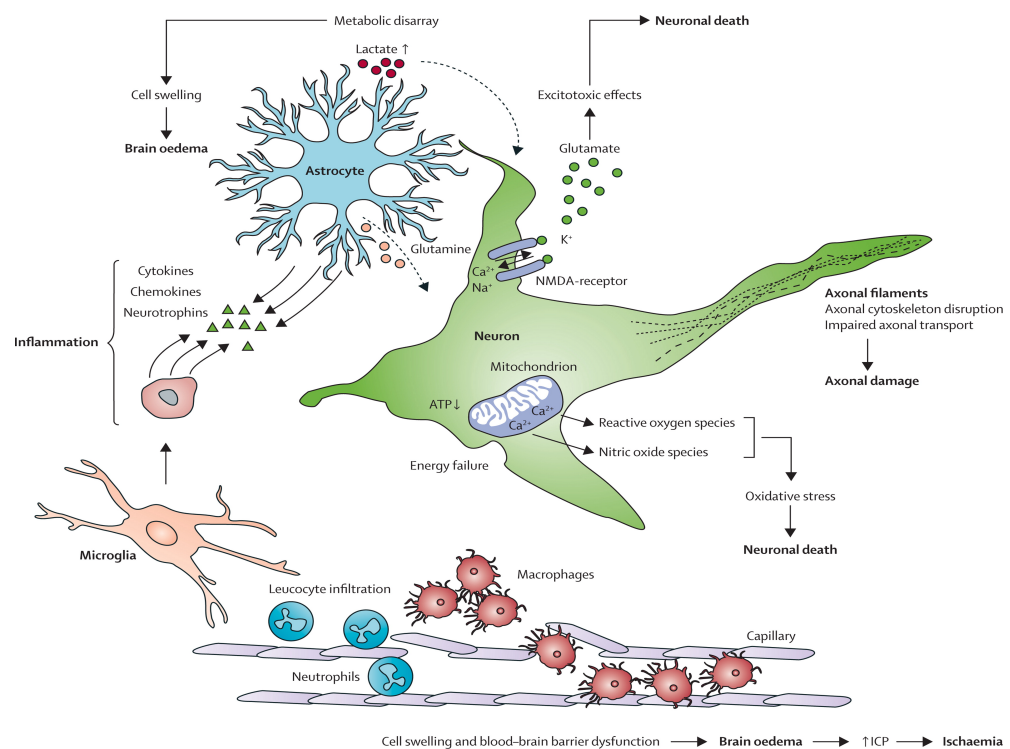
Figure 4 - Pathophysiology of traumatic brain injury

In addition, regardless of origin, TBI sufferers experience a relatively stereotyped array of symptoms associated with the injury: dizziness, confusion, and sometimes loss of consciousness (especially in severe injury). Even after the initial injury is managed and resolved, approximately 70–80% of TBI patients develop long-lasting effects such as changes in personality and cognition, anxiety and depressive-like behaviors (Whitnall *et al.*, 2006; Kiraly and Kiraly, 2007; Draper and Ponsford, 2008; Ponsford *et al.*, 2008). TBI also increases the risk for certain neurodegenerative conditions. For instance, repeated concussive TBI has been associated with the development of chronic traumatic encephalopathy (CTE) in athletes. Furthermore, both repeated and single TBI show a strong association with increased Alzheimer's disease (AD) risk or earlier AD onset. Correlations with Parkinson's disease and amyotrophic lateral sclerosis (ALS) have also been reported, but the supporting evidence is not as strong as for CTE and AD (Gyoneva and Ransohoff, 2015).

Considering the high prevalence of TBI and its association with serious neurological problems and risk for neurodegenerative diseases, there is a strong impetus to develop new TBI therapies that not only promote cell survival immediately after the injury but also address the development of secondary pathology. Because of the close association between neuropathology and inflammation in space and time, the latter has emerged as an important target for the amelioration of TBI (Das *et al.*, 2012; Giunta *et al.*, 2012; Woodcock and Morganti-Kossmann, 2013).

1.2.1 CLASSIFICATION OF TRAUMATIC BRAIN INJURY

Glasgow Coma Scale (GCS) is the most widely used scoring procedure for mental and neurological status following head injury in the U.S. and most English-speaking countries (Gouvier *et al.*, 1987). Its score is based on the sum of three components: eye opening response, verbal response, and best motor response. For instance, if an individual at the accident scene opened eyes to voice, used inappropriate words, and demonstrated a flexion response to motor stimulation, the scoring would be E + V + M = 3 + 3 + 4 = 10 (see following table).

Category		Best Response
Eye opening		
Spontaneous		4
To speech		3
To pain		2
None		1
Verbal	(Modified for Infants)	
Oriented	Babbles	5
Confused	Irritable	4
Inappropriate words	Cries to pain	3
Moans	Moans	2
None	None	1
Motor		
Follows commands		6
Localizes to pain		5
Withdraws to pain		4
Abnormal flexion		3
Abnormal extension		2
None		1
Glasgow Coma Score		
Best possible score		15
Worst possible score		3
If tracheally intubated then verbal designated with "T"		
Best possible score while intubated		10T
Worst possible score while intubated		2T

Table 1 – Glasgow Coma Scale.

This in turn produces a graded score in the moderate severity range. The GCS can be further subdivided into mild injury (GCS = 13 to 15), moderate injury (GCS = 9 to 12), and severe injury (GCS = 3 to 8). The clinical features of mild injury are loss of consciousness for 20 minutes, no focal neurological signs, no intracranial mass lesion, and no intracranial surgery. Regardless of mental state, a focal computed tomography (CT) lesion places the patient into the moderate category. A coma duration of at least 6 h places the patient into the severe category, regardless of mental state.

In terms of outcome, the most commonly used current scales are the Glasgow Outcome Scale and the **Rancho Los Amigos Level of Cognitive Functioning Scale** (see following table).

Levels	Clinical Signs
I. No response	Unresponsive to any stimulus
II. Generalized response	Nonpurposeful responses, usually to pain only
III. Localized responses	Purposeful; may follow simple commands
IV. Confused, agitated	Confused, disoriented, aggressive; unable to perform self-care
V. Confused, inappropriate	Nonagitated; appears alert; responds to commands; verbally inappropriate; does not learn
VI. Confused, appropriate	Can relearn old skills; serious memory defects; some awareness of self and others
VII. Automatic, appropriate	Oriented; robot-like in daily activities; minimal confusion; lacks insight or planning ability
VIII. Purposeful, appropriate	Alert and oriented; independent in living skills; capable of driving; defects may remain in judgment, stress tolerance and abstract reasoning may not be at preinjury cognitive ability

Table 2 - Rancho Los Amigos Level of Cognitive Functioning Scale.

The Rancho Scale is widely used by rehabilitation facilities after the patient leaves the neurosurgical intensive care unit or neurosurgical floor for postacute care. Generally, a final grading using the Rancho Scale is made prior to the patient's discharge from a brain injury rehabilitation unit if such is required (Gouvier *et al.*, 1987).

1.2.2 EPIDEMIOLOGY, INCIDENCE AND CAUSES

Each year, approximately 30 million injury-related emergency department (ED) visits, hospitalizations, and deaths occur in the United States. Of the injury hospitalizations, approximately 16% included TBI as a primary or secondary diagnosis. Of the injury deaths, approximately one-third included a TBI as a direct or underlying cause of death. In 2010, CDC estimated that TBIs accounted for approximately 2.5 million ED visits, hospitalizations, and deaths in the United States, either as an isolated injury or in combination with other injuries. Of these persons, approximately 87% (2,213,826) were treated in and released from EDs, another 11% (283,630) were hospitalized and discharged, and approximately 2% (52,844) died.
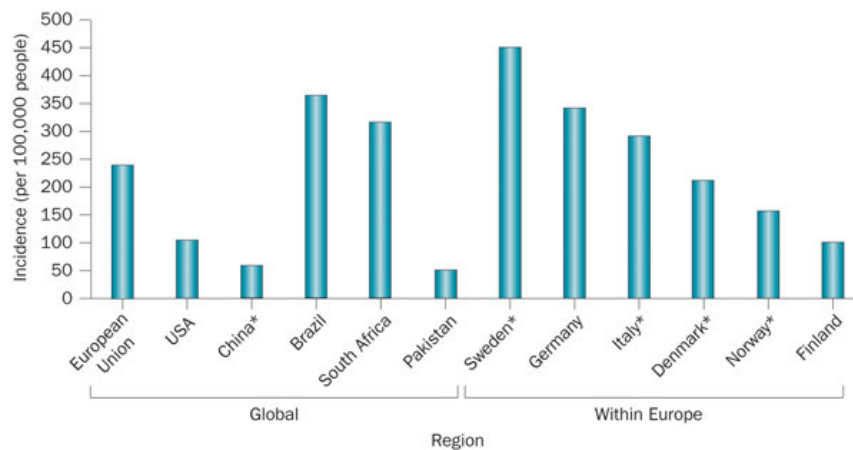
Figure 5 - Estimates of the global incidence of TBI

These figures, however, underestimate the occurrence of TBIs, as they do not account for those persons who did not receive medical care, had outpatient or office-based visits, or those who received care at a federal facility (i.e., persons serving in the U.S. military or seeking care at a Veterans Affairs hospital) (Faul, 2010). Department of Defense data revealed that from 2000 through 2011 235,046 service members (or 4.2% of the 5,603,720 who served in

the Army, Air Force, Navy, and Marine Corps) were diagnosed with a TBI (CDC, NIH, DoD, and VA Leadership Panel, 2013). In the United States, children aged 0–4 years, adolescents aged 15–19 years, and older adults aged ≥75 years are the groups most likely to have a TBI-related ED visit or hospitalization (Faul, 2010). Adults' aged ≥75 years have the highest rates of TBI-related hospitalizations and deaths among all age groups. Overall, males account for approximately 59% of all reported TBI-related medical visits in the United States (Faul, 2010). As shown in Table 4, during 2002—2010, the leading causes of TBI-related ED visits were falls, being struck by or against an object, and motor-vehicle traffic crashes. The leading causes of TBI-related hospitalizations were falls, motor-vehicle traffic incidents, and assaults.

For TBI-related deaths, the leading causes were motor-vehicle traffic incidents, suicides, and falls (Coronado *et al.*, 2012). The proportion of TBIs occurring during sports and recreation-related activities are undetermined because of limitations of the data source. However, according to the National Electronic Injury Surveillance System – All Injury Program, during 2001–2009 (CDC, 2011) the activities associated with the greatest estimated number of TBI-related ED visits were bicycling, football, playground activities, basketball, and soccer among persons younger than 19 years.

Mechanism of injury	ED visits	Hospitalizations	Deaths
Falls	658,668	66,291	10,944
Struck by or against an object	304,797	6,808	372
Motor vehicle traffic	232,240	53,391	14,795
Assault/Homicide	179,408	15,032	5,665
Self-inflicted/ Suicide	*	*	14,713
Other	122,667	25,478	4,990
Unknown	97,018	113,172	0

*Estimate not reported because of small numbers

Figure 6 – Major causes of TBI

1.2.3 PHARMACOTHERAPY

Despite substantial investments by government, philanthropic, and commercial sources over the past several decades, TBI remains an unmet medical need and a major source of disability and mortality in both developed and developing societies.

1.2.3.1 Acetylcholinesterase inhibitors

Central acetylcholinesterase inhibitors (AChEI) increase synaptic acetylcholine by inhibiting its breakdown in the synaptic cleft. Studies of these compounds for the treatment of patients with TBI suggest they may have potentially beneficial effects—particularly in patients with chronic moderate and severe TBI who have persistent cognitive deficits—by increasing synaptic ACh levels (Tenovuo, 2005). Beneficial effects have been reported in pre-clinical TBI studies with AChEI, including positive effects on acute injury processes with reduced TBI-induced neuronal death, preservation of neurons in the CA1 hippocampal region, reduced blood–brain barrier (BBB) disruption, decreased vasogenic brain edema, and preserved neurologic and motor function (Chen *et al.*, 1998; Ballesteros *et al.*, 2008)..

1.2.3.2 Amantadine

Amantadine (1-adamantamine hydrochloride) is a tricyclic amine used for the prophylaxis and treatment of influenza A and was serendipitously discovered to have modest efficacy for the treatment of Parkinson disease (PD). The anti-parkinsonian mechanism of action is not fully understood, but research has suggested that amantadine increases extracellular dopamine (DA) concentrations either by blocking DA reuptake or facilitating DA synthesis (Bales *et al.*, 2009). Amantadine may also have post-synaptic effects on DA circuits by increasing DA receptor density (Gianutsos *et al.*, 1985). One study showed that amantadine treatment, starting 1 day after a closed controlled cortical impact model of TBI in rats and continuing for 18 days after injury, resulted in modest improvement in Morris water maze (MWM) latencies (Dixon *et al.*, 1999).

1.2.3.3 Cyclosporine A/FK 506

Cyclosporine A (CsA) inhibits opening of the mitochondrial permeability transition pore after TBI, thereby maintaining mitochondrial membrane potential. Many studies in animal models of TBI have suggested that this action of CsA confers benefit by preserving mitochondrial function and reducing reactive oxygen species (Brustovetsky and Dubinsky, 2000; Sharov *et al.*, 2007) Inhibition of the protein phosphatase calcineurin via the immunophilin effects of CsA also has beneficial effects on axonal injury and learning and memory (Van Den Heuvel *et al.*, 2004; Setkowicz and Guzik, 2007). Similarly, immunosuppressive effects, also mediated by calcineurin inhibition, may further confer benefit after TBI or mediate potential side effects. The related compound, FK 506, inhibits calcineurin and exhibits

immunosuppressive effects but does not inhibit opening of the mitochondrial permeability transition pore (Singleton *et al.*, 2001).

1.2.3.4 Progesterone

Progesterone is a steroid that is made in the brain, in addition to its synthesis in the reproductive organs and adrenal glands. Progesterone has pleiotropic effects, and thus has multiple candidates for mechanisms of action with regard to its potential therapeutic efficacy in TBI (Hammond *et al.*, 1983). Multiple pre-clinical models of TBI have demonstrated neuroprotective properties of progesterone and have shown that it enhances behavioral and functional outcomes, decreases cerebral edema, apoptosis, pro-inflammatory cytokines, and other markers of inflammation, and prevents neuronal cell death (Liu *et al.*, 2009). Progesterone also enhances myelination, neurogenesis, and impacts aquaporin expression, and modulates neurotrophin expression, among other actions (Koenig *et al.*, 1995; Liu *et al.*, 2009).

1.2.3.5 Simvastatin/other statins

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMGA) reductase inhibitors, reduce serum cholesterol but also have potent effects in the brain relevant to mechanisms of TBI injury and recovery. Such effects target mechanisms that influence both the acute and chronic phases of TBI (Li *et al.*, 2009; Chauhan and Gatto, 2010). There is pre-clinical evidence of beneficial effects including those on acute injury processes such as brain edema, BBB integrity, cerebral blood flow, neuroinflammation, axonal injury, and cell death, in addition to effects on key facets of regeneration such as trophic factor production. A variety of molecular outcomes are influenced including TUNEL staining, CREB, Akt, eNOS, FOXO1, NF-κB, GSK3, cytokines, BrdU labeling, blood vessel formation, and vascular endothelial growth factor (Chen *et al.*, 2009a).

1.2.3.6 N-acetylcysteine (NAC)

NAC is FDA-approved as an antidote for acetaminophen overdose and as a mucolytic for cystic fibrosis and other bronchopulmonary diseases. In animal models of TBI, NAC has shown strong antioxidant activity by increasing glutathione levels and decreasing markers of oxidative damage (Hicdonmez *et al.*, 2006). NAC also showed anti-inflammatory activity by decreasing the activation of NF-κB, while lowering interleukin (IL)-1β, tumor necrosis factor

(TNF)-α, and intercellular adhesion molecule (ICAM)-1 levels (Yi and Hazell, 2005; Chen *et al.*, 2008). It is unclear how the anti-inflammatory action of NAC is related to its antioxidant activity. NAC has been shown to reduce lesion volume while simultaneously reducing levels of the putative neuroprotective enzyme heme oxidase (Yi and Hazell, 2005; Chen *et al.*, 2008).

1.2.3.7 Growth hormone (GH)

GH is a polypeptide that is synthesized, stored, and secreted by somatotrophic cells within the lateral wings of the anterior pituitary gland. GH deficiency/insufficiency (GHD/GHI) is the most common anterior pituitary abnormality after TBI. Manipulating the GH axis has been shown to improve motor function, enhance learning and memory retention after TBI in rats, and to improve spatial learning and memory in a mouse model of AD (Saatman *et al.*, 1997; Doulah *et al.*, 2009).

CHAPTER 2: THE ROLE OF NEUROINFLAMMATION

2.1 CYTOKINE RESPONSES TO INFLAMMATION

Cytokines are small and nonstructural proteins with no amino acid sequence motif, their biological activities allow us in turn to group them into different classes: exit 18 cytokines called interleukin (IL), some of these promote inflammation and are named pro-inflammatory cytokines such as IL1β and IL1α, IL6, IL8 and TNFα; whereas other cytokines suppress the activity of pro-inflammatory cytokines and are called anti-inflammatory cytokines such as IL-4, IL-10, TGFβ. The hypothesis that some cytokines function primarily induce inflammation while others suppresses inflammation is essential to cytokine biology and to clinical medicine.

Cytokines are secreted by a variety of immune cells such as T-lymphocytes and macrophages, as well as b non-immune cells such as fibroblasts; the physiological effects mediated by cytokines comprise the stimulation or inhibition of cell growth, cytotoxicity/apoptosis, antiviral activity and inflammatory responses. The main function of cytokines is the regulation of T-cell differentiation from undifferentiated cells to T-helper 1 and 2, regulatory T cells, and T-helper 17 cells (Steinman, 2007). These regulatory proteins include ILs, interferons (IFNs) and TNFs. Many of these cytokines have already been shown to be produced by neurons or glia in CNS disorders in which they are notably increased.
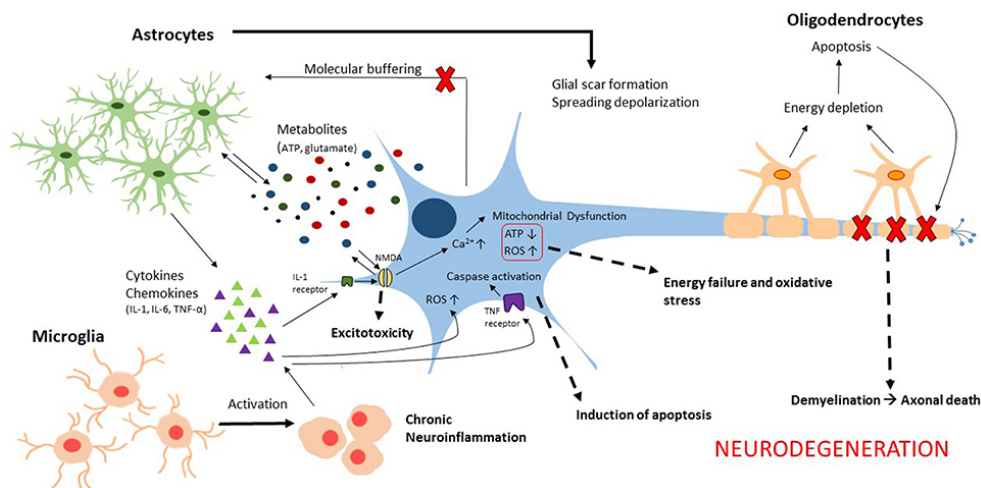
Figure 7 – Neuroinflammation following TBI

The cytokine class of inflammatory mediators is secreted by microglia and astrocytes and their production is increased in inflammatory states, moreover, they act by modulating the intensity and duration of the immune response. Pro-inflammatory cytokines and chemokines up-regulate microbicidal activity of neutrophils, and they can be considered as additional immunomodulatory agents to treat serious or refractory infections in humans.

Through cytokines IL-1 initiate the immune response, having a crucial role in the onset and expansion of a complex hormonal and cellular inflammatory cascade; the IL-1 family of cytokines includes IL-1α and IL-1β, which generate cell activation upon binding with specific membrane receptors and has been documented that IL-1 play a role in neuronal degeneration. In astrocytes, IL-1 induces IL-6 production, stimulates iNOS activity (Hausmann, 2003), enhances neuronal acetylcholinesterase activity, microglial activation and additional IL-1 production, and astrocyte activation.

Another important pro-inflammatory cytokine is the IL-6, a multifunctional cytokine that plays an important role in host defense (Okada *et al.*, 2004), and possess main effects during the inflammatory response (Roxburgh and McMillan, 2016). IL-6 is associated to the neuropoietin cytokine's family and it possess direct and indirect neurotrophic effects on neurons (Teng and Tang, 2006); moreover, IL-6 promotes astrogliosis (Morales *et al.*, 2010), activates microglia (Inoue, 2002), and stimulates the release of acute phase molecules.

2.1.1 INFLAMMATORY MEDIATOR: ROLE OF TNF α

Through all the cytokines involved in the secondary damage of SCI and TBI, TNF-α plays a crucial role in fact it is release shortly after injury, it can accumulate rapidly at the site of injury and it is produced by a number of different cell populations, such as neutrophils, macrophages and microglia, astrocytes and T cells (Yan *et al.*, 2001). Several cell types are able to produce TNFα, including macrophages after its activation, dendritic cells, monocytes, NK cells, CD4+ T cells, CD8+ T cells, microglia and astrocytes. Macrophages/monocytes are able to produce TNFα in the acute phase of inflammation and this cytokines drives several range of signalling events within cells, leading to necrosis or apoptosis.

Several biological functions are ascribed to the TNFα and for this reason the mechanism of action is somewhat complex; although it inhibits the growth of tumor cells and it has an enhancing effect on the proliferation of normal cells (Mandi *et al.*, 1991) TNF-α is takes part in septic shock, autoimmunity and inflammatory disorders. The major role of TNFα is explicated as mediator in resistance against infections; moreover, it was postulated that TNF

plays a pathological role in a number of autoimmune pathology such as graft vs host rejection or rheumatoid arthritis. Moreover, TNFα possess potent pro-inflammatory effects that are associated to its capacity to generate endothelial cell adhesion molecules and subsequently support neutrophil adherence to vascular endothelium. Neutrophils are exquisite targets of TNF-α that, under certain conditions, strengthens their expression of adhesion molecules, induces their degranulation and successive release of lysosomal enzymes, causing the production of highly reactive oxygen species. TNF-α-induced the migration of neutrophils mediating the production of chemotactic factors, including IL-8, this testifies cytokine networking involvement in inflammatory cell recruitment and an active role in inflammation.

TNFα works by binding and clustering high-affinity receptors that are present in a great numbers on most cell membranes (Loetscher *et al.*, 1991), the ligand/receptor complex is easily internalised via clathrin-coated pits and ends up in secondary lysozymes where it is degraded. Interestingly the binding of TNFα to the 75 kDa TNFR-2 is not sufficient to reach cytotoxicity, but rather binding to the 55 kDa TNFR-1 is sufficient to reach TNFα mediated cell killing. TNFα exerts its effects by activating several secondary proteins that provoke a variety of responses within the cell such as activation of gene transcription and/or production of reactive oxygen or nitrogen radicals (e.g., NO). Activated proteins include Gprotein, transcription factors such as NF-κB and AP-1 and serine and cysteine proteases, known as caspases. Many members of the TNF receptor superfamily have intracellular "death domains," which represent protein interaction domains each consisting of 65–80 amino acids; these proteins participate in TNFα mediated apoptosis process; many evidence demonstrated that TNF–TNFR interactions are implicated in the pathogenesis of CNS disorders such as EAE and MS. These interactions are able to monitor the disease outcome by modifying immune response and the interactions between CNS-resident cells and effector immune cells in the CNS.

However, recent studies showed a dual nature for TNF-α not only neurotoxic but also it can be neuroprotective; a study conducted with transgenic mice for TNF-α receptors demonstrated that the mice lacking for TNF-α showed more tissue loss and functional deficits compared to wild-type mice, implying that TNF-α mediated a neuroprotective effect (Kim *et al.*, 2001). The beneficial or deleterious effects of TNF-α dependent when it is being released and on cellular populations that is acting on, the conflicting actions of TNF-α described above reflects a growing view of inflammation as a "dual-edged sword," having neurotoxic and neuroprotective properties (Bethea, 2000).

Thus, comprehension of their profile, kinetics of expression and interactions between TNF-α ligands and their TNFRs on different CNS residents and infiltrating immune cells, would aid to better design strategies to control neuroinflammation and CNS autoimmunity. Blockers of TNF α have been acknowledged for human use in treating TNF-linked autoimmune and inflammatory disorders. Pathways downstream of receptor ligation supply critical points for interjection for planning new therapeutic strategies.

2.2 MICROGLIA ACTIVATION

Moreover, another important mediators of inflammation that respond rapidly to disturbances within the microenvironment by change in morphology are the microglia, the expression 'activated microglia' is used to define cells that change their immunophenotype and their morphology after a specific stimuli; the principal role of microglia at the lesion site is a rapid phagocytosis of fragments and induction of apoptosis (Shuman *et al.*, 1997). The different response of microglia *in vitro* suggest that these cells may elicit unique functional properties, and consequently control the inflammatory response at the injury site. Microglial activation has been well-known in the spinal cord tissue that receive a trauma and has been shown to occur from caudal to lumbar enlargement, based on that there are papers supporting the role of microglia in pain after injury and showing activation of microglia post-SCI.

Microglia activate the innate immune system and are key regulators of inflammatory processes in CNS pathologies such as trauma and neurodegenerative diseases participation in both acute and chronic phase of the inflammatory responses. Activated microglia secrete cytotoxic substances including various cytokines such as TNF-α, IL-1, reactive free radicals, and nitric oxide. However, the principal effects of microglia at the levels of the lesion core are probably rapid phagocytosis of debris rather than induction of apoptosis. Microglia when activated can cause neuronal and glial toxicity through the release of cytokines, free radicals, eicosanoids, activated neutrophils and macrophages (Schwartz, 2000). On the other hand, microglia activation lead to beneficial effects producing growth factors that are fundamental for neuronal and tissue restoration. Moreover, it has been demonstrated that transplantation of peripherally activated macrophages has beneficial effects on spinal cord regeneration.

2.3 APOPTOSIS

In the last decade the generation of apoptotic process after SCI and TBI was also confirmed, apoptosis can be triggered by a variety of insults including cytokines, inflammatory injury, free radical damage and excitotoxicity. The apoptotic process after SCI and TBI is activated

in neurons, oligodendrocytes, microglia, and perhaps, astrocytes; apoptosis in microglia contributes to inflammatory secondary injury.

Two main pathways of apoptosis—extrinsic or receptor-dependent and intrinsic or receptor-independent—have been well characterized, and both appear to be active in SCI and TBI; the extrinsic or receptor-dependent pathway is mediated by Fas ligand and Fas receptor (Leskovar *et al.*, 2000) and/or inducible nitric oxide synthase production by macrophages (Satake *et al.*, 2000), while intrinsic or receptor-independent pathway is mediated via direct caspase-3 proenzyme activation (Citron *et al.*, 2000) and/or mitochondrial damage, release of cytochrome c and activation of the inducer caspase-9, pathways of caspase-mediated apoptotic death (Hartley *et al.*, 2000).
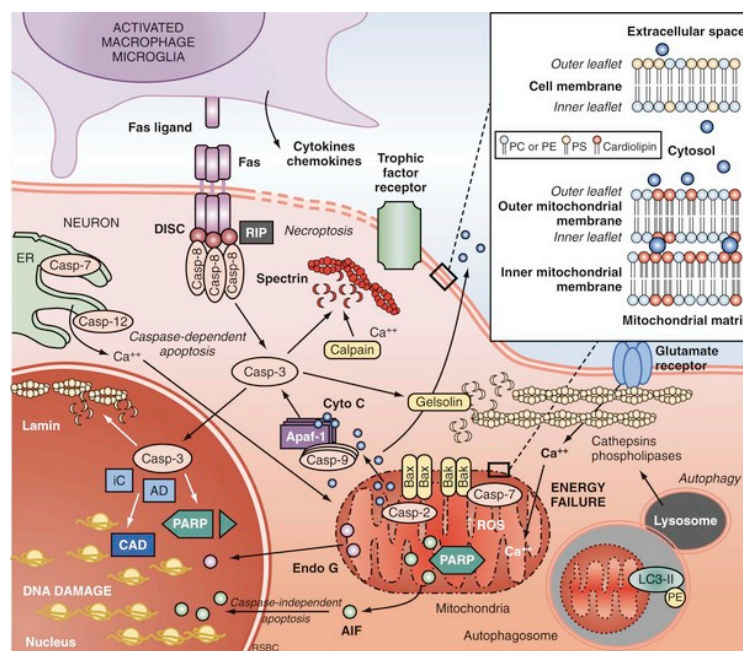
Figure 8 – Apoptosis following brain injury

Receptor-dependent apoptosis is evoked by extracellular signals, the most significant of which is TNF, so the term of "extrinsic" pathway; tumor necrosis factor is known to rapidly accumulate in the injured spinal cord, and activation of the Fas receptor of neurons, microglia, and oligodendrocytes induces a programmed sequence of caspase activation. Moreover, additional control of cell death/survival is provided by the balance between major proapoptotic proteins such as Bax, Bad, and Bid and antiapoptotic proteins such as Bcl-XL and Bcl-2.

Apoptotic process that is activated in the secondary injury in SCI has recently come under close study, and the precise contribution and potential therapeutic implications of apoptosis in

SCI could be help to generate new therapeutic approach to treat the secondary events associated to spinal cord injury.

2.4 INFLAMMATORY/IMMUNOLOGIC RESPONSE

The inflammatory and immunological response to injury within the CNS, is different than that which is occurring in other tissues (Schwartz *et al.*, 1999). The inflammatory and immunologic responses to injury involve activation of innate immune cells that provide immediate defense against inflammatory stimuli and in turn help to recruit cells of the adaptive immune system (i.e., T and B lymphocytes). The activation of immune system is driven by interactions involving presentation of antigen and release of various inflammatory mediators (Ling *et al.*, 2003). Also, cells present in the injury site may sequester debris and carry CNS antigens to secondary lymphoid organs (Karman *et al.*, 2004), where trigger lymphocyte activation. Recent studies in mice showed that the number of activated T and B cells increases in the spleen and bone marrow within 24 hours of trauma (Ankeny *et al.*, 2006).

2.4.1 LYMPHOCYTES INFILTRATION

Under normal conditions, activated T cells can cross the BBB and enter the CNS parenchyma. In contrast with other inflammatory cells enrolled after a trauma, the number of lymphocytes remains low (Schnell *et al.*, 1999); however, T-lymphocytes play an important role in the CNS immune system, since on activation, T-lymphocytes may kill target cells and produce cytokines (Kierdorf *et al.*, 2010).

Once lymphocytes enter to the lesion site, they persist indeterminately (Sroga *et al.*, 2003; Ankeny *et al.*, 2006), whereas T and B cell increase in the lesion site at least 9 weeks post-injury (Bilgen *et al.*, 2002; Whetstone *et al.*, 2003), suggesting that cytokine/chemokine gradients exist chronically and regulate integrin expression on endothelia and cells (Lee *et al.*, 2000; Babcock *et al.*, 2003). These chemokine gradients and adhesion molecules represent molecular targets for manipulating the effects of intraspinal lymphocytes after SCI (Eng and Lee, 2003; Gonzalez *et al.*, 2003; Bao *et al.*, 2004); the progressive increase in lymphocyte numbers may also be justified by lymphocyte activation and proliferation within the injured centre of spinal cord.
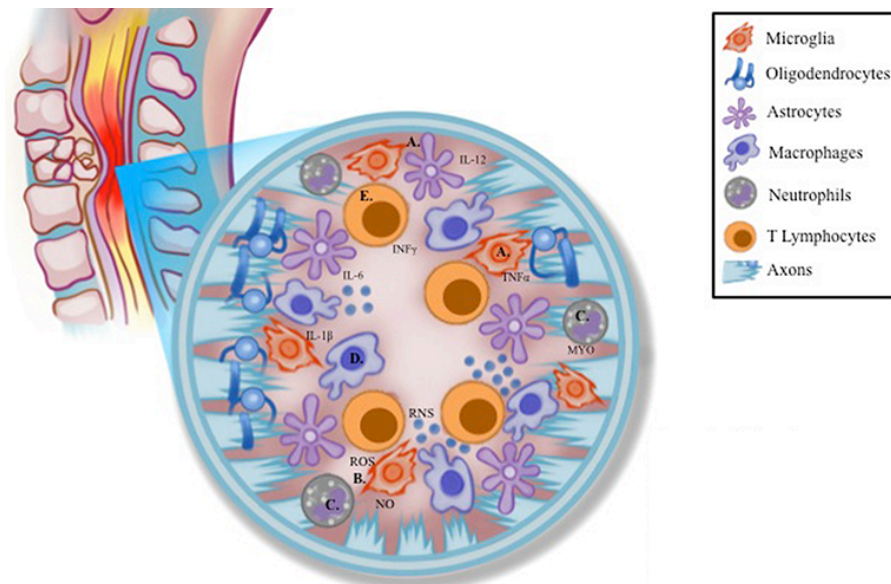
Figure 9 - Cellular immune response in SCI

Moreover, induction of immune response could be generating as impaired nerve transmission; increasing production of pro-inflammatory cytokines in chronic phase of SCI could worsen the damage increasing the axonal injury and demyelination. Furthermore, there are evidences that autoreactive lymphocytes promote neuronal survival *in vivo* through activation not only of autoreactive-T-cell but also through activation other non- CNS-reactive T cells or B cells such as resident microglia and infiltrating macrophages.

Thus, because lymphocytes remains for long term at the site of the lesion, new strategy of treatment could orientate on this cells that posses a fundamental role in regulating degenerative and regenerative processes after injury.

2.5 DUAL ROLE OF INFLAMMATION IN SPINAL CORD AND BRAIN INJURY

Based on manifold experimental and clinical studies published in recent years, there is conflicting evidence on the role of the neuroinflammatory response in the injured CNS. Many of the formerly designated "proinflammatory" mediators have shown to possess potential effects in mediating deleterious as well as repair processes in the CNS.

"Classic" inflammatory cytokines like TNF have been historically determined as harmful mediators based on in vitro data of neurotoxicity and in vivo data showing neuroprotection by

pharmacological inhibition of TNF in various models of neuroinflammation and neurodegeneration (Shohami *et al.*, 1999)

Moreover, many pro-inflammatory mediators have been found to induce neurotrophin production after brain injury, as demonstrated for cytokines (e.g., IL-1β, IL-6), chemokines (e.g., IL-8), and inflammatory complement activation fragments (e.g., C3a) (Stahel, 2004).

In addition to neurotrophin induction, the neuropoietic cytokines like IL-6 have been shown to possess additional mechanisms of neuroprotection in TBI and SCI models, by mediating the generation of antioxidants, such as metallothioneins (Penkowa *et al.*, 2003a; Penkowa *et al.*, 2003b).

Altogether, in order to reconcile the apparently conflicting reports of beneficial and deleterious effects of various pro-inflammatory mediators, the exact timing and extent of mediator production and activation must be taken into account, as well as the presence of additional factors which may take over redundant functions, e.g., in neuropathology models with use of genetically engineered mice. Thus, appropriate context of concomitant factors and the kinetics and localization of inflammatory mediator expression and activation will determine the harmful or protective properties in the context of neuroinflammation.

CHAPTER 3: AUTOPHAGY AND mTOR

3.1 AUTOPHAGY

The term 'autophagy', derived from the Greek meaning 'eating of self', was first coined by Christian de Duve over 40 years ago, and was largely based on the observed degradation of mitochondria and other intra-cellular structures within lysosomes of rat liver perfused with the pancreatic hormone, glucagon (Glick *et al.*, 2010). The mechanism of glucagon-induced autophagy in the liver is still not fully understood at the molecular level, other than that it requires cyclic AMP induced activation of protein kinase-A and is highly tissue-specific (Yin *et al.*, 2008). In recent years the scientific world has 'rediscovered' autophagy, with major contributions to molecular understanding and appreciation of the physiological significance of this process coming from numerous laboratories (Mizushima, 2007; Xie and Klionsky, 2007; Levine and Kroemer, 2008; Nakatogawa *et al.*, 2009).

Although the importance of autophagy is well recognized in mammalian systems, many of the mechanistic breakthroughs in delineating how autophagy is regulated and executed at the molecular level have been made in yeast (Saccharomyces cerevisiae) (Xie and Klionsky, 2007; Nakatogawa *et al.*, 2009). Currently, 32 different autophagy-related genes (Atg) have been identified by genetic screening in yeast and, significantly, many of these genes are conserved in slime mould, plants, worms, flies and mammals, emphasizing the importance of the autophagic process in responses to starvation across phylogeny.

3.2 MAJOR TYPES OF AUTOPHAGY

Based on the type of cargo delivery, there are three types of autophagy systems in mammals macroautophagy (autophagy), microautophagy, and chaperone-mediated autophagy.

3.2.1 MACROAUTOPHAGY (AUTOPHAGY)

Whole regions of the cytosol are sequestered and delivered to lysosomes for degradation. Cargo sequestration occurs in the autophagosome, a double-membrane vesicle that forms through the elongation and sealing of a *de novo* generated membrane (Ohsumi and Mizushima, 2004).

This limiting membrane originates from a tightly controlled series of interactions between more than 10 different proteins which resemble the conjugation steps that mediate protein ubiquitinization (Cuervo, 2010). Formation of the limiting membrane also requires the interaction between a protein and a specific lipid molecule, regulated by conjugating enzymes.
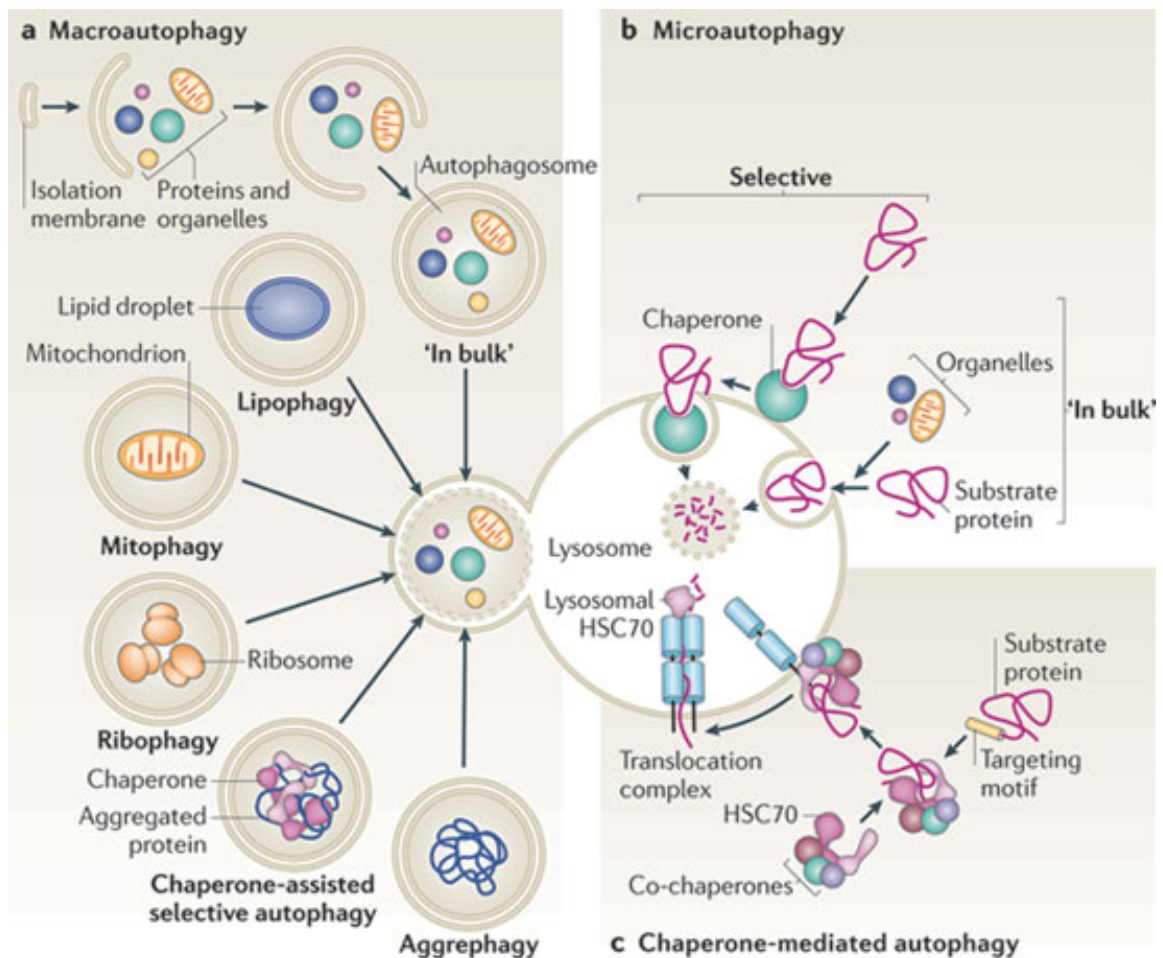
Figure 10 *Different types of autophagies*

3.2.2 MICROAUTOPHAGY

Microautophagy is the direct uptake of soluble or particulate cellular constituents into lysosomes. It translocates cytoplasmic substances into the lysosomes for degradation via direct invagination, protrusion, or septation of the lysosomal limiting membrane. In other words, microautophagy involves direct invagination and fusion of the vacuolar/lysosomal membrane under nutrient limitation. The limiting/sequestering membrane is the lysosomal membrane, which invaginates to form tubules that pinch off into the lysosomal lumen. Microautophagy of soluble components, as in macroautophagy (autophagy), is induced by nitrogen starvation and rapamycin. Microautophagy is controlled by the TOR and EGO signaling complexes, resulting in direct uptake and degradation of the vacuolar boundary membrane (Uttenweiler *et al.*, 2007). Hence, this process could compensate for the enormous influx of membrane caused by autophagy.

It seems that microautophagy is required for the maintenance of organelle size and membrane composition rather than for cell survival under nutrient restriction. Uttenweiler et colleagues have identified the vacuolar transporter chaperone, VTC complex, required for microautophagy (Uttenweiler *et al.*, 2007). This complex is present on the endoplasmic reticulum and vacuoles, and at the cell periphery. Deletion of the VTC complex blocks microautophagic uptake into vacuoles.

3.2.3 CHAPERONE-MEDIATED AUTOPHAGY

Chaperone-mediated autophagy (CMA) has been characterized in higher eukaryotes but not in yeast. Because of the particular characteristics of this type of delivery, only soluble proteins, but not whole organelles, can be degraded through CMA (Cuervo, 2010). CMA is dependent on the constitutively expressed heat shock cognate 70 (Hsc70), shares 80% homology with the heat shock protein 70 (Hsp70), and identifies peptide sequences of cytoplasmic substrates; thus, it is more selective than autophagy in its degradation (Hoffman *et al.*, 2012).

CMA serves to balance dysregulated energy, and is maximally activated by nutrient/metabolic and oxidative/nitrosative stresses. Cross talk between CMA and autophagy is likely. CMA differs from the other two types of autophagies with respect to the mechanism for cargo selection and delivery to the lysosomal lumen for degradation. In other words, CMA is involved in the delivery of cargo, which does not require the formation of intermediate vesicles, membrane fusion, or membrane deformity of any type. Instead, the substrates are translocated from the cytosol directly into the lysosomal lumen across the membrane in a

process mediated by a translocation protein complex that requires the substrate unfolding. A chaperone protein binds first to its cytosolic target substrate, followed by a receptor on the lysosomal membrane at the site of protein unfolding.

This protein is subsequently translocated into the lysosome for its degradation. In this system the substrate proteins are selectively targeted one-by-one to the lysosomes, and are then translocated across the lysosomal membrane. Selectivity and direct lysosomal translocation have thus become trademarks of CMA. An essential requirement for a protein to become a CMA substrate is the presence of a pentapeptide motif biochemically related to KFERQ in its amino acid sequence (Dice, 1990). During CMA, proteins are directly imported into lysosomes via the lysosomal membrane protein type 2a (LAMP-2a) transporter assisted by the cytosolic and lysosomal Hsc70 chaperone that recognizes the KFERQ-like motif. Substrates of CMA carry signal peptides for sorting into lysosomes, similar to other protein transport mechanisms across membranes.

CMA is a generalized form of autophagy present in almost all cell and tissue types. All the CMA substrate proteins are soluble cytosolic proteins containing a targeting motif biochemically related to the pentapeptide KFERQ. This motif, present in ~30% of the proteins in the cytosol, is recognized by a cytosolic chaperone, the heat shock cognate protein of 73 kDa (cyt-Hsc70). The interaction with chaperone, modulated by the Hsc70 co-chaperones, targets the substrate to the lysosomal membrane, where it interacts with the LAMP-2a (Cuervo and Dice, 1996). Substrates are required to be unfolded before translocation into the lysosomal lumen.

Several cytosolic chaperones associated with the lysosomal membrane have been proposed, which assist in the unfolding (Agarraberes and Dice, 2001). Translocation of the substrate requires the presence of a variant of Hsc70, lys-Hsc70, in the lysosomal lumen. This is followed by the rapid proteolysis of the substrate by residual lysosomal proteases (half-life of 5–10 minutes in the lysosomal lumen).

3.2.4 SELECTIVE AUTOPHAGIES

There are specific types of autophagy in which specific proteins or cell organelles are delivered to the autophagosome/lysosome for degradation.

1. **Aggrephagy**: selective degradation of cellular aggregates, especially proteins (Overbye *et al.*, 2007).

2. **Axophagy**: degradation of axons (Yue, 2007).

3. **Glyophagy**: degradation of glycogen particles (Jiang *et al.*, 2011).

4. **Lipophagy**: selective degradation of lipid droplets (Singh *et al.*, 2009).

5. **Mitophagy**: selective degradation of mitochondria (Kanki, 2010; Coto-Montes *et al.*, 2012).

6. **Nucleophagy**: selective degradation of parts of the nucleus (Mijaljica *et al.*, 2010).

7. **Pexophagy**: selective degeneration of peroxisomes; dependent on PEX3 and PEX4 proteins (Klionsky, 1997).

8. **Reticulophagy**: selective degradation of rough endoplasmic reticulum to balance its expansion by unfolded proteins (Klionsky *et al.*, 2007).

9. **Ribophagy**: selective degradation of the 60S ribosomal subunit (Kraft *et al.*, 2008).

10. **Xenophagy**: defense against intracellular pathogens (Shpilka and Elazar, 2012).

11. **Zymophagy**: degradation of zymogen granules (Vaccaro, 2012).

3.3 mTOR SIGNALLING PATHWAYS

The target of rapamycin (TOR), an evolutionarily conserved Ser/Thr protein kinase, forms the catalytic core of at least two functionally distinct complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2) (Bhaskar and Hay, 2007; Jacinto and Lorberg, 2008; Cybulski and Hall, 2009; Dunlop and Tee, 2009). These complexes contain shared and distinct partner proteins and control a myriad of cellular processes in response to diverse environmental cues.

mTORC1 and mTORC2 contain shared and unique partners, the molecular functions of which remain poorly understood. Each complex contains mTOR, mLST8/GβL, and deptor (Bhaskar and Hay, 2007; Jacinto and Lorberg, 2008; Cybulski and Hall, 2009; Dunlop and Tee, 2009).
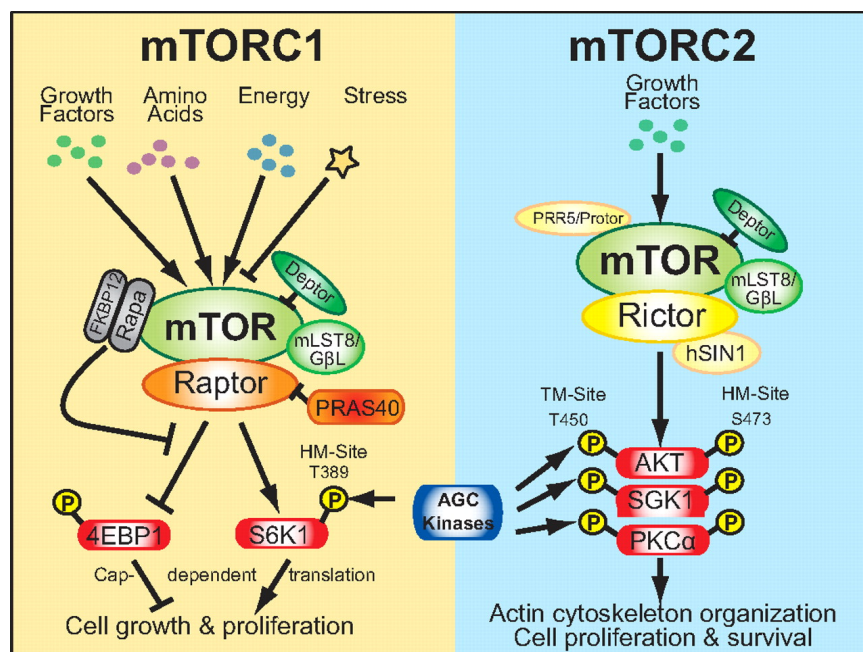
Figure 11 – Graphical representation of mTOR1 and mTORC2

mLST8/GβL binds the mTOR kinase domain in both complexes but appears more critical for mTORC2 assembly and signaling (Guertin *et al.*, 2006). Deptor functions as an inhibitor of both complexes (Laplante and Sabatini, 2009; Peterson *et al.*, 2009). Other partner proteins distinguish the two complexes. mTORC1 contains exclusively raptor (Kog1 in budding yeast) and PRAS40. Raptor functions as a scaffolding protein that links the mTOR kinase with mTORC1 substrates to promote mTORC1 signaling. PRAS40 functions in an incompletely defined and controversial regulatory capacity as an mTORC1 inhibitor, competitive substrate, or both (3). Thus, disagreement exists in the field as to whether PRAS40 represents a core mTORC1 partner or an interacting substrate. In contrast, mTORC2 contains exclusively rictor (rapamycin-insensitive companion of mTOR) (Avo3 in S. cerevisiae), mSin1 (Avo1 in S.

cerevisiae), and PRR5/protor. Rictor and mSin1 promote mTORC2 assembly and signaling; the function of PRR5/protor remains obscure (Bhaskar and Hay, 2007; Jacinto and Lorberg, 2008; Cybulski and Hall, 2009; Dunlop and Tee, 2009).

3.3.1 FUNCTIONS OF MTORC1

mTORC1 senses and integrates diverse extra- and intracellular signals to promote anabolic and inhibit catabolic cellular processes. Growth factors and nutrients (e.g. amino acids, energy) promote mTORC1-dependent protein synthesis, cell growth (increase in cell mass/size), cell proliferation, and cell metabolism (Dunlop and Tee, 2009; Ma and Blenis, 2009). Conversely, insufficient levels of these factors, or signals of cell stress, blunt mTORC1 action to maintain cellular biosynthetic rates appropriate for suboptimal cellular conditions (Reiling and Sabatini, 2006; Dunlop and Tee, 2009; Ma and Blenis, 2009). Reduced mTORC1 signaling also promotes macroautophagy (Chang *et al.*, 2009). Cell growth, cell cycle progression, and cell proliferation represent evolutionarily conserved TORC1 functions (Fingar and Blenis, 2004). Strikingly, mTORC1-dependent control of cell size extends to control of organ and organismal size (Ruvinsky and Meyuhas, 2006). Mechanisms underlying mTORC1-mediated inhibition of cell growth and proliferation remain incompletely defined but likely involve reduced protein synthesis.

3.3.2 FUNCTIONS OF MTORC2

The serine/threonine protein kinase Akt (also known as protein kinase B) represents the first identified substrate of mTORC2. Akt promotes cell proliferation, cell survival, and cell migration and controls various metabolic processes (Sarbassov *et al.*, 2005; Manning and Cantley, 2007). Control of actin cytoskeleton organization represents the first noted function of mTORC2 (Sarbassov *et al.*, 2004; Feldman *et al.*, 2009). Recently, a role for mTORC2 in control of cell size and cell cycle progression was also reported (Rosner *et al.*, 2009).

3.4 CROSSTALK BETWEEN AUTOPHAGY AND OXIDATIVE STRESS

It has long been known that the conditions that regulate the activity of the autophagic process are also associated with changes in the production of reactive oxygen species (ROS) and reactive nitrate species (RNS) in cells. ROS/RNS are a range of oxygen-derived molecules formed by the incomplete reduction of oxygen during oxidative metabolism and have both specific mechanisms of production and intracellular targets (Murphy, 2006). The most important biologically are $O_2^{\bullet-}$ and H_2O_2, since both can be formed by controlled mechanisms in cells and are cell-signaling molecules. $O2^{\bullet-}$ and H_2O_2 can interact with NO to generate nitrating species, such as $ONOO^-$, and oxidized lipids to produce reactive lipid species (RLS) (Beckman *et al.*, 1990; Deen *et al.*, 2002). A major endogenous source of both $O2^{\bullet-}$ and $H2O2$ is the mitochondrial electron-transport chain (mETC), where continuous electron leakage to O_2 occurs during aerobic respiration (Murphy, 2009). In addition to the mETC, low levels of ROS are produced by membrane-localized NADPH oxidases (NOXs), peroxisomes and the cytochrome p450 system (Hanukoglu, 2006; Schrader and Fahimi, 2006; Lambeth, 2007).

It is important to recognize that the ROS cannot be considered as discrete or independent redox messengers since they can be interconverted.
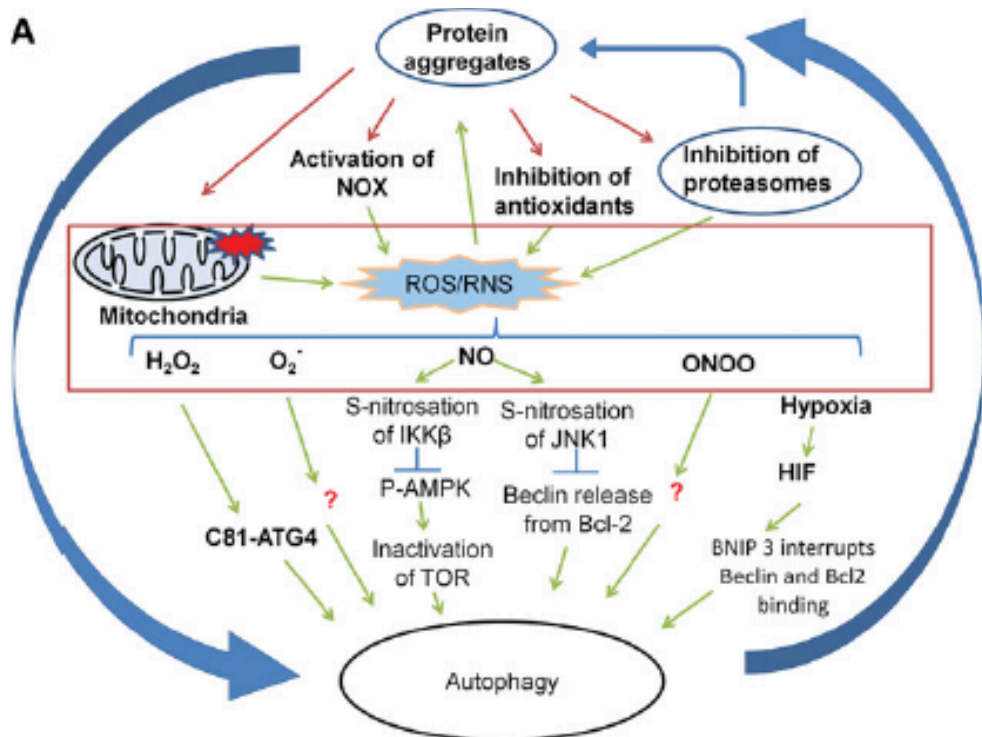
Figure 12 – Correlations between ROS/RNS production and autophagy

For example, starvation not only increases ROS levels in the cell, but also stimulates autophagy . Both $O_2^{\bullet-}$ and H_2O_2 have been shown to mediate autophagy induction . For example, H_2O_2 can modify mitochondrial proteins damaging the electron-transfer process, so that they now generate intramitochondrial $O_2^{\bullet-}$ (Scherz-Shouval *et al.*, 2007; Bensaad *et al.*, 2009; Chen *et al.*, 2009b). In a further example, oxidative modification of the nitric oxide synthases (NOSs) can cause disruption of electron transfer within the enzymes, inhibiting the formation of nitric oxide and generating $O_2^{\bullet-}$ (Forstermann, 2006; Velayutham *et al.*, 2011). In a biological setting, it is then likely that the cell is responding to the combined effects of ROS/RNS acting at different sites within the autophagic process. Mitochondria generate ROS from a number of different redox centres in the respiratory chain and other metabolic pathways. The primary ROS generated from mitochondria is $O_2^{\bullet-}$, which can then be converted into other ROS such as H_2O_2 or $ONOO^-$. At low levels, it is thought that mitochondrial ROS play a role in cell signalling, but, at higher levels, mitochondrial proteins are susceptible to damage because of the concentration of both oxidizable lipids and abundant redox-active proteins which can amplify oxidative damage (Ballinger *et al.*, 2000; Murphy, 2009). It is then essential to regulate autophagy at multiple levels, including removal of defective proteins generating uncontrolled ROS and the entire organelle by mitophagy. Not surprisingly, the mitochondrion and mitochondrially produced ROS are emerging as important players in autophagy and mitophagy (Scherz-Shouval *et al.*, 2007; Scherz-Shouval and Elazar, 2011).
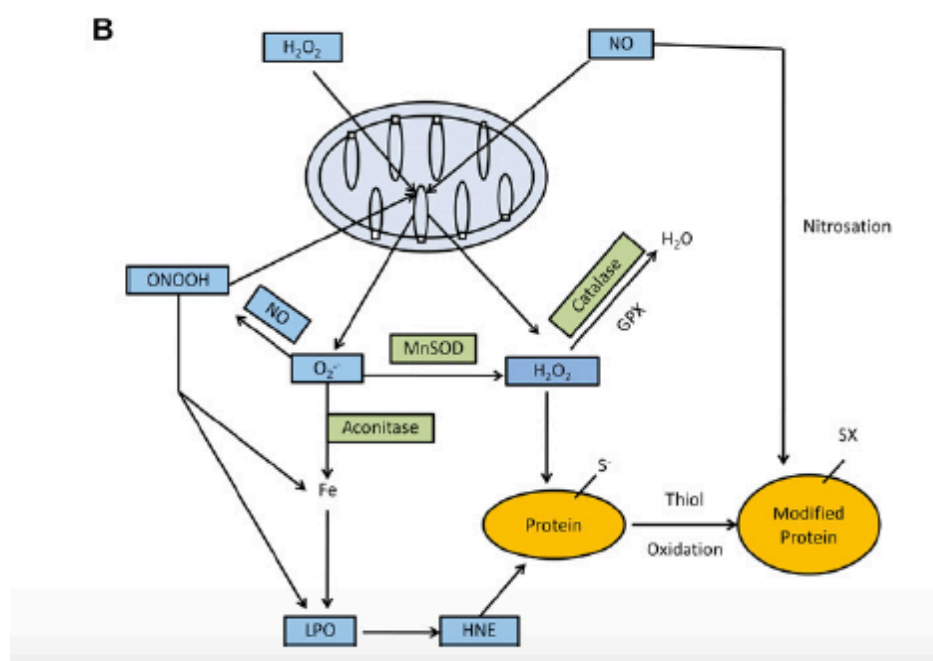
Figure 13 - Mitochondrial production of ROS/RNS

The reduction of superoxide and generation of H_2O_2 is controlled by the superoxide dismutases (SODs). The mitochondrial form is located in the matrix and contains a manganese prosthetic group and is known as SOD2 or MnSOD. Mitochondrial MnSOD-deficient cells exhibit decreased oxygen consumption and increased $O_2^{\bullet-}$ production, suggesting a key role in the response to pathological stressors . For example, in response to acute alcohol binge in mice, MnSOD overexpression prevents, and MnSOD deficiency exacerbates, NOS expression, plasma nitrites/nitrates, nitration of complex I and V proteins, and mtDNA (mitochondrial DNA) depletion (Williams *et al.*, 1998; Larosche *et al.*, 2010; Zhang *et al.*, 2010).

Accumulation of autophagosomes and p62 occurs in motor neurons in ALS patients' spinal cords, as well as in experimental animal models (Sasaki, 2011; Zhang *et al.*, 2011a). Mitochondrial ROS production and oxidation of mitochondrial lipids have been shown to play a role in autophagy. In yeast, rapamycin induces ROS, fatty acid modification, autophagy and mitophagy. Resveratrol and, to a lesser extent, the soluble antioxidant NAC (N-acetyl-L-cysteine) inhibit these effects (Kissova *et al.*, 2006). In mammalian cells, starvation-induced autophagy is associated with increased oxidative stress, and both H_2O_2 and $O_2^{\bullet-}$ have been shown to induce autophagy. In addition to mitochondrially generated ROS, NOX activities have also been shown to play an important role in antibacterial phagosome autophagy, neutrophil autophagy and ER stress autophagy (Scherz-Shouval *et al.*, 2007; Chen *et al.*, 2009b; Huang and Brumell, 2009).

3.5 CROSSTALK BETWEEN AUTOPHAGY AND INFLAMMATION

The signaling pathways that regulate inflammatory processes now apparently have a role in the regulation of autophagy and *vice-versa*. In addition to classical signals such as starvation and energy exhaustion, several pathogen-associated molecular patterns (PAMPs) have been shown to promote autophagic activation (Bertin *et al.*, 2008; Levine *et al.*, 2011).

Recent observations have revealed a relationship between autophagic proteins and inflammasome-associated proinflammatory cytokine maturation in macrophages (Nakahira *et al.*, 2011; Zhou *et al.*, 2011b). Inflammasomes are cytosolic multiprotein complexes that constitute a novel inflammatory signaling mechanism and which govern the maturation and secretion of select proinflammatory cytokines, such as IL-1β, IL-18, and IL-33 (Schroder and Tschopp, 2010). Cytosolic receptors of the NOD-like receptor (NLR) family (i.e., NLRP3 and NLRP1) interact with binding partners to form inflammasome complexes. NLRP3 interacts with an adaptor protein (apoptosis-associated speck like protein containing CARD (ASC)), which recruits and activates the procaspase-1 by proteolytic cleavage (Schroder and Tschopp, 2010).

Proinflammatory cytokine secretion (IL-1β and IL-18) was enhanced in *atg16l1* or *atg7* deleted macrophages in response to LPS (Saitoh *et al.*, 2008). In contrast, *atg16l1* or *atg7* deficiency did not affect TNF and IFN-β production or NF-κB pathway activation in macrophages stimulated with LPS (Saitoh *et al.*, 2008).
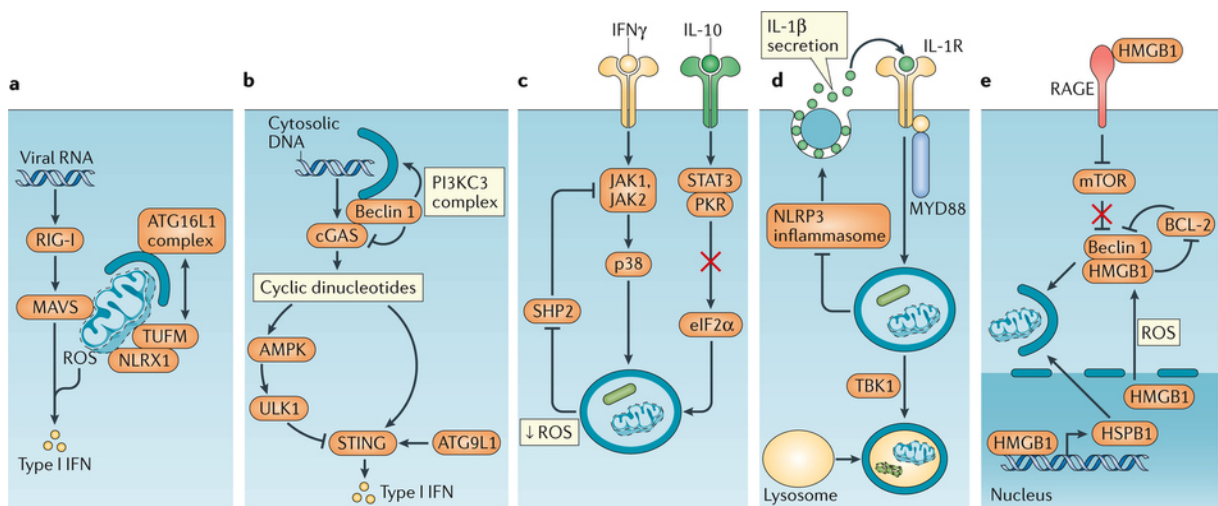
Figure 14 - Intersection between autophagy and cytokines

Furthermore, atg16l1-deleted mice displayed increased susceptibility to a murine model of colitis, which could be ameliorated by anti-IL-18 therapy (Saitoh *et al.*, 2008). In recent studies, increased activation of IL-1β and IL-18 has also been observed in macrophages and monocytes isolated from mice genetically deficient in Beclin 1 and LC3B (Nakahira *et al.*, 2011).

Cytokine activation in response to LPS and ATP in wild-type macrophages, as well as the amplification observed in LC3B or Beclin 1-deficient macrophages, required the NLRP3 inflammasome pathway (Nakahira *et al.*, 2011; Zhou *et al.*, 2011a). The mechanism by which autophagy deficiency enhanced NLRP3 inflammasome pathway activation was mediated by deregulation of mitochondrial homeostasis, including the enhanced production of mitochondrial ROS and increased mitochondrial membrane permeability transition (Nakahira *et al.*, 2011; Zhou *et al.*, 2011a). The pathway to caspase-1-dependent IL-18 secretion in macrophages was further shown to be blocked by mitochondrial targeting antioxidants (Nakahira *et al.*, 2011).

These experiments, taken together, suggest that autophagic proteins dampen inflammasome pathway activation by stabilizing mitochondria and/or maintaining mitochondrial quality control through autophagy. Further research in this area may uncover additional mechanisms. Taken together these studies suggest an important role for autophagic proteins in the dampening of proinflammatory responses, and that warrants further investigation in models of inflammatory disease.

3.6 CROSSTALK BETWEEN AUTOPHAGY AND APOPTOSIS PATHWAYS

Notably, autophagy is often observed in dying cells. In many settings, this represents an attempt of the cell to mitigate the stress before resorting to the irreversible and final solution of apoptosis. In other settings, activation of autophagy might reflect a crosstalk between the two processes,(Rubinstein and Kimchi, 2012). Indeed, autophagy is induced in response to many stresses that ultimately lead to apoptosis, including organelle dysfunction, metabolic stress and pathogen infection. If the stress is resolved, cells typically restore autophagy to baseline levels and return to their initial state. However, if the stress persists and autophagy can no longer support cell survival, cells might respond by activating apoptosis in order to ensure their controlled and efficient elimination, without triggering local inflammation (Rubinstein and Kimchi, 2012). Therefore, it is conceivable that the formation of regulatory ties between autophagy and apoptosis (and vice versa) conferred an evolutionary advantage to cells, as it enabled a more controlled and precise response to a given stress signal. Thus, although autophagy and apoptosis undoubtedly represent distinct cellular processes with fundamentally different biochemical and morphological features, the protein networks that control their regulation and execution are highly interconnected (Rubinstein and Kimchi, 2012).
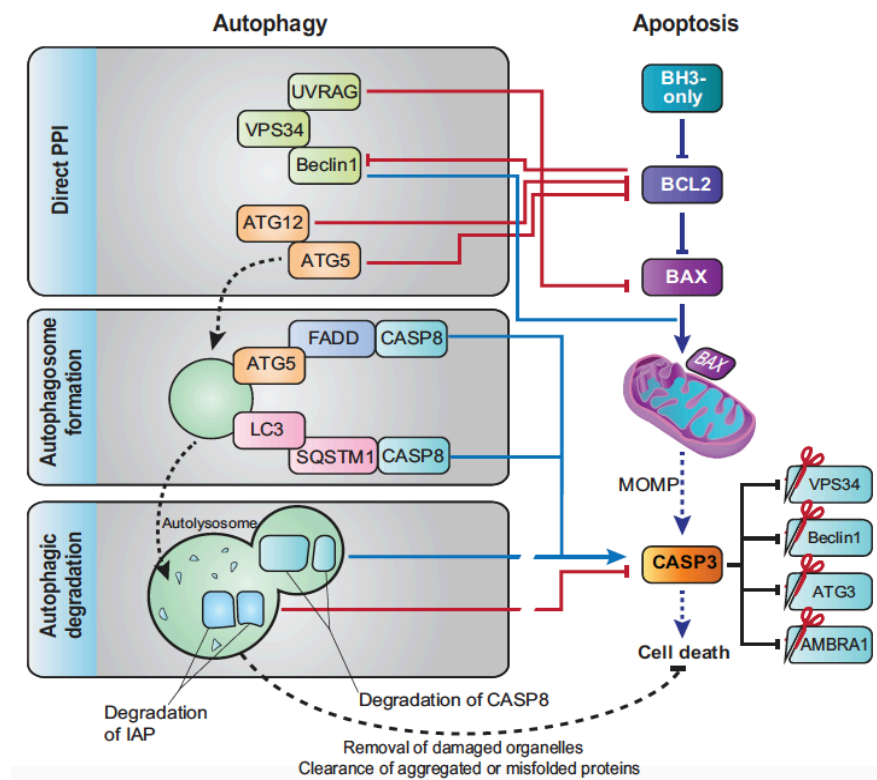

Figure 15 – Crosstalk between autophagy and apoptosis.

The regulation of apoptosis by autophagy can be subdivided into three main mechanistic paradigms: (1) regulation of apoptosis by specific autophagy proteins that can directly regulate apoptosis in a manner that is not necessarily related to their canonical role in autophagy signalling; (2) activation of caspases on autophagosomal membranes that can serve as a platform for caspase activation. (3) regulation of apoptosis by autophagic degradation that is dependent on the function of proteins that are involved both in the early (autophagosome formation) and late (lysosomal fusion and cargo degradation) stages of autophagy. A key characteristic of this paradigm is the dependence on an active autophagic flux, which requires lysosomal activity (Rubinstein and Kimchi, 2012).

The best-studied example for this type of cross regulation is the dual function played by BCL2 in the inhibition of both pathways. BCL2 has been identified as a direct binding partner of Beclin 1, leading to decreased autophagic activity. In resting cells, BCL2 is constitutively bound to Beclin 1, thereby allowing for only low (basal) levels of autophagy. Under autophagy inducing conditions, BCL2 dissociates from Beclin 1, resulting in increased autophagy (Pattingre *et al.*, 2005)

Several mechanisms that control the dissociation of BCL2 from Beclin 1 under autophagy-inducing conditions have emerged. First, the identification of Beclin 1 as a BH3-only protein suggested that other members of the BH3-only family competitively displace BCL2 from Beclin 1 (Maiuri *et al.*, 2007). Indeed, Maiuri and colleagues have found that knockdown of the BH3-only protein BAD reduces the extent of autophagy in response to starvation, whereas its ectopic expression is sufficient to induce autophagy (Maiuri *et al.*, 2007). Moreover, they showed that starvation leads to an increase in the interaction between BclXL and BAD, which correlates with decreased binding of BclXL to Beclin 1 and elevated levels of autophagy, supporting a competitive type of interaction. Regulation of the BCL2–Beclin-1 interaction by BAD (Maiuri *et al.*, 2007), as well as by other BH3-only proteins, including Noxa, represents an additional layer of crosstalk, in which pro-apoptotic BH3-only proteins can act as positive regulators of autophagy by displacing BCL2 from Beclin 1 (Rashmi *et al.*, 2008; Zhang *et al.*, 2008; Chang *et al.*, 2010). Interestingly, Luo and colleagues recently discovered that overexpression of the BH3-only protein BIM does not affect the interaction between BCL2 and Beclin 1. Instead, BIM functions as an inhibitor of autophagy, by anchoring Beclin 1 to microtubules (Luo *et al.*, 2012). Phosphorylation of Beclin 1 and BCL2 constitutes another post-translational mechanism that controls the dissociation of BCL2 from Beclin 1. Multi-site phosphorylation of BCL2 by c-Jun N-terminal protein kinase 1 (JNK1, also known as

MAPK8) has been shown to substantially reduce the affinity of BCL2 for Beclin 1, leading to activation of autophagy in response to starvation or ceramide treatment (Wei *et al.*, 2008a; Pattingre *et al.*, 2009). Likewise, phosphorylation of Beclin 1 within its BH3 domain by death-associated protein kinase (DAP kinase) was shown to induce autophagy by promoting its dissociation from BclXL. Notably, both JNK1 and DAPK have been implicated in the regulation of apoptosis (Bialik and Kimchi, 2006; Zalckvar *et al.*, 2009).

One of the most intriguing aspects of the crosstalk between apoptosis and autophagy is the elegant way in which cells balance between the 'economical' solution of utilising a single protein (e.g. BCL2) to regulate two pathways, and the need to maintain a complex and individual regulation of each pathway. In the case of BCL2, this is achieved, in part, through differential binding affinities and spatial separation of apoptotic and autophagic components. For example, Wei and colleagues found that at early time points of amino acid starvation, JNK1-mediated phosphorylation of BCL2 leads to its rapid dissociation from Beclin 1 and subsequent induction of pro-survival autophagy (Wei *et al.*, 2008a). Because pro-apoptotic proteins, such as BAX, bind BCL2 with higher affinity than Beclin 1, this low level of BCL2 phosphorylation is insufficient to displace them from BCL2. However, if the stress of starvation persists, accumulation of hyper-phosphorylated BCL2 eventually results in the dissociation of BCL2 from BAX and the activation of apoptosis (Bassik *et al.*, 2004; Wei *et al.*, 2008b). Thus, differential-binding affinities, fine-tuned by evolution, provide a way to achieve sequential activation of autophagy and apoptosis with the use of a single protein regulator. Spatial separation of proteins to different cellular compartments provides an additional way to achieve independent regulation of autophagy and apoptosis.
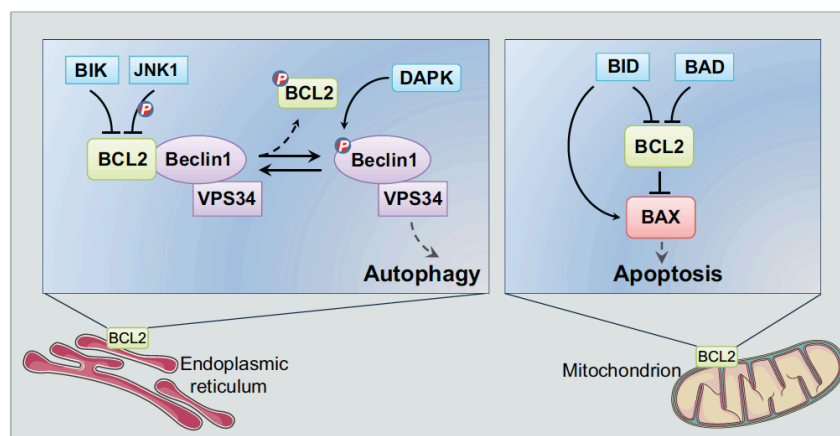
Figure 16 - Compartmentalization of BCL2 to different cellular organelles allows independent control of autophagy and apoptosis by a single protein regulator

Two distinct cellular pools of BCL2 at the endoplasmic reticulum (ER) and mitochondria appear to regulate autophagy and apoptosis, respectively (Pattingre *et al.*, 2005). A localised group of ER proteins ensures the partitioning of BCL2 to the autophagy pathway at this organelle. For example, regulation of autophagy by binding of BCL2 to Beclin 1 at the ER is facilitated by the ER protein nutrient-deprivation autophagy factor-1 (NAF1), and is inhibited by the ER-localised BH3-only protein BIK (Chang *et al.*, 2010).

Moreover, JNK1 specifically phosphorylates the ER pool of BCL2 during starvation to induce autophagy (Wei *et al.*, 2008b). Conversely, interactions between BCL2, BH3-only proteins and BAX or BAK at the mitochondrion control the activation of apoptosis. Thus, compartmentalizations of BCL2, together with organelle specific sets of interacting proteins, enable the dynamic and independent regulation of autophagy and apoptosis. Nevertheless, it has been suggested that mitochondria-localised BCL2 is also involved in inhibition of autophagy, by sequestering a mitochondrial pool of AMBRA1, a positive regulator of Beclin 1 (Adams and Cory, 2007; Martinou and Youle, 2011; Strappazzon *et al.*, 2011).

3.7 TARGETING mTOR AS AN EMERGING PHARMACOLOGICAL STRATEGY FOR CNS INJURIES

Original studies on axonal regeneration revealed that, unlike the peripheral nervous system (PNS), the adult human CNS environment contains extracellular inhibitory factors that limit the ability of injured CNS neurons to repair themselves after a traumatic injury (Tsang *et al.*, 2007). These studies have led to the current view that stimulation of intrinsic signaling pathways might overcome the inhibitory extracellular CNS environment to promote functional recovery following injury (Tsang *et al.*, 2007).

Several recent evidences have revealed that activation of the intrinsic growth signal in adult CNS neurons can significantly reduce neuronal death and promote repair and regeneration. In a rat model of TBI, phosphorylation of mTOR and its downstream targets (p70S6K, S6 and 4E-BP1) increased within 30 min of a moderate injury to the parietal cortex and lasted up to 24 h (Chen *et al.*, 2007). This increase in phosphorylation and activation of mTOR pathway could provide a mechanism to respond to, and recover from, the TBI. In fact, Park et al. demonstrated that activation of mTOR signaling pathway in adult retinal ganglion cells (RGCs) induced robust axon regeneration after optical nerve injury (Park *et al.*, 2008). Moreover, a follow-up study from the same research group using corticospinal tract (CST) axonal injury models demonstrated that mTOR activity was also a crucial regulator of the regenerative capacity in corticospinal neurons (Liu *et al.*, 2010). The CST is essential for voluntary movements and CST axons are often injured during SCI. These investigators showed that conditional deletion of PTEN enhances axon regeneration from both spared axons (compensatory sprouting) and injured axons (regenerative growth). The regenerating injured axons were able to pass through the lesion site and form synapses past these sites. Therefore, upregulation of mTOR pathway might be sufficient to promote axon regeneration in the adult CNS after brain injuries and SCI (Park *et al.*, 2008; Liu *et al.*, 2010).

ATP is an important regulator of signaling pathways and is thought to have a central role in recovery after CNS injuries; therefore, Hu et al. investigated the role of ATP-induced alterations in mTOR signaling pathway for repair of SCI using a rat compression model (Hu *et al.*, 2010). Exogenous administration of ATP significantly increased protein and mRNA levels as well as the phosphorylation and activation of mTOR pathway components in the spinal cord. Activation of mTOR pathway was associated with improvements in locomotor function recovery after the injury and increases in expression of neuronal genes, nestin,

neuronal nuclei (NeuN) and neurofilament 200 (NF200). These findings suggest that stimulation of mTOR pathway provides beneficial effects for locomotor functional defects associated with SCI. Nevertheless, mTOR activity is crucial for axonal regenerative response in adult CNS injuries.

In addition to neuroprotection and axon regeneration, stimulation of mTOR activity might be beneficial in other aspects associated with TBI. For instance, mTOR activity reduces the cytotoxic effect of TBI-induced glutamate excitotoxicity. mTOR activity is required for glutamate transporter expression in cultured astrocytes (Wu *et al.*, 2010). Given that the amount of glutamate transporter is associated with glutamate clearance, it is possible that pharmacological activation of mTOR reduces glutamate-induced excitotoxicity induced by TBI. Moreover, mTOR activity is required for insulin-induced neuronal differentiation of neural progenitors in primary culture (Han *et al.*, 2008). These data suggest that modulation of mTOR promotes neurogenesis. Therefore, pharmacological activation of mTOR pathway might significantly improve the condition of patients with traumatic CNS injuries.

Interestingly, inhibition of mTOR with rapamycin in animal models of TBI is beneficial for ameliorating TBI-associated symptoms, including epilepsy and adverse inflammatory responses (Erlich *et al.*, 2007; Wong, 2010; Pignataro *et al.*, 2011; Sunnen *et al.*, 2011). TBI-induced epilepsy is characterized by numerous abnormalities, including molecular, cellular and synaptic activities in the brain (Wong, 2010). mTOR might also be involved in these cellular processes, which cause epileptogenesis following brain injuries. First, mTOR regulates various cellular functions, such as protein synthesis and synaptic plasticity, that might cause abnormally excited electrical signals, thereby contributing to epileptogenesis in the injured brain. Second, tuberous sclerosis protein complex (TSC) is one of the most common genetic causes of epilepsy. It is caused by mutation of the genes TSC1/2, which encode the upstream negative regulators of mTOR (Tsang *et al.*, 2008). The role of mTOR in epilepsy pathogenesis has been demonstrated using the mTOR inhibitor rapamycin in a variety of mouse models of epilepsy (Ljungberg *et al.*, 2009; Wong, 2010). Therefore, pharmacological inhibition of mTOR to alleviate TBI-induced epilepsy could represent a novel anti-epileptogenic therapy for patients with TBI.

In addition to epilepsy, the secondary brain injury is frequently associated with neuro-inflammatory responses owing to activation of immune cells, such as microglia and

astrocytes. These cause secondary neuronal damage by releasing cytotoxic molecules, including reactive oxygen species (ROS) and cytokines (Shohami *et al.*, 1997). In addition, early inflammatory response (within hours) contributes to the later stages of brain injuries (Erlich *et al.*, 2007; DeLegge and Smoke, 2008). As a potent immunosuppressant, rapamycin has been investigated for its neuroprotective effects in closed head injury TBI models. Rapamycin injection within 4 h following brain injury reduced microglia and/or macrophage activation, increased survival of neurons and significantly improved brain functional recovery(Erlich *et al.*, 2007). Taken together, these encouraging preclinical studies suggest that pharmacological modulation of mTOR pathway is an attractive therapeutic strategy for treatment of traumatic CNS injuries.

CHAPTER 4: MTOR INHIBITORS

Since the discovery of mTOR, much research has been done on the subject, using inhibitors to understand its biological functions (Xu *et al.*, 2008; Vilar *et al.*, 2011). The clinical results from targeting this pathway were not as straight forward as thought at first. Those results have changed the course of clinical research in this field (Vilar *et al.*, 2011).

4.1 FIRST GENERATION OF MTOR INHIBITORS: RAPAMYCIN AND RAPALOGS

Rapamycin (sirolimus) is a macrocyclic antibiotic produced by the bacterium Streptomyces hygroscopicus found in the soil of Easter Island.

Initially, rapamycin was developed as an antifungal drug against *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* (Faivre et al., 2006). Few years later, its immunosuppressive properties were detected. Later studies led to the establishment of rapamycin as a major immunosuppressant against transplant rejection, along with cyclosporine A (Strimpakos *et al.*, 2009). By using rapamycin in combination with cyclosporin A, it enhanced the rejection prevention in renal transplantation. Therefore, it was possible to use lower doses of cyclosporine which minimized toxicity of the drug (Faivre et al., 2006).

In the 1980s, rapamycin was evaluated by the Developmental Therapeutic Branch of the National Cancer Institute (NCI). It was discovered that rapamycin had an anticancer activity and was a non-cytotoxic agent with cytostatic activity against several human cancer types (Faivre et al., 2006).
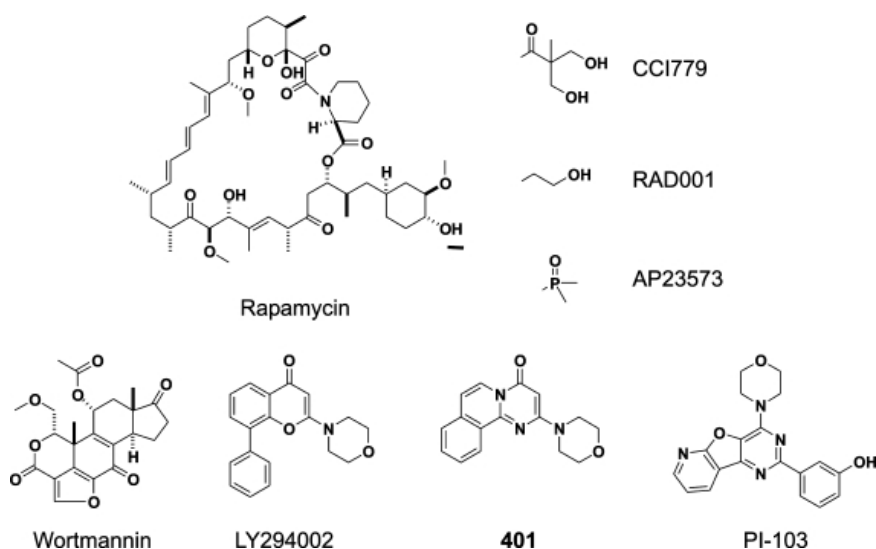
Figure 17- Chemical structure of rapamycin, rapalogs and mTOR kinase inhibitors

However, due to unfavorable pharmacokinetic properties, the development of mTOR inhibitors for the treatment of cancer was not successful at that time (Yuan *et al.*, 2009). Since then, rapamycin has also shown to be effective for preventing coronary artery re-stenosis and for the treatment of neurodegenerative diseases (Faivre et al., 2006).

The development of rapamycin as an anticancer agent began again in the 1990s with the discovery of temsirolimus (CCI-779). This was a novel soluble rapamycin derivative that had a favorable toxicological profile in animals. More rapamycin derivatives with improved pharmacokinetics and reduced immunosuppressive effects have since then been developed for the treatment of cancer (Faivre et al., 2006). Rapamycin analogs have similar therapeutic effects as rapamycin. However, they have improved hydrophilicity and can be used for oral and intravenous administration. In 2012 National Cancer Institute listed more than 200 clinical trials testing the anticancer activity of rapalogs either as monotherapy or as a part of combination therapy for many cancer types(Tsang *et al.*, 2007; Zaytseva *et al.*, 2012).

One of the most important reason for the limited success is that there is a feedback loop between mTORC1 and AKT in certain tumor cells. It seems that mTORC1 inhibition by rapalogs fails to repress a negative feedback loop that results in phosphorylation and activation of AKT. These limitations have led to the development of the second generation of mTOR inhibitors (Sutherlin *et al.*, 2011; Zaytseva *et al.*, 2012).

Treatment with mTOR inhibitors can be complicated by adverse events. The most frequently occurring adverse events are stomatitis, rash, anemia, fatigue, decreased appetite, nausea, and diarrhea. Additionally, interstitial lung disease is an adverse event of particular importance. mTORi-induced ILD often is asymptomatic (with ground glass abnormalities on chest CT) or mild symptomatic (with a non-productive cough), but can be very severe as well (Willemsen *et al.*, 2016).

4.1.1. RAPALOGS

Rapalogs, which are the first generation mTOR inhibitors, have proven effective in a range of preclinical models. However, the success in clinical trials can limited to only a few rare cancers. Animal and clinical studies show that rapalogs are primarily cytostatic, and therefore effective as disease stabilizers rather than for regression. The response rates in solid tumors where rapalogs have been used as a single-agent therapy have been modest. Due to partial mTOR inhibition as mentioned before, rapalogs are not sufficient for achieving a broad and robust anticancer effect, at least when used as monotherapy (Brachmann *et al.*, 2009; Wander *et al.*, 2011; Zhang *et al.*, 2011b; Tanneeru and Guruprasad, 2012).

Temsirolimus is pro-drug of rapamycin. It is approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA), for the treatment of renal cell carcinoma (RCC). Temsirolimus has higher water solubility than rapamycin and is therefore administrated by intravenous injection. It was approved in May 30, 2007, by FDA for the treatment of advanced RCC (Vignot *et al.*, 2005; Yuan *et al.*, 2009).

Everolimus is the second novel Rapamycin analog. From March 30, 2009 to May 5, 2011 the U.S. FDA approved everolimus for the treatment of advanced renal cell carcinoma after failure of treatment with sunitinib or sorafenib, subependymal giant cell astrocytoma (SEGA) associated with Tuberous Sclerosis (TS), and Progressive neuroendocrine tumors of pancreatic origin (PNET). In July and August 2012, two new indications were approved, for advanced hormone receptor-positive, HER2-negative breast cancer in combination with exemestane, and pediatric and adult patients with SEGA. In 2009 and 2011, it was also approved throughout the European Union for advanced breast cancer, pancreatic neuroendocrine tumours, advanced renal cell carcinoma, and SEGA in patients with tuberous sclerosis (Strimpakos *et al.*, 2009).

Deforolimus, or ridaforolimus, is the newest rapamycin analog and it is not a prodrug. Like temsirolimus, it can be administrated intravenously, and oral formulation is being estimated for treatment of sarcoma. It was not on market in June 2012, since FDA wanted more human testing on it due to its effectiveness and safety (Strimpakos *et al.*, 2009; Yuan *et al.*, 2009).

4.2 SECOND GENERATION MTOR INHIBITORS: KU0063794

The second generation of mTOR inhibitors is known as ATP-competitive mTOR kinase inhibitors. mTORC1/mTORC2 dual inhibitors are designed to compete with ATP in the catalytic site of mTOR. They inhibit all of the kinase-dependent functions of mTORC1 and mTORC2 and therefore, block the feedback activation of PI3K/AKT signaling, unlike rapalogs that only target mTORC1. These types of inhibitors have been developed and several of them are being tested in clinical trials. Like rapalogs, they decrease protein translation, attenuate cell cycle progression, and inhibit angiogenesis in many cancer cell lines and also in human cancer. In fact they have been proven to be more potent than rapalogs (Zaytseva *et al.*, 2012).

Theoretically, the most important advantages of these mTOR inhibitors is the considerable decrease of AKT phosphorylation on mTORC2 blockade and in addition to a better inhibition on mTORC1 (Vilar *et al.*, 2011). However, some drawbacks exist. Even though these compounds have been effective in rapamycin-insensitive cell lines, they have only shown limited success in KRAS driven tumors. This suggests that combinational therapy may by necessary for the treatment of these cancers. Another drawback is also their potential toxicity. These facts have raised concerns about the long term efficacy of these types of inhibitors (Zaytseva *et al.*, 2012).
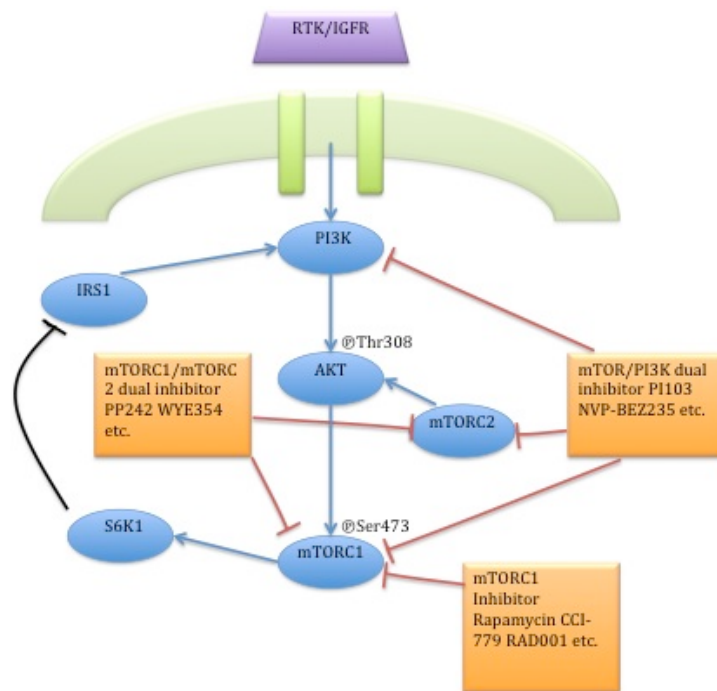
Figure 18 - Action point of first and second generation mTOR inhibitors

The close interaction of mTOR with the PI3K pathway has also led to the development of mTOR/PI3K dual inhibitors (Zaytseva *et al.*, 2012). Compared with drugs that inhibit either mTORC1 or PI3K, these drugs have the benefit of inhibiting mTORC1, mTORC2, and all the catalytic isoforms of PI3K. Targeting both kinases at the same time reduces the upregulation of PI3K, which is typically produced with an inhibition on mTORC1 (Vilar *et al.*, 2011). The inhibition of the PI3K/mTOR pathway has been shown to potently block proliferation by inducing G1 arrest in different tumor cell lines. Strong induction of apoptosis and autophagy has also been seen. Despite good promising results, there is preclinical evidence that some types of cancers may be insensitive to this dual inhibition. The dual PI3K/mTOR inhibitors are also likely to have increased toxicity.
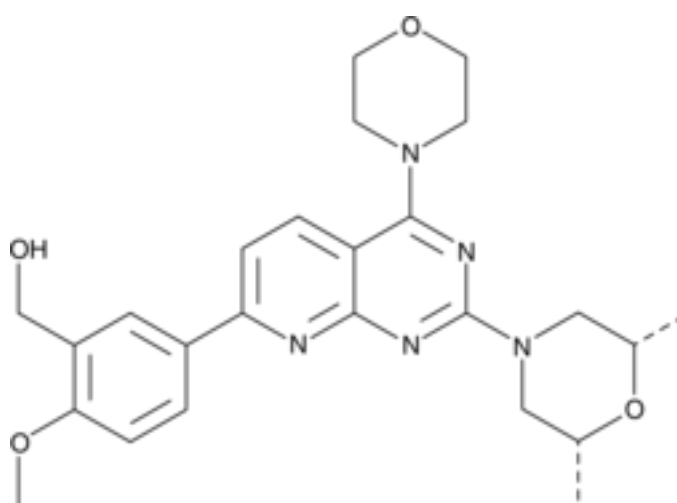
Figure 19 –Chemical structure of KU0063794

KU0063794 is a second-generation mTOR inhibitor targeting mTORC1 and mTORC2, including p70S6K, 4E-BP1 and Akt. Specifically, inhibits the phosphorylation of S6K1 and 4E-BP1, which are downstream substrates of mTORC1, and it inhibits Akt phosphorylation on Ser473, which is the target of mTORC2 (Garcia-Martinez *et al.*, 2009; Zhang *et al.*, 2013). In a recent study it has been demonstrated that KU0063794 decreasing the viability and growth of renal cell carcinoma cell lines, Caki-1 and 786-O, and showed anti-fibrotic activity in Keloid disease (Syed *et al.*, 2013; Zhang *et al.*, 2013).

CHAPTER 5: MATERIAL AND METHODS

5.1 MATERIALS AND METHODS FOR SCI STUDY

5.1.1 IN-VIVO PROCEDURES

5.1.1.1 ANIMALS

Male adult CD1 mice (male 25–30 g; Envigo, Italy) were accommodated in a controlled environment and provided with standard rodent food and water. Mice were housed in stainless steel cages in a room kept at 22 ± 1 °C with a 12-h light, 12-h dark cycle. The animals were acclimatized to their location for 1 week and had *ad libitum* access to water and rodent standard diet. The study was permitted by the University of Messina Review Board for the care of animals. All animal experiments complied with regulations in Italy (D.M. 116192) as well as the EU regulations (O.J. of E.C. L 358/1 12/18/1986).

5.1.1.2 SPINAL CORD INJURY

Surgical anesthesia was induced by ketamine and xylazine (2.6 and 0.16 mg/kg body weight respectively) administered intraperitoneally (i.p.). A longitudinal cut was made on the midline of the back and the paravertebral muscles were uncovered. The spinal cord was uncovered via a four-level T6–T7 laminectomy; injury was produced by extradural compression of the spinal cord by an aneurysm clip with a closing force of 24 g. In all damaged groups, the spinal cord was compressed for 1 min. Sham animals were just subjected to laminectomy. After surgery, 1 ml of saline was administered subcutaneously to replace the blood volume lost during the operation. Throughout recuperation from anaesthesia, mice were sited on a warm heating pad and covered with a warm towel. Food and water were offered to the mice *ad libitum*. During this period, the animal's bladders were physically voided twice a day until the mice were able to regain normal bladder function.

5.1.1.3 EXPERIMENTAL GROUPS

Mice were randomly allocated into the following groups:

- Sham + vehicle group: mice were subjected to the surgical procedures as the above groups except that the aneurysm clip was not applied.

- Sham + Rapamicyn (1mg/kg): same as the sham plus intraperitoneal administration of Rapamicyn (1mg/kg) 1 and 6 h after injury.

- Sham + Temsirolimus (0,6mg/kg): same as the sham plus intraperitoneal administration of Temsirolimus (0,6mg/kg) 1 and 6 h after injury.

- Sham + KU0063794 (8mg/kg): same as the sham plus intraperitoneal administration of KU0063794 (8mg/kg) 1 and 6 h after injury.

- SCI + vehicle group: mice were subjected to SCI plus intraperitoneal administration of vehicle (saline).

- SCI + Rapamicyn (1mg/kg): same as the SCI plus intraperitoneal administration of Rapamicyn (1mg/kg) 1 and 6 h after injury.

- SCI + Temsirolimus (0,6mg/kg): same as the SCI plus intraperitoneal administration of Temsirolimus (0,6mg/kg) 1 and 6 h after injury.

- SCI + KU0063794 (8mg/kg): same as the SCI plus intraperitoneal administration of KU0063794 (8mg/kg) 1 and 6 h after injury.

Ten mice from each group for each parameter were killed at 24 hours after SCI in order to evaluate the various considerations.

5.1.1.4 TISSUE PROCESSING AND HISTOLOGY

Briefly, paraffin tissue sections (thickness, 7 μm) were deparaffinized with xylene, stained with hematoxylin and eosin, and studied using light microscopy (AxioVision, Zeiss, Milan, Italy) by an experienced histopathologist. Damaged neurons were counted and the histopathologic alterations of the grey matter were scored on a six-point scale (Lang-Lazdunski *et al.*, 2003): 0, no lesion detected; 1, grey matter contained one to five eosinophilic neurons; 2, grey matter contained five to 10 eosinophilic neurons; 3, grey matter contained more than 10 eosinophilic neurons; 4, small infarction (less than one third of the grey matter area); 5, moderate infarction (one third to one half of the grey matter area); 6, large infarction (more than half of the grey matter area). The scores from all the sections of every spinal cord were averaged to give a final score for distinct mice. All the histological analyses were completed in a blinded fashion.

5.1.1.5 GRADING OF MOTOR DISTURBANCE

Recovery from motor impairment was classified using the Basso mouse scale (BMS) open-field score (Basso *et al.*, 2006). After injury, the motor function of mice exposed to compression trauma was evaluated once a day for 10 days. The evaluations were completed by two blind observers for totally analyzed groups. The BMS scale ranges from 0 (indicating complete paralysis) to 9 (indicating normal hind limb function), and rates locomotion on such aspects of hind limb function as weight support, stepping ability, coordination and toe clearance. The BMS score was determined for ten mice in each group.

5.1.1.6 IMMUNOHISTOCHEMICAL LOCALIZATION OF COX2, iNOS, BAX, BCL-2 AND GFAP.

At the end of the experiment, 24 h following trauma, spinal cord tissues were taken and fixed for 24 hours in paraformaldehyde mix (4% in PBS 0.1 M) at room temperature, dehydrated by graded ethanol and xylene and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Thereafter, 7 μm sections thick were cut from the paraffin-embedded tissue. Following deparaffinization with xylene and graded ethanol, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. Slices were permeablized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin and avidin binding sites were blocked by progressive incubation for 15 min with biotin and avidin (Vector Laboratories, Burlingame, CA, USA), respectively. Subsequently, slices were incubated overnight with anti-COX2 mouse polyclonal antibody (Cayman 1:500 in PBS, v/v), or anti-iNOS mouse polyclonal antibody (BD Trasduction 1:500 in PBS, v/v), anti-Bax rabbit polyclonal antibody (SantaCruz Biotechnology 1:500 in PBS, v/v), anti-Bcl-2 rabbit polyclonal antibody (SantaCruz Biotechnology 1:500 in PBS, v/v), and with anti-glial fibrillary acidic protein (anti-GFAP) mouse monoclonal antibody (1:500; Cell Signaling Technology). Sections were cleaned with PBS and incubated with peroxidase-conjugated bovine anti-mouse immunoglobulin G (IgG) secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2,000 Jackson Immuno Research, West Grove, PA, USA). Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG or biotin-conjugated goat anti-mouse IgG and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA, USA). To verify the binding specificity for COX2, iNOS, Bax, Bcl-2 and GFAP, control slices were also incubated with single the primary antibody (no secondary) or with just the secondary antibody (no primary). In these controls, no positive staining was detected,

indicating that the immunoreaction was positive in all the experiments. The immunohistochemical pictures were collected by Zeiss microscope using Axio Vision software. For graphic representation of densitometric analysis, we measured the intensity of positive staining (brown staining) by computer-assisted color image analysis (Leica QWin V3, UK). The percentage area of immunoreactivity (determined by the number of positive pixels) was expressed as percent of total tissue area (red staining). Replicates for each experimental condition and histochemical staining were obtained from each mouse in each experimental group. In sham-operated mice, the central areas of equivalent tissue sections were taken as reference points, and a comparable number of optical fields were counted. (Shea, 1994). All the histological examination was carried out without knowledge of the treatments.

5.1.1.7 WESTERN BLOT ANALYSIS FOR NNOS, FAS-LIGAND, IL-1β, TNF-A AND β-ACTIN.

Cytosolic and nuclear extracts were made as previously described with minor modifications (Bethea *et al.*, 1998). Spinal cord tissue from each mouse were suspended in extraction Buffer A containing 0.2mM PMSF, 0.15mM pepstatin A, 20mM leupeptin, 1mM sodium orthovanadate, homogenized at the maximum setting for 2 min, and centrifuged at 12000 x rpm for 4 min at 4°C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were resuspended in Buffer B containing 1% Triton X-100, 150mM NaCl, 10mM Tris–HCl pH 7.4, 1mM EGTA, 1mM EDTA, 0.2mM PMSF, 20mM leupeptin, 0.2mM sodium orthovanadate. After centrifugation 10 min at 12000 rpm at 4°C, the supernatants containing the nuclear protein were stored at -80 C for further analysis. The levels of nNOS, Fas-ligand, IL-1β, TNF-α and β-actin were calculated in cytosolic fraction from spinal cord tissue collected after 24 h following SCI. The filters were blocked with 1x PBS, 5% (w/v) non fat dried milk (PM) for 40 minutes at room temperature and successively probed with specific anti-nNOS (1:1000; BD transduction), anti-Fas-Ligand (1:500; Santa Cruz Biotechnology), anti-IL-1β (1:500; Santa Cruz Biotechnology), anti-TNF-α (1:1000; AbCam), in 1x PBS, 5% (w/v) non fat dried milk, 0.1% Tween-20 (PMT) at 4 ºC overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson Immuno Research, West Grove, PA) for 1 hour at room temperature. To establish that blots were loaded with equivalent amounts of proteic lysates, they were similarly incubated in the presence of the antibody against β-actin (1:5000; Santa Cruz Biotechnology). The relative expression of the protein bands of for nNOS (155kDa), Fas-ligand (31kDa), IL-1β (17kDa), TNF-α (17kDa) and β-

actin (42 kDa) were detected with enhanced chemiluminescence detection system reagent according to manufacturer's instructions (SuperSignal West Pico Chemiluminescent Substrate, Pierce). The relative expression of the protein bands was calculated by densitometry with Bio-Rad ChemiDoc™ XRS + software and standardized to β-actin levels. Images of blot signals (8-bit/600-dpi resolution) were imported to analysis software (Image Quant TL, v2003). A preparation of commercially available molecular weight markers made of proteins of molecular weight 10 to 250 kDa was used to define molecular weight positions and as indication of concentrations for each molecular weight.

5.1.2 EX-VIVO PROCEDURES

5.1.2.1 PREPARATION OF SPINAL CORD ORGANOTYPIC SLICE CULTURES

Spinal cord slice cultures were taken from mouse spinal cord at postnatal day 6 as previously described by Esposito et al (Esposito *et al.*, 2012). Briefly, the dorsal skin as well as the musculature of the trunk were disconnected nearby the midline, after decapitation with large bandage scissors. Successively, the dura mater was incised after a total longitudinal laminectomy and the spinal cord was separated from the denticulate ligaments and immediately sited in ice-cold dissecting media (pH 7.15). Residues of the adjacent dura mater were removed under microscopic control. Next, the spinal cord was cut into transverse slices of 400-μm thickness by a tissue chopper (McIlwain, ON, Canada) to make the spinal cord organotypic slice cultures and positioned into a sterilized petri dish with Earle's balanced salt solution. To acquire reliable data with analysis of cell death, we cultured just thoracic slices that were very consistent in cross-sectional dimension and in this way each animal generated up to eight usable slices. Ultimately, spinal slices were transferred into Millicell-CM cultured plate inserts (Millipore, Billerica, MA, USA). The inserts were located into wells of a 6-well plate containing 1.5 ml of antibiotic-free medium, containing: 50% MEM with Earle's balanced salt solution and glutamine; 25% Hank's balanced salt solution; and 25% horse serum supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) sodium salt and 6 mg/ml D-glucose (Gibco, Carlsbad, CA, USA). Slices were incubated at 37°C for 7 days and the medium was changed every two days. Organotypic cultures were examined day-to-day to observe general structural integrity (white and gray matter) and neurite outgrowth.

5.1.2.2 KU0063794 TREATMENTS

7 days after stabilization of development, cultures were distributed into the following groups:

1) Control (CTR): spinal cord slices were cultured with standard culture medium and treated only with vehicle.

2) Damage: spinal cord slices were sagittally cut with a blade under microscopic control (Esposito *et al.*, 2012).

3) Damage + KU0063794 (KU0063794): spinal cord slices were sagittally cut, as previously described, and KU0063794 0.5μM, was placed in culture medium 1 h before injury.

KU0063794 was left in a culture medium for 24 hours after injury. Spinal cord slices were then used for 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and nitrite production.

5.1.2.3 VIABILITY OF ORGANOTYPIC CULTURES BY TETRAZOLIUM DYE

The MTT colorimetric assay was used to measure cell growth and viability, as previously described by Abe et Matsuki (Abe and Matsuki, 2000). Twenty-four hours after mechanical damage, viability of organotypic cultures was calculated by using a mitochondria-dependent dye for live cells (tetrazolium dye; MTT) to formazan. Cultures were incubated at 37°C with MTT (0.2 mg/ml) for one hour. Culture medium was aspired by aspiration and the cells were lysed with dimethyl sulfoxide (DMSO; 100 μl). The extent of reduction of MTT to formazan within cells was quantified by the measurement of optical density at 550 nm (OD_{550}) with the microplate reader.

5.1.2.4 MEASUREMENT OF NITRITE LEVELS

Total nitrite levels, as an indicator of nitric oxide (NO) synthesis, were measured in the supernatant. The nitrate in the medium was reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and β-nicotinamide adenine dinucleotide 3-phosphate (160 mM) at room temperature for three hours. The entire nitrite concentration was then measured using the Griess reaction by addition 100 μl Griess reagent (0.1% (w/v) N-(1-Naphthyl) ethylenediamine dihydrochloride in H_2O and 1% (w/v) sulfanilamide in 5% (v/v) concentrated H_3PO_4; volume 1:1) to the 100-μl sample. OD_{550} was measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Tecan, Männedorf,

Switzerland). Nitrite concentrations were analysed by comparison with OD_{550} of standard solutions of sodium nitrite prepared in H_2O.

5.1.3 MATERIALS

Except otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd (Milan, Italy). All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl; Baxter, Italy) or 10% dimethyl sulfoxide.

5.1.4 STATISTICAL EVALUATION

All values in the figures and text are expressed as mean ± standard error of the mean (SEM) of N observations. For the *ex vivo* studies N represents the number of animals studied. In the experiments involving histology and immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. A *p*-value of less than 0.05 was considered significant. The results were analyzed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons.

5.2. MATERIALS AND METHODS FOR TBI STUDY

5.2.1 ANIMALS

Male CD1 mice (25- 30g, Envigo Milan, Italy), aged 10–12 weeks, were used for all studies. Mice were housed in individual cages (five per cage) and maintained under 12:12 h light–dark cycle at 21 ± 1°C and 50 ± 5% humidity. Standard laboratory diet and tap water were available ad libitum. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192) as well as with the EEC regulations (O.J: of E.C.L 358/1 12/18/1986).

5.2.2 CONTROLLED CORTICAL IMPACT (CCI) EXPERIMENTAL TBI.

A number of animal models have been developed to induce brain trauma. Of these, the most commonly used are weight-drop injury, fluid percussion injury (FPI) and CCI. The use of TBI models has resulted in an increased understanding of the pathophysiology of TBI, including changes in molecular and cellular pathways and neurobehavioral outcomes. CCI models utilize a pneumatic pistol to laterally deform the exposed dura and provide controlled impact and quantifiable biomechanical parameters. This model produces graded, reproducible brain injury. Dependent on the severity of injury, CCI results in an ipsilateral injury with cortical contusion, hemorrhage and blood-brain barrier disruption. CCI injury reproduces changes reported in clinical head injuries such as cortical contusion, brain edema, subarachnoid hemorrhage, elevated intracerebral pressure, reduced cortical perfusion, decreased cerebral blood flow and neuro-endocrine and metabolic changes. The predominantly focal brain injury caused by CCI makes this model to a useful tool for studying the pathophysiology of the secondary processes induced by focal brain injury (Xiong *et al.*, 2013). TBI was induced in mice (n=10 per group) by controlled cortical impactor. The mice were anesthetized under intra-peritoneal Ketamine + Xylazine (2.6/0.16 mg/kg of body weight, respectively). A craniotomy was made in the right hemisphere encompassing bregma and lambda and between the sagittal suture and the coronal ridge with a Micro motor hand piece and drill (UGO Basile S.R.L., Comerio VA, Italy). The resulting bone flap was removed, and the craniotomy enlarged further with cranial rongeurs. A cortical contusion was produced on the exposed cortex using a controlled impactor device Impact OneTM Stereotaxic impactor for CCI (myNeurolab.com, Richmond). Briefly, the impacting shaft was extended, and the impact tip was centered and lowered over the craniotomy site until it touched the dura mater. Then, the rod was retracted and the impact tip was advanced farther to produce a brain injury of

moderate severity for mice (tip diameter, 4 mm; cortical contusion depth, 3 mm; impact velocity, 1.5 m/sec). Immediately after injury, the skin incision was closed with nylon sutures, and 2% lidocaine jelly was applied to the lesion site to minimize any possible discomfort.

5.2.3 EXPERIMENTAL GROUPS

Mice were randomly allocated into the following groups:

- Sham + vehicle group: mice were subjected to the surgical procedures as the above groups except for TBI.
- Sham + Rapamicyn (1mg/kg): same as the sham plus intraperitoneal administration of Rapamicyn (1mg/kg) 1 and 6 h after injury.
- Sham + KU0063794 (8mg/kg): same as the sham plus intraperitoneal administration of KU0063794 (8mg/kg) 1 and 6 h after injury.
- TBI + vehicle group: mice were subjected to TBI plus intraperitoneal administration of vehicle (saline).
- TBI + Rapamicyn (1mg/kg): same as the TBI plus intraperitoneal administration of Rapamicyn (1mg/kg) 1 and 6 h after injury.
- TBI + KU0063794 (8mg/kg): same as the TBI plus intraperitoneal administration of KU0063794 (8mg/kg) 1 and 6 h after injury.

As describe below, mice (n = 10 from each group for each parameters) were sacrificed at 24 h after TBI in order to evaluate the various parameter. In a separate set of experiments, other 10 animals for each group were observed after TBI in order to evaluate the behavioural testing.

5.2.4 BEHAVIOURAL TESTING

TBI mice display motor and cognitive deficits. Thus, the present behavioural tests involved analyses of motor asymmetry (Elevated Biased Swing Test and Rotarod test). Training for the rotarod test was initiated at 1 week, respectively, before the CCI injury, whereas no training was required for EBST. The retard treadmill (Accuscan, Inc., Columbus, OH, USA) provided a motor balance and coordination assessment. Data were generated by averaging the scores (total time spent on treadmill divided by 5 trials) for each animal during training and testing days. Each animal was placed in a neutral position on a cylinder (3 cm and 1 cm diameter for rats and mice, respectively) then the rod was rotated with the speed accelerated linearly from 0 rpm to 24 rpm within 60 s, and the time spent on the retard was recorded automatically. The maximum score given to an animal was fixed to 60. For training, animals were given 5 trials

each day and declared having reached the criterion when they scored 60 in 3 consecutive trials. For testing, animals were given 3 trials and the average score on these 3 trials was used as the individual rotarod score. The EBST provided a motor asymmetry parameter and involved handling the animal by its tail and recording the direction of the biased body swings. The EBST consisted of 20 trials with the number of swings ipsilateral and contralateral to the injured hemisphere recorded and expressed in percentage to determine the biased swing activity.

5.2.5 QUANTIFICATION OF INFARCT VOLUME

The mice were anesthetized with ketamine and decapitated. Their brains were carefully removed. The brains were cut into 5 coronal slices of 2 mm thickness. Slices were incubated in 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 30 min and immersion fixed in 10% buffered formalin solution. TTC stains the viable brain tissue red, while infracted tissue remains unstained (Bederson *et al.*, 1986; Schomacher *et al.*, 2008). For quantification of infracted area and volumes, the brain slices were photographed using a digital camera (Canon 4x, Canon Inc, China) and then Image analysis was performed on a personal computer with an image analysis software program (using an ImageJ for Mac). To compensate for the effect of brain oedema the corrected infarct volume was calculated as previously described in detail (Schabitz *et al.*, 2000). Corrected infarct area equals left hemisphere area minus (right hemisphere area minus infarct area). Values are given as mean±S.D. The corrected total infarct volume was calculated by summing the infarct area in each slice and multiplying it by slice thickness (2 mm).

5.2.6 TISSUE PROCESSING AND HISTOLOGY

Tissue segments containing the lesion (1 cm on each side of the lesion) were paraffin embedded and cut into 5-μm-thick sections. Tissue sections were deparaffinized with xylene stained with Haematoxylin/Eosin (H&E) and studied using light microscopy (Dialux 22 Leitz). The segments of each brain contained the lesion (1 cm on each side of the lesion), were evaluated by an experienced histopathologist. Damaged neurons were counted and the histopathologic changes of the gray matter were scored on a 6-point scale (Kawai and Akira, 2007): 0, no lesion observed, 1, gray matter contained 1–5 eosinophilic neurons; 2, gray

matter contained 5–10 eosinophilic neurons; 3, gray matter contained more than 10 eosinophilic neurons; 4, small infarction (less than one third of the gray matter area); 5, moderate infarction; (one third to one half of the gray matter area); 6, large infarction (more than half of the gray matter area). The scores from all the sections from each brain were averaged to give a final score for individual mice. All the histological studies were performed in a blinded fashion.

5.2.7 WESTERN BLOT ANALYSES FOR IκBα, NF-κB, COX-2, INOS, β-ACTIN AND LAMIN A/C

Cytosolic and nuclear extracts were made as previously described with minor modifications (Bethea *et al.*, 1998). Brain tissue from each mouse were suspended in extraction Buffer A containing 0.2mM PMSF, 0.15mM pepstatin A, 20mM leupeptin, 1mM sodium orthovanadate, homogenized at the maximum setting for 2 min, and centrifuged at 12000 x rpm for 4 min at 4°C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were resuspended in Buffer B containing 1% Triton X-100, 150mM NaCl, 10mM Tris–HCl pH 7.4, 1mM EGTA, 1mM EDTA, 0.2mM PMSF, 20mM leupeptin, 0.2mM sodium orthovanadate. After centrifugation 10 min at 12000 rpm at 4°C, the supernatants containing the nuclear protein were stored at -80 C for further analysis. The levels of IκBα, NF-κB, Cox-2 and iNOS were calculated in cytosolic and nuclear fractions from brain tissue collected after 24 h following TBI. The filters were blocked with 1x PBS, 5% (w/v) non fat dried milk (PM) for 40 minutes at room temperature and successively probed with specific anti-IκBα (1:500; Santa Cruz Biotechnology), anti-NF-κB (1:500; Santa Cruz Biotechnology), anti-Cox-2 (1:1,000; Cayman Chemicals), anti-iNOS (1:1,000; BD Biosciences), in 1x PBS, 5% (w/v) non fat dried milk, 0.1% Tween-20 (PMT) at 4 ºC overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson Immuno Research, West Grove, PA) for 1 hour at room temperature. To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibody against β-actin or lamin A/C (1:5,000; Santa Cruz Biotechnology). The relative expression of the protein bands of for IκBα (41kDa), NF-κB (65kDa), Cox-2 (75kDa) and iNOS (130kDa) were detected with enhanced chemiluminescence detection system reagent according to manufacturer's instructions (SuperSignal West Pico Chemiluminescent

Substrate, Pierce). The relative expression of the protein bands was calculated by densitometry with Bio-Rad ChemiDoc™ XRS + software and standardized to β-actin or lamin a/c levels. Images of blot signals (8-bit/600-dpi resolution) were imported to analysis software (Image Quant TL, v2003). A preparation of commercially available molecular weight markers made of proteins of molecular weight 10 to 250 kDa was used to define molecular weight positions and as indication of concentrations for each molecular weight.

5.2.8 IMMUNOFLUORESCENCE FOR BDNF, NT3, NEUN

After deparaffinization and rehydration, detection of BDNF, NT3 and NeuN was carried out after boiling in 0.1 M citrate buffer for 1 min. Non-specific adsorption was minimized by incubating the section in 2% (vol/vol) normal goat serum in PBS for 20 min. Sections were incubated with polyclonal rabbit anti-BDNF (1:100, v/v Santa Cruz Biotechnology), or with polyclonal rabbit anti-NT3 (1:100, v/v Santa Cruz, Biotechnology), or with monosclonal mouse anti-NeuN (1:100, v/v Santa Cruz Biotechnology), antibody in a humidified chamber for O/N at 37 °C. Sections were washed with PBS and were incubated with secondary antibody FITC-conjugated anti-mouse Alexa Fluor-488 antibody (1:2000 v/v Molecular Probes, UK) or with TEXAS RED-conjugated anti-rabbit Alexa Fluor-594 antibody (1:1000 in PBS, v/v Molecular Probes, UK) for 1 h at 37 °C. Sections were washed and for nuclear staining 4′,6′-diamidino-2-phenylindole (DAPI; Hoechst, Frankfurt; Germany) 2 μg/ml in PBS was added. Sections were observed and photographed using Cells were observed at x20 magnification using a Leica DM2000 microscope (Leica). All images were digitalized at a resolution of 8 bits into an array of 2560 × 1920 pixels. Optical sections of fluorescence specimens were obtained using a HeNe laser (543 nm), a laser UV (361–365 nm) and an argon laser (458 nm) at a 1-min, 2-s scanning speed with up to 8 averages; 1.5-μm sections were obtained using a pinhole of 250. Examining the most brightly labeled pixels and applying settings that allowed clear visualization of structural details while keeping the highest pixel intensities close to 200 established contrast and brightness. The same settings were used for all images obtained from the other samples that had been processed in parallel. Digital images were cropped and figure montages prepared using Adobe Photoshop 7.0 (Adobe Systems; Palo Alto, CA).

5.2.9 MATERIALS

Except otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd (Milan, Italy). All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl; Baxter, Italy) or 10% dimethyl sulfoxide.

5.2.10 STATISTICAL EVALUATION

All values in the figures and text are expressed as mean ± standard error of the mean (SEM) of N observations. In the experiments involving histology and immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. A p-value of less than 0.05 was considered significant. The results were analyzed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons.

CHAPTER 6: RESULTS

6.1 RESULTS FOR SCI STUDY

6.1.1 IN VIVO STUDY

6.1.1.1 The severity of tissue damage following SCI is decreased in KU0063794 and Temsirolimus treatment mice

Twenty-four hours after SCI, the sections obtained from each group were stained with hematoxylin and eosin (H&E) for histologic assessment of contusion areas. A substantial damage to the spinal cord, at the level of the perilesional area, considered by the presence of edema as well as alteration of the white matter, was observed in SCI mice group (Fig. 20B, see histological score 20F) compared to sham operated mice (Figure 20A). As shown in figure 20D and relative quantification in figure 20F, a decrease in the severity of trauma was observed in mice treated with Temsirolimus. Indeed, treatment with the KU0063794 (Fig. 20E, see histological score 20F) reduced histological alterations more effectively than the treatment with Temsirolimus or Rapamicyn (Fig. 20C, 20D see histological score 20F). To evaluate whether histological damage to the spinal cord was associated with a loss of motor function, the BMS open-field score was used. Motor function was not impaired in sham mice. Mice subjected to SCI showed significant deficits in hind limb movement (Figure 20G). The treatment of SCI-operated mice with KU0063794 lead to a significant improvement of the neurological score in comparison with the treatment with Temsirolimus or Rapamicyn. Moreover, the motor function improvement observed after the treatment with KU0063794 significantly persisted up to 10 days after SCI.
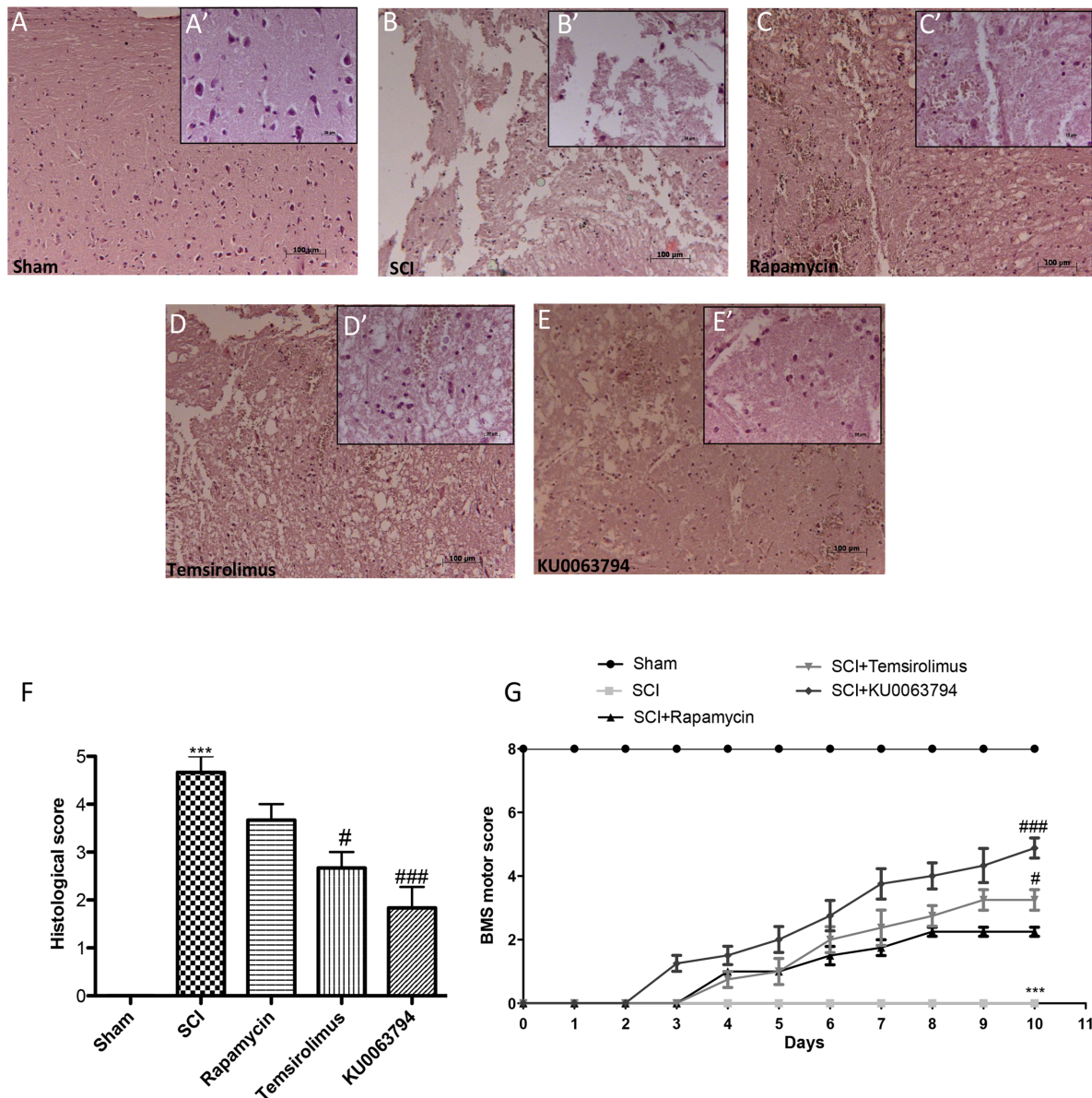
Figure 20 *The severity of tissue damage following SCI is decreased in KU0063794 and Temsirolimus treatment mice*

As showed in figure 20, an extensive damage to the spinal cord, was observed in SCI mice group (Figure 20B) compared to sham operated mice (Figure 20A). Figure 20 D and relative quantification in figure 20F, showed a decrease in the severity of trauma in mice treated with Temsirolimus. Indeed, treatment with the KU0063794 (Figure 20E) reduced histological alterations more effectively than the treatment with Temsirolimus or Rapamicyn (Figure 20C). Also, mice subjected to SCI showed significant deficits in hind limb movement (Figure 20G). In the KU0063794-treated mice group, the neurological score better improved than Temsirolimus-treated or Rapamicyn –treated mice group, and persisted up to 10 days after SCI (Figure 20G). The figures are representative of at least three experiments performed on different experimental days. Figures are representative of all the animals in each group. Values are given as mean ± SEM of 20 animals for each group. #P < 0.05 vs SCI group; ***P< 0.001 vs Sham group; ###P< 0.001 vs SCI group.

6.1.1.2 KU0063794 and Temsirolimus modulates COX2 and iNOS expression and nNOS formation after SCI

Two important landmarks of secondary tissue loss following SCI injury are the expression of inducible nitric oxide (iNOS) and Cyclo-oxygenase-2 (COX-2). For this reason iNOS and COX-2 expression were evaluated by immunohistochemical analysis in the spinal cord sections 24 h after SCI. Spinal cord sections obtained from sham-operated mice did no stain either for COX2 and iNOS (Figure 21A and 21B respectively, see densitometry analysis K). A substantial increase in COX2 and iNOS expression was observed in spinal cord section obtained at 24 h after SCI (Figure 21C and 21D respectively, see densitometry analysis 21K), while Temsirolimus treatment significantly reduced the degree of positive staining (Figure 21G and 21H respectively, see densitometry analysis 21K). However, in KU0063794-treated mice group the decrease of positive staining is more effectively than the treatment with Temsirolimus or Rapamicyn (Figure 21I and 21J respectively, see densitometry analysis 21K). Besides, to investigate whether the KU0063794 had also effect on neuronal nitric oxide synthase (nNOS) we assessed by western blot the nNOS expression. As show in figure 21L we observed a basal levels of nNOS in the spinal cord section obtained from Sham-operated animals (see densitometry analysis L'). On the contrary, at 24 hours after SCI a loss of nNOS was detected. The treatment with KU0063794 significantly restored the levels of nNOS up to that of uninjured mice.
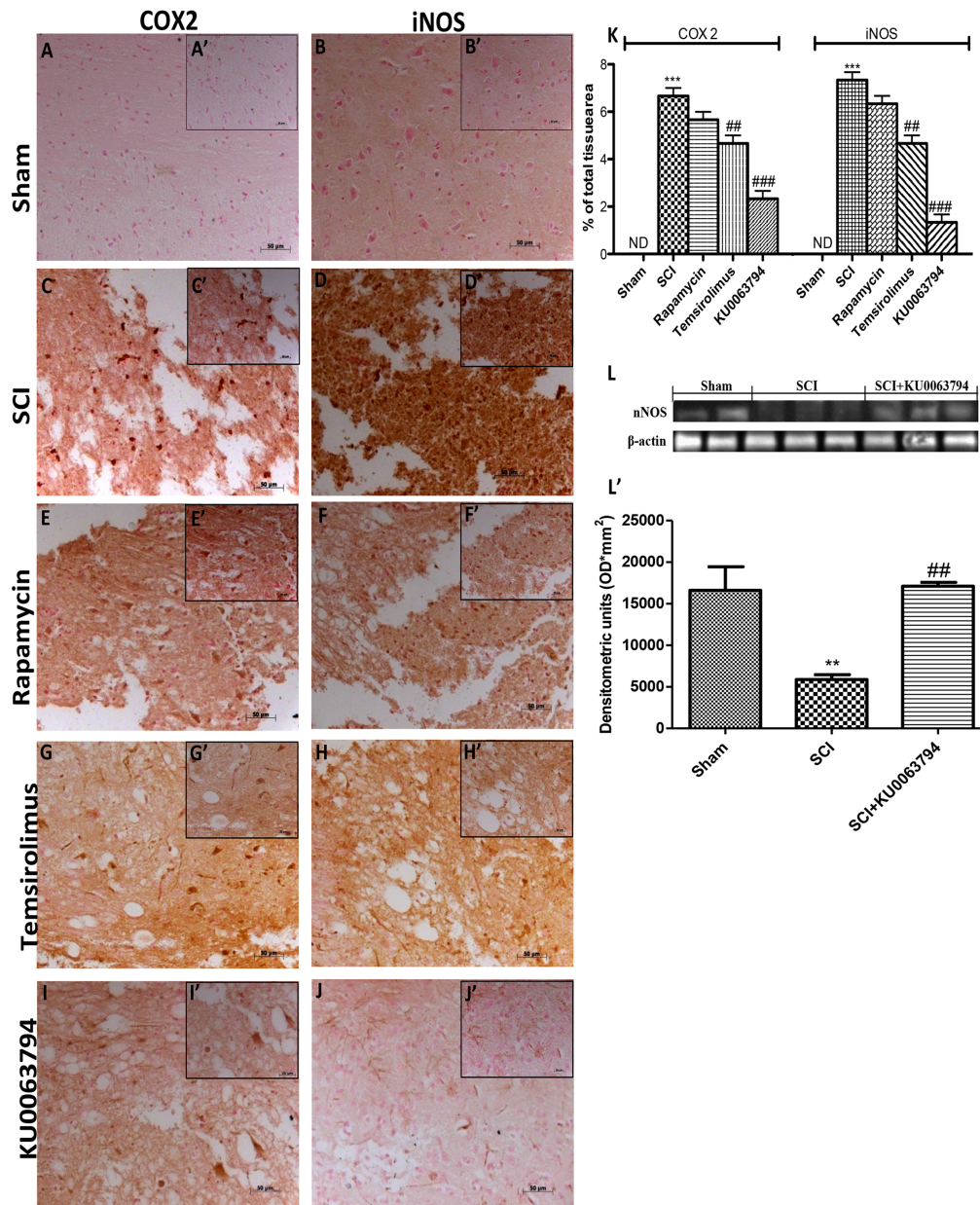
Figure 21 KU0063794 and Temsirolimus modulates COX2 and iNOS expression and nNOS formation after SCI

Spinal cord sections obtained from sham-operated mice did no stain either for COX2 and iNOS (Figure 21A,B). A substantial increase in COX2 and iNOS expression was observed in spinal cord section obtained at 24 h after SCI, while Temsirolimus treatment significantly reduced the degree of positive staining (Figure 21G,H). However, in KU0063794-treated mice group the decrease of positive staining is more effectively than the treatment with Temsirolimus or Rapamicyn (Figure 21 I,J). Additionally as show in figure 21L we observed a basal levels of nNOS in the spinal cord section obtained from Sham-operated animals. On the contrary, at 24 hours after SCI a loss of nNOS was detected. The treatment with KU0063794 significantly restored the levels of nNOS up to that of uninjured mice. The figures are representative of at least three experiments performed on different experimental days. Figures are representative of all the animals in each group. Values are given as mean ± SEM of 20 animals for each group. ##$P < 0.01$ vs SCI group; **$P < 0.01$ vs Sham group; ***$P < 0.001$ vs Sham group; ###$P < 0.001$ vs SCI group.

-73-

6.1.1.3 Effect of KU0063794 and Temsirolimus on astrocyte activation and cytokines production

The primary mechanical damage to the spinal cord initiates a secondary damage that includes microglia and astrocytes activation that release a large number of pro-inflammatory cytokines. To evaluate if in astrocytes, KU0063794, may induce indirectly, by activating mTORC2, the activation of p-Akt and cell survival we investigated GFAP, IL-1β and TNF-α production, both inflammation sign. A substantial increase in GFAP expression was found in spinal cord tissues collected at 24 h after SCI (Fig. 22B see relative densitometric analysis shown in Fig. 22F). Spinal activation of astrocytes was significantly attenuated in KU0063794-treated mice (Fig. 22E see relative densitometric analysis shown in Fig. 22F) compared to Temsirolimus-treated or Rapamicyn –treated mice group (Fig. 22C and 22D see relative densitometric analysis shown in Fig. 22F). Furthermore, western blot analysis revealed a markedly increase of expression of IL-1β and TNF-α production in spinal tissues collected at 24 h after SCI compared to sham group. KU0063794 treatment significantly diminished the post-SCI expression of IL-1β and TNF-α (Fig. 22G and Fig. 22H and relative densitometric analysis shown in Fig. 22G' and Fig. 22H').
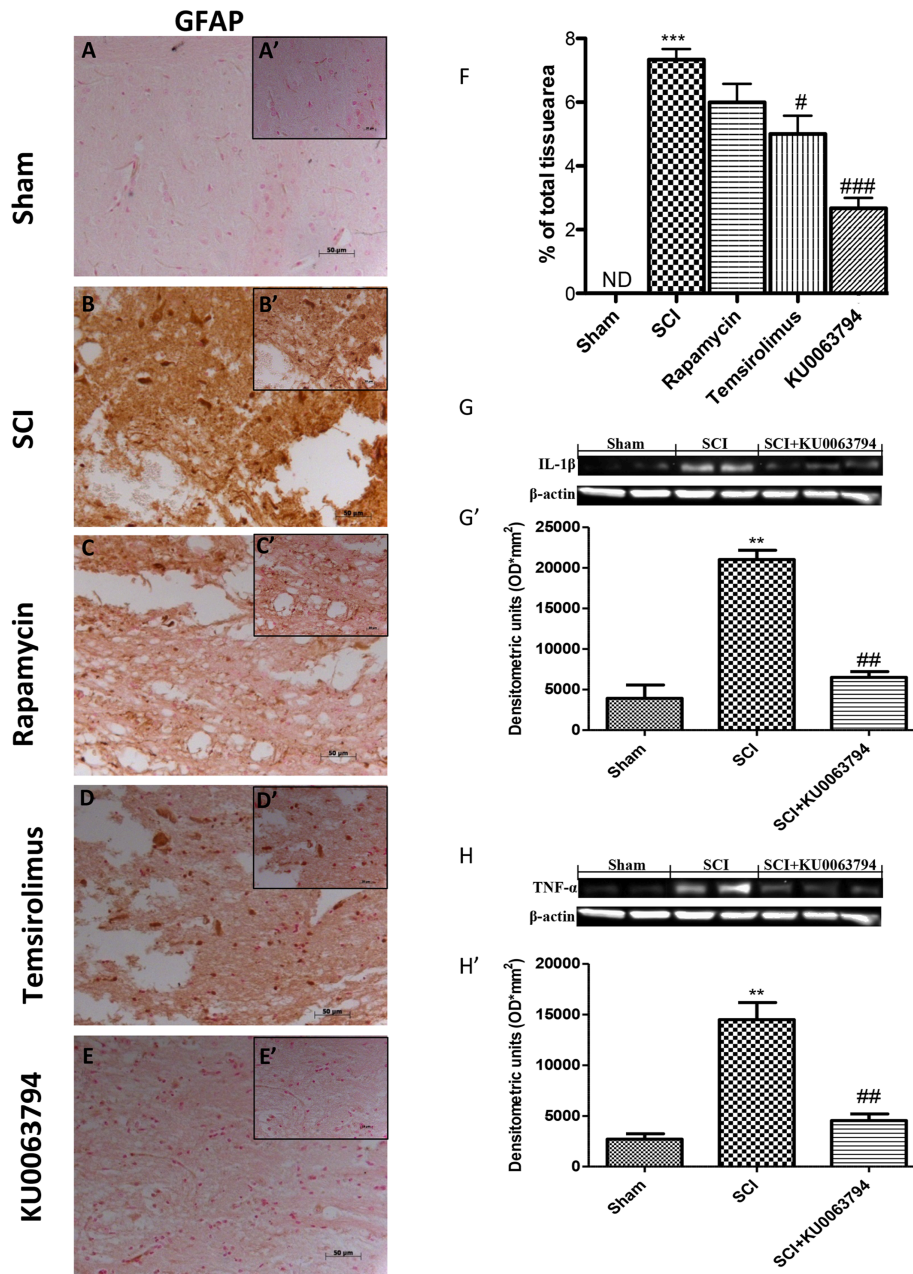
Figure 22 Effect of KU0063794 and Temsirolimus on astrocyte activation and cytokines production

As showed in panel 22, a substantial increase in GFAP expression was found in spinal cord tissues collected at 24 h after SCI (Figure 22B). Spinal activation of astrocytes was significantly attenuated in KU0063794-treated mice (Figure 22E) compared to Temsirolimus-treated or Rapamicyn –treated mice group (Figure 22C,D). Furthermore, western blot analysis revealed a markedly increase of expression of IL-1β and TNF-α production in spinal tissues collected at 24 h after SCI compared to sham group. KU0063794 treatment significantly diminished the post-SCI expression of IL-1β and TNF-α (Figure 22G,H). The figures are representative of at least three experiments performed on different experimental days. Figures are representative of all the animals in each group. Values are given as mean ± SEM of 20 animals for each group. #$P < 0.05$ vs SCI group; ##$P < 0.01$ vs SCI group; **$P < 0.01$ vs Sham group; ***$P < 0.001$ vs Sham group; ###$P < 0.001$ vs SCI group;

6.1.1.4 Effect of KU0063794 and Temsirolimus on apoptosis pathway

The connection between autophagy and apoptosis is a increasing area of research. The molecular relationship between autophagy and cell death are intricate, complex and still poorly understood. To test whether spinal cord damage was associated with autophagy and apoptosis we determined Bax and Bcl-2 expression by immunohistochemical staining. Immunohistochemistry for Bax and Bcl-2 showed that spinal cord sections from sham-operated mice did not stain for Bax (Figure 23A), whereas SCI-operated mice exhibited positive staining for Bax (Figure 23C). Temsirolimus treatment significantly reduced the degree of positive staining (Figure 23G). However, in KU0063794-treated mice group the decrease of positive staining is more effectively than the treatment with Temsirolimus or Rapamicyn (Figure 23G and 23I see densitometry analysis K). Viceversa, spinal cord sections from sham-operated mice demonstrated Bcl-2 positive staining (Figure 23B), whereas in SCI mice, the staining was significantly reduced (Figure 23D). Treatment with Temsirolimus significantly increased the degree of positive staining (Figure 23D). However, in KU0063794-treated mice group the increase of positive staining for Bcl-2 is more effectively than the treatment with Temsirolimus or Rapamicyn (Figure 23F and 23H see densitometry analysis 23K). In addiction, we investigated the expression of Fas-Ligand a mediator of apoptosis. A low basal expression of Fas-Ligand was detected in spinal cord samples from sham-operated mice, whereas Fas-Ligand levels was substantially increased in SCI mice (Figure 23L, see densitometry analysis 23L'). As showed by densitometric analysis KU0063794 drastically decreased Fas-Ligand expression.
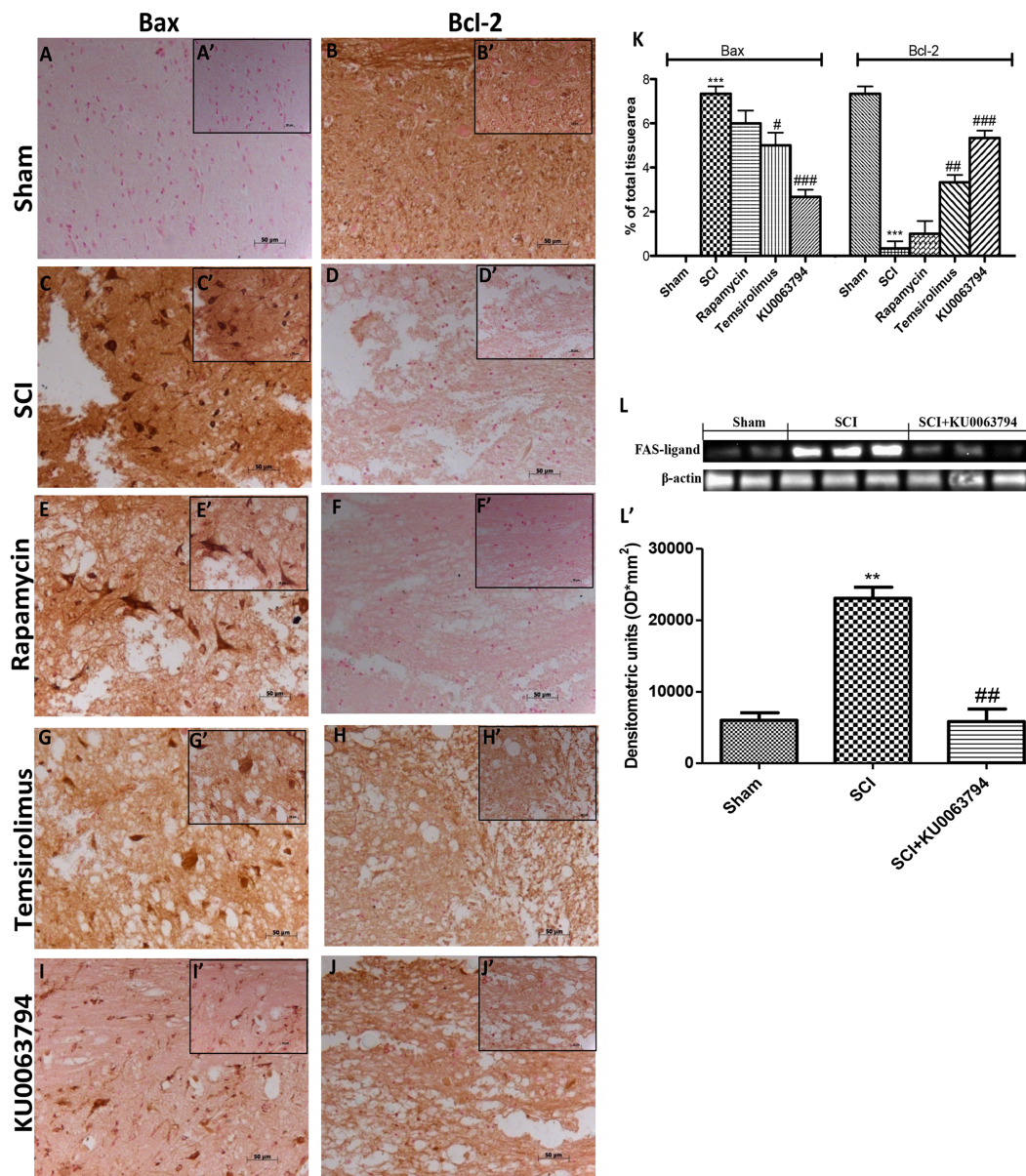
Figure 23 Effect of KU0063794 and Temsirolimus on apoptosis pathway

Immunohistochemistry for Bax and Bcl-2 showed that spinal cord sections from sham-operated mice did not stain for Bax (Figure 23A), whereas SCI-operated mice exhibited positive staining for Bax (Figure 23C). Temsirolimus treatment significantly reduced the degree of positive staining (Figure 23G). However, in KU0063794-treated mice group the decrease of positive staining is more effectively than the treatment with Temsirolimus or Rapamicyn (Figure 23G,I). Viceversa, spinal cord sections from sham-operated mice demonstrated Bcl-2 positive staining (Figure 23B), whereas in SCI mice, the staining was significantly reduced (Figure 23D). Treatment with Temsirolimus significantly reduced the degree of positive staining (Figure 23D). However, in KU0063794-treated mice group the increase of positive staining for Bcl-2 is more effectively than the treatment with Temsirolimus or Rapamicyn (Figure 23F,H). Moreover, western blot analysis showed a low basal expression of Fas-Ligand in spinal cord samples from sham-operated mice, whereas Fas-Ligand levels was substantially increased in SCI mice (Figure 23L). As showed by densitometric analysis KU0063794 drastically decreased Fas-Ligand expression. The figures are representative of at least three experiments performed on different experimental days. Figures are representative of all the animals in each group. Values are given as mean ± SEM of 20 animals for each group. #P < 0.05 vs SCI group; ##P < 0.01 vs SCI group; **P < 0.01 vs Sham group; ***P< 0.001 vs Sham group; ###P< 0.001 vs SCI group;

6.1.2 Ex vivo study

6.1.2.1 Effect of KU0063794 on cell viability and nitrite (NO_2^-) concentration in spinal cord slices

Slices were effectively cultured for up to 7 days. Viable cells within the slices, recognized using MTT tetrazolium dye, were visualized under light microscopy. The level of cell death was evaluated in each slice at 24 hours after damage. Slices subject to mechanical damage showed a significantly reduced viability compared to the uninjured group (Fig. 24A). Pre-treatment with KU0063794 0.5μM, 1 hour before injury, considerably reduced cell death compared to the injured group. Also, we investigated the levels of nitrite liberated into the culture medium by Griess reagent. The untreated control group released a very low levels of NO2-; instead, damage significantly enhanced the levels of NO2- production (Figure 24B). KU0063794 treatment decreased the injury-induced NO2- production.
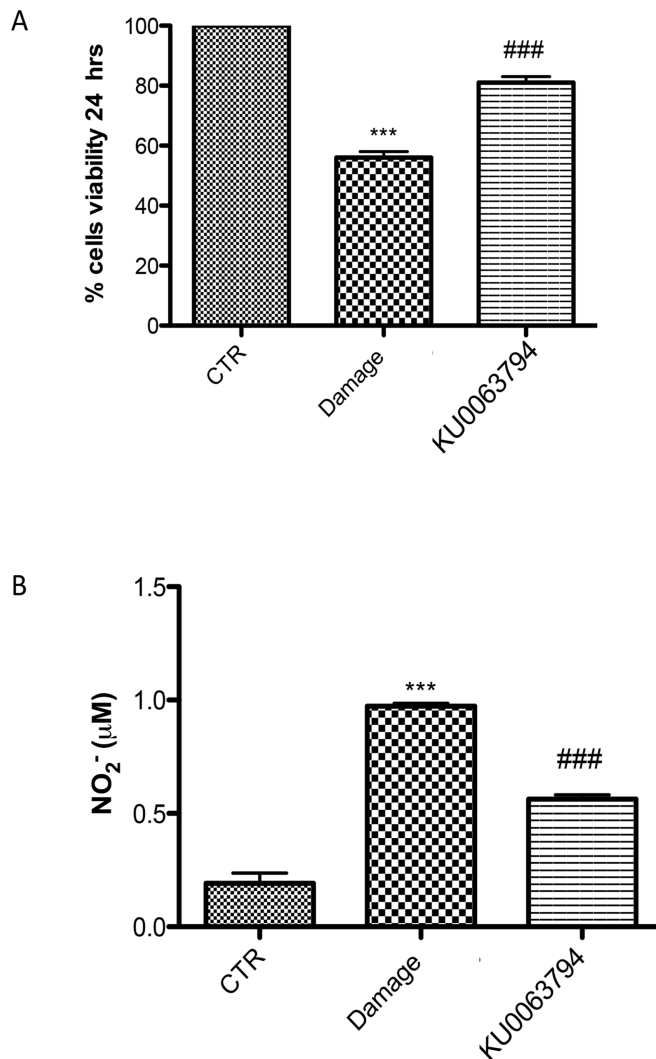
Figure 24 Effect of KU0063794 on cell viability and nitrite (NO2-) concentration in spinal cord slices

The level of cell death assessed in each slice at 24 hours after damage showed that slices subject to mechanical damage showed a significantly reduced viability compared to the uninjured group (Figure 24A). Pre-treatment with and KU0063794 1 hour before injury, significantly reduced cell death compared to the injured group. Moreover, the untreated control group released a very low levels of NO2-; instead, damage significantly enhanced the levels of NO2- production (Figure 24B). KU0063794 treatment decreased the injury-induced NO2- production. The figures are representative of at least three experiments performed on different experimental days. Figures are representative of all the animals in each group. Values are given as mean ± SEM of 20 animals for each group. ***P< 0.001 vs Sham group; ###P< 0.001 vs SCI group;

6.2 RESULTS FOR TBI STUDY

6.2.1 Effect of KU0063794 on brain edema, infarction and locomotor activity following TBI

Brain water content is a sensitive measure of cerebral edema. This measure indicates pathology associated with endothelial cell activation and endothelial dysfunction. Water content was significantly different between groups overall with levels significantly higher in animals subjected to TBI compared to sham controls. The increased water content in the ipsilateral brain induced by TBI was significantly decreased by Rapamycin as well as by KU0063794 treatments at 24 h post-injury.

Directly related to overall brain injury, measurement of brain infarctions is a standard method to evaluate brain injury after trauma. To evaluate the effect of Rapamycin and KU0063794 on brain infarctions in the TBI, we performed TTC staining (Figure 25 A1, A2, A3, A4). As showed in figure 25B and 25C, the infarction area and the infarct volume were significantly reduced after treatment with Rapamycin (1 mg/kg) as well as KU0063794 (8mg/kg). Moreover, to investigate the relationship between neurological deficit and motor function in the setting of TBI, the mice were subjected, 24 h after TBI, to the EBST and the rotarod test. Mice subjected to moderate injury showed significant hippocampal damage and behavioral deficits but low mortality. CCI-injured mice displayed a range of impairments in locomotor tasks as showed in figure D and E. Rapamycin (1 mg/kg) as well as KU0063794 (8mg/kg) treatment groups gradually and significantly improved latency compared to TBI group.
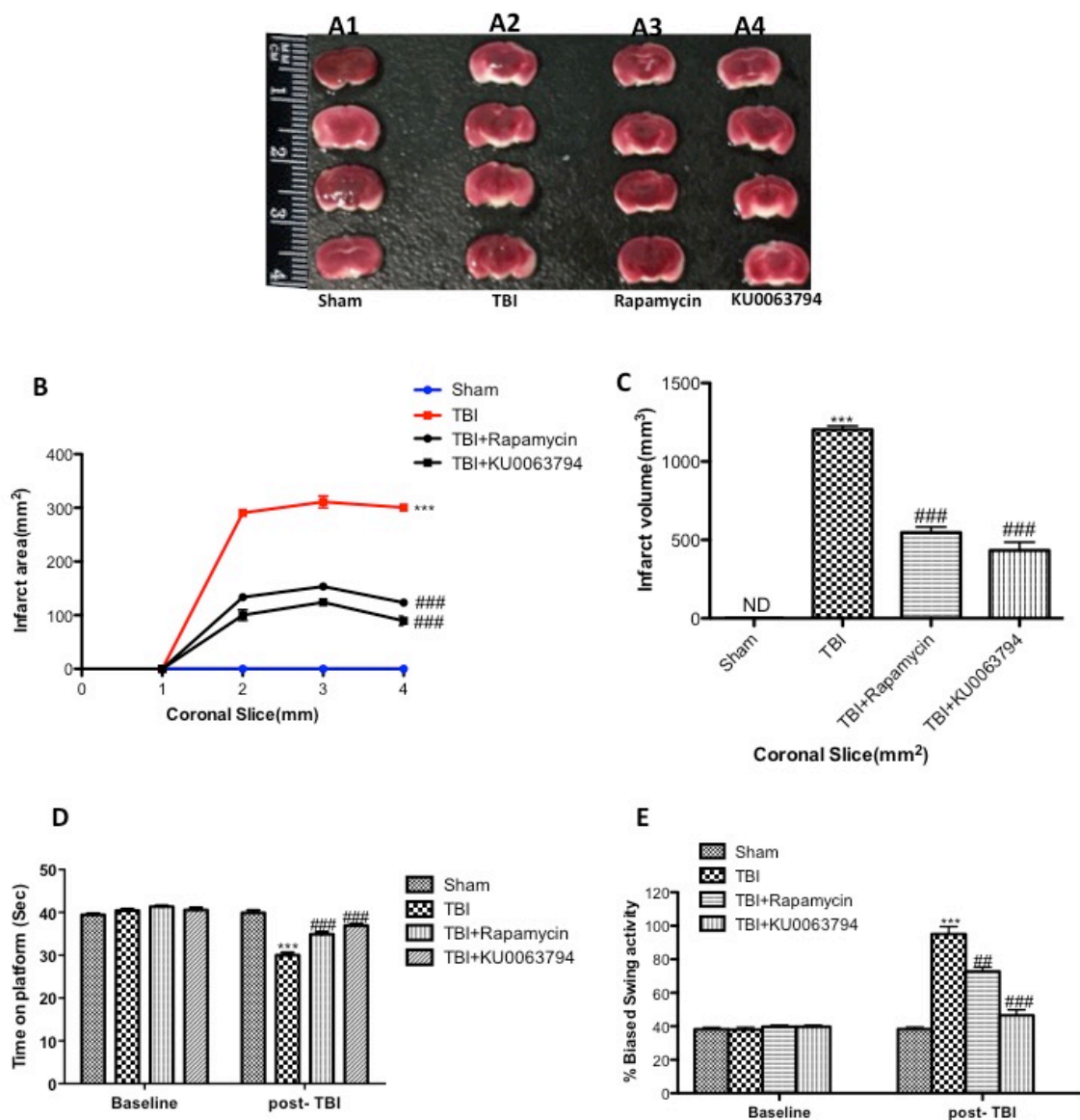
Figure 25 Effect of KU0063794 on brain edema, infarction and locomotor activity following TBI

Representative TTC stained brain section (four out of the six consecutive sections from cranial to caudate region) corresponding to largest infraction from each group (Figure 25A). Brain sections (2 mm thick) were stained with TTC at 24 hours after TBI and show significant difference after KU0063794 treatment in terms of area (Figure 25B) and volume (Figure 25C) of infarctions. The figures are representative of at least three experiments performed on different experimental days. TBI determined a range of impairments in locomotor tasks, as showed by the rotarod test (Figure 25D) and the EBST (Figure 25E), after seven days. Both groups of animals that received Rapamycin or KU0063794 were significantly less impaired in EBST and rotarod tests compared with the TBI group. KU0063794-treated mice displayed significant improvement in their behavioral performance as revealed by decreased biased swing activity in the EBST (Figure 25E) and increased time on the rotating rod in the rotarod test (Figure 25D). Each data are expressed as mean $\pm$ SEM from N = four male CD mice for each group. A P value of less than 0.05 was considered significant. ***P <0.001 versus sham, ##P <0.01 versus TBI, ###P <0.001 versus TBI.

6.2.2 Effect of KU0063794 treatment on histological parameters

A histological examination of brain sections at the level of the perilesional area, stained 24 hours after injury, revealed significant damage in the TBI group, such as prominent and thickened blood vessels, ischemic changes and gliosis in the brain parenchyma (Figure 26B, see densitometry analysis 26E) compared to sham mice (Figure 26A, see densitometry analysis 26E). Rapamycin (1mg/kg) treatment attenuated the development of inflammation at 24 hours after TBI; moreover, a strong and important protection on the severity of trauma was observed in the tissue collected from KU0063794-treated mice (8mg/kg) (Figure 26C and 26D respectively, see densitometry analysis 26E).
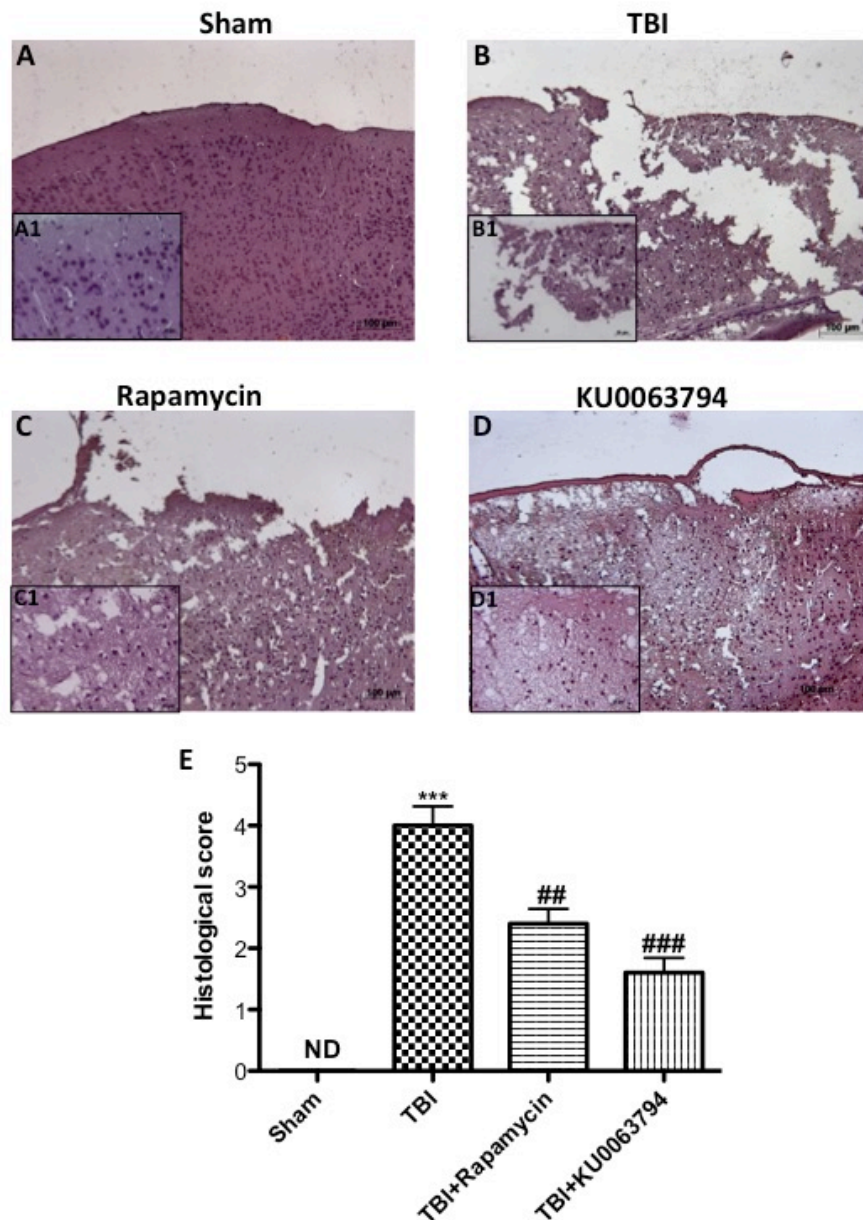
Figure 26- Effect of KU0063794 treatment on histological parameters

Brain sections from TBI mice (Figure 26B, see densitometry analysis E) demonstrated brain tissue injury and inflammatory cell infiltration. Rapamycin treatment did not attenuate completely the development of acute brain injury at one and six hours after TBI (Figure 26C, see densitometry analysis 26E). On the contrary, KU0063794 treatment reduced the degree of brain injury and the inflammatory cells infiltration (Figure 26D, see densitometry analysis 26E). Figure is representative of at least three experiments performed on different experimental days. ***P <0.001 versus sham, ##P <0.01 versus TBI, ###P <0.001 versus TBI.

6.2.3 Effect of KU0063794 on IκBα degradation, NFκBp65 translocation, iNOS and COX-2 expressions

To investigate whether the cellular mechanism through KU0063794 could attenuate inflammatory processes we assessed by Western blot analysis of the ipsilateral hemisphere after TBI, using an IκBα and an NFκB-specific antibodies. The results showed a basal expression of IκBα in the brain from sham-mice (Figure 27A see densitometric analysis A1), while IκBα expression was significantly reduced in mice subject to TBI as showed in Figure 27A (see densitometric analysis A1). Rapamycin treatment blunted the degradation of IκBα but KU0063794 was more able to restore significantly IκBα degradation (Figure 27A, see densitometry analysis A1). Moreover, p65 subunit translocation was increased after TBI in the nuclear brain homogenates, compared with sham-group. Indeed, treatment with the KU0063794 (Figure 27B, see histological score B1) reduced p65 subunit translocation more effectively than the treatment with Rapamicyn (Figure 27B, see densitometrc analysis B1).

Additionaly, to determine the role of •NO produced during TBI, iNOS expression was evaluated by Western blot analysis. A significant increase in iNOS expression was observed in the contused area from mice subjected to TBI (Figure 27C see densitometry analysis C1). Consequently, Rapamycin (1mg/kg) reduced TBI-induced iNOS expression (Figure 27C see densitometry analysis C1); on the other hand, a more significant decrease in iNOS expression was observed after KU0063794 treatment (Figure 27C see densitometry analysis C1). Similarly, COX-2 expression was induced by TBI compared to the sham group (Figure 27D see densitometry analysis D1). Both treatments with Rapamycin and KU0063794 lowered COX-2 expression (Figure 27D see densitometry analysis D1).
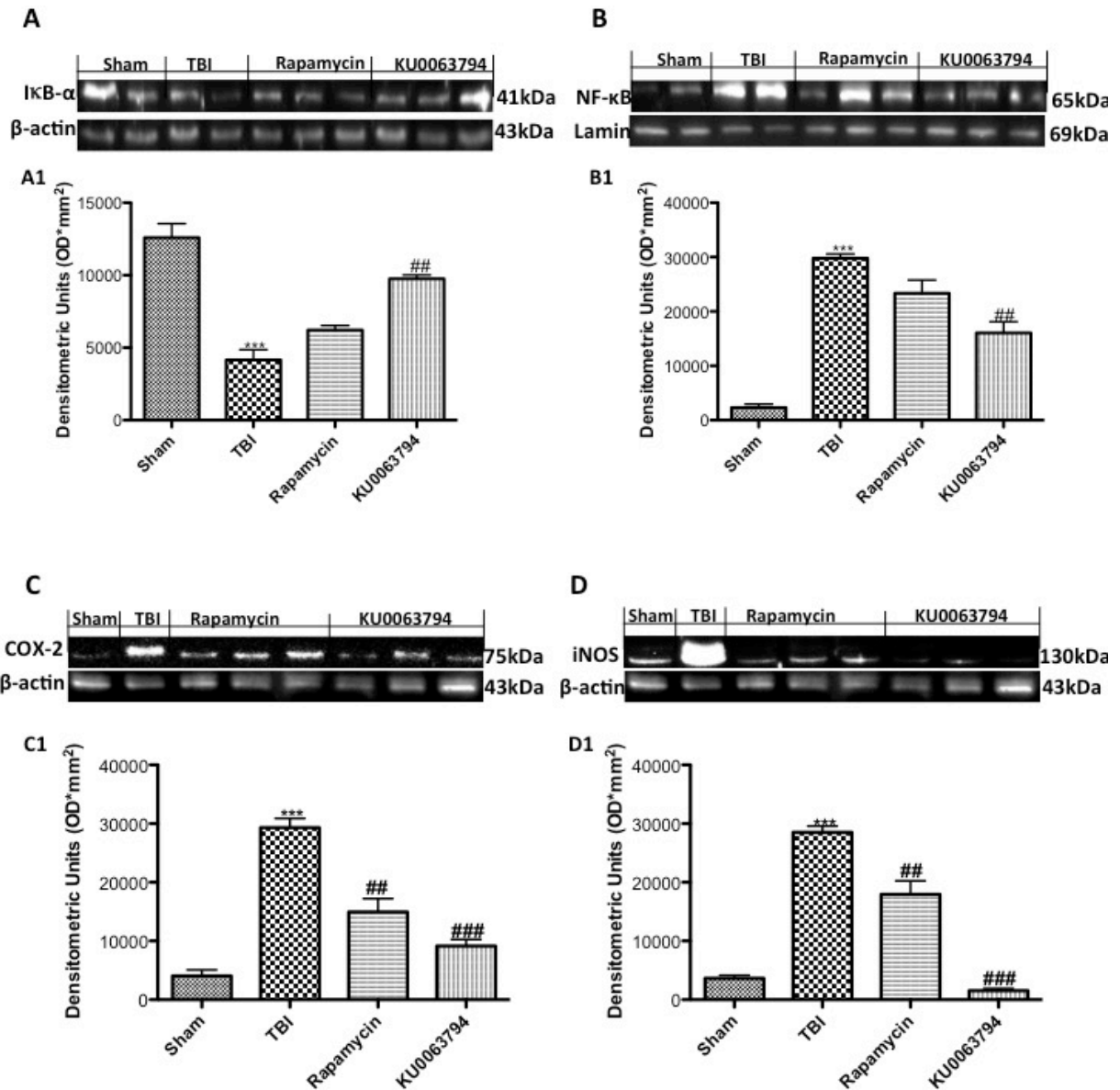
Figure 27 - Effect of KU0063794 on IκBα degradation, NFκBp65 translocation, iNOS and COX-2 expressions

Degradation of IκBα was significantly blocked by Rapamycin (1mg/kg) and KU0063794 (8mg/kg) treatment (Figure 27A). Moreover, KU0063794 (8mg/kg) treatment resulted in an inhibition of nuclear translocation of p65 (Figure 27B). Translocation of NFκB is a critical step in the coupling of extracellular stimuli to the transcriptional activation of specific target genes. A significant increase in inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 (Figure 27C and Figure 27D, respectively) was observed in the injured area from TBI mice compared with the Sham mice. KU0063794-treated mice had notably reduced expression of pro-inflammatory enzymes (Figure 27C and Figure 27D, respectively). Data show one representative blot from three independent experiments with similar results. Mean ± SEM of four to five animals per group. One-way ANOVA, followed by Bonferroni's multiple comparison test. ***$P<0.001$ vs sham, ##$P<0.01$ vs TBI, ###$P<0.001$ vs TBI.

6.2.4 Effect of KU0063794 on TBI-induced apoptotis

The dialogue between autophagy and cell death pathways influences the normal clearance of dying cells. Unfortunately, the exact connection was still poorly understood. To test whether TBI was associated with autophagy and apoptosis we determined Bax and Bcl-2 expression by immunohistochemical staining. Immunohistochemistry for Bax and Bcl-2 showed that brain sections from sham-operated mice did not stain for Bax (Figure 28A), whereas TBI-operated mice exhibited positive staining for Bax (Figure 28B). Rapamycin treatment significantly reduced the degree of positive staining (Figure 28C). However, in KU0063794-treated mice group the decrease of positive staining is more effectively than the treatment with Rapamicyn (Figure 28D, see densitometry analysis I). Viceversa, brain sections from sham-operated mice demonstrated Bcl-2 positive staining (Figure 28E), whereas in TBI mice, the staining was significantly reduced (Figure 28F). However, in KU0063794-treated mice group the increase of positive staining for Bcl-2 is more effectively than the treatment with Rapamicyn (Figure 28G, see densitometry analysis I).
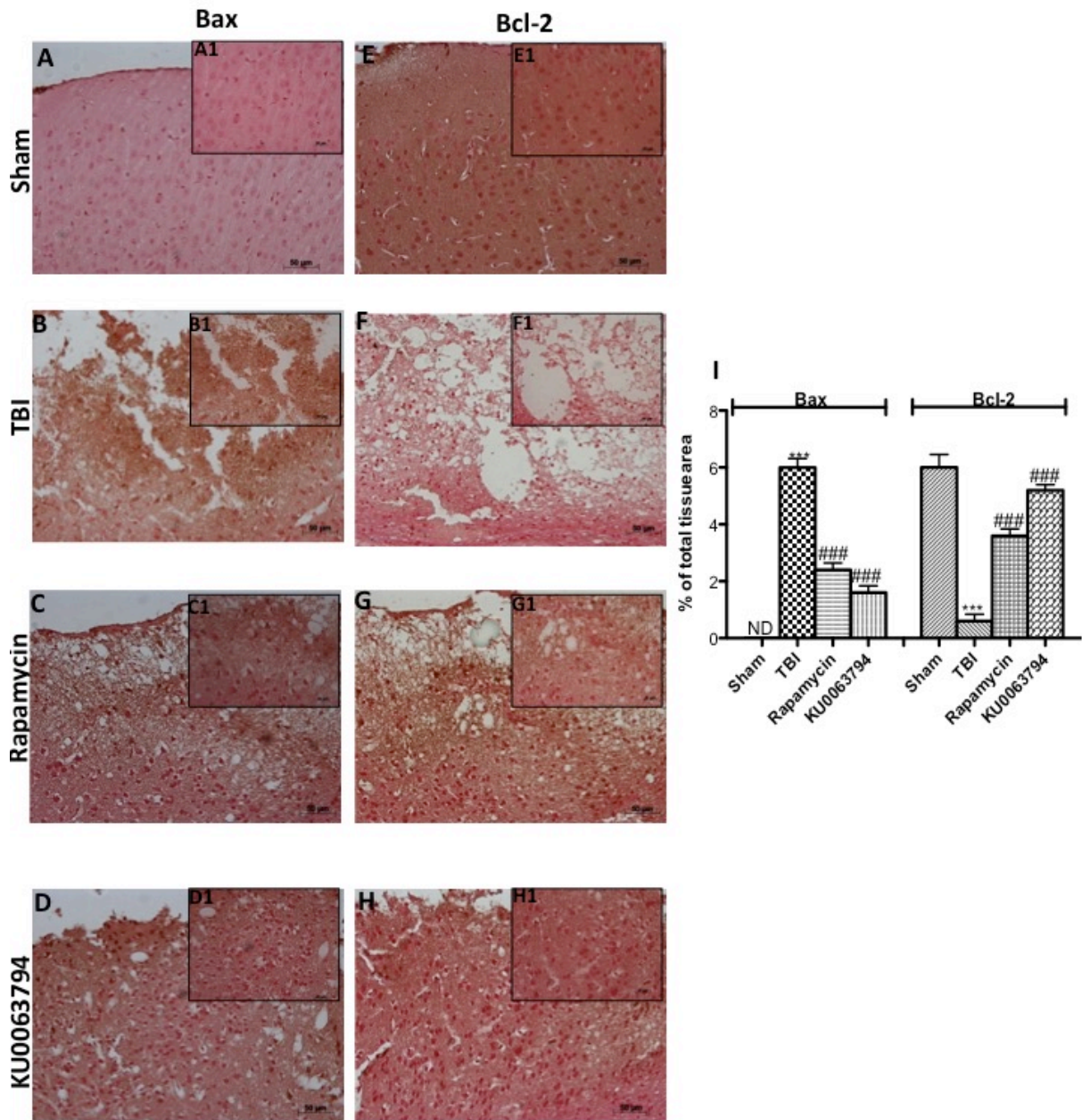
Figure 28 - Effect of KU0063794 on TBI-induced apoptotis

Immunohistochemistry for Bax and Bcl-2 showed that brain sections from sham-operated mice did not stain for Bax (Figure 28A), whereas TBI-operated mice exhibited positive staining for Bax (Figure 28B). Rapamycin treatment significantly reduced the degree of positive staining (Figure 28C). However, in KU0063794-treated mice group the decrease of positive staining is more effectively than the treatment with Rapamycin (Figure 28D). Viceversa, brain sections from sham-operated mice demonstrated Bcl-2 positive staining (Figure 28E), whereas in TBI mice, the staining was significantly reduced (Figure 28F). Treatment with Rapamycin significantly reduced the degree of positive staining (Figure 28G). However, in KU0063794-treated mice group the increase of positive staining for Bcl-2 is more effectively than the treatment with Rapamicyn (Figure 28H). The figures are representative of at least three experiments performed on different experimental days. Figures are representative of all the animals in each group. Values are given as mean ± SEM of 5 animals for each group. ***$P < 0.001$ vs Sham group; ###$P < 0.001$ vs TBI group;

6.2.5 Effect of KU0063794 on TBI-induced activation of astrocytes and microglia

Activation of astrocytes and microglia have a key role in the pathogenesis of TBI. Accordingly, we used an immunohistochemical approach to assess the presence of GFAP and Iba-1 in brain sections collected 24h after TBI. Immunohistochemistry for GFAP and Iba-1 showed that brain sections from sham-operated mice did not stain for GFAP and IBA (Figure 29 A and 29 E), whereas TBI-operated mice exhibited positive staining for GFAP and IBA (Figure 29 B and 29 F). Rapamycin treatment significantly reduced the degree of both positive staining (Figure 29 C and 29 G). However, in KU0063794-treated mice group the decrease of positive staining for GFAP and IBA is more effectively than the treatment with Rapamicyn (Figure 29 D and 29 H, see densitometry analysis 29 I).

Figure 29 - Effect of KU0063794 on TBI-induced activation of astrocytes and microglia

Immunohistochemistry for GFAP and Iba-1 showed that brain sections from sham-operated mice did not stain for GFAP (Figure 29A) and Iba-1 (Figure 29E), whereas TBI-operated mice exhibited positive staining for GFAP (Figure 29B) and Iba-1 (Figure 29F). Rapamycin treatment significantly reduced the degree of positive staining for GFAP (Figure 29C) and Iba-1 (Figure 29D). However, in KU0063794-treated mice group the decrease of positive staining is more effectively than the treatment with Rapamycin (Figure 29D and 29H, see densitometry analysis 29I). The figures are representative of at least three experiments performed on different experimental days. Figures are representative of all the animals in each group. Values are given as mean ± SEM of 5 animals for each group. ***P< 0.001 vs Sham group; ###P< 0.001 vs TBI group;

6.2.6 Effect of KU0063794 on TBI-induced pro-inflammatory cytokines production

Microglia and astrocytes activation release a large number of pro-inflammatory cytokines. To evaluate the effect of KU0063794 on pro-inflammatory cytokine production we investigated IL-1β and TNF-α production, both inflammation sign, by immunohistochemical approach. Immunohistochemistry showed that brain sections from sham-operated mice did not stain for IL-1β and TNF-α (Figure 30 A and 30 E), whereas 24h following TBI, mice exhibited positive staining for IL-1β and TNF-α (Figure 30 B and 30 F). Rapamycin treatment (1 mg/kg) significantly reduced the degree of both positive staining (Figure 30 C and 30 G). However, in mice treated with KU0063794 (8 mg/kg) the decrease of positive staining for IL-1β and TNF-α is more effectively than the treatment with Rapamicyn (Figure 30 D and 30 H, see densitometry analysis 30 I).
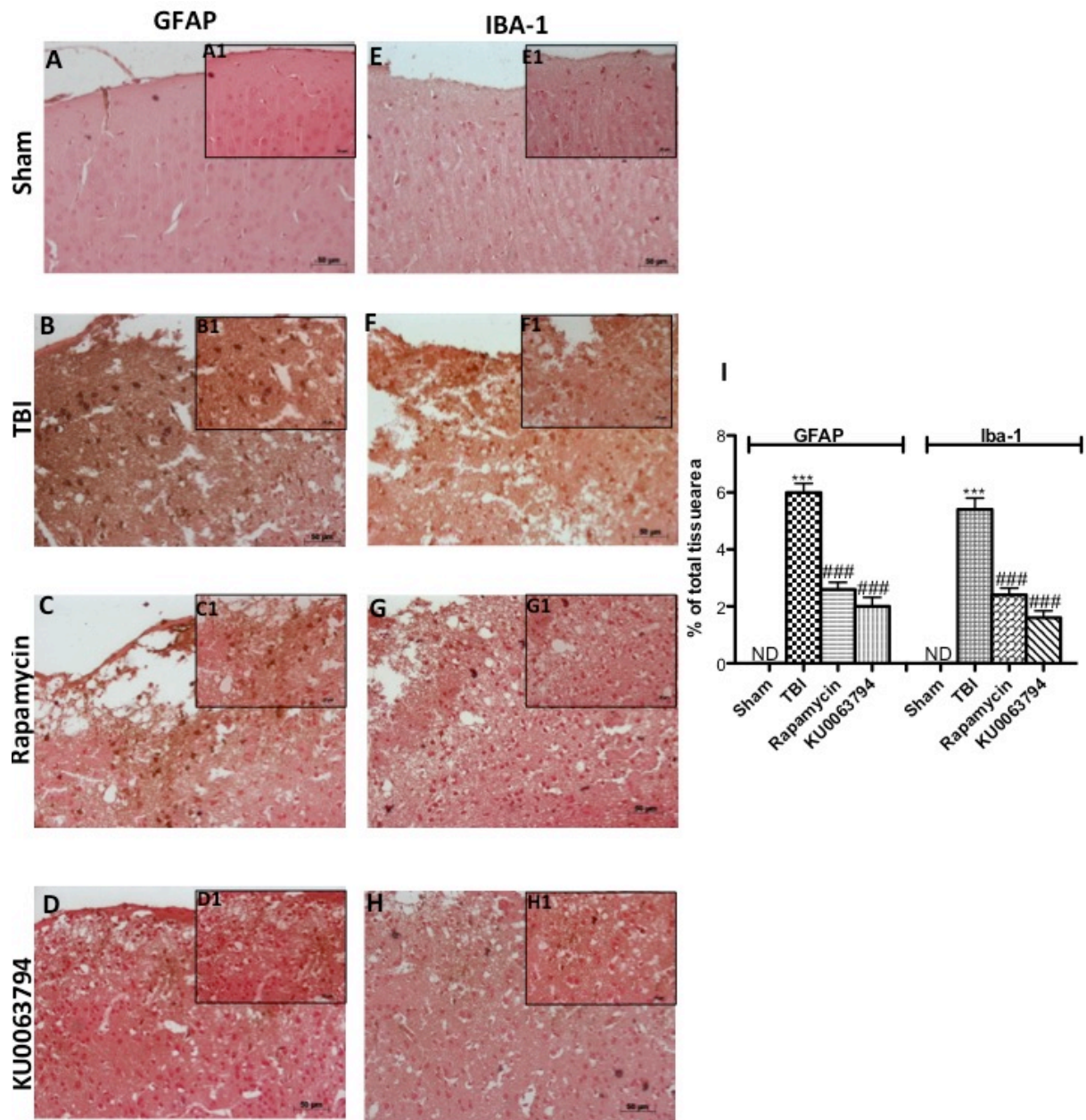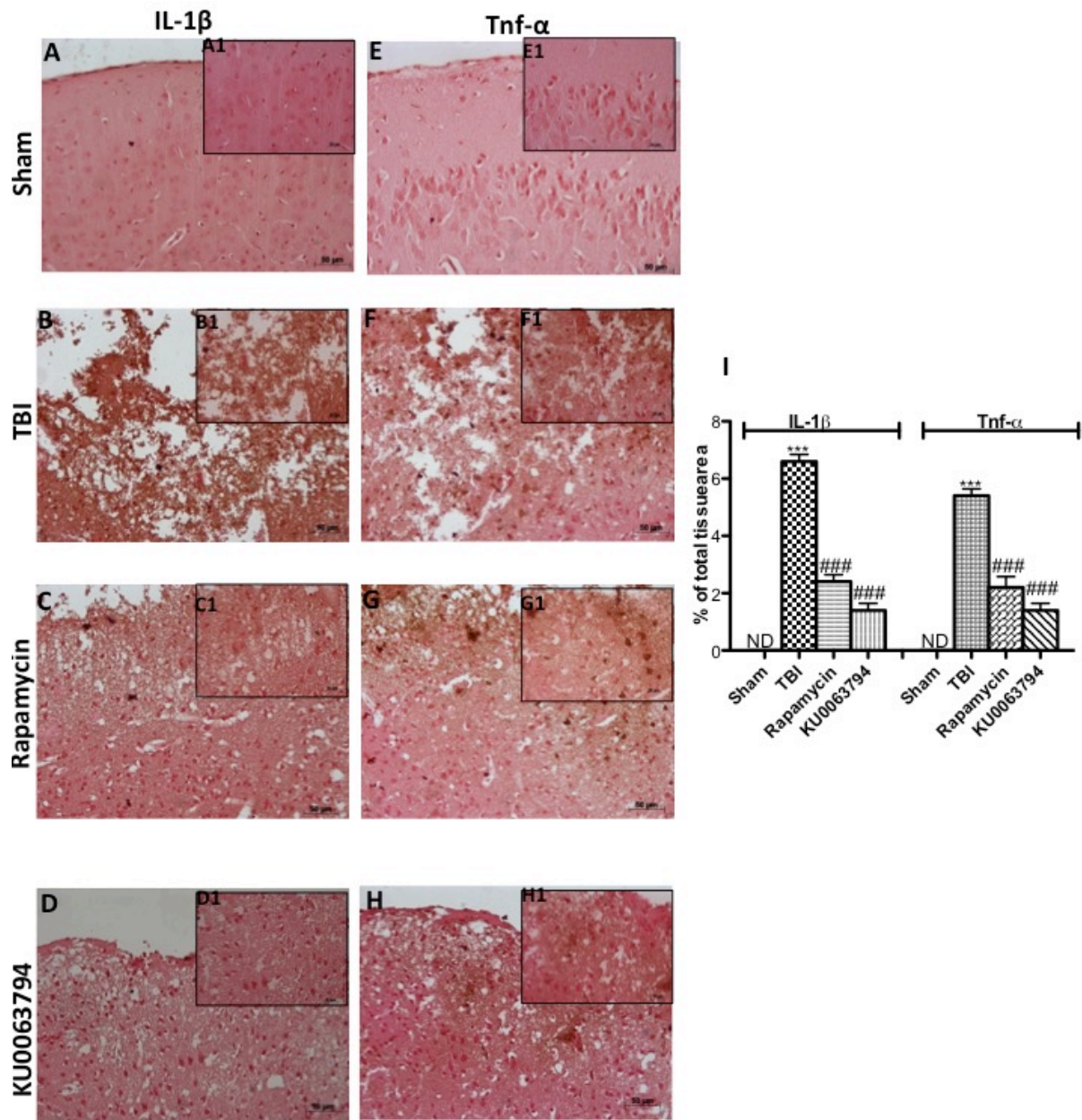
Figure 30 - Effect of KU0063794 on TBI-induced pro-inflammatory cytokines production

Immunohistochemistry for IL-1β and TNF-α showed that brain sections from sham-operated mice did not stain for IL-1β (Figure 30A) and TNF-α (Figure 30E), whereas TBI-operated mice exhibited positive staining for IL-1β (Figure 30B) and TNF-α (Figure 30F). Rapamycin treatment significantly reduced the degree of positive staining for IL-1β (Figure 30C) and TNF-α (Figure 30D). However, in KU0063794-treated mice group the decrease of positive staining is more effectively than the treatment with Rapamycin (Figure 30 D and 30 H, see densitometry analysis 30I). The figures are representative of at least three experiments performed on different experimental days. Figures are representative of all the animals in each group. Values are given as mean ± SEM of 5 animals for each group. ***P< 0.001 vs Sham group; ###P< 0.001 vs TBI group;

6.2.7 Effect of KU0063794 on neurotrophic factor release following TBI

Recent evidence have been demonstrated that neuroprotective effect of brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) was mediated by autophagy (Chen *et al.*, 2013a). For this reason to test whether KU0063794 modulates the inflammatory process through regulation of the neurotrophic factors levels, we have examined BDNF and NT-3 by immunofluorescence. Immunofluorescence showed that BDNF and NT-3 expression decreased at 24 h after TBI (Figure 31 B and 31 F) compared with sham-operated mice (Figure A and E). Rapamycin treatment (1 mg/kg) significantly increased the degree of both positive staining (Figure 31 C and 31 G). However, in mice treated with KU0063794 (8 mg/kg) the increase of positive staining for BDNF and NT-3 is more effectively than the treatment with Rapamicyn (Figure 31 D and 31 H, see densitometry analysis 31 I and 31 J).
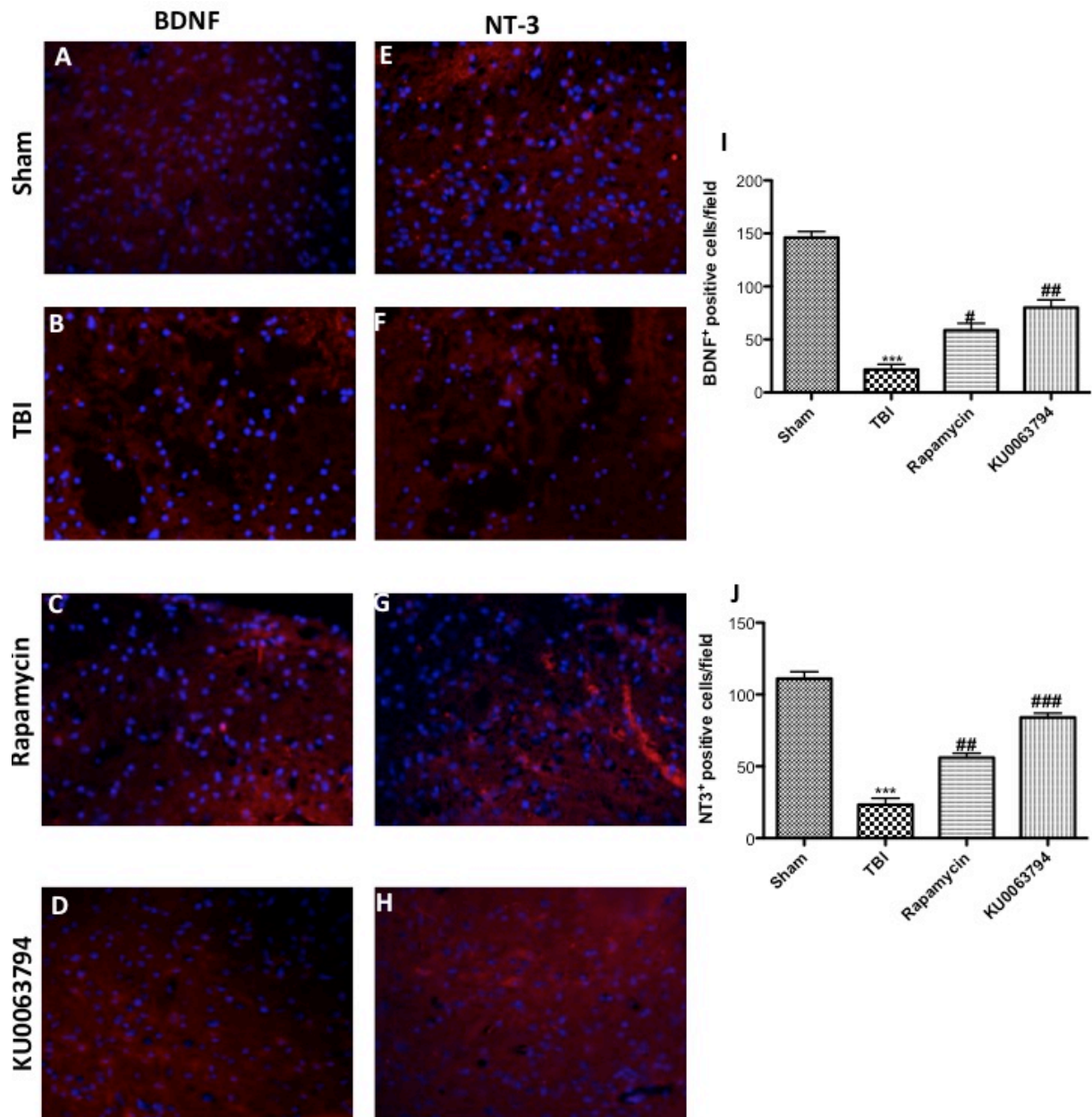
Figure 31 - Effect of KU0063794 on neurotrophic factor release following TBI

Immunofluorescence for BDNF and NT-3 showed BDNF and NT-3 expression decreased at 24 h after TBI (Figure 31 B and 31 F) compared with sham-operated mice (Figure 31 A and 31 E). Rapamycin treatment (1 mg/kg) significantly increased the degree of both positive staining (Figure 31 C and 31 G). However, in mice treated with KU0063794 (8 mg/kg) the increase of positive staining for BDNF and NT-3 is more effectively than the treatment with Rapamicyn (Figure 31 D and 31 H, see densitometry analysis 31 I and 31 J). The figures are representative of at least three experiments performed on different experimental days. Figures are representative of all the animals in each group. Values are given as mean ± SEM of 5 animals for each group. ***P< 0.001 vs Sham group; #P< 0.05 vs TBI group; ##P< 0.01 vs TBI group; ###P< 0.001 vs TBI group;

6.2.8 Effect of KU0063794 on TBI-induced neuronal loss

Recent evidence demonstrates that both acute and delayed neuronal injury occurs after traumatic brain injury (Sato *et al.*, 2001). Immunofluorescence showed that NeuN expression significantly decreased at 24 h after TBI (Figure 32 B) compared with sham-operated mice (Figure 32 A). Rapamycin treatment (1 mg/kg) significantly increased the degree of NeuN positive staining (Figure 32 C). However, in mice treated with KU0063794 (8 mg/kg) the increase of positive staining NeuN is more effectively than the treatment with Rapamicyn (Figure 32 D see densitometry analysis 32 E).
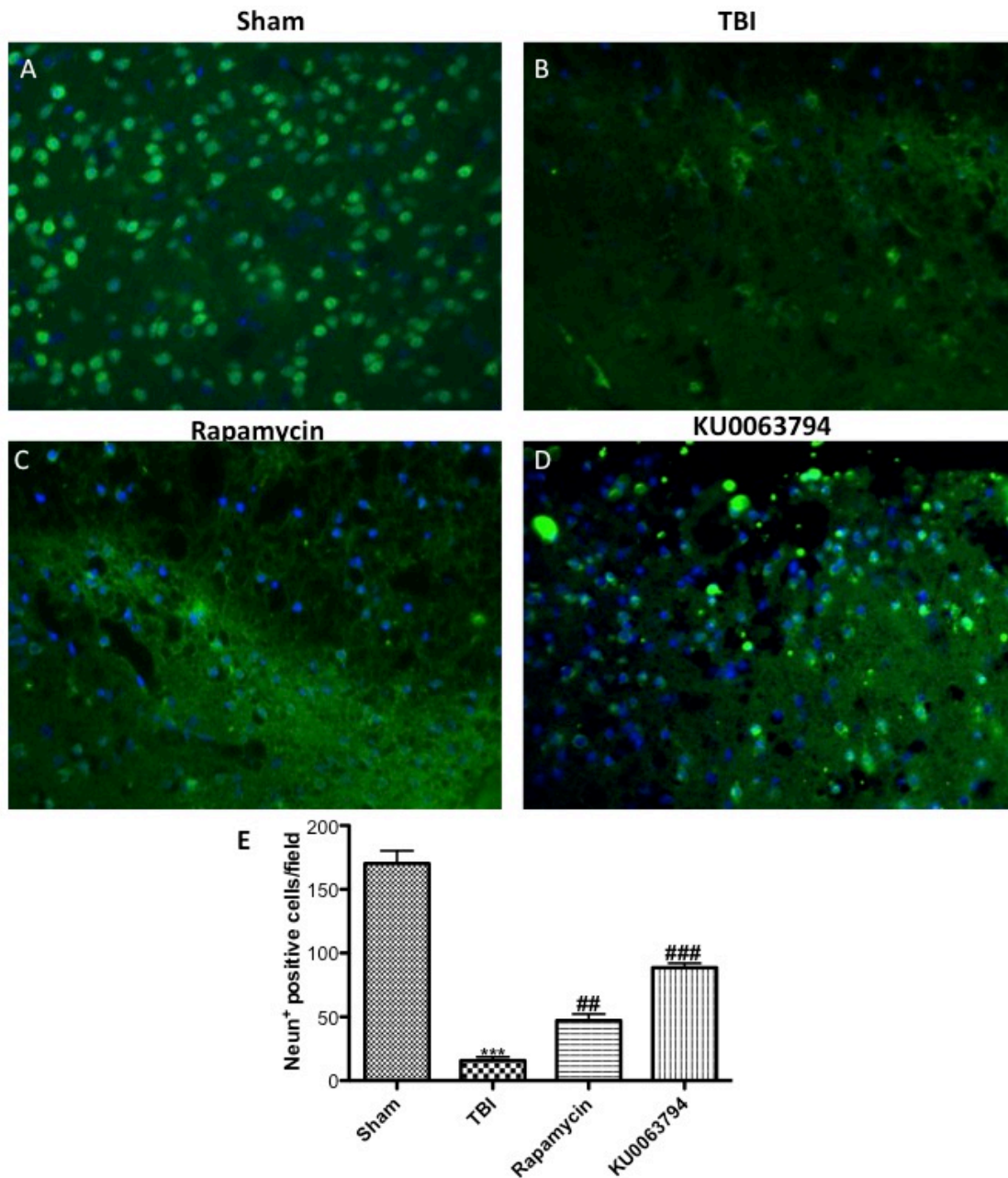
Figure 32 - Effect of KU0063794 on TBI-induced neuronal loss

Immunofluorescence for NeuN showed a significantly decreased at 24 h after TBI (Figure 32 B) compared with sham-operated mice (Figure 32 A). Rapamycin treatment (1 mg/kg) significantly increased the degree of NeuN positive staining (Figure 32 C). However, in mice treated with KU0063794 (8 mg/kg) the increase of positive staining for NeuN is more effectively than the treatment with Rapamicyn (Figure 32 D see densitometry analysis 32 E). The figures are representative of at least three experiments performed on different experimental days. Figures are representative of all the animals in each group. Values are given as mean ± SEM of 5 animals for each group. ***P< 0.001 vs Sham group; ##P< 0.01 vs TBI group; ###P< 0.001 vs TBI group;

CHAPTER 7: DISCUSSION AND CONCLUSION

TBI and SCI are physical injuries most often caused by sports, motor accidents and combat (Nolan and Burton, 1998; Silva *et al.*, 2014). In both cases, the primary insult leads to unavoidable damage in the epicenter immediately after the event, while the secondary damage progresses for days and encompasses the areas surrounding the primary injury (Dirnagl *et al.*, 1999; Masel and DeWitt, 2010). Within minutes of an acute insult, secondary injury starts with energy failure that results in shutdown of Na^+/K^+-ATPase leading to ionic balance that promotes edema. Excitotoxicity (increased release and decreased reuptake of glutamate leading to overactivation of ionotropic glutamate receptors) also starts within minutes of an insult to CNS. Glutamate receptor activation allows lethal amounts of Ca^{2+} to enter the cell that activate proteases, nucleases and enzymes that form free radicals which are toxic to neurons (Obrenovitch and Urenjak, 1997; Hazell, 2007). Following these events, the second line of pathophysiological events that include inflammation, oxidative/nitrosative stress and endoplasmic reticulum (ER) stress starts within hours. Activation of microglia releases pro-inflammatory cytokines that attracts blood-borne macrophages and neutrophils to extravasate into CNS. All these cells release pro-inflammatory cytokines and ROS and RNS, which are highly toxic to neurons (Manzanero *et al.*, 2013). In addition, activation of NADPH oxidase forms ROS and curtails functioning of anti-oxidant enzymes like SOD and catalase promotes oxidative stress. Energy failure also leads to ineffective folding of proteins in ER lumen leading to accumulation of unfolded/misfolded proteins that activates ER stress pathways. Inflammation, oxidative/nitrosative stress and ER stress are simultaneous and potentiate each other .

Autophagy, a highly complex process that cells induced in response to a wide range of stressful conditions in order to maintain cellular homeostasis was also been connected in

various neuronal damage models. However, the role of autophagy in SCI is still controversial and its interrelationship with inflammation and apoptosis remains unclear (Kroemer *et al.*, 2010).

The connection between autophagy and inflammation are complex. Each regulates the other by different mechanism such as toll-like receptors (TLRs) and NOD-like receptor (NLRs) can elicit autophagy for pathogen clearance (Saitoh and Akira, 2010). Moreover, recently studies, have hypotized that autophagy acts by at least two means to protect cells from excessive long lasting inflammation: indirectly by allowing efficient clearance of damaged organelles and directly by suppressing proinflammatory complexes (Lapaquette *et al.*, 2015).

Previous study conducted by Chen et colleagues have been demonstrated that treatment with rapamycin enhanced autophagy, has anti-inflammatory and neuroprotective effect and improved motor function suggesting that it can be applied during the acute phase after SCI (Chen *et al.*, 2013b). Based on these observations we performed studies in the attempt to determine if the new second generation mTOR inhibitor targeting mTORC1 and mTORC2, KU0063794, could be more efficient as a novel anti-inflammatory treatment respect first generation mTOR inhibitor targeting only mTORC1, Rapamicyn and Temsirolimus.

For that purpose, in this thesis, we used an experimental mouse model of SCI and TBI.

SCI was induced by extradural compression of the spinal cord (T6-T7) using an aneurysm clip with a closing force of 24 g via a four-level T5-T8 laminectomy and using spinal cord organotypic slice culture. Also TBI was induced in mice by a controlled cortical impactor that reproduces changes reported in clinical head injuries such as cortical contusion, brain edema, subarachnoid hemorrhage, elevated intracerebral pressure, reduced cortical perfusion, decreased cerebral blood flow and neuro-endocrine and metabolic changes (Morales *et al.*, 2005).

In the first step, using H&E staining, we analyzed the severity of both traumas at the level of the perilesional area. Our results clearly established important damage in the spinal cord and brain tissue collected from SCI or TBI animals compared with sham-operated mice. Protection against tissue damage, edema formation and infarct area was observed in the group of mice treated with Temsirolimus or Rapamicyn. Indeed, treatment with KU0063794 reduced histological alterations more effectively than the treatment with both first generations of mTOR inhibitors.

Moreover, neurological deficit and motor function was evaluated after SCI and TBI. In mice subjected to SCI or TBI and than treated with KU0063794, we detected a significantly increased neurological and motor recovery than Temsirolimus-treated or Rapamicyn-treated mice group.

It has been demonstrated that on a molecular level, autophagy and NF-κB pathways share common upstream signals and regulators and can control each other through positive or negative feedback loops, thus ensuring homeostatic responses (Trocoli and Djavaheri-Mergny, 2011). In our studies, we found that IκBα expression was significantly reduced in mice subject to TBI but Ku0063794 (8mg/kg) treatment blunted the degradation of IκBα and conseguent p65 nuclear traslocation better than Rapamycin (1mg/kg) treatment.

Moreover, previous studies indicated an improved iNOS and peroxynitrite in both injured spinal cord and brain tissue. In fact, NO production can contribute to cell death, tissue damage and degeneration observed in SCI and TBI. Moreover, high PGE2 concentrations reflecting an increased activity of Cox-2 in the damaged spinal cord (Bal-Price and Brown, 2001; Xu *et al.*, 2001). Thus, in this study we analyzed the expression of iNOS and COX-2 and nNOS. Our results demonstrated that Rapamycin and Temsirolimus treatment significantly reduced the degree of Cox-2 and iNOS, but in KU0063794-treated mice group, the decrease of both

pro-inflammatory enzymes expression was more effectively. In addition, we assessed that the expression of nNOS was significantly restored after treatment with KU0063794.

After SCI and TBI, reactive astrocytes contributes to the inhibitory environment within the injured spinal cord and brain, denoted by increased immunoreactivity of GFAP and Iba-1 as well as gene expression of pro-inflammatory cytokines and their receptors, including IL-1β, TNF-α is significantly increased (Hausmann, 2003; Esposito and Cuzzocrea, 2011). Our results demonstrate that activation of astrocytes and microglia was significantly attenuated in KU0063794-treated mice compared to Temsirolimus-treated or Rapamicyn–treated mice group as well as KU0063794 treatment significantly diminished the post-SCI expression of IL-1β and TNF-α.

Proinflammatory cytokines stimulate other biochemical signals leading to the degeneration of myelin and apoptosis of neurons that cause the neurological deficit (Nesic *et al.*, 2001; Dong *et al.*, 2003). We report in this thesis that in KU0063794-treated mice group the decrease of positive staining for Bax was more effectively than the treatment with Temsirolimus or Rapamicyn, conversely, Bcl-2 was expressed much more in mice treated with KU0063794. Additionally, FasL signaling plays a central role in SCI (Ackery *et al.*, 2006). We found that KU0063794 treatment lead to a substantial reduction of FasL activation.

More recent studies have suggested a significant role for neurotrophic factors like BDNF, and NT-3 in regeneration after CNS injury. (Nakamura and Bregman, 2001) In the current work we have demonstrated that treatment with Rapamycin (1mg/kg) significantly restore neurotrophic factors levels after TBI but KU0063794 (8mg/kg) is more effective than Rapamycin. Moreover, we found a significantly decrease of neuronal loss following KU0063794 treatment.

At least, to better understand the role of KU0063794, we used an *ex vivo* model of organotypic spinal cord slices cultures. This model offers the advantage of improved knowledge about the mechanism of action of a dual mTORC1 and mTORC2 inhibitor.

To validate our data in this study, using spinal cord organotypic slice cultures, we showed a significant decrease in cell death and in the injury-induced NO_2^- production following KU0063794 pretreatment.

The necessity for emerging new therapeutics for SCI and TBI treatment and the current scarcity of specific therapy for this indication underscore the importance of connection and characterization of novel neuroprotective compounds. Despite the increasing knowledge about the role of autophagy in modulation of inflammation, this field is still only in its beginnings.

Taken together, our data demonstrate that modulation of autophagy may represent a promising therapeutic approach for a wide range of inflammatory conditions such as SCI and TBI. However, considering the multiple roles of autophagy, further investigations are needed to examine in which situations stimulated autophagy is beneficial and does not generate detrimental side effects.

ACKNOWLEDGEMENTS

Reference

Abe, K., Matsuki, N., 2000. Measurement of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction activity and lactate dehydrogenase release using MTT. Neuroscience research. 38, 325-329

Ackery, A., Robins, S., Fehlings, M.G., 2006. Inhibition of Fas-mediated apoptosis through administration of soluble Fas receptor improves functional outcome and reduces posttraumatic axonal degeneration after acute spinal cord injury. Journal of neurotrauma. 23, 604-616,10.1089/neu.2006.23.604.

Acton, P.A., Farley, T., Freni, L.W., Ilegbodu, V.A., Sniezek, J.E., Wohlleb, J.C., 1993. Traumatic spinal cord injury in Arkansas, 1980 to 1989. Archives of physical medicine and rehabilitation. 74, 1035-1040

Adams, J.M., Cory, S., 2007. Bcl-2-regulated apoptosis: mechanism and therapeutic potential. Curr Opin Immunol. 19, 488-496,10.1016/j.coi.2007.05.004.

Agarraberes, F.A., Dice, J.F., 2001. A molecular chaperone complex at the lysosomal membrane is required for protein translocation. Journal of cell science. 114, 2491-2499

Anderson, K.D., 2004. Targeting recovery: priorities of the spinal cord-injured population. Journal of neurotrauma. 21, 1371-1383,10.1089/neu.2004.21.1371.

Ankeny, D.P., Lucin, K.M., Sanders, V.M., McGaughy, V.M., Popovich, P.G., 2006. Spinal cord injury triggers systemic autoimmunity: evidence for chronic B lymphocyte activation and lupus-like autoantibody synthesis. Journal of neurochemistry. 99, 1073-1087,10.1111/j.1471-4159.2006.04147.x.

Azari, M.F., Profyris, C., Zang, D.W., Petratos, S., Cheema, S.S., 2005. Induction of endogenous neural precursors in mouse models of spinal cord injury and disease. European journal of neurology. 12, 638-648,Doi 10.1111/J.1468-1331.2005.01066.X.

Babcock, A.A., Kuziel, W.A., Rivest, S., Owens, T., 2003. Chemokine expression by glial cells directs leukocytes to sites of axonal injury in the CNS. The Journal of neuroscience : the official journal of the Society for Neuroscience. 23, 7922-7930

Bains, M., Hall, E.D., 2012a. Antioxidant therapies in traumatic brain and spinal cord injury. Bba-Mol Basis Dis. 1822, 675-684,10.1016/j.bbadis.2011.10.017.

Bains, M., Hall, E.D., 2012b. Antioxidant therapies in traumatic brain and spinal cord injury. Biochimica et biophysica acta. 1822, 675-684,10.1016/j.bbadis.2011.10.017.

Bal-Price, A., Brown, G.C., 2001. Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. The Journal of neuroscience : the official journal of the Society for Neuroscience. 21, 6480-6491

Bales, J.W., Wagner, A.K., Kline, A.E., Dixon, C.E., 2009. Persistent cognitive dysfunction after traumatic brain injury: A dopamine hypothesis. Neuroscience and biobehavioral reviews. 33, 981-1003,10.1016/j.neubiorev.2009.03.011.

Ballesteros, J., Guemes, I., Ibarra, N., Quemada, J.I., 2008. The effectiveness of donepezil for cognitive rehabilitation after traumatic brain injury: a systematic review. The Journal of head trauma rehabilitation. 23, 171-180,10.1097/01.HTR.0000319935.99837.96.

Ballinger, S.W., Patterson, C., Yan, C.N., Doan, R., Burow, D.L., Young, C.G., Yakes, F.M., Van Houten, B., Ballinger, C.A., Freeman, B.A., Runge, M.S., 2000. Hydrogen peroxide- and peroxynitrite-induced mitochondrial DNA damage and dysfunction in vascular endothelial and smooth muscle cells. Circ Res. 86, 960-966

Bao, F., Chen, Y., Dekaban, G.A., Weaver, L.C., 2004. Early anti-inflammatory treatment reduces lipid peroxidation and protein nitration after spinal cord injury in rats. Journal of neurochemistry. 88, 1335-1344

Bareyre, F.M., Kerschensteiner, M., Raineteau, O., Mettenleiter, T.C., Weinmann, O., Schwab, M.E., 2004. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. Nature neuroscience. 7, 269-277,10.1038/nn1195.

Bassik, M.C., Scorrano, L., Oakes, S.A., Pozzan, T., Korsmeyer, S.J., 2004. Phosphorylation of BCL-2 regulates ER Ca2+ homeostasis and apoptosis. Embo Journal. 23, 1207-1216,10.1038/sj.emboj.7600104.

Basso, D.M., Fisher, L.C., Anderson, A.J., Jakeman, L.B., McTigue, D.M., Popovich, P.G., 2006. Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. Journal of neurotrauma. 23, 635-659,10.1089/neu.2006.23.635.

Beattie, M.S., Bresnahan, J.C., Komon, J., Tovar, C.A., Van Meter, M., Anderson, D.K., Faden, A.I., Hsu, C.Y., Noble, L.J., Salzman, S., Young, W., 1997. Endogenous repair after spinal cord contusion injuries in the rat. Experimental neurology. 148, 453-463,Doi 10.1006/Exnr.1997.6695.

Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A., Freeman, B.A., 1990. Apparent Hydroxyl Radical Production by Peroxynitrite - Implications for Endothelial Injury from Nitric-Oxide and Superoxide. Proceedings of the National Academy of Sciences of the United States of America. 87, 1620-1624,Doi 10.1073/Pnas.87.4.1620.

Bederson, J.B., Pitts, L.H., Germano, S.M., Nishimura, M.C., Davis, R.L., Bartkowski, H.M., 1986. Evaluation of 2, 3, 5-Triphenyltetrazolium Chloride as a Stain for Detection and Quantification of Experimental Cerebral Infarction in Rats. Stroke; a journal of cerebral circulation. 17, 1304-1308

Bensaad, K., Cheung, E.C., Vousden, K.H., 2009. Modulation of intracellular ROS levels by TIGAR controls autophagy. Embo Journal. 28, 3015-3026,10.1038/emboj.2009.242.

Bertin, S., Samson, M., Pons, C., Guigonis, J.M., Gavelli, A., Baque, P., Brossette, N., Pagnotta, S., Ricci, J.E., Pierrefite-Carle, V., 2008. Comparative Proteomics Study Reveals That Bacterial CpG Motifs Induce Tumor Cell Autophagy in Vitro and in Vivo. Mol Cell Proteomics. 7, 2311-2322,10.1074/mcp.M800100-MCP200.

Bethea, J.R., 2000. Spinal cord injury-induced inflammation: a dual-edged sword. Progress in brain research. 128, 33-42,10.1016/S0079-6123(00)28005-9.

Bethea, J.R., Castro, M., Keane, R.W., Lee, T.T., Dietrich, W.D., Yezierski, R.P., 1998. Traumatic spinal cord injury induces nuclear factor-kappaB activation. The Journal of neuroscience : the official journal of the Society for Neuroscience. 18, 3251-3260

Betz, C., Hall, M.N., 2013. Where is mTOR and what is it doing there? The Journal of cell biology. 203, 563-574,10.1083/jcb.201306041.

Bhalala, O.G., Srikanth, M., Kessler, J.A., 2013. The emerging roles of microRNAs in CNS injuries. Nat Rev Neurol. 9, 328-339,10.1038/nrneurol.2013.67.

Bhaskar, P.T., Hay, N., 2007. The two TORCs and Akt. Dev Cell. 12, 487-502,10.1016/j.devcel.2007.03.020.

Bialik, S., Kimchi, A., 2006. The death-associated protein kinases: Structure, function, and beyond. Annu Rev Biochem. 75, 189-210,10.1146/annurev.biochem.75.103004.142615.

Bilgen, M., Dogan, B., Narayana, P.A., 2002. In vivo assessment of blood-spinal cord barrier permeability: serial dynamic contrast enhanced MRI of spinal cord injury. Magnetic resonance imaging. 20, 337-341

Blagosklonny, M.V., 2011. Rapamycin-induced glucose intolerance Hunger or starvation diabetes. Cell cycle. 10, 4217-4224,10.4161/cc.10.24.18595.

Brachmann, S., Fritsch, C., Maira, S.M., Garcia-Echeverria, C., 2009. PI3K and mTOR inhibitors - a new generation of targeted anticancer agents. Curr Opin Cell Biol. 21, 194-198,10.1016/j.ceb.2008.12.011.

Bracken, M.B., Collins, W.F., Freeman, D.F., Shepard, M.J., Wagner, F.W., Silten, R.M., Hellenbrand, K.G., Ransohoff, J., Hunt, W.E., Perot, P.L., Jr., et al., 1984. Efficacy of methylprednisolone in acute spinal cord injury. Jama. 251, 45-52

Bracken, M.B., Freeman, D.H., Jr., Hellenbrand, K., 1981. Incidence of acute traumatic hospitalized spinal cord injury in the United States, 1970-1977. American journal of epidemiology. 113, 615-622

Bracken, M.B., Shepard, M.J., Collins, W.F., Holford, T.R., Young, W., Baskin, D.S., Eisenberg, H.M., Flamm, E., Leo-Summers, L., Maroon, J., et al., 1990. A randomized, controlled trial of

methylprednisolone or naloxone in the treatment of acute spinal-cord injury. Results of the Second National Acute Spinal Cord Injury Study. The New England journal of medicine. 322, 1405-1411,10.1056/NEJM199005173222001.

Bracken, M.B., Shepard, M.J., Holford, T.R., Leo-Summers, L., Aldrich, E.F., Fazl, M., Fehlings, M., Herr, D.L., Hitchon, P.W., Marshall, L.F., Nockels, R.P., Pascale, V., Perot, P.L., Jr., Piepmeier, J., Sonntag, V.K., Wagner, F., Wilberger, J.E., Winn, H.R., Young, W., 1997. Administration of methylprednisolone for 24 or 48 hours or tirilazad mesylate for 48 hours in the treatment of acute spinal cord injury. Results of the Third National Acute Spinal Cord Injury Randomized Controlled Trial. National Acute Spinal Cord Injury Study. Jama. 277, 1597-1604

Bruce, J.H., Norenberg, M.D., Kraydieh, S., Puckett, W., Marcillo, A., Dietrich, D., 2000. Schwannosis: Role of gliosis and proteoglycan in human spinal cord injury. Journal of neurotrauma. 17, 781-788,Doi 10.1089/Neu.2000.17.781.

Brustovetsky, N., Dubinsky, J.M., 2000. Limitations of cyclosporin A inhibition of the permeability transition in CNS mitochondria. The Journal of neuroscience : the official journal of the Society for Neuroscience. 20, 8229-8237

Bunge, R.P., Puckett, W.R., Becerra, J.L., Marcillo, A., Quencer, R.M., 1993. Observations on the pathology of human spinal cord injury. A review and classification of 22 new cases with details from a case of chronic cord compression with extensive focal demyelination. Advances in neurology. 59, 75-89

Chang, N.C., Nguyen, M., Germain, M., Shore, G.C., 2010. Antagonism of Beclin 1-dependent autophagy by BCL-2 at the endoplasmic reticulum requires NAF-1. Embo Journal. 29, 606-618,10.1038/emboj.2009.369.

Chang, Y.Y., Juhasz, G., Goraksha-Hicks, P., Arsham, A.M., Mallin, D.R., Muller, L.K., Neufeld, T.P., 2009. Nutrient-dependent regulation of autophagy through the target of rapamycin pathway. Biochemical Society transactions. 37, 232-236,10.1042/BST0370232.

Chauhan, N.B., Gatto, R., 2010. Synergistic benefits of erythropoietin and simvastatin after traumatic brain injury. Brain research. 1360, 177-192,10.1016/j.brainres.2010.09.010.

Chen, A., Xiong, L.J., Tong, Y., Mao, M., 2013a. Neuroprotective effect of brain-derived neurotrophic factor mediated by autophagy through the PI3K/Akt/mTOR pathway. Mol Med Rep. 8, 1011-1016,10.3892/mmr.2013.1628.

Chen, G., Shi, J., Hu, Z., Hang, C., 2008. Inhibitory effect on cerebral inflammatory response following traumatic brain injury in rats: a potential neuroprotective mechanism of N-acetylcysteine. Mediators of inflammation. 2008, 716458,10.1155/2008/716458.

Chen, G., Zhang, S., Shi, J., Ai, J., Qi, M., Hang, C., 2009a. Simvastatin reduces secondary brain injury caused by cortical contusion in rats: possible involvement of TLR4/NF-kappaB pathway. Experimental neurology. 216, 398-406,10.1016/j.expneurol.2008.12.019.

Chen, H.C., Fong, T.H., Hsu, P.W., Chiu, W.T., 2013b. Multifaceted effects of rapamycin on functional recovery after spinal cord injury in rats through autophagy promotion, anti-inflammation, and neuroprotection. The Journal of surgical research. 179, e203-210,10.1016/j.jss.2012.02.023.

Chen, S.Y., Atkins, C.M., Liu, C.L.L., Alonso, O.F., Dietrich, W.D., Hu, B.R., 2007. Alterations in mammalian target of rapamycin signaling pathways after traumatic brain injury. J Cerebr Blood F Met. 27, 939-949,10.1038/sj.jcbfm.9600393.

Chen, Y., Azad, M.B., Gibson, S.B., 2009b. Superoxide is the major reactive oxygen species regulating autophagy. Cell death and differentiation. 16, 1040-1052,10.1038/cdd.2009.49.

Chen, Y., Shohami, E., Bass, R., Weinstock, M., 1998. Cerebro-protective effects of ENA713, a novel acetylcholinesterase inhibitor, in closed head injury in the rat. Brain research. 784, 18-24

Citron, B.A., Arnold, P.M., Sebastian, C., Qin, F., Malladi, S., Ameenuddin, S., Landis, M.E., Festoff, B.W., 2000. Rapid upregulation of caspase-3 in rat spinal cord after injury: mRNA, protein, and cellular localization correlates with apoptotic cell death. Experimental neurology. 166, 213-226,10.1006/exnr.2000.7523.

Coronado, V.G., McGuire, L.C., Sarmiento, K., Bell, J., Lionbarger, M.R., Jones, C.D., Geller, A.I., Khoury, N., Xu, L.K., 2012. Trends in Traumatic Brain Injury in the US and the public health response: 1995-2009. J Safety Res. 43, 299-307,10.1016/j.jsr.2012.08.011.

Coto-Montes, A., Boga, J.A., Rosales-Corral, S., Fuentes-Broto, L., Tan, D.X., Reiter, R.J., 2012. Role of melatonin in the regulation of autophagy and mitophagy: a review. Molecular and cellular endocrinology. 361, 12-23,10.1016/j.mce.2012.04.009.

Crowe, M.J., Bresnahan, J.C., Shuman, S.L., Masters, J.N., Beattie, M.S., 1997. Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys (vol 3, pg 73, 1997). Nature medicine. 3, 240-240

Cuervo, A.M., 2010. Chaperone-mediated autophagy: selectivity pays off. Trends in endocrinology and metabolism: TEM. 21, 142-150,10.1016/j.tem.2009.10.003.

Cuervo, A.M., Dice, J.F., 1996. A receptor for the selective uptake and degradation of proteins by lysosomes. Science. 273, 501-503

Cybulski, N., Hall, M.N., 2009. TOR complex 2: a signaling pathway of its own. Trends in biochemical sciences. 34, 620-627,10.1016/j.tibs.2009.09.004.

Das, M., Mohapatra, S., Mohapatra, S.S., 2012. New perspectives on central and peripheral immune responses to acute traumatic brain injury. Journal of neuroinflammation. 9,Artn 236

10.1186/1742-2094-9-236.

Deen, W.M., Tannenbaum, S.R., Beckman, J.S., 2002. Protein tyrosine nitration and peroxynitrite - Comment. Faseb Journal. 16, 1144-1144

DeLegge, M.H., Smoke, A., 2008. Neurodegeneration and inflammation. Nutr Clin Pract. 23, 35-41,Doi 10.1177/011542650802300135.

Devivo, M.J., 2012. Epidemiology of traumatic spinal cord injury: trends and future implications. Spinal cord. 50, 365-372,10.1038/sc.2011.178.

DeVivo, M.J., Chen, Y., 2011. Trends in new injuries, prevalent cases, and aging with spinal cord injury. Archives of physical medicine and rehabilitation. 92, 332-338,10.1016/j.apmr.2010.08.031.

Dice, J.F., 1990. Peptide sequences that target cytosolic proteins for lysosomal proteolysis. Trends in biochemical sciences. 15, 305-309

Dirnagl, U., Iadecola, C., Moskowitz, M.A., 1999. Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci. 22, 391-397,Doi 10.1016/S0166-2236(99)01401-0.

Dixon, C.E., Kraus, M.F., Kline, A.E., Ma, X., Yan, H.Q., Griffith, R.G., Wolfson, B.M., Marion, D.W., 1999. Amantadine improves water maze performance without affecting motor behavior following traumatic brain injury in rats. Restorative neurology and neuroscience. 14, 285-294

Dong, H., Fazzaro, A., Xiang, C., Korsmeyer, S.J., Jacquin, M.F., McDonald, J.W., 2003. Enhanced oligodendrocyte survival after spinal cord injury in Bax-deficient mice and mice with delayed Wallerian degeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience. 23, 8682-8691

Doulah, A.H., Rohani, A.H., Khaksari Haddad, M., Motamedi, F., Farbood, Y., Badavi, M., Malek, M., Sarkaki, A., 2009. The effect of peripheral administration of growth hormone on AD-like cognitive deficiency in NBM-lesioned rats. Neuroscience letters. 466, 47-51,10.1016/j.neulet.2009.09.016.

Draper, K., Ponsford, J., 2008. Cognitive functioning ten years following traumatic brain injury and rehabilitation. Neuropsychology. 22, 618-625,10.1037/0894-4105.22.5.618.

Dunlop, E.A., Tee, A.R., 2009. Mammalian target of rapamycin complex 1: Signalling inputs, substrates and feedback mechanisms. Cell Signal. 21, 827-835,10.1016/j.cellsig.2009.01.012.

Duran, I., Siu, L.L., Oza, A.M., Chung, T.B., Sturgeon, J., Townsley, C.A., Pond, G.R., Seymour, L., Niroumand, M., 2006. Characterisation of the lung toxicity of the cell cycle inhibitor temsirolimus. European journal of cancer. 42, 1875-1880,10.1016/j.ejca.2006.03.015.

Eng, L.F., Lee, Y.L., 2003. Response of chemokine antagonists to inflammation in injured spinal cord. Neurochemical research. 28, 95-100

Erlich, S., Alexandrovich, A., Shohami, E., Pinkas-Kramarski, R., 2007. Rapamycin is a neuroprotective treatment for traumatic brain injury. Neurobiol Dis. 26, 86-93,10.1016/j.nbd.2006.12.003.

Esposito, E., Cuzzocrea, S., 2011. Anti-TNF therapy in the injured spinal cord. Trends in pharmacological sciences. 32, 107-115,10.1016/j.tips.2010.11.009.

Esposito, E., Paterniti, I., Meli, R., Bramanti, P., Cuzzocrea, S., 2012. GW0742, a high-affinity PPAR-delta agonist, mediates protection in an organotypic model of spinal cord damage. Spine. 37, E73-78,10.1097/BRS.0b013e3182276d88.

Faden, A.I., Jacobs, T.P., Holaday, J.W., 1981. Thyrotropin-releasing hormone improves neurologic recovery after spinal trauma in cats. The New England journal of medicine. 305, 1063-1067,10.1056/NEJM198110293051806.

Faivre, S., Kroemer, G., Raymond, E., 2006. Current development of mTOR inhibitors as anticancer agents. Nature Reviews Drug Discovery. 5, 671-688,10.1038/nrd2062.

Fang, Y.M., Westbrook, R., Hill, C., Boparai, R.K., Arum, O., Spong, A., Wang, F.Y., Javors, M.A., Chen, J., Sun, L.Y., Bartke, A., 2013. Duration of Rapamycin Treatment Has Differential Effects on Metabolism in Mice. Cell metabolism. 17, 456-462,10.1016/j.cmet.2013.02.008.

Faul, M., Xu, L., Wald, M.M., and Coronado, V.G., 2010. Traumatic brain injury in the United States: Emergency department visits, hospitalizations, and deaths 2002–2006. Atlanta, GA: Centers for Disease Control and Prevention, National Center for Injury Prevention

and Control,

Fawcett, J.W., 2006. Overcoming inhibition in the damaged spinal cord. Journal of neurotrauma. 23, 371-383,Doi 10.1089/Neu.2006.23.371.

Feldman, M.E., Apsel, B., Uotila, A., Loewith, R., Knight, Z.A., Ruggero, D., Shokat, K.M., 2009. Active-Site Inhibitors of mTOR Target Rapamycin-Resistant Outputs of mTORC1 and mTORC2. Plos Biol. 7, 371-383,ARTN e1000038

10.1371/journal.pbio.1000038.

Ferrari, G., Greene, L.A., 1998. Promotion of neuronal survival by GM1 ganglioside. Phenomenology and mechanism of action. Annals of the New York Academy of Sciences. 845, 263-273

Fingar, D.C., Blenis, J., 2004. Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. Oncogene. 23, 3151-3171,10.1038/sj.onc.1207542.

Forstermann, U., 2006. Janus-faced role of endothelial NO synthase in vascular disease: uncoupling of oxygen reduction from NO synthesis and its pharmacological reversal. Biol Chem. 387, 1521-1533,10.1515/BC.2006.190.

Foster, K.G., Fingar, D.C., 2010. Mammalian target of rapamycin (mTOR): conducting the cellular signaling symphony. The Journal of biological chemistry. 285, 14071-14077,10.1074/jbc.R109.094003.

Garcia-Martinez, J.M., Moran, J., Clarke, R.G., Gray, A., Cosulich, S.C., Chresta, C.M., Alessi, D.R., 2009. Ku-0063794 is a specific inhibitor of the mammalian target of rapamycin (mTOR). The Biochemical journal. 421, 29-42,10.1042/BJ20090489.

Geisler, F.H., Coleman, W.P., Grieco, G., Poonian, D., Sygen Study, G., 2001. The Sygen multicenter acute spinal cord injury study. Spine. 26, S87-98

Gianutsos, G., Chute, S., Dunn, J.P., 1985. Pharmacological changes in dopaminergic systems induced by long-term administration of amantadine. European journal of pharmacology. 110, 357-361

Giunta, B., Obregon, D., Velisetty, R., Sanberg, P.R., Borlongan, C.V., Tan, J., 2012. The immunology of traumatic brain injury: a prime target for Alzheimer's disease prevention. Journal of neuroinflammation. 9,Artn 185

10.1186/1742-2094-9-185.

Glick, D., Barth, S., Macleod, K.F., 2010. Autophagy: cellular and molecular mechanisms. Journal of Pathology. 221, 3-12,10.1002/path.2697.

Gonzalez, R., Glaser, J., Liu, M.T., Lane, T.E., Keirstead, H.S., 2003. Reducing inflammation decreases secondary degeneration and functional deficit after spinal cord injury. Experimental neurology. 184, 456-463

Gouvier, W.D., Blanton, P.D., Laporte, K.K., Nepomuceno, C., 1987. Reliability and Validity of the Disability Rating-Scale and the Levels of Cognitive-Functioning Scale in Monitoring Recovery from Severe Head-Injury. Archives of physical medicine and rehabilitation. 68, 94-97

Griffin, M.R., Opitz, J.L., Kurland, L.T., Ebersold, M.J., O'Fallon, W.M., 1985. Traumatic spinal cord injury in Olmsted County, Minnesota, 1935-1981. American journal of epidemiology. 121, 884-895

Guertin, D.A., Stevens, D.M., Thoreen, C.C., Burds, A.A., Kalaany, N.Y., Moffat, J., Brown, M., Fitzgerald, K.J., Sabatini, D.M., 2006. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKC alpha but not S6K1. Dev Cell. 11, 859-871,10.1016/j.devcel.2006.10.007.

Guest, J.D., Hiester, E.D., Bunge, R.P., 2005. Demyelination and Schwann cell responses adjacent to injury epicenter cavities following chronic human spinal cord injury. Experimental neurology. 192, 384-393,10.1016/j.expneurol.2004.11.033.

Guha, A., Tator, C.H., Piper, I., 1987. Effect of a calcium channel blocker on posttraumatic spinal cord blood flow. Journal of neurosurgery. 66, 423-430,10.3171/jns.1987.66.3.0423.

Gyoneva, S., Ransohoff, R.M., 2015. Inflammatory reaction after traumatic brain injury: therapeutic potential of targeting cell-cell communication by chemokines. Trends in pharmacological sciences. 36, 471-480,10.1016/j.tips.2015.04.003.

Hagen, E.M., Eide, G.E., Rekand, T., Gilhus, N.E., Gronning, M., 2010. A 50-year follow-up of the incidence of traumatic spinal cord injuries in Western Norway. Spinal cord. 48, 313-318,10.1038/sc.2009.133.

Hall, E.D., Yonkers, P.A., Andrus, P.K., Cox, J.W., Anderson, D.K., 1992. Biochemistry and pharmacology of lipid antioxidants in acute brain and spinal cord injury. Journal of neurotrauma. 9 Suppl 2, S425-442

Hammond, G.L., Hirvonen, J., Vihko, R., 1983. Progesterone, androstenedione, testosterone, 5 alpha-dihydrotestosterone and androsterone concentrations in specific regions of the human brain. Journal of steroid biochemistry. 18, 185-189

Han, J., Wang, B., Mao, Z.F., Gao, Y., Zhao, Y.H., Zhang, J., Chen, B., Wang, X., Dai, J.W., 2008. Mammalian target of rapamycin (mTOR) is involved in the neuronal differentiation of neural progenitors induced by insulin. Mol Cell Neurosci. 39, 118-124,10.1016/j.mcn.2008.06.003.

Hanukoglu, I., 2006. Antioxidant protective mechanisms against reactive oxygen species (ROS) generated by mitochondrial P450 systems in steroidogenic cells. Drug Metab Rev. 38, 171-196,10.1080/03602530600570040.

Hartley, C.J., Reddy, A.K., Madala, S., Martin-McNulty, B., Vergona, R., Sullivan, M.E., Halks-Miller, M., Taffet, G.E., Michael, L.H., Entman, M.L., Wang, Y.X., 2000. Hemodynamic changes in apolipoprotein E-knockout mice. Am J Physiol Heart Circ Physiol. 279, H2326-2334

Hausmann, O.N., 2003. Post-traumatic inflammation following spinal cord injury. Spinal cord. 41, 369-378,10.1038/sj.sc.3101483.

Hazell, A.S., 2007. Excitotoxic mechanisms in stroke: An update of concepts and treatment strategies. Neurochemistry international. 50, 941-953,10.1016/j.neuint.2007.04.026.

Hicdonmez, T., Kanter, M., Tiryaki, M., Parsak, T., Cobanoglu, S., 2006. Neuroprotective effects of N-acetylcysteine on experimental closed head trauma in rats. Neurochemical research. 31, 473-481,10.1007/s11064-006-9040-z.

Hill, C.E., Beattie, M.S., Bresnahan, J.C., 2001. Degeneration and sprouting of identified descending supraspinal axons after contusive spinal cord injury in the rat. Experimental neurology. 171, 153-169,10.1006/exnr.2001.7734.

Hoffman, W.H., Shacka, J.J., Andjelkovic, A.V., 2012. Autophagy in the brains of young patients with poorly controlled T1DM and fatal diabetic ketoacidosis. Experimental and molecular pathology. 93, 273-280,10.1016/j.yexmp.2011.10.007.

Horky, L.L., Galimi, F., Gage, F.H., Horner, P.J., 2006. Fate of endogenous stem/progenitor cells following spinal cord injury. J Comp Neurol. 498, 525-538,10.1002/cne.21065.

Hu, L.Y., Sun, Z.G., Wen, Y.M., Cheng, G.Z., Wang, S.L., Zhao, H.B., Zhang, X.R., 2010. ATP-MEDIATED PROTEIN KINASE B Akt/MAMMALIAN TARGET OF RAPAMYCIN mTOR/P70 RIBOSOMAL S6 PROTEIN p70S6 KINASE SIGNALING PATHWAY ACTIVATION PROMOTES IMPROVEMENT OF LOCOMOTOR FUNCTION AFTER SPINAL CORD INJURY IN RATS. Neuroscience. 169, 1046-1062,10.1016/j.neuroscience.2010.05.046.

Huang, J., Brumell, J.H., 2009. NADPH oxidases contribute to autophagy regulation. Autophagy. 5, 887-889,10.1073/pnas.0811045106.

Hurlbert, R.J., 2000. Methylprednisolone for acute spinal cord injury: an inappropriate standard of care. Journal of neurosurgery. 93, 1-7

Hurlbert, R.J., 2001. The role of steroids in acute spinal cord injury: an evidence-based analysis. Spine. 26, S39-46

Hurlbert, R.J., 2006. Strategies of medical intervention in the management of acute spinal cord injury. Spine. 31, S16-21; discussion S36,10.1097/01.brs.0000218264.37914.2c.

Inoue, K., 2002. Microglial activation by purines and pyrimidines. Glia. 40, 156-163,10.1002/glia.10150.

Jacinto, E., Lorberg, A., 2008. TOR regulation of AGC kinases in yeast and mammals. Biochemical Journal. 410, 19-37,10.1042/BJ20071518.

Jiang, S., Wells, C.D., Roach, P.J., 2011. Starch-binding domain-containing protein 1 (Stbd1) and glycogen metabolism: Identification of the Atg8 family interacting motif (AIM) in Stbd1 required for interaction with GABARAPL1. Biochemical and biophysical research communications. 413, 420-425,10.1016/j.bbrc.2011.08.106.

Johnson, R.L., Gabella, B.A., Gerhart, K.A., McCray, J., Menconi, J.C., Whiteneck, G.G., 1997. Evaluating sources of traumatic spinal cord injury surveillance data in Colorado. American journal of epidemiology. 146, 266-272

Jones, L.L., Margolis, R.U., Tuszynski, M.H., 2003. The chondroitin sulfate proteoglycans neurocan, brevican, phosphacan, and versican are differentially regulated following spinal cord injury. Experimental neurology. 182, 399-411,10.1016/S0014-4886(03)00087-6.

Jones, L.L., Yamaguchi, Y., Stallcup, W.B., Tuszynski, M.H., 2002. NG2 is a major chondroitin sulfate proteoglycan produced after spinal cord injury and is expressed by macrophages and oligodendrocyte progenitors. Journal of Neuroscience. 22, 2792-2803

Jones, T.B., McDaniel, E.E., Popovich, P.G., 2005. Inflammatory-mediated injury and repair in the traumatically injured spinal cord. Current pharmaceutical design. 11, 1223-1236,Doi 10.2174/1381612053507468.

Kakulas, B.A., 1999. A review of the neuropathology of human spinal cord injury with emphasis on special features. The journal of spinal cord medicine. 22, 119-124

Kalia, L.V., Kalia, S.K., Salter, M.W., 2008. NMDA receptors in clinical neurology: excitatory times ahead. Lancet Neurol. 7, 742-755,Doi 10.1016/S1474-4422(08)70165-0.

Kanki, T., 2010. Nix, a receptor protein for mitophagy in mammals. Autophagy. 6, 433-435

Kanno, H., Ozawa, H., Sekiguchi, A., Yamaya, S., Tateda, S., Yahata, K., Itoi, E., 2012. The role of mTOR signaling pathway in spinal cord injury. Cell cycle. 11, 3175-3179,10.4161/cc.21262.

Karman, J., Ling, C., Sandor, M., Fabry, Z., 2004. Initiation of immune responses in brain is promoted by local dendritic cells. Journal of immunology. 173, 2353-2361

Kawai, T., Akira, S., 2007. Signaling to NF-kappa B by Toll-like receptors. Trends Mol Med. 13, 460-469,10.1016/j.molmed.2007.09.002.

Khalatbary, A.R., Tiraihi, T., Boroujeni, M.B., Ahmadvand, H., Tavafi, M., Tamjidipoor, A., 2010. Effects of epigallocatechin gallate on tissue protection and functional recovery after contusive spinal cord injury in rats. Brain research. 1306, 168-175,10.1016/j.brainres.2009.09.109.

Kierdorf, K., Wang, Y., Neumann, H., 2010. Immune-mediated CNS damage. Results and problems in cell differentiation. 51, 173-196,10.1007/400_2008_15.

Kim, G.M., Xu, J., Xu, J., Song, S.K., Yan, P., Ku, G., Xu, X.M., Hsu, C.Y., 2001. Tumor necrosis factor receptor deletion reduces nuclear factor-kappaB activation, cellular inhibitor of apoptosis protein 2 expression, and functional recovery after traumatic spinal cord injury. The Journal of neuroscience : the official journal of the Society for Neuroscience. 21, 6617-6625

Kiraly, M.A., Kiraly, S.J., 2007. Traumatic brain injury and delayed sequelae: A review - Traumatic brain injury and mild traumatic brain injury (Concussion) are precursors to later-onset brain disorders, including early-onset dementia. TheScientificWorldJournal. 7, 1768-1776,10.1100/tsw.2007.269.

Kirshblum, S.C., Burns, S.P., Biering-Sorensen, F., Donovan, W., Graves, D.E., Jha, A., Johansen, M., Jones, L., Krassioukov, A., Mulcahey, M.J., Schmidt-Read, M., Waring, W., 2011. International standards for neurological classification of spinal cord injury (Revised 2011). Journal of Spinal Cord Medicine. 34, 535-546,10.1179/204577211X13207446293695.

Kissova, I., Deffieu, M., Samokhvalov, V., Velours, G., Bessoule, J.J., Manon, S., Camougrand, N., 2006. Lipid oxidation and autophagy in yeast. Free Radical Bio Med. 41, 1655-1661,10.1016/j.freeradbiomed.2006.08.012.

Klionsky, D.J., 1997. Protein transport from the cytoplasm into the vacuole. The Journal of membrane biology. 157, 105-115

Klionsky, D.J., Cuervo, A.M., Seglen, P.O., 2007. Methods for monitoring autophagy from yeast to human. Autophagy. 3, 181-206

Koenig, H.L., Schumacher, M., Ferzaz, B., Thi, A.N., Ressouches, A., Guennoun, R., Jung-Testas, I., Robel, P., Akwa, Y., Baulieu, E.E., 1995. Progesterone synthesis and myelin formation by Schwann cells. Science. 268, 1500-1503

Kraft, C., Deplazes, A., Sohrmann, M., Peter, M., 2008. Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease. Nature cell biology. 10, 602-610,10.1038/ncb1723.

Kraus, J.F., Franti, C.E., Riggins, R.S., Richards, D., Borhani, N.O., 1975. Incidence of traumatic spinal cord lesions. Journal of chronic diseases. 28, 471-492

Kroemer, G., Marino, G., Levine, B., 2010. Autophagy and the integrated stress response. Molecular cell. 40, 280-293,10.1016/j.molcel.2010.09.023.

Kwon, B.K., Fisher, C.G., Dvorak, M.F., Tetzlaff, W., 2005. Strategies to promote neural repair and regeneration after spinal cord injury. Spine. 30, S3-13

Lambeth, J.D., 2007. Nox enzymes, ROS, and chronic disease: An example of antagonistic pleiotropy. Free Radical Bio Med. 43, 332-347,10.1016/j.freeradbiomed.2007.03.027.

Lang-Lazdunski, L., Blondeau, N., Jarretou, G., Lazdunski, M., Heurteaux, C., 2003. Linolenic acid prevents neuronal cell death and paraplegia after transient spinal cord ischemia in rats. Journal of vascular surgery. 38, 564-575

Lapaquette, P., Guzzo, J., Bretillon, L., Bringer, M.A., 2015. Cellular and Molecular Connections between Autophagy and Inflammation. Mediators of inflammation. 2015, 398483,10.1155/2015/398483.

Laplante, M., Sabatini, D.M., 2009. mTOR signaling at a glance. Journal of cell science. 122, 3589-3594,10.1242/jcs.051011.

Larosche, I., Letteron, P., Berson, A., Fromenty, B., Huang, T.T., Moreau, R., Pessayre, D., Mansouri, A., 2010. Hepatic Mitochondrial DNA Depletion after an Alcohol Binge in Mice: Probable Role of Peroxynitrite and Modulation by Manganese Superoxide Dismutase. Journal of Pharmacology and Experimental Therapeutics. 332, 886-897,10.1124/jpet.109.160879.

Lee, S.M., Yune, T.Y., Kim, S.J., Park, D.W., Lee, Y.K., Kim, Y.C., Oh, Y.J., Markelonis, G.J., Oh, T.H., 2003. Minocycline reduces cell death and improves functional recovery after traumatic spinal cord injury in the rat. Journal of neurotrauma. 20, 1017-1027,10.1089/089771503770195867.

Lee, Y.L., Shih, K., Bao, P., Ghirnikar, R.S., Eng, L.F., 2000. Cytokine chemokine expression in contused rat spinal cord. Neurochemistry international. 36, 417-425

Leskovar, A., Moriarty, L.J., Turek, J.J., Schoenlein, I.A., Borgens, R.B., 2000. The macrophage in acute neural injury: changes in cell numbers over time and levels of cytokine production in mammalian central and peripheral nervous systems. The Journal of experimental biology. 203, 1783-1795

Levine, B., Kroemer, G., 2008. Autophagy in the pathogenesis of disease. Cell. 132, 27-42,10.1016/j.cell.2007.12.018.

Levine, B., Mizushima, N., Virgin, H.W., 2011. Autophagy in immunity and inflammation. Nature. 469, 323-335,10.1038/nature09782.

Li, B., Mahmood, A., Lu, D., Wu, H., Xiong, Y., Qu, C., Chopp, M., 2009. Simvastatin attenuates microglial cells and astrocyte activation and decreases interleukin-1beta level after traumatic brain injury. Neurosurgery. 65, 179-185; discussion 185-176,10.1227/01.NEU.0000346272.76537.DC.

Ling, C., Sandor, M., Fabry, Z., 2003. In situ processing and distribution of intracerebrally injected OVA in the CNS. Journal of neuroimmunology. 141, 90-98

Liu, K., Lu, Y., Lee, J.K., Samara, R., Willenberg, R., Sears-Kraxberger, I., Tedeschi, A., Park, K.K., Jin, D., Cai, B., Xu, B.G., Connolly, L., Steward, O., Zheng, B.H., He, Z.G., 2010. PTEN deletion enhances the regenerative ability of adult corticospinal neurons. Nature neuroscience. 13, 1075-U1064,10.1038/nn.2603.

Liu, L., Wang, J., Zhao, L., Nilsen, J., McClure, K., Wong, K., Brinton, R.D., 2009. Progesterone increases rat neural progenitor cell cycle gene expression and proliferation via extracellularly regulated kinase and progesterone receptor membrane components 1 and 2. Endocrinology. 150, 3186-3196,10.1210/en.2008-1447.

Ljungberg, M.C., Sunnen, C.N., Lugo, J.N., Anderson, A.E., D'Arcangelo, G., 2009. Rapamycin suppresses seizures and neuronal hypertrophy in a mouse model of cortical dysplasia. Dis Model Mech. 2, 389-398,10.1242/dmm.002386.

Loetscher, H., Gentz, R., Zulauf, M., Lustig, A., Tabuchi, H., Schlaeger, E.J., Brockhaus, M., Gallati, H., Manneberg, M., Lesslauer, W., 1991. Recombinant 55-kDa tumor necrosis factor (TNF) receptor. Stoichiometry of binding to TNF alpha and TNF beta and inhibition of TNF activity. The Journal of biological chemistry. 266, 18324-18329

Luo, S.Q., Garcia-Arencibia, M., Zhao, R., Puri, C., Toh, P.P.C., Sadiq, O., Rubinsztein, D.C., 2012. Bim Inhibits Autophagy by Recruiting Beclin 1 to Microtubules. Molecular cell. 47, 359-370,10.1016/j.molcel.2012.05.040.

Ma, X.J.M., Blenis, J., 2009. Molecular mechanisms of mTOR-mediated translational control. Nat Rev Mol Cell Bio. 10, 307-318,10.1038/nrm2672.

Maiuri, M.C., Le Toumelin, G., Criollo, A., Rain, J.C., Gautier, F., Juin, P., Tasdemir, E., Pierron, G., Troulinaki, K., Tavernarakis, N., Hickman, J.A., Geneste, O., Kroemer, G., 2007. Functional and physical interaction between Bcl-X-L and a BH3-like domain in Beclin-1. Embo Journal. 26, 2527-2539,10.1038/sj.emboj.7601689.

Mandi, Y., Endresz, V., Krenacs, L., Regely, K., Degre, M., Beladi, I., 1991. Tumor necrosis factor production by human granulocytes. International archives of allergy and applied immunology. 96, 102-106

Manning, B.D., Cantley, L.C., 2007. AKT/PKB signaling: Navigating downstream. Cell. 129, 1261-1274,10.1016/j.cell.2007.06.009.

Manzanero, S., Santro, T., Arumugam, T.V., 2013. Neuronal oxidative stress in acute ischemic stroke: Sources and contribution to cell injury. Neurochemistry international. 62, 712-718,10.1016/j.neuint.2012.11.009.

Martinou, J.C., Youle, R.J., 2011. Mitochondria in Apoptosis: Bcl-2 Family Members and Mitochondrial Dynamics. Dev Cell. 21, 92-101,10.1016/j.devcel.2011.06.017.

Masel, B.E., DeWitt, D.S., 2010. Traumatic Brain Injury: A Disease Process, Not an Event. Journal of neurotrauma. 27, 1529-1540,10.1089/neu.2010.1358.

Menon, S., Manning, B.D., 2008. Common corruption of the mTOR signaling network in human tumors. Oncogene. 27 Suppl 2, S43-51,10.1038/onc.2009.352.

Mijaljica, D., Prescott, M., Devenish, R.J., 2010. The intricacy of nuclear membrane dynamics during nucleophagy. Nucleus. 1, 213-223,10.4161/nucl.1.3.11738.

Miller, R.A., Harrison, D.E., Astle, C.M., Fernandez, E., Flurkey, K., Han, M., Javors, M.A., Li, X.N., Nadon, N.L., Nelson, J.F., Pletcher, S., Salmon, A.B., Sharp, Z.D., Van Roekel, S., Winkleman, L., Strong, R., 2014. Rapamycin-mediated lifespan increase in mice is dose and sex dependent and metabolically distinct from dietary restriction. Aging Cell. 13, 468-477,10.1111/acel.12194.

Mizushima, N., 2007. Autophagy: process and function. Genes & development. 21, 2861-2873,10.1101/gad.1599207.

Morales, D.M., Marklund, N., Lebold, D., Thompson, H.J., Pitkanen, A., Maxwell, W.L., Longhi, L., Laurer, H., Maegele, M., Neugebauer, E., Graham, D.I., Stocchetti, N., McIntosh, T.K., 2005. Experimental models of traumatic brain injury: Do we really need to build a better mousetrap? Neuroscience. 136, 971-989,10.1016/j.neuroscience.2005.08.030.

Morales, I., Farias, G., Maccioni, R.B., 2010. Neuroimmunomodulation in the pathogenesis of Alzheimer's disease. Neuroimmunomodulation. 17, 202-204,10.1159/000258724.

Morganti-Kossmann, M.C., Satgunaseelan, L., Bye, N., Kossmann, T., 2007. Modulation of immune response by head injury. Injury-International Journal of the Care of the Injured. 38, 1392-1400,10.1016/j.injury.2007.10.005.

Murphy, M.P., 2006. Induction of mitochondrial ROS production by electrophilic lipids: a new pathway of redox signaling? Am J Physiol-Heart C. 290, H1754-H1755,10.1152/ajpheart.00040.2006.

Murphy, M.P., 2009. How mitochondria produce reactive oxygen species. Biochemical Journal. 417, 1-13,10.1042/BJ20081386.

Nakahira, K., Haspel, J.A., Rathinam, V.A.K., Lee, S.J., Dolinay, T., Lam, H.C., Englert, J.A., Rabinovitch, M., Cernadas, M., Kim, H.P., Fitzgerald, K.A., Ryter, S.W., Choi, A.M.K., 2011. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat Immunol. 12, 222-U257,10.1038/ni.1980.

Nakamura, M., Bregman, B.S., 2001. Differences in neurotrophic factor gene expression profiles between neonate and adult rat spinal cord after injury. Experimental neurology. 169, 407-415,10.1006/exnr.2001.7670.

Nakatogawa, H., Suzuki, K., Kamada, Y., Ohsumi, Y., 2009. Dynamics and diversity in autophagy mechanisms: lessons from yeast. Nat Rev Mol Cell Bio. 10, 458-467,10.1038/nrm2708.

Naseem, M., Parvez, S., 2014. Role of melatonin in traumatic brain injury and spinal cord injury. TheScientificWorldJournal. 2014, 586270,10.1155/2014/586270.

Nesic, O., Xu, G.Y., McAdoo, D., High, K.W., Hulsebosch, C., Perez-Pol, R., 2001. IL-1 receptor antagonist prevents apoptosis and caspase-3 activation after spinal cord injury. Journal of neurotrauma. 18, 947-956,10.1089/089771501750451857.

Nolan, K.A., Burton, L.A., 1998. Incidence of the Fuld WAIS-R profile in traumatic brain injury and Parkinson's disease. Arch Clin Neuropsych. 13, 425-432

Obrenovitch, T.P., Urenjak, J., 1997. Altered glutamatergic transmission in neurological disorders: From high extracellular glutamate to excessive synaptic efficacy. Prog Neurobiol. 51, 39-87,Doi 10.1016/S0301-0082(96)00049-4.

Ohsumi, Y., Mizushima, N., 2004. Two ubiquitin-like conjugation systems essential for autophagy. Seminars in cell & developmental biology. 15, 231-236

Okada, S., Nakamura, M., Mikami, Y., Shimazaki, T., Mihara, M., Ohsugi, Y., Iwamoto, Y., Yoshizaki, K., Kishimoto, T., Toyama, Y., Okano, H., 2004. Blockade of interleukin-6 receptor suppresses reactive astrogliosis and ameliorates functional recovery in experimental spinal cord injury. Journal of neuroscience research. 76, 265-276,10.1002/jnr.20044.

Overbye, A., Fengsrud, M., Seglen, P.O., 2007. Proteomic analysis of membrane-associated proteins from rat liver autophagosomes. Autophagy. 3, 300-322

Park, K.K., Liu, K., Hu, Y., Smith, P.D., Wang, C., Cai, B., Xu, B.G., Connolly, L., Kramvis, I., Sahin, M., He, Z.G., 2008. Promoting Axon Regeneration in the Adult CNS by Modulation of the PTEN/mTOR Pathway. Science. 322, 963-966,10.1126/science.1161566.

Pattingre, S., Bauvy, C., Carpentier, S., Levade, T., Levine, B., Codogno, P., 2009. Role of JNK1-dependent Bcl-2 Phosphorylation in Ceramide-induced Macroautophagy. Journal of Biological Chemistry. 284, 2719-2728,10.1074/jbc.M805920200.

Pattingre, S., Tassa, A., Qu, X.P., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D., Levine, B., 2005. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell. 122, 927-939,10.1016/j.cell.2005.07.002.

Penkowa, M., Camats, J., Giralt, M., Molinero, A., Hernandez, J., Carrasco, J., Campbell, I.L., Hidalgo, J., 2003a. Metallothionein-I overexpression alters brain inflammation and stimulates brain repair in transgenic mice with astrocyte-targeted interleukin-6 expression. Glia. 42, 287-306,10.1002/glia.10208.

Penkowa, M., Camats, J., Hadberg, H., Quintana, A., Rojas, S., Giralt, M., Molinero, A., Campbell, I.L., Hidalgo, J., 2003b. Astrocyte-targeted expression of interleukin-6 protects the central nervous system during neuroglial degeneration induced by 6-aminonicotinamide. Journal of neuroscience research. 73, 481-496,10.1002/jnr.10681.

Peterson, T.R., Laplante, M., Thoreen, C.C., Sancak, Y., Kang, S.A., Kuehl, W.M., Gray, N.S., Sabatini, D.M., 2009. DEPTOR Is an mTOR Inhibitor Frequently Overexpressed in Multiple Myeloma Cells and Required for Their Survival. Cell. 137, 873-886,10.1016/j.cell.2009.03.046.

Pettigrew, D.B., Shockley, K.P., Crutcher, K.A., 2001. Disruption of spinal cord white matter and sciatic nerve geometry inhibits axonal growth in vitro in the absence of glial scarring. Bmc Neurosci. 2,Artn 8

Doi 10.1186/1471-2202-2-8.

Phung, T.L., Ziv, K., Dabydeen, D., Eyiah-Mensah, G., Riveros, M., Perruzzi, C., Sun, J., Monahan-Earley, R.A., Shiojima, I., Nagy, J.A., Lin, M.I., Walsh, K., Dvorak, A.M., Briscoe, D.M., Neeman, M., Sessa, W.C., Dvorak, H.F., Benjamin, L.E., 2006. Pathological angiogenesis is induced by sustained Akt signaling and inhibited by rapamycin. Cancer cell. 10, 159-170,10.1016/j.ccr.2006.07.003.

Pignataro, G., Capone, D., Polichetti, G., Vinciguerra, A., Gentile, A., Di Renzo, G., Annunziato, L., 2011. Neuroprotective, immunosuppressant and antineoplastic properties of mTOR inhibitors: current and emerging therapeutic options. Curr Opin Pharmacol. 11, 378-394,10.1016/j.coph.2011.05.003.

Pitts, L.H., Ross, A., Chase, G.A., Faden, A.I., 1995. Treatment with thyrotropin-releasing hormone (TRH) in patients with traumatic spinal cord injuries. Journal of neurotrauma. 12, 235-243,10.1089/neu.1995.12.235.

Pointillart, V., Gense, D., Gross, C., Bidabe, A.M., Gin, A.M., Rivel, J., Caille, J.M., Senegas, J., 1993. Effects of nimodipine on posttraumatic spinal cord ischemia in baboons. Journal of neurotrauma. 10, 201-213,10.1089/neu.1993.10.201.

Pointillart, V., Petitjean, M.E., Wiart, L., Vital, J.M., Lassie, P., Thicoipe, M., Dabadie, P., 2000. Pharmacological therapy of spinal cord injury during the acute phase. Spinal cord. 38, 71-76

Ponsford, J., Draper, K., Schonberger, M., 2008. Functional outcome 10 years after traumatic brain injury: Its relationship with demographic, injury severity, and cognitive and emotional status. J Int Neuropsych Soc. 14, 233-242,10.1017/S1355617708080272.

Price, C., Makintubee, S., Herndon, W., Istre, G.R., 1994. Epidemiology of traumatic spinal cord injury and acute hospitalization and rehabilitation charges for spinal cord injuries in Oklahoma, 1988-1990. American journal of epidemiology. 139, 37-47

Raineteau, O., Fouad, K., Bareyre, F.M., Schwab, M.E., 2002. Reorganization of descending motor tracts in the rat spinal cord. European Journal of Neuroscience. 16, 1761-1771,10.1046/j.1460-9568.2002.02243.x.

Raineteau, O., Schwab, M.E., 2001. Plasticity of motor systems after incomplete spinal cord injury. Nat Rev Neurosci. 2, 263-273,Doi 10.1038/35067570.

Rashmi, R., Pillai, S.G., Vijayalingam, S., Ryerse, J., Chinnadurai, G., 2008. BH3-only protein BIK induces caspase-independent cell death with autophagic features in Bcl-2 null cells. Oncogene. 27, 1366-1375,10.1038/sj.onc.1210783.

Reiling, J.H., Sabatini, D.M., 2006. Stress and mTORture signaling. Oncogene. 25, 6373-6383,10.1038/sj.onc.1209889.

Rosner, M., Fuchs, C., Siegel, N., Valli, A., Hengstschlager, M., 2009. Functional interaction of mammalian target of rapamycin complexes in regulating mammalian cell size and cell cycle. Human molecular genetics. 18, 3298-3310,10.1093/hmg/ddp271.

Roxburgh, C.S., McMillan, D.C., 2016. Therapeutics targeting innate immune/inflammatory responses through the interleukin-6/JAK/STAT signal transduction pathway in patients with cancer. Translational research : the journal of laboratory and clinical medicine. 167, 61-66,10.1016/j.trsl.2015.08.013.

Rubinstein, A.D., Kimchi, A., 2012. Life in the balance - a mechanistic view of the crosstalk between autophagy and apoptosis. Journal of cell science. 125, 5259-5268,10.1242/jcs.115865.

Ruvinsky, I., Meyuhas, O., 2006. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. Trends in biochemical sciences. 31, 342-348,10.1016/j.tibs.2006.04.003.

Saatman, K.E., Contreras, P.C., Smith, D.H., Raghupathi, R., McDermott, K.L., Fernandez, S.C., Sanderson, K.L., Voddi, M., McIntosh, T.K., 1997. Insulin-like growth factor-1 (IGF-1) improves both neurological motor and cognitive outcome following experimental brain injury. Experimental neurology. 147, 418-427,10.1006/exnr.1997.6629.

Saitoh, T., Akira, S., 2010. Regulation of innate immune responses by autophagy-related proteins. The Journal of cell biology. 189, 925-935,10.1083/jcb.201002021.

Saitoh, T., Fujita, N., Jang, M.H., Uematsu, S., Yang, B.G., Satoh, T., Omori, H., Noda, T., Yamamoto, N., Komatsu, M., Tanaka, K., Kawai, T., Tsujimura, T., Takeuchi, O., Yoshimori, T., Akira, S., 2008. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1 beta production. Nature. 456, 264-U268,10.1038/nature07383.

Samantaray, S., Das, A., Thakore, N.P., Matzelle, D.D., Reiter, R.J., Ray, S.K., Banik, N.L., 2009. Therapeutic potential of melatonin in traumatic central nervous system injury. Journal of pineal research. 47, 134-142,10.1111/j.1600-079X.2009.00703.x.

Sarbassov, D.D., Ali, S.M., Kim, D.H., Guertin, D.A., Latek, R.R., Erdjument-Bromage, H., Tempst, P., Sabatini, D.M., 2004. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Current Biology. 14, 1296-1302

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L., Sabatini, D.M., 2006. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Molecular cell. 22, 159-168,10.1016/j.molcel.2006.03.029.

Sarbassov, D.D., Guertin, D.A., Ali, S.M., Sabatini, D.M., 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 307, 1098-1101,10.1126/science.1106148.

Sasaki, S., 2011. Autophagy in Spinal Cord Motor Neurons in Sporadic Amyotrophic Lateral Sclerosis. J Neuropath Exp Neur. 70, 349-359,10.1097/NEN.0b013e3182160690.

Satake, K., Matsuyama, Y., Kamiya, M., Kawakami, H., Iwata, H., Adachi, K., Kiuchi, K., 2000. Nitric oxide via macrophage iNOS induces apoptosis following traumatic spinal cord injury. Brain research. Molecular brain research. 85, 114-122

Sato, M., Chang, E., Igarashi, T., Noble, L.J., 2001. Neuronal injury and loss after traumatic brain injury: time course and regional variability. Brain research. 917, 45-54,Doi 10.1016/S0006-8993(01)02905-5.

Schabitz, W.R., Sommer, C., Zoder, W., Kiessling, M., Schwaninger, M., Schwab, S., 2000. Intravenous brain-derived neurotrophic factor reduces infarct size and counterregulates Bax and Bcl-2 expression after temporary focal cerebral ischemia. Stroke; a journal of cerebral circulation. 31, 2212-2217

Scherz-Shouval, R., Elazar, Z., 2011. Regulation of autophagy by ROS: physiology and pathology. Trends in biochemical sciences. 36, 30-38,Doi 10.1016/J.Tibs.2010.07.007.

Scherz-Shouval, R., Shvets, E., Fass, E., Shorer, H., Gil, L., Elazar, Z., 2007. Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. Embo Journal. 26, 1749-1760,10.1038/sj.emboj.7601623.

Schnell, L., Fearn, S., Klassen, H., Schwab, M.E., Perry, V.H., 1999. Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal cord. The European journal of neuroscience. 11, 3648-3658

Schomacher, M., Muller, H.D., Sommer, C., Schwab, S., Schabitz, W.R., 2008. Endocannabinoids mediate neuroprotection after transient focal cerebral ischemia. Brain research. 1240, 213-220,10.1016/j.brainres.2008.09.019.

Schrader, M., Fahimi, H.D., 2006. Peroxisomes and oxidative stress. Bba-Mol Cell Res. 1763, 1755-1766,10.1016/j.bbamcr.2006.09.006.

Schroder, K., Tschopp, J., 2010. The Inflammasomes. Cell. 140, 821-832,10.1016/j.cell.2010.01.040.

Schwartz, M., 2000. Autoimmune involvement in CNS trauma is beneficial if well controlled. Progress in brain research. 128, 259-263,10.1016/S0079-6123(00)28023-0.

Schwartz, M., Moalem, G., Leibowitz-Amit, R., Cohen, I.R., 1999. Innate and adaptive immune responses can be beneficial for CNS repair. Trends in neurosciences. 22, 295-299

Setkowicz, Z., Guzik, R., 2007. Injections of vehicle, but not cyclosporin A or tacrolimus (FK506), afford neuroprotection following injury in the developing rat brain. Acta neurobiologiae experimentalis. 67, 399-409

Sharov, V.G., Todor, A., Khanal, S., Imai, M., Sabbah, H.N., 2007. Cyclosporine A attenuates mitochondrial permeability transition and improves mitochondrial respiratory function in cardiomyocytes isolated from dogs with heart failure. Journal of molecular and cellular cardiology. 42, 150-158,10.1016/j.yjmcc.2006.09.013.

Shea, T.B., 1994. Technical report. An inexpensive densitometric analysis system using a Macintosh computer and a desktop scanner. BioTechniques. 16, 1126-1128

Shohami, E., Gallily, R., Mechoulam, R., Bass, R., BenHur, T., 1997. Cytokine production in the brain following closed head injury: Dexanabinol (HU-211) is a novel TNF-alpha inhibitor and an effective neuroprotectant. Journal of neuroimmunology. 72, 169-177,Doi 10.1016/S0165-5728(96)00181-6.

Shohami, E., Ginis, I., Hallenbeck, J.M., 1999. Dual role of tumor necrosis factor alpha in brain injury. Cytokine Growth F R. 10, 119-130,Doi 10.1016/S1359-6101(99)00008-8.

Short, D.J., El Masry, W.S., Jones, P.W., 2000. High dose methylprednisolone in the management of acute spinal cord injury - a systematic review from a clinical perspective. Spinal cord. 38, 273-286

Shpilka, T., Elazar, Z., 2012. Essential role for the mammalian ATG8 isoform LC3C in xenophagy. Molecular cell. 48, 325-326,10.1016/j.molcel.2012.10.020.

Shuman, S.L., Bresnahan, J.C., Beattie, M.S., 1997. Apoptosis of microglia and oligodendrocytes after spinal cord contusion in rats. Journal of neuroscience research. 50, 798-808

Silva, N.A., Sousa, N., Reis, R.L., Salgado, A.J., 2014. From basics to clinical: A comprehensive review on spinal cord injury. Prog Neurobiol. 114, 25-57,10.1016/j.pneurobio.2013.11.002.

Silver, J., Miller, J.H., 2004. Regeneration beyond the glial scar. Nat Rev Neurosci. 5, 146-156,10.1038/nrn1326.

Singh, R., Kaushik, S., Wang, Y., Xiang, Y., Novak, I., Komatsu, M., Tanaka, K., Cuervo, A.M., Czaja, M.J., 2009. Autophagy regulates lipid metabolism. Nature. 458, 1131-1135,10.1038/nature07976.

Singleton, R.H., Stone, J.R., Okonkwo, D.O., Pellicane, A.J., Povlishock, J.T., 2001. The immunophilin ligand FK506 attenuates axonal injury in an impact-acceleration model of traumatic brain injury. Journal of neurotrauma. 18, 607-614,10.1089/089771501750291846.

Sparks, C.A., Guertin, D.A., 2010. Targeting mTOR: prospects for mTOR complex 2 inhibitors in cancer therapy. Oncogene. 29, 3733-3744,10.1038/onc.2010.139.

Sribnick, E.A., Matzelle, D.D., Ray, S.K., Banik, N.L., 2006. Estrogen treatment of spinal cord injury attenuates calpain activation and apoptosis. Journal of neuroscience research. 84, 1064-1075,10.1002/jnr.21016.

Sroga, J.M., Jones, T.B., Kigerl, K.A., McGaughy, V.M., Popovich, P.G., 2003. Rats and mice exhibit distinct inflammatory reactions after spinal cord injury. The Journal of comparative neurology. 462, 223-240,10.1002/cne.10736.

Stahel, P.F., 2004. The role of complement-mediated neuroinflammation in traumatic brain injury. Journal of neurochemistry. 90, 25-25

Steinman, L., 2007. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. Nature medicine. 13, 139-145,10.1038/nm1551.

Stirling, D.P., Khodarahmi, K., Liu, J., McPhail, L.T., McBride, C.B., Steeves, J.D., Ramer, M.S., Tetzlaff, W., 2004. Minocycline treatment reduces delayed oligodendrocyte death, attenuates axonal dieback, and improves functional outcome after spinal cord injury. The Journal of neuroscience : the official journal of the Society for Neuroscience. 24, 2182-2190,10.1523/JNEUROSCI.5275-03.2004.

Strappazzon, F., Vietri-Rudan, M., Campello, S., Nazio, F., Florenzano, F., Fimia, G.M., Piacentini, M., Levine, B., Cecconi, F., 2011. Mitochondrial BCL-2 inhibits AMBRA1-induced autophagy. Embo Journal. 30, 1195-1208,10.1038/emboj.2011.49.

Strimpakos, A.S., Karapanagiotou, E.M., Saif, M.W., Syrigos, K.N., 2009. The role of mTOR in the management of solid tumors: An overview. Cancer treatment reviews. 35, 148-159,10.1016/j.ctrv.2008.09.006.

Sunnen, C.N., Brewster, A.L., Lugo, J.N., Vanegas, F., Turcios, E., Mukhi, S., Parghi, D., D'Arcangelo, G., Anderson, A.E., 2011. Inhibition of the mammalian target of rapamycin blocks epilepsy progression in NS-Pten conditional knockout mice. Epilepsia. 52, 2065-2075,10.1111/j.1528-1167.2011.03280.x.

Surkin, J., Smith, M., Penman, A., Currier, M., Harkey, H.L., 3rd, Chang, Y.F., 1998. Spinal cord injury incidence in Mississippi: a capture-recapture approach. The Journal of trauma. 45, 502-504

Sutherlin, D.P., Bao, L., Berry, M., Castanedo, G., Chuckowree, I., Dotson, J., Folks, A., Friedman, L., Goldsmith, R., Gunzner, J., Heffron, T., Lesnick, J., Lewis, C., Mathieu, S., Murray, J., Nonomiya, J., Pang, J., Pegg, N., Prior, W.W., Rouge, L., Salphati, L., Sampath, D., Tian, Q.P., Tsui, V., Wan, N.C., Wang, S.M., Wei, B.Q., Wiesmann, C., Wu, P., Zhu, B.Y., Olivero, A., 2011. Discovery of a Potent, Selective, and Orally Available Class I Phosphatidylinositol 3-Kinase (PI3K)/Mammalian Target of Rapamycin (mTOR) Kinase Inhibitor (GDC-0980) for the Treatment of Cancer. Journal of medicinal chemistry. 54, 7579-7587,10.1021/jm2009327.

Syed, F., Sanganee, H.J., Singh, S., Bahl, A., Bayat, A., 2013. Potent dual inhibitors of TORC1 and TORC2 complexes (KU-0063794 and KU-0068650) demonstrate in vitro and ex vivo anti-keloid scar activity. The Journal of investigative dermatology. 133, 1340-1350,10.1038/jid.2012.483.

Tanneeru, K., Guruprasad, L., 2012. Ligand-based 3-D pharmacophore generation and molecular docking of mTOR kinase inhibitors. J Mol Model. 18, 1611-1624,10.1007/s00894-011-1184-3.

Tator, C.H., 2006. Review of treatment trials in human spinal cord injury: issues, difficulties, and recommendations. Neurosurgery. 59, 957-982; discussion 982-957,10.1227/01.NEU.0000245591.16087.89.

Teng, F.Y., Tang, B.L., 2006. Axonal regeneration in adult CNS neurons--signaling molecules and pathways. Journal of neurochemistry. 96, 1501-1508,10.1111/j.1471-4159.2006.03663.x.

Tenovuo, O., 2005. Central acetylcholinesterase inhibitors in the treatment of chronic traumatic brain injury-clinical experience in 111 patients. Progress in neuro-psychopharmacology & biological psychiatry. 29, 61-67,10.1016/j.pnpbp.2004.10.006.

Thuret, S., Moon, L.D.F., Gage, F.H., 2006. Therapeutic interventions after spinal cord injury. Nat Rev Neurosci. 7, 628-643,10.1038/nrn1955.

Thurman, D.J., Burnett, C.L., Jeppson, L., Beaudoin, D.E., Sniezek, J.E., 1994. Surveillance of spinal cord injuries in Utah, USA. Paraplegia. 32, 665-669,10.1038/sc.1994.107.

Topal, N.B., Hakyemez, B., Erdogan, C., Bulut, M., Koksal, O., Akkose, S., Dogan, S., Parlak, M., Ozguc, H., Korfali, E., 2008. MR imaging in the detection of diffuse axonal injury with mild traumatic brain injury. Neurological research. 30, 974-978,10.1179/016164108X323799.

Totoiu, M.O., Keirstead, H.S., 2005. Spinal cord injury is accompanied by chronic progressive demyelination. J Comp Neurol. 486, 373-383,10.1002/cne.20517.

Trocoli, A., Djavaheri-Mergny, M., 2011. The complex interplay between autophagy and NF-kappa B signaling pathways in cancer cells. Am J Cancer Res. 1, 629-649

Tsang, C.K., Qi, H.Y., Liu, L.F., Zheng, X.F.S., 2007. Targeting mammalian target of rapamycin (mTOR) for health and diseases. Drug discovery today. 12, 112-124,10.1016/j.drudis.2006.12.008.

Tsang, C.K., Qi, H.Y., Liu, L.F., Zheng, X.F.S., 2008. Targeting mammalian target of rapamycin (mTOR) for health and diseases (vol 12, pp 112, 2007). Drug discovery today. 13, 824-824,10.1016/j.drudis.2008.06.003.

Uttenweiler, A., Schwarz, H., Neumann, H., Mayer, A., 2007. The vacuolar transporter chaperone (VTC) complex is required for microautophagy. Molecular biology of the cell. 18, 166-175,10.1091/mbc.E06-08-0664.

Vaccaro, M.I., 2012. Zymophagy: selective autophagy of secretory granules. International journal of cell biology. 2012, 396705,10.1155/2012/396705.

Van Den Heuvel, C., Donkin, J.J., Finnie, J.W., Blumbergs, P.C., Kuchel, T., Koszyca, B., Manavis, J., Jones, N.R., Reilly, P.L., Vink, R., 2004. Downregulation of amyloid precursor protein (APP) expression following post-traumatic cyclosporin-A administration. Journal of neurotrauma. 21, 1562-1572,10.1089/neu.2004.21.1562.

Velayutham, M., Hemann, C., Zweier, J.L., 2011. Removal of H2O2 and generation of superoxide radical: Role of cytochrome c and NADH. Free Radical Bio Med. 51, 160-170,10.1016/j.freeradbiomed.2011.04.007.

Vignot, S., Faivre, S., Aguirre, D., Raymond, E., 2005. MTOR-targeted therapy of cancer with rapamycin derivatives. Annals of Oncology. 16, 525-537,10.1093/annonc/mdi113.

Vilar, E., Perez-Garcia, J., Tabernero, J., 2011. Pushing the Envelope in the mTOR Pathway: The Second Generation of Inhibitors. Molecular cancer therapeutics. 10, 395-403,10.1158/1535-7163.MCT-10-0905.

Wander, S.A., Hennessy, B.T., Slingerland, J.M., 2011. Next-generation mTOR inhibitors in clinical oncology: how pathway complexity informs therapeutic strategy. Journal of Clinical Investigation. 121, 1231-1241,10.1172/JCI44145.

Wang, W., Guo, Z., Xu, Z., Meng, Q., Chen, C., Zhang, Y., Cao, X., 2015. Effect of pollen typhae on inhibiting autophagy in spinal cord injury of rats and its mechanisms. International journal of clinical and experimental pathology. 8, 2375-2383

Waters, R.L., Adkins, R.H., Yakura, J.S., 1991. Definition of Complete Spinal-Cord Injury. Paraplegia. 29, 573-581

Wei, Y.J., Pattingre, S., Sinha, S., Bassik, M., Levine, B., 2008a. JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. Molecular cell. 30, 678-688,10.1016/j.molcel.2008.06.001.

Wei, Y.J., Sinha, S., Levine, B., 2008b. Dual role of JNK1-mediated phosphorylation of Bcl-2 in autophagy and apoptosis regulation. Autophagy. 4, 949-951

Weidner, N., Ner, A., Salimi, N., Tuszynski, M.H., 2001. Spontaneous corticospinal axonal plasticity and functional recovery after adult central nervous system injury. Proceedings of the National Academy of Sciences of the United States of America. 98, 3513-3518,Doi 10.1073/Pnas.051626798.

Wells, J.E., Hurlbert, R.J., Fehlings, M.G., Yong, V.W., 2003. Neuroprotection by minocycline facilitates significant recovery from spinal cord injury in mice. Brain : a journal of neurology. 126, 1628-1637,10.1093/brain/awg178.

Whetstone, W.D., Hsu, J.Y., Eisenberg, M., Werb, Z., Noble-Haeusslein, L.J., 2003. Blood-spinal cord barrier after spinal cord injury: relation to revascularization and wound healing. Journal of neuroscience research. 74, 227-239,10.1002/jnr.10759.

Whitnall, L., McMillan, T.M., Murray, G.D., Teasdale, G.M., 2006. Disability in young people and adults after head injury: 5-7 year follow up of a prospective cohort study. J Neurol Neurosur Ps. 77, 640-645,10.1136/jnnp.2005.078246.

Wilkinson, J.E., Burmeister, L., Brooks, S.V., Chan, C.C., Friedline, S., Harrison, D.E., Hejtmancik, J.F., Nadon, N., Strong, R., Wood, L.K., Woodward, M.A., Miller, R.A., 2012. Rapamycin slows aging in mice. Aging Cell. 11, 675-682,10.1111/j.1474-9726.2012.00832.x.

Willemsen, A.E.C.A.B., Grutters, J.C., Gerritsen, W.R., van Erp, N.P., van Herpen, C.M.L., Tol, J., 2016. mTOR inhibitor-induced interstitial lung disease in cancer patients: Comprehensive review and a practical management algorithm. International Journal of Cancer. 138, 2312-2321,10.1002/ijc.29887.

Williams, M.D., Van Remmen, H., Conrad, C.C., Huang, T.T., Epstein, C.J., Richardson, A., 1998. Increased oxidative damage is correlated to altered mitochondrial function in heterozygous manganese superoxide dismutase knockout mice. Journal of Biological Chemistry. 273, 28510-28515,Doi 10.1074/Jbc.273.43.28510.

Wong, M., 2010. Mammalian target of rapamycin (mTOR) inhibition as a potential antiepileptogenic therapy: From tuberous sclerosis to common acquired epilepsies. Epilepsia. 51, 27-36,10.1111/j.1528-1167.2009.02341.x.

Woodcock, T., Morganti-Kossmann, M.C., 2013. The role of markers of inflammation in traumatic brain injury. Front Neurol. 4,Artn 18

10.3389/Fneur.2013.00018.

Wu, X.F., Kihara, T., Akaike, A., Niidome, T., Sugimoto, H., 2010. PI3K/Akt/mTOR signaling regulates glutamate transporter 1 in astrocytes. Biochemical and biophysical research communications. 393, 514-518,10.1016/j.bbrc.2010.02.038.

Wullschleger, S., Loewith, R., Hall, M.N., 2006. TOR signaling in growth and metabolism. Cell. 124, 471-484,10.1016/j.cell.2006.01.016.

Xie, Z.P., Klionsky, D.J., 2007. Autophagosome formation: Core machinery and adaptations. Nature cell biology. 9, 1102-1109,10.1038/ncb1007-1102.

Xiong, Y., Mahmood, A., Chopp, M., 2013. Animal models of traumatic brain injury. Nat Rev Neurosci. 14, 128-142,10.1038/nrn3407.

Xu, J., Kim, G.M., Chen, S., Yan, P., Ahmed, S.H., Ku, G., Beckman, J.S., Xu, X.M., Hsu, C.Y., 2001. iNOS and nitrotyrosine expression after spinal cord injury. Journal of neurotrauma. 18, 523-532,10.1089/089771501300227323.

Xu, W.M., Kang, C., Delgado, M., Perrin-Ninkovic, S.M., Papa, P.W., Riggs, J.R., Sapienza, J., Albers, R.J., Lee, B.G.S., Harris, R.L., Mortensen, D.S., Worland, P., Sankar, S., 2008. A Novel mTOR Kinase Inhibitor Causes Growth Inhibition, Cell Cycle Arrest, Apoptosis and Autophagic Cell Death in Mantle Cell Lymphoma Cell Lines: A Distinct Profile from Rapamycin. Blood. 112, 560-560

Yamamoto, S., Yamamoto, N., Kitamura, T., Nakamura, K., Nakafuku, M., 2001. Proliferation of parenchymal neural progenitors in response to injury in the adult rat spinal cord. Experimental neurology. 172, 115-127,10.1006/exnr.2001.7798.

Yan, P., Li, Q., Kim, G.M., Xu, J., Hsu, C.Y., Xu, X.M., 2001. Cellular localization of tumor necrosis factor-alpha following acute spinal cord injury in adult rats. Journal of neurotrauma. 18, 563-568,10.1089/089771501300227369.

Yang, H., Lu, P., McKay, H.M., Bernot, T., Keirstead, H., Steward, O., Gage, F.H., Edgerton, V.R., Tuszynski, M.H., 2006. Endogenous neurogenesis replaces oligodendrocytes and astrocytes after primate spinal cord injury. Journal of Neuroscience. 26, 2157-2166,10.1523/JNEUROSCI.4070-05.2005.

Yi, J.H., Hazell, A.S., 2005. N-acetylcysteine attenuates early induction of heme oxygenase-1 following traumatic brain injury. Brain research. 1033, 13-19,10.1016/j.brainres.2004.10.055.

Yin, X.M., Ding, W.X., Gao, W.T., 2008. Autophagy in the liver. Hepatology. 47, 1773-1785,10.1002/hep.22146.

Young, W., 1991. Methylprednisolone treatment of acute spinal cord injury: an introduction. Journal of neurotrauma. 8 Suppl 1, S43-46

Yuan, R.R., Kay, A., Berg, W.J., Lebwohl, D., 2009. Targeting tumorigenesis: development and use of mTOR inhibitors in cancer therapy. J Hematol Oncol. 2,Artn 45

10.1186/1756-8722-2-45.

Yue, Z., 2007. Regulation of neuronal autophagy in axon: implication of autophagy in axonal function and dysfunction/degeneration. Autophagy. 3, 139-141

Yune, T.Y., Kim, S.J., Lee, S.M., Lee, Y.K., Oh, Y.J., Kim, Y.C., Markelonis, G.J., Oh, T.H., 2004. Systemic administration of 17beta-estradiol reduces apoptotic cell death and improves functional recovery following traumatic spinal cord injury in rats. Journal of neurotrauma. 21, 293-306,10.1089/089771504322972086.

Zalckvar, E., Berissi, H., Mizrachy, L., Idelchuk, Y., Koren, I., Eisenstein, M., Sabanay, H., Pinkas-Kramarski, R., Kimchi, A., 2009. DAP-kinase-mediated phosphorylation on the BH3 domain of beclin 1 promotes dissociation of beclin 1 from Bcl-X-L and induction of autophagy. Embo Rep. 10, 285-292,10.1038/embor.2008.246.

Zaytseva, Y.Y., Valentino, J.D., Gulhati, P., Evers, B.M., 2012. mTOR inhibitors in cancer therapy. Cancer letters. 319, 1-7,10.1016/j.canlet.2012.01.005.

Zhang, H., Berel, D., Wang, Y., Li, P., Bhowmick, N.A., Figlin, R.A., Kim, H.L., 2013. A comparison of Ku0063794, a dual mTORC1 and mTORC2 inhibitor, and temsirolimus in preclinical renal cell carcinoma models. PloS one. 8, e54918,10.1371/journal.pone.0054918.

Zhang, H.F., Bosch-Marce, M., Shimoda, L.A., Tan, Y.S., Baek, J.H., Wesley, J.B., Gonzalez, F.J., Semenza, G.L., 2008. Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. Journal of Biological Chemistry. 283, 10892-10903,10.1074/jbc.M800102200.

Zhang, X.J., Li, L.A., Chen, S., Yang, D.H., Wang, Y., Zhang, X., Wang, Z., Le, W.D., 2011a. Rapamycin treatment augments motor neuron degeneration in SOD1(G93A) mouse model of amyotrophic lateral sclerosis. Autophagy. 7, 412-425,10.4161/auto.7.4.14541.

Zhang, Y.J., Duan, Y.W., Zheng, X.F.S., 2011b. Targeting the mTOR kinase domain: the second generation of mTOR inhibitors. Drug discovery today. 16, 325-331,10.1016/j.drudis.2011.02.008.

Zhang, Y.Q., Zhang, H.M., Shi, Y., Lustgarten, M., Li, Y., Qi, W.B., Zhang, B.X., Van Remmen, H., 2010. Loss of manganese superoxide dismutase leads to abnormal growth and signal transduction in mouse embryonic fibroblasts. Free Radical Bio Med. 49, 1255-1262,10.1016/j.freeradbiomed.2010.07.006.

Zhou, R.B., Yazdi, A.S., Menu, P., Tschopp, J., 2011a. A role for mitochondria in NLRP3 inflammasome activation. Nature. 469, 221-225,10.1038/nature09663.

Zhou, R.B., Yazdi, A.S., Menu, P., Tschopp, J., 2011b. A role for mitochondria in NLRP3 inflammasome activation (vol 469, pg 221, 2011). Nature. 475,10.1038/nature10156.