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Abstract

Parkinson´s Disease (PD) is the second most prevalent neurodegenerative brain disorder worldwide. Nevertheless, there is lack of certainty on the pathophysiology of the neurodegenerative mechanisms underlying PD. Several neurotoxins, rotenone among them, have been shown to induce parkinsonism-like brain degeneration and are widely used in cellular and animal models of PD.

In spite of an extensive association of PD with dopaminergic neuron degeneration of the *substantia nigra pars compacta*, other brain areas like the cerebellum have been more recently implicated in the pathology of the disease. Therefore, we used a rotenone/cerebellar granule neurons (CGN) model to study not only the potential of creatine (as ergogenic compound), epicatechin and kaempferol (as antioxidant compounds) to afford neuroprotection against rotenone neurotoxicity but also to better understand the cellular mechanisms underlying this neurotoxicity.

Our results revealed a strong protection by creatine against rotenone-induced CGN death, while kaempferol did not afford a significant protection and epicatechin elicited at most a very weak protection. On the contrary, kaempferol also antagonized the protective effect of creatine. These results lend support to the potential use of creatine in PD therapeutics, and alert for the avoidance of the consumption of foods or infusions with high content in kaempferol.

Furthermore, we noted that rotenone triggered an energetic failure in CGN as a primary event, supported not only by the protection afforded by creatine but also by a deregulation in calcium homeostasis thought voltage operate calcium channels type L and N-Methyl-D-aspartate receptors stimulation and store-operated calcim entry inhibition, by the elevation of AMP-kinase active levels and by mitochondrial membrane depolarization. Rotenone also promoted an enhanced production of reactive oxygen species and a weak nitrosative stress, but only as a later event in the development of CGN death.

In conclusion, our results support a role for creatine in affording neuroprotection against rotenone neurotoxicity, through a mechanim that prevent an energetic failure.

Keywords: Parkinson´s disease; cerebellar granule neurons; rotenone; creatine; epicatechin; kaempferol; energetic failure.

Resumo

A doença de Parkinson é a segunda doença neurodegenerativa mais prevalente a nível mundial. Apesar disso, são ainda pouco conhecidos os mecanismos neurodegenerativos que estão por detrás desta doença. Varias neurotoxinas, nas quais se inclui a rotenona, têm vindo a ser demonstradas como indutoras de degenerescência cerebral de tipo-Parkinsonismo e são, por isso, vastamente utilizadas em modelos celulares e animais da doença de Parkinson.

Apesar da extensiva associação entra a doença de Parkinson e a degenerescência dos neurónios dopaminérgicos da *substantia nigra pars compacta*, mais recentemente, outras áreas cerebrais, nomeadamente o cerebelo, têm sido implicadas na patogénese da doença. Portanto, neste trabalho, foi utilizado um modelo celular de neurónios granulares do cerebelo expostos à neurotoxina rotenona. Este modelo foi utilizado, não só para estudar a capacidade da creatina (como composto ergogénico) e de epicatequina e kaempferol (como antioxidantes) em proteger contra a neurotoxicidade despoletada pela rotenona, mas também para melhor entender os mecanismos subjacentes a esta neurotoxicidade.

Os resultados obtidos revelaram que a creatina apresenta uma elevada protecção contra a morte celular, induzida pela rotenona nos neurónios granulares do cerebelo, enquanto o kaempferol não ofereceu qualquer protecção e a epicatequina, por sua vez, promoveu uma protecção demasiado fraca. Pelo contrário, o kaempferol demostrou um efeito antagónico relativamente à creatina. Os resultados suportam, assim, o potencial uso da creatina na terapêutica da doença de Parkinson e alertam para que se evite o consumo de comidas e infusões com um elevado conteúdo em kaempferol nesta mesma terapêutica.

Para além disso, os nossos resultados revelaram que a rotenona como evento primário conduz a uma falência energética nos neurónios granulares do cerebelo. Esta conclusão é suportada, não só pela protecção exercida pela creatina, mas também pela observação de uma desregulação nos níveis citosólicos de cálcio através de uma estimulação dos canais de cálcio do tipo L operados por voltagem e dos receptores N-Metil-D-aspartato e pela inibição da entrada capacitativa de cálcio; pelo aumento dos níveis da AMP-quinase activa e ainda pela despolarização da membrana mitocondrial.

A rotenona promoveu, por fim, a produção de espécies reactivas de oxigénio e um stresse nitrosativo fraco, no decurso da morte dos neurónios granulares do cerebelo.

Em suma, os resultados suportam a utilização da creatina como composto neuroprotector contra a neurotoxicidade exercida pela rotenona, através da prevenção da ocorrência de uma falência energética.

Palavras-chave: Doença de Parkinson; neurónios granulares do cerebelo; rotenona; creatina; epicatequina; kaempferol; falência energética.

Table of contents

Abbreviations, acronyms and symbols

$[Ca^{2+}]_i$	Intracellular calcium concentration
$\Delta\psi m$	Mitochondrial membrane potential
6-OHDA	6-hydroxydopamine
ADP	Adenosine diphosphate
AMC	7-amino-4-methyl-coumarin
AMPK	Adenosine monophosphate-protein kinase
ATP	Adenosine triphosphate
BB-CK	Cytosolic brain-type creatine kinase
Ca^{2+}	Calcium
CGN	Cerebellar granule neurons
CK	Creatine kinase
CPA	Cyclopiazonic acid
CTC	Cerebellothalamocortical
CyCK	Cytosolic form of creatine kinase
DA	Dopaminergic
DCF	2',7'-Dichlorofluorescein
DIV	Days in vitro
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
fMRI	Functional magnetic resonance imaging
fura-2 AM	Fura-2-acetoxymethyl ester
GSH	Glutathione
H_2DCFDA	2',7'-Dichlorodihydrofluorescein diacetate
HD	Huntington's disease
L-DOPA	Levodopa
L-VOCC	Voltage operate calcium channel type L
MCB	Monochlorobimane
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

mPTP	Mitochondrial permeability transition pore
MtCK	Mitochondrial creatine kinase
MTT	3-(4,5-dimethyl-thiazole-2yl)-2,5-diphenyltetrazolium bromide
Na^+	Sodium
Na^+/K^+-ATPase	Sodium potassium ATPase
$NADP^+$	Oxidized nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NCX	Na^+/Ca^{2+} exchanger
NMDA	N-Methyl-D-aspartate
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCr	Phosphocreatine
PD	Parkinson's disease
PET	Positron emission tomography
PI	Propidium iodide
PMCA	Plasma membrane Ca^{2+} ATPase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SERCA	Ca^{2+}-ATPase of sarco-endoplasmic reticulum
sMtCK	Mitochondrial sarcomeric muscle form of creatine kinase
SNc	*Substantia nigra pars compacta*
SOCE	Store-operated calcim entry
SPECT	Single-photon emission computed tomography
TG	Thapsigargin
TMRE	Tetramethylrhodamine, ethyl ester
TMS	Transcranial magnetic stimulation
TRPC-1	Transient receptor potential C1
uMtCK	Mitochondrial ubiquitous brain form of creatine kinase
VOCC	Voltage-operated calcium channels
α-syn	α-synuclein

I.Introduction

1.1.Parkinson´s disease

Parkinson's disease (PD) is a neurodegenerative disorder characterized, in part, by motor disturbances, including tremor, rigidity, bradykinesia, postural instability, and rest tremor originating from loss of dopaminergic (DA) neurons of the *substantia nigra pars compacta* (SNc) (Dauer and Przedborski, 2003). In the later stages of the disease autonomic and sensorimotor dysfunction, cognitive decline, depression and sleep disturbances also occur and become clinically relevant (Marsden, 1990). Non-motor functional deficits often precede the major motor symptoms by a number of years and it has been suggested that they are indicative of neurodegeneration that originates in the brain stem and progresses throughout the brain (Braak et al., 2003).

PD is the second most prevalent neurodegenerative brain disorder, affecting 1 to 2% of the population above 65 years of age and its prevalence increases to approximately 4% in individuals above 85 years of age (Bekris et al., 2010; de Rijk et al., 2000).

PD pathology is not restricted to the DA system; degeneration of cholinergic, serotinergic, noradrenergic, peptidergic and DA brainstem nuclei, together with the presence of proteinaceous intraneuronal inclusions in the soma or dendrites (termed Lewy bodies and Lewy neuritis respectively) of neurons in the central, autonomic and enteric nervous system (Braak et al., 2003; Braak et al., 2006; Braak et al., 2007; Hornykiewicz, 1975; Jellinger, 1991; Klos et al., 2006) have been implicated in disease´s pathology. In fact, in the last decades the traditional focus on neurons has been changed. Indeed, it is increasingly recognized that degenerating neurons in PD, such as DA neurons of the nigrostriatal pathway, do not survive after isolation. These neurons receive a variety of afferents and are surrounded by a large number of non-dopaminergic neurons like GABAergic and cholinergic neurons and non-neuronal cells such as astrocytes and microglia. Thus, it is the current belief that the neurodegeneration in PD occurs in response to a mixture of deleterious mechanisms taking place both inside and outside of degenerating neurons.

While a fraction of PD occurrence is related to mutations in genes, over 90% of PD is sporadic, that is, it occurs in the absence of any obvious genetic linkage. Many evidences suggest that there is not much difference between the sporadic and the rare familial forms of PD. Specific mutations in nuclear genes encoding α-synuclein (α-syn), DJ-1, LRRK2, PINK1, and parkin as well as within the mtDNA were identified in familial forms of PD, giving the possibility, by manipulating their expression levels in

cellular and animal models, to investigate their physiological function and early pathogenic changes that may lead to neurodegeneration (reviewed in (Cali et al., 2012)).

The most significant pathological features of PD are mitochondrial dysfunction, oxidative stress (Dexter et al., 1994; Jenner and Olanow, 1998), altered protein handling, and inflammatory response (Hirsch et al., 1998; McGeer et al., 1988a; McGeer et al., 1988b), which are considered to lead to cell dysfunction and death mainly by apoptosis or autophagy (Schapira and Jenner, 2011).

1.1.1. The involvement of cerebellum in PD

As mentioned before, the pathology of PD is not restricted to DA degeneration. Recent studies have implicated other cerebral zones and because of the relevance of this work we highlight the cerebellum.

The cerebellum is a structure located in the posterior fossa of the skull, dorsal to the pons and substantia nigra (Figure 1.1). Although the cerebellum accounts for approximately 10% of the brain's volume, it contains over 50% of the total number of neurons in the brain (Larsell, 1947). The cerebellum is involved in several functions related to movement such as maintenance of balance and posture; coordination of voluntary movements; motor learning and in certain cognitive functions, such as language. Although motor commands are not initiated in the cerebellum; rather, the cerebellum modifies the motor commands of the descending pathways to make movements more adaptive and accurate (Knierim, 2012).

Recently, the application of medical techniques such as transcranial magnetic stimulation (TMS), single-photon emission computed tomography (SPECT), positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) have demonstrated the occurrence of significative alterations in the cerebellum of PD and Parkinsonism patients (Brockmann et al., 2012; Cao et al., 2011; Kimura et al., 2011; Koch et al., 2009; Ni et al., 2010; Wu et al., 2009a; Wu et al., 2009b).

Ni et al, (2012) applying TMS in the cerebellum verified decreased excitability of the cerebellothalamocortical (CTC) pathway in PD. The authors concluded that the CTC pathway is involved in the generation or transmission of postural tremor in PD.

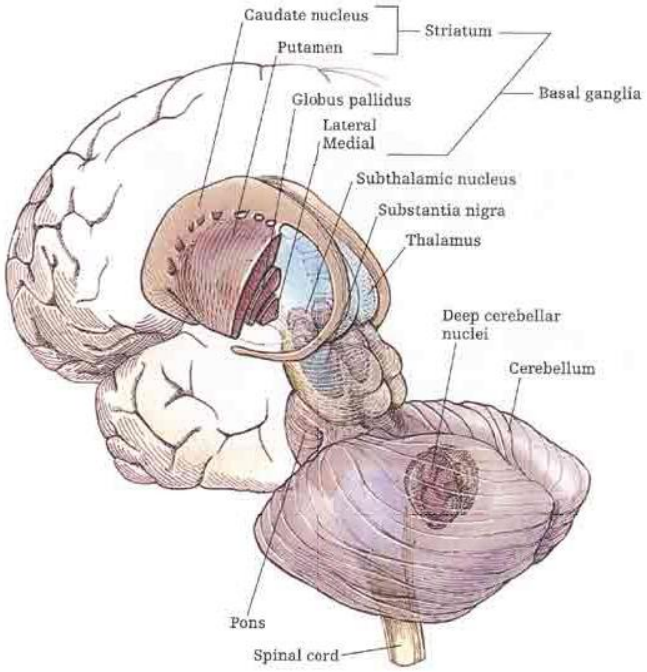

Figure 1.1: Schematic representation of specific anatomy areas of the brain.

Other authors (Kimura et al., 2011) compared the regional cerebral blood flow of patients with different types of Parkinsonism with PD patients and controls. They observed a decreased regional cerebral blood flow in the cingulate gyrus and thalamus in progressive supranuclear palsy patients, whereas Parkinson variant of multiple system atrophy showed decreased regional cerebral blood flow in the cerebellum. These findings suggest that parkinsonian disorders show a distinct SPECT pattern in the frontal cortex, thalamus, and cerebellum. Therefore these measurements may be helpful in screening for the differential diagnosis of parkinsonian syndrome.

Since resting state brain activity in PD can give clues to the pathophysiology of the disorder, Wu et al (2009a) used a regional homogeneity method to investigate PD-related modulations of neural activity in the resting state. The authors verified a decreased regional homogeneity in extensive brain regions, including the putamen, thalamus, and supplementary motor area; and increased in some other areas, including the cerebellum, primary sensorimotor cortex, and premotor area. Later the same authors (Wu et al., 2009b) concluded that PD patients at off-state had significantly decreased functional connectivity in the supplementary motor area, left dorsal lateral prefrontal cortex and left putamen, and had increased functional connectivity in the left

cerebellum, left primary motor cortex, and left parietal cortex compared to normal subjects. The authors concluded that a disrupted pattern of functional connectivity of the motor network in PD is an important factor contributing to some motor deficits in PD, such as akinesia.

1.1.2. Neutoxin models of PD

The possible involvement of oxidative stress/mitochondrial dysfunction as an etiological factor of PD is further supported by studies with specific neurotoxins that are extremely potent inducers of Parkinsonism in humans and animals. The best studied of these toxins are 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium (MPP+), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which selectively destroys catecholaminergic neurons. Both toxins have been shown to generate hydroxyl radicals in the caudate of treated animals (reviewed in (Cannon and Greenamyre, 2010)). The rotenone model has also been used to study PD; rotenone is a pesticide and a complex I inhibitor, which induces DA cell loss in cell culture (Testa et al., 2005) and in animal models (Alam and Schmidt, 2002; Betarbet et al., 2000). An inhibition of mitochondrial complex I by rotenone may not only enhance reactive oxygen species (ROS) production but also lead to mitochondrial dysfunction, such as a decrease in adenosine triphosphate (ATP) production and mitochondrial membrane depolarization (Barrientos and Moraes, 1999; Li et al., 2003; Sherer et al., 2003a). Complex I has been suggested as a strong modulator of the mitochondrial permeability transition pore (mPTP), which is responsible for a critical step in the mitochondria-dependent apoptotic pathway (Batandier et al., 2004; Chauvin et al., 2001; Fontaine et al., 1998; Fontaine and Bernardi, 1999). The mPTP is a complex and large conductance channel; its opening provokes mitochondrial membrane depolarization, release of cytochrome c and sequential activation of caspases that eventually lead to apoptotic cell death (Green and Reed, 1998). In addition, rotenone can cause PD type neuropathology and movement abnormalities (Hoglinger et al., 2006; Sherer et al., 2003a; Sherer et al., 2003b).

1.1.2.1. Cerebellar granule neurons as Parkinsonism model

The cerebellum is essential for fine motor control of movement and posture, and its dysfunction disrupts balance and impairs control of speech, limb and eye

movements. The developing cerebellum consists mainly of three types of neuronal cells: granule cells in the external germinal layer, Purkinje cells, and neurons of the deep nuclei (Figure 1.2).

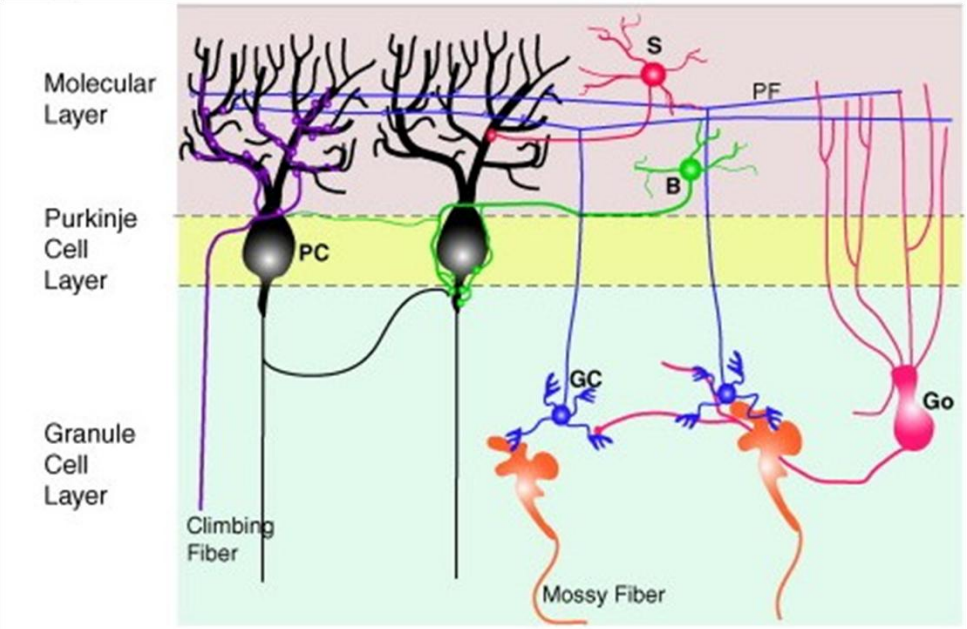

Figure 1.2: Cellular organization of the cerebellar cortex. The cerebellar cortex has only three layers. The molecular layer contains two main types of interneurons, the basket (B) and stellate (S) cells. The Purkinje cell layer contains the cell bodies of Purkinje cells (PC), whose dendrites arborize in the molecular layer; these are the only neurons projecting out of the cerebellar cortex. The deepest layer, the granule cell layer, contains the cell bodies of granule cells (GC) and other interneurons, such as Golgi cells (Go), Lugaro cells and unipolar brush cells (not depicted here). Granule cells are glutamatergic interneurons that extend a single axon through the internal granular layer (IGL), and Purkinje cell layer (PCL) that splits into two branches, the parallel fibers (PF), in the molecular layer, forming synaptic contacts on Purkinje cell dendritic spines. There are two main types of extracerebellar axons projecting to the cerebellar cortex: the mossy fibers that contact the granule cells and have several origins in the hindbrain and spinal cord, and the climbing fibers that project to Purkinje cells and originate from the inferior olivary nucleus in the brainstem (adapted from (Chedotal, 2010)).

Granule cells of the cerebellum are the largest homogeneous neuronal population of mammalian brain (Chambers and Sprague, 1955a; Chambers and Sprague, 1955b; Saab and Willis, 2003). Due to their postnatal generation and the feasibility of well characterized primary *in vitro* cultures, cerebellar granule neurons (CGN) are well accepted as a model for the study of cellular and molecular correlates of neurodegeneration/neuroprotection (Contestabile, 2002).

These glutamatergic neurons are extremely sensitive to calcium (Ca^{2+}) deregulation, in particular mediated by voltage operate Ca^{2+} channel type L (L-VOCC)

activity (Franklin and Johnson, 1992; Franklin and Johnson, 1994). The major role in maintaining cytosolic Ca^{2+} concentrations within the optimal range for the survival and normal excitability of CGN in culture is by Ca^{2+} influx through L-VOCC (Franklin and Johnson, 1992; Franklin and Johnson, 1994; Gutierrez-Martin et al., 2005).

CGN have been widely used as PD model. Rotenone has been shown to induce cell death of CGN (Isaev et al., 2004). As little as 5 nM rotenone increased mitochondrial superoxide levels and potentiated glutamate-induced cytosolic Ca^{2+} deregulation, the first irreversible stage of necrotic cell death (Yadava and Nicholls, 2007).

Although 6-OHDA is a neurotoxin specific for catecholamine neurons in both the central and peripheral nervous systems, cultured rat CGN have been demonstrated to be another useful *in vitro* system for studying the mechanism of 6-OHDA-induced neurotoxicity as a PD model. Relatively low concentrations (μM) of 6-OHDA induce apoptosis of CGN via activation of a caspase-3-like proteases (Dodel et al., 1999). In parallel, 6-OHDA was also demonstrated to produce free radicals in these neurons (Lin et al., 2003) as well as enhance excitation of CGN by glutamate (Garber-Goldsman et al., 1986).

On these grounds, CGN have acquired a special position in modern neuroscience as one reliable model for the study of neural development, function and, in particular for this work, PD pathology.

1.1.3. Cell death in PD

There are several mechanisms by which cells could die. Until Kerr first used the term apoptosis (Kerr et al., 1972), all cells were thought to die by necrosis. Necrosis is characterized by cytoplasmic swelling, irreversible plasma membrane damage, and organelle breakdown (Fiers et al., 1999; Grooten et al., 1993). DNA in necrotic cells is usually degraded randomly by extracellular DNAse I present in culture serum that has not been heat-inactivated (Napirei et al., 2004), or by lysosomal DNAse II (Tsukada et al., 2001), giving rise to a smear of DNA (Higuchi, 2003). The cellular contents leak into the extracellular environment, where they may act as a "danger signal", and consequently necrosis is usually associated with inflammation (Proskuryakov et al., 2003). In addition, there is now sufficient evidence that necrosis, a process traditionally regarded as "passive," may also be genetically programmed. After signalling- or

damage-induced lesions, necrosis can include signs of controlled processes such as mitochondrial dysfunction, enhanced generation of ROS, ATP depletion, proteolysis by calpains and cathepsins, and early plasma membrane rupture. Furthermore, the inhibition of specific proteins involved in regulating apoptosis or autophagy can shift the type of cell death to necrosis (Festjens et al., 2006; Golstein and Kroemer, 2007).

For a long time the term "programmed cell death" (PCD) was synonymous of apoptosis. Today is known that apoptosis (or Type I cell death) is merely one type of PCD. Apoptosis is the major cell death pathway used to remove unwanted and harmful cells in a "clean" manner (Ellis et al., 1991), as during apoptosis the membrane integrity remains intact with the contents of the dying cell enclosed within apoptotic bodies, without releasing them into the extracellular space. DNA in apoptotic cells is degraded specifically, giving rise to a characteristic laddering pattern on the gels (Higuchi, 2003; Napirei et al., 2004; Tsukada et al., 2001).

Another type of PCD, autophagic cell death (or Type II cell death), has received a significant attention from PD researchers in the past few years. Autophagy is recognized by the formation of autophagosomes, double membrane autophagic vacuoles that eventually fuse with lysosomes to form autolysosomes. Swallowed contents and the inner membrane of the autophagosome are subsequently degraded by lysosomal hydrolases (Levine and Klionsky, 2004). Various forms of environmental stress induce autophagy, which eventually results in either caspase-dependent (Guimaraes et al., 2003; Xue et al., 1999) or caspase-independent cell death (Gozuacik and Kimchi, 2004; Xue et al., 1999; Yanagisawa et al., 2003).

Nevertheless the same stimulus can trigger multiple cell death pathways, depending on its intensity and duration, brain region and cell type, and also depending on the bioenergetics state of the cell (Eguchi et al., 1997; Young et al., 2004). All these three types of cell death have been identified in PD, predominating apoptosis (Kostrzewa, 2000) and autophagy (Anglade et al., 1997; Schapira and Jenner, 2011).

1.2.Brain bioenergetics alterations in PD

Cellular mechanisms regulating energy utilization must function properly to sustain the cell's life. Dysfunction in mitochondria has been widely associated with PD. Therefore, and because mitochondria plays a central role in energy production, bioenergetics alterations have been associated with PD.

The shortage of mitochondrial supply of ATP and other energy-rich metabolites could be counterbalanced by cellular enzymes such as creatine kinase (CK) and adenosine monophosphate-protein kinase (AMPK), especially in locations of high energy consumption like the cytosol.

1.2.1. Creatine Kinase

CK is an enzyme that rapidly catalyses the conversion of creatine and consumes ATP to produce phosphocreatine (PCr) and adenosine diphosphate (ADP), being an important enzyme in producing and buffering energy stores in excitable cells (Schlattner et al., 2006).

In fact, large amounts of energy are required to maintain the signalling activities of the cells in the central nervous system. Energy consumption in the brain is largely due to the maintenance of brain function-related processes, for example, for maintenance of membrane potential by the sodium potassium ATPase (Na^+/K^+-ATPase), Ca^{2+} homeostasis by the Ca^{2+}-ATPase, neurotransmitters synthesis, secretion and recycling, intracellular signalling, and axonal as well as dendritic transport (Ames, 2000). Indeed, it has been proposed that mechanisms to facilitate energy transfer within cells that require fluctuating high energy levels, such as brain, include the juxtaposition of intracellular sites of ATP generation with sites of ATP consumption, as well as the transfer of high-energy phosphates between these sites by the CK/PCr system (Burklen et al., 2006).

The PCr/CK energy pathway represents an extremely efficient energy buffering system for two major reasons. First, PCr has a slightly higher diffusion capacity than ATP, allowing for a more efficient energy delivery system to different subcellular locations. Second, the subcellular localization of cytosolic and mitochondrial CK couples areas of high energy consumption with energy production. Thus, the CK/PCr essentially serves as a spatial ''energy shuttle'' or energy circuit within the cell (Wallimann et al., 1992).

There are four isoforms of CK based on its tissue expression (muscle or brain) and subcellular distribution (cytosolic or mitochondrial). In the brain, the dimeric cytosolic form of CK (CyCK) is called brain-type CK (BB-CK). The octameric mitochondrial CK (MtCK) is classified into two forms: sarcomeric muscle form (sMtCK) and brain form called ubiquitous MtCK (uMtCK) (Booth and Clark, 1978; Schlattner et al., 2006). Both

MtCKs are located in the mitochondrial intermembrane space, along the entire inner membrane and also at peripheral contact sites where inner and outer membranes are in close proximity (Biermans et al., 1990). There, MtCK can directly transphosphorylate intramitochondrially produced ATP into PCr (Jacobus, 1985), which is then exported into the cytosol (Figure 1.3).

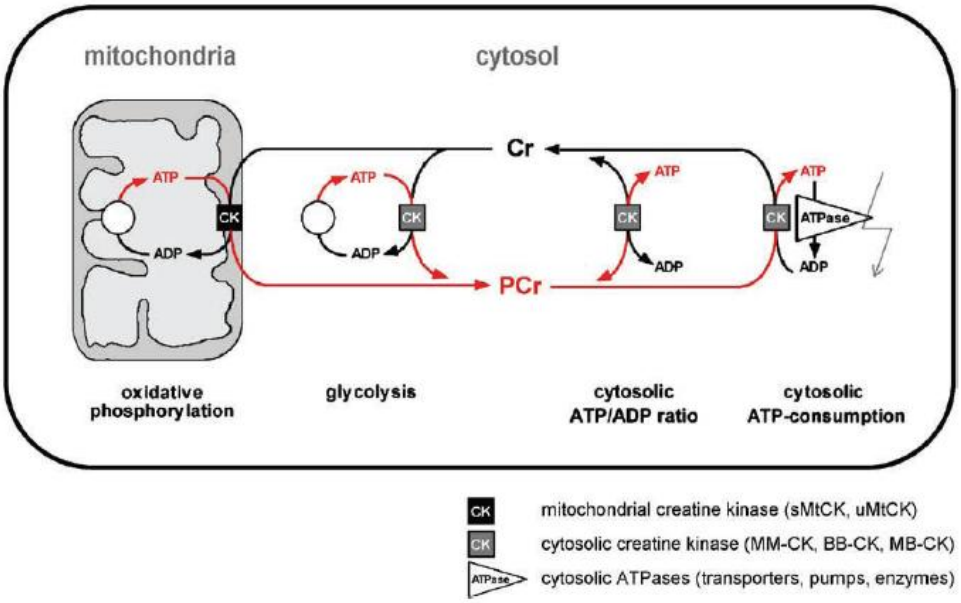

Figure 1.3: The creatine kinase/phosphocreatine system. Compartment-specific isoenzymes of creatine kinase (CK) are found in mitochondria (sMtCK, uMtCK, left) and cytosol (BB-CK, right). They are either associated with ATP-delivering processes (oxidative phosphorylation or glycolysis, left) and ATPconsuming processes (ATPases, to maintain local ATP/ADP ratios, right), or occur in soluble form (to maintain global cytosolic ATP/ADP ratios, center right). A large cytosolic phosphocreatine (PCr) pool of up to 30 mM is built up by CK from creatine (Cr), using ATP from oxidative phosphorylation (e.g., in heart) or glycolysis (e.g., in fast-twitch glycolytic muscle). The large phosphocreatine pool is then used as a temporal energy buffer to maintain constant global and local ATP/ ADP ratios over a wide range of workloads. The higher diffusibility of phosphocreatine, as compared to ATP, together with localized CK isoenzymes, is used for spatial energy buffering, i.e., for an energy shuttle between ATP-providing or-consuming processes. The latter seems to be most important for cells that are polarized and/or have very high or localized ATP consumption. Adapted from (Schlattner et al., 2006).

CK isoenzymes are highly susceptible to oxidative stress, an important pathophysiological condition associated with the progress of neurodegeneration in PD (Schlattner et al., 2006; Wang et al., 2001). In this scenario, MtCK has been suggested to be more vulnerable than CyCK due to its mitochondrial localization (Koufen and Stark, 2000). In line with this, it has been demonstrated a compromised CK system in

common neurodegenerative diseases (Aksenov et al., 2000; Ferrante et al., 2000; Wendt et al., 2002).

1.2.1.1. Creatine

Creatine is a constituent of a normal diet of protein-based foods, such as meat, milk, and nuts. It is not considered an essential nutrient, as kidneys, liver, pancreas, and possibly brain cells are able to synthesize this compound endogenously from the amino acids arginine, glycine, and methionine (Andres et al., 2008; Beard and Braissant, 2010; Wyss and Kaddurah-Daouk, 2000).

The primary physiological function of creatine is to buffer energy concentrations in tissues with significant and fluctuating energy demands, especially in muscles and brain (Wyss and Schulze, 2002). Until recently, the interest in creatine has been largely centered primarily on its use as an ergogenic aid to enhance sports performance (Benzi, 2000; Froiland et al., 2004). Nevertheless, it is becoming increasingly evident that administration of creatine might be beneficial in diseases with impaired bioenergetics like PD (Andreassen et al., 2001b; Baker and Tarnopolsky, 2003; Bender et al., 2005; Bender et al., 2006; Bender et al., 2008a; Brustovetsky et al., 2001; Burklen et al., 2006; Shefner et al., 2004).

Creatine produced dose-dependent neuroprotective effects against MPTP toxicity, reducing the loss of dopamine within the striatum and the loss of DA neurons in the SNc (Matthews et al., 1999). In addition, it has been shown that creatine may stabilize MtCK, and prevent activation of the mPTP (O'Gorman et al., 1997).

Due to its neuroprotective effects, creatine is now in clinical trials for the treatment of PD and Huntington's disease (HD) (Hersch et al., 2006; NINDS-NET-PD, 2006). A placebo-controlled randomized pilot trial of creatine supplementation in PD showed that creatine improved patient mood and led to a smaller dose increase of dopaminergic therapy but had no effect on overall Unified Parkinson's Disease Rating Scale scores or dopamine transporter SPECT (Bender et al., 2006). However, a phase II futility trial in PD showed approximately a 50% improvement in Unified Parkinson's Disease Rating Scale at one year, and the compound was judged to be non futile. Due to this finding, creatine is now under further investigation in a phase III clinical trial by the NET-PD investigators (NINDS-NET-PD, 2006).

More recently, oral creatine supplementation showed to attenuate levodopa (L-DOPA)-induced dyskinesia, a motor complication that arises in Parkinson patients after a chronic treatment with L-DOPA, in 6-OHDA-lesioned rats (Valastro et al., 2009). However, the molecular mechanisms underlying the neuroprotective actions of creatine are under current scientific debate. For example, stimulation by creatine of hippocampal Na^+,K^+-ATPase activity via N-Methyl-D-aspartate (NMDA)–calcineurin pathway is a very recent novel finding (Rambo et al., 2012), suggesting that the role of creatine in neuronal bioenergetics modulation can be more complex than currently assumed.

The safety of creatine supplementation has been reviewed extensively, and it has been concluded that creatine supplementation does not have any deleterious effects in humans (Bender et al., 2008b; Hersch et al., 2006; Mihic et al., 2000). These evidences clearly show the potential of creatine in PD's therapy and in other neurological diseases.

1.2.2. AMP-Kinase

AMPK is a heterotrimeric complex of a catalytic subunit (α) and two regulatory subunits (β and γ). AMPK is activated by phosphorylation of the α subunit at Thr172 (Hawley et al., 1996), being the downstream component of a protein kinase cascade. Once activated, AMPK switches-on catabolic pathways that generate ATP, while switching-off energy-consuming anabolic processes, thereby, acting as a key cellular modulator for the maintenance of the energy balance within cells. AMPK activation has been demonstrated to be regulated not only by cellular AMP/ATP ratio, but also by Ca^{2+} concentration and ROS (Hawley et al., 2005; Jung et al., 2008; Park et al., 2006; Weekes et al., 1994). When activated, AMPK phosphorylates several intracellular protein targets, like acetyl-CoA carboxylase, nitric oxide synthase and peroxisome proliferator-activated receptor gamma coactivator-1-α (Chen et al., 2000; Fryer et al., 2000; Suwa et al., 2003).

It has been reported that AMP-activated protein kinase is also activated by MPTP and other investigators have shown that overexpression of AMPK increased cell viability (Choi et al., 2010; Culmsee et al., 2001). These and other studies suggest that activation of AMPK may prevent neuronal cell death in neurodegenerative disease like PD. This possibility is also supported by recently published experimental data. Wu et al (2011) have demonstrated that AMPK and/ or mammalian silent information regulator 2 are required in resveratrol mediated autophagy induction, leading to neuronal survival

on PD cellular models. On the other hand, Arsikin et al (2012) have demonstrated that the neurotoxin 6-OHDA induces cytotoxic autophagy in SH-SY5Y neuroblastoma cells through the oxidative stress-dependent activation of AMPK and subsequent inhibition of the main autophagy repressor mTOR. Thus, these studies reveal that depending on the cellular insult the activation of AMPK can play either a neuroprotector or neurotoxic role.

1.3. Oxidative stress in the brain

The imbalance between normal cellular and environmental-induced production of ROS and reactive nitrogen species (RNS) and the ability of cells to efficiently defend against them (by the natural antioxidant defence system) is called oxidative stress. Oxidative stress produced in the body is toxic, could damage DNA, lipids and proteins leading to necrosis, ATP depletion, and prevention of controlled apoptotic death (Beal, 2005).

Since the brain uses 20% of the inspired oxygen and 80-90% of the consumed oxygen to produce energy during oxidative phosphorylation and the cefaloraquid liquid has lower antioxidant potency than the blood plasma, it is not surprising that neuronal cells are particularly sensitive to oxidative stress. During oxidative phosphorylation, neurons in the brain are extremely vulnerable to oxidative damage because of their high metabolic activity, low antioxidant capacity (Nunomura et al., 2006), non-replicative nature and the presence of high levels of polyunsaturated fatty acids which are readily oxidized (Perry et al., 2002). Additional chalenge is the presence of the blood-brain barrier, which protects the brain from toxins by limiting their diffusion into neurons and glia but also prevents/reduces the uptake of some blood-circulating antioxidants like vitamin E, into the brain. In this regard, it is to be noted that neurons may be exposed to ROS/RNS produced intracellularly and extracellularly, respectively, by neuronal cells and non-neuronal cells, such as microglia and endothelial cells of blood vessels.

1.3.1. Oxidative stress in PD

The etiology of PD is unknown although mitochondrial dysfunction, oxidative and nitrosative stress have been implicated in the mechanisms associated with PD pathogenesis (Danielson and Andersen, 2008; Dawson and Dawson, 2003; Fahn and Cohen, 1992; Fiskum et al., 2003; Hashimoto et al., 2003; Jenner, 2003; Kanthasamy et

al., 2010; Tsang and Chung, 2009). However, it is not clear whether oxidative stress is a consequence of dysfunctional and dying neurons or plays a causal role in this neurodegenerative disease.

Postmortem tissues from PD patients have provided experimental evidences supporting that a deficient function of complex I of the mitochondrial electron-transport chain in SNc, i.e. 30–40% decrease in its activity, may be one of the central causes of sporadic PD (Dawson and Dawson, 2003). The decreased activity could be due to underproduction of certain complex I subunits, complex I disassembly, or self-damage by ROS produced during its function (Beal, 2004; Keeney et al., 2006; Schapira, 2001).

More evidences of oxidative stress and PD come from examination of human PD brain showing oxidative damage to DNA and protein (protein carbonyls) besides immunocytochemical evidences for oxidative modifications of proteins by nitration, nitrosylation and glycation (Alam et al., 1997; Beal, 2002; Brown and Borutaite, 2004; Castellani et al., 1996; Floor and Wetzel, 1998; Good et al., 1998; Kikuchi et al., 2002; Zhang et al., 1999), and also by increased levels of lipid peroxidation (Dalfo et al., 2005). Along with the increase in oxidative damage, decreased levels of the antioxidant glutathione (GSH) were also found in the SNc of PD patients (Pearce et al., 1997; Perry et al., 1982; Perry and Yong, 1986).

1.3.1.1. Flavonoids in PD: Kaempferol and Epicatechin

In the last decade flavonoids have been described as important neuroprotectors against PD (Gagne et al., 2003; Pan et al., 2003; Panickar et al., 2009; Tan et al., 2008; Vauzour et al., 2010). This neuroprotection has been mainly attributed to their antioxidant and anti-inflammatory capacities (Aquilano et al., 2008; Dajas et al., 2003; Gutierrez-Merino et al., 2011). In the present study, taking into account the experimental data pointing out the occurrence of oxidative stress in the brain of patients with PD, we have studied the ability of the flavonoids kaempferol and epicatechin to afford protection against rotenone-induced neuronal death.

Kaempferol is a natural flavonoid that exists in propolis (Scheller et al., 1990), tea (Tsaknis and Lalas, 2005), Gingko biloba (Smith and Luo, 2003) and other plant sources, which have been considered as a potent antioxidant and anti-inflammatory compound (Comalada et al., 2006; Martini et al., 2004; Wang et al., 2006). Ginkgo biloba extracts, quercetin and kaempferol have been reported to rescue PC12 cells

exposed to MPP+-induced oxidative stress and also to reverse neurotoxin effect (Gagne et al., 2003). More recently, the neuroprotective effect of kaempferol against MPTP was confirmed in a mouse model (Li and Pu, 2011). Kaempferol derivatives have also been recognized with protective potential in PD; they have been shown to prevent oxidative stress–induced cell death in a genetic (DJ-1)–dependent manner (Qu et al., 2009). Very recently, kaempferol was implicated in the enhancement of mitochondrial turnover by autophagy, revealing to be antiapoptotic and antioxidant in rotenone models of PD (Filomeni et al., 2012).

Epicatechin is a catechin present in cocoa (Dreosti, 2000; Sanchez-Rabaneda et al., 2003), green tea (Graham, 1992) and red wine (Damianaki et al., 2000). This flavonoid has been widely demonstrated to act as a cellular antioxidant, free radical scavenger and iron chelating (Lee et al., 2003; Pan et al., 2003; Xu et al., 2004), being neuroprotector in PD, not only by itself but also as a constituent of the mentioned sources of polyphenols (Datla et al., 2007; Gao et al., 2012; Levites et al., 2001). For instance, green tea polyphenols have demonstrated to elicit neuroprotection in cell cultures and animal models, such as the prevention of neurotoxin-induced neurodegeneration of the striatum and DA neurons of the SNc (Panickar et al., 2009). Recently, Vauzour et al. (2010) have reported that the flavonoids catechin, epicatechin and quercetin and polyphenols like caffeic acid and p-coumaric acid afford protection against the neurotoxicity of 5-S-cysteinyl-dopamine, which has been proposed to contribute to the progression of the brain neurodegeneration in PD.

1.4. Calcium homeostasis in brain

Intracellular Ca^{2+} regulates a wide array of cellular processes and is important for signal transduction. Many biological processes of great importance for the neuronal function are extremely dependent on cytosolic Ca^{2+} concentration, such as secretion of neurotransmitters and synaptic plasticity (Trifaro and Vitale, 1993), intracellular signaling pathways that mediate the metabolic extracellular neuronal stimuli (Berridge et al., 2000) and development of neurites (Benowitz and Routtenberg, 1997). Ca^{2+} plays a central role not only in normal and healthy neurons but is also involved in the many cellular processes (e.g. oxidative stress, mitochondrial impairment, proteasomal dysfunction, excitotoxicity, neuroinflammation, apoptosis) that can lead to cell death in

PD (Berridge et al., 2000; Bueler, 2010; Hegde and Upadhya, 2011; Lau and Tymianski, 2010; Witte et al., 2010).

The concentration of cytosolic free Ca^{2+} in resting neurons (≈100 nM) is 10,000 fold lower than the concentration of Ca^{2+} in the extracellular space (≈1.2 mM) (Gleichmann and Mattson, 2011). This concentration gradient leads to a significant increase in cytosolic Ca^{2+} after depolarization, rendering Ca^{2+} regulation a critical process in neurons. To maintain Ca^{2+} homeostasis, Ca^{2+} entering neurons is rapidly sequestered in intracellular organelles, such as the mitochondria and the endoplasmic reticulum (ER), or pumped back across the plasma membrane concentration gradient, all of which require the consumption of high levels of energy in the form of ATP (Gleichmann and Mattson, 2011). In neurons the Ca^{2+} transport systems of the plasma membrane most important in the control of the homeostasis of cytosolic Ca^{2+} are voltage-operated calcium channels (VOCC), some ionotropic receptors (with the NMDA receptors playing an outstanding role in the brain), the plasma membrane Ca^{2+} ATPase (PMCA) and the Na^{+}/Ca^{2+} exchanger (NCX) (Gutierrez-Merino, 2008). Ca^{2+} influx through L-VOCC has a major role in maintaining cytosolic Ca^{2+} concentrations within the optimal range 70 to 200 nM for the survival and normal excitability of CGN in culture (Gutierrez-Martin et al., 2005).

The sustained impairment of intracellular Ca^{2+} homeostasis in neurons associated with the oxidative stress induced by an elevated production of ROS is a common metabolic feature in brain neurodegenerative diseases. Moreover, cytosolic Ca^{2+} concentration can be regarded as a major bioenergetic marker for neuronal activity and neuronal survival, as it has been shown that a sustained low cytosolic Ca^{2+} concentration elicits apoptosis in neurons in culture (Franklin and Johnson, 1992; Franklin and Johnson, 1994), and also that a steadily elevated cytosolic Ca^{2+} concentration induces rapid cell death by necrosis mediated by activation of calpains (Choi, 1988; Franklin and Johnson, 1992; Garcia-Bereguiain et al., 2008; Gutierrez-Martin et al., 2005; Orrenius et al., 1989).

1.4.1. Alterations of calcium homeostasis in PD

In spite of the occurrence of degeneration in other brain areas, DA region is one of the most affect in PD patients. Many characteristics of DA neurons have been point out that these are neurons with high energy requirements (Surmeier et al., 2011a).

Unlike the vast majority of neurons in the brain, adult SNc DA neurons are autonomously active, generating broad slow action potentials regularly (2–4 Hz) in the absence of synaptic input (Grace and Bunney, 1983). This pacemaking activity is believed to be important in maintaining ambient DA levels in regions that are innervated by these neurons, particularly the striatum (Romo and Schultz, 1990). Whereas the majority of neurons rely exclusively on monovalent cation channels (like sodium (Na^+)) to drive pacemaking, SNc DA neurons also express ion channels that allow extracellular Ca^{2+} to enter the cytoplasm (Ping and Shepard, 1996; Puopolo et al., 2007), which lead to elevated intracellular Ca^{2+} concentrations (Chan et al., 2007; Wilson and Callaway, 2000). The use of Ca^{2+} rather than Na^+ ions for pacemaking involves more energy costs for neurons in order to maintain a safe intracellular Ca^{2+}. The Ca^{2+} channels involved in this pacemaking activity have a distinctive $Ca_v1.3$ pore-forming subunit (Chan et al., 2007). $Ca_v1.3$ Ca^{2+} channels are relatively rare (Sinnegger-Brauns et al., 2009), but they are found at an extraordinary high density in SNc DA neurons (Guzman et al., 2009; Khaliq and Bean, 2010; Puopolo et al., 2007).

It is now accepted that Ca^{2+} entry through plasma membrane $Ca_v1.3$ Ca^{2+} channels during activity is either pumped back across the plasma membrane (by PMCA and/or NCX) or rapidly sequestered in the ER or mitochondria (Surmeier et al., 2011a) (Figure 1.4). Both processes waste cellular energy stored in the form of ATP. The metabolic demand created by these ATP-dependent steps in Ca^{2+} homeostasis should increase oxidative phosphorylation in mitochondria and the production of damaging ROS (Guzman et al., 2010). This places SNc DA neurons in a stressful working situation, as ROS damage mitochondrial proteins such as complex I and mtDNA, reducing the efficiency of oxidative phosphorylation (Harman, 1972; Wallace, 2005). In extreme cases, the stress on mitochondria induces mPTP opening, swelling and the release of cytochrome c and other pro-apoptotic proteins such as apoptosis inducing factor (Nicholls, 2002). In parallel, ROS are capable of damaging ER proteins, elevating the concentration of misfolded proteins that need to be degraded by proteasomes and autophagosomes (Kaufman, 1999). The unfolded protein response triggered by this elevation in misfolded proteins should further reduce ER production of proteins and potentially lead to the release of pro-apoptotic factors such as C/EBP homologous protein (CHOP) (Chan et al., 2009; Oyadomari and Mori, 2004). The role of mitochondria in Ca^{2+} homeostasis could further compromise their ability to generate ATP, leading to a functionally important drop in cytosolic ATP levels (Nicholls, 2002).

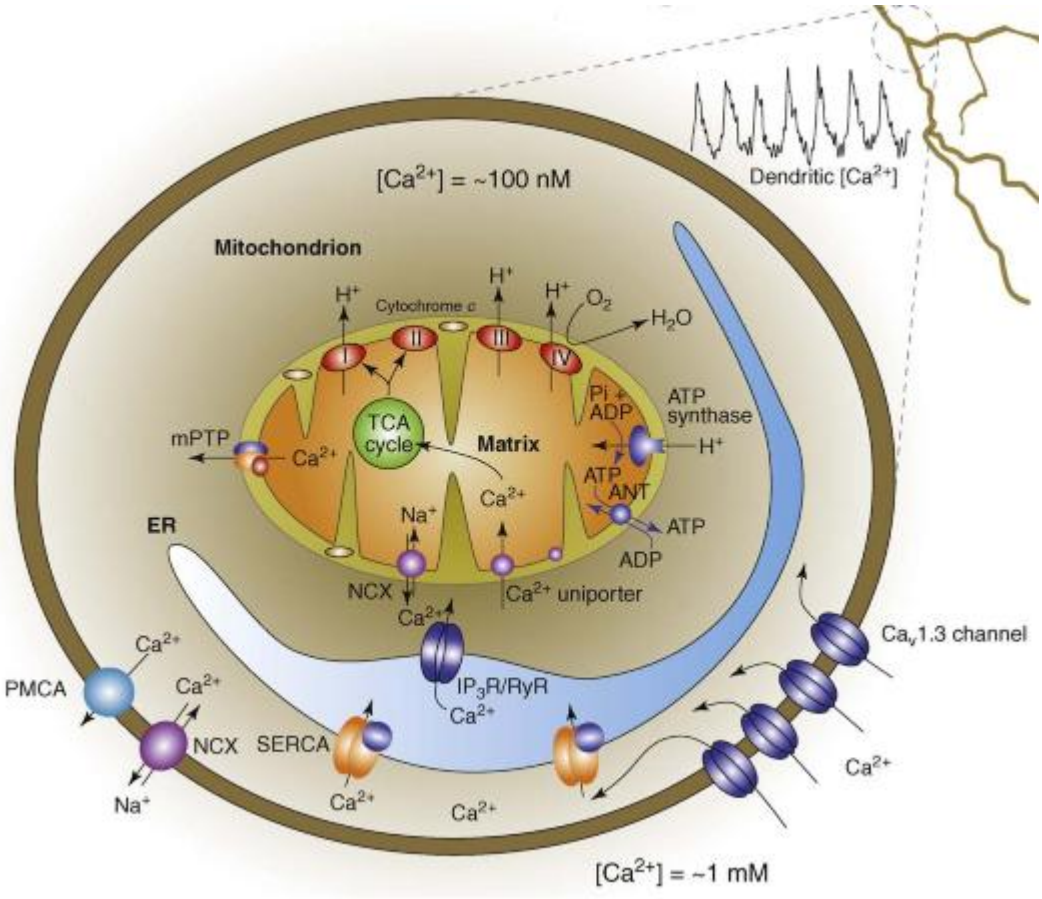

Figure 1.4: Ca^{2+} transport in SNc DA neurons. The steep concentration gradient for Ca^{2+} enables it to cross the plasma membrane readily into cells through open pores such as L-type Ca^{2+} channels ($Ca_v1.3$ VOCC-subtype). Once inside neurons, it is either transported back across the plasma membrane (by PMCA or NCX) or sequestered in intracellular organelles (i.e. mitochondria and ER). The ER uses high-affinity smooth ER Ca^{2+} (SERCA) pumps that depend upon ATP to take Ca^{2+} from the cytoplasm into the ER lumen. Ca^{2+} flows back into the cytoplasm after the opening of inositol trisphosphate receptors (IP_3R) and ryanodine receptors (RyR) also located in the ER membrane. Mitochondria are often found in close apposition to the ER and plasma membrane, creating a region of high (but localized) Ca^{2+} concentration that drives Ca^{2+} into the matrix of mitochondria through a Ca^{2+} uniporter. Ca^{2+} can leave the mitochondrion through a number of mechanisms. The dominant mitochondrial Ca^{2+}-efflux pathway in neurons is through mitochondrial NCXs. Ca^{2+} release through higher conductance ion channels, such as the mitochondrial permeability transition pore (mPTP), has also been proposed. The mPTP is known to have two conductance states: a low-conductance state that is reversible and participates in physiological Ca^{2+} handling, and a high conductance state that is irreversible and leads to mitochondrial swelling and loss of molecules such as cytochrome c that trigger apoptosis (adapted from (Chan et al., 2009)).

Genetic mutations or environmental toxins such as rotenone could further compromise mitochondrial or ER function, rendering them more vulnerable to Ca^{2+} stress (Surmeier et al., 2011b). By rushing the decline in ER and mitochondrial function

and the accelerated loss of SNc DA neurons, these genetic and environmental factors could be seen as "causing" PD.

1.5. Aims

The major aim of this work was to assess the potential of the ergogenic molecule creatine and the flavonoids epicatechin and kaempferol (and possible synergism between these compounds) to afford neuroprotection of CGN in culture against rotenone, a toxin used to induce parkinsonism-like neurodegeneration.

Due to its energetic capacity, creatine has been shown to be a neuroprotector in some neurodegenerative diseases such as HD and PD, being at the time in phase III clinical trials (Hersch et al., 2006; NINDS-NET-PD, 2006). Moreover, we wanted to evaluate the benefits/inconveniences of co-administration of creatine with flavonoids like kaempferol and epicatechin that have been proposed to be bioactive compounds accounting, at least in part, for the beneficial effects of green tea against the progress of neurodegeneration in PD treatment.

In addition, as the cellular mechanisms underlying the neurotoxicity exerted by rotenone in CGN are not well understood, a second major goal of this work is to use creatine and the above mentioned flavonoids as tools to shed light on the relative relevance of the different mechanisms proposed to mediate brain neurodegeneration in PD.

To achieve these major goals, we have defined the experimental strategy and partial objectives schematically presented in figure 1.5.

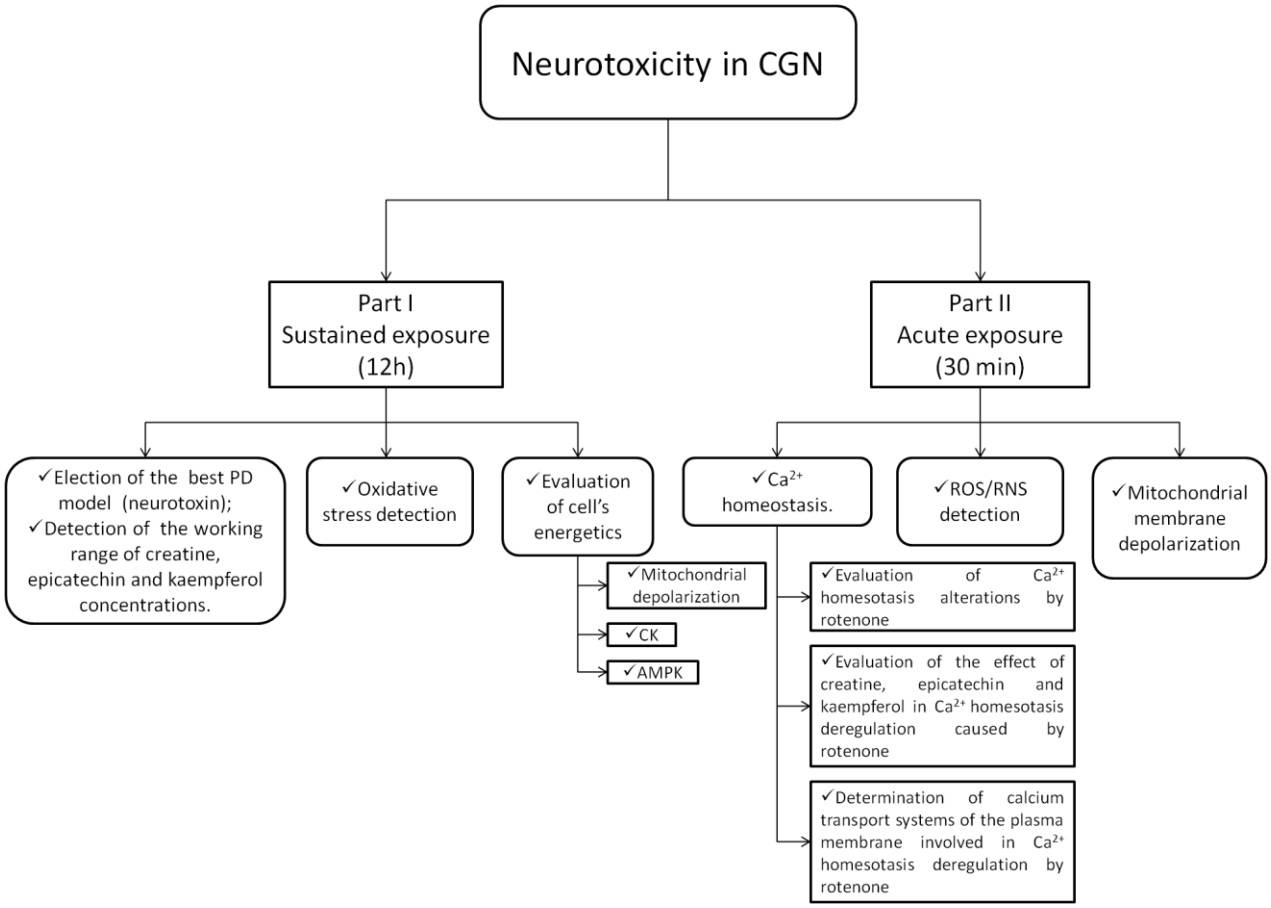

Figure1.5: Schematic representation of the experimental strategy and partial objectives of this research work.

II. Material and Methods

2.1. Equipment and reagents

2.1.1. Scientific equipment

- Laminar Flow Cabinet Nüve LN 090
- CO_2 incubator Heal Force - HF 90
- Microcentrifuge Eppendorf 5415R
- Ultracentrifuge Beckman Coulter Optima™ L-90K
- Spectrophotometer PG Intruments T70
- Fluorescence Spectrophotometer Perkin Elmer 650-40
- Varioskan Flash spectral scanning multimode reader Thermo Scientific
- Epifluorescence microscope Nikon Diaphot 300
- Microscope camera Hamamatsu ORCA-R^2
- Microscope Wheel Filters Lambda 10-2
- Electrophoresis system and western blotting BIO-RAD

2.1.2. Chemicals and reagents

All antibodies were used at a dilution in the range recommended in the product datasheets. The primary antibodies used are described in Table 2.1, and the secondary antibodies labeled with fluorophores are described in Table 2.2.

Western blotting reagents anti-goat IgG horseradish peroxidase anti-rabbit IgG horseradish peroxidase and SuperSignal West Dura Extended Duration Substrate were purchased from Pierce (Rockford, IL, USA).

All other reagents and chemicals were supplied by Sigma-Aldrich, Roche or Merck (Darmstadt, Germany).

Table 2.1- Informative description of the primary antibodies used in Western blot experiments.

Antibody	**Produced in:**	**Trading house**	**Reference**
Caspase-3	Rabbit	Calbiochem	PC679
Nitrotyrosines	Mouse	Calbiochem-Merck KGaA	CC22.8C7.3
Creatine Kinase-B	Goat	Santa Cruz Biotechnology	sc-15157
Phospho-AMPKα	Rabbit	Cell Signaling Technology®	2535
Cathepsin D	Goat	Santa Cruz Biotechnology	sc-6494

Table 2.2- Informative description of the secondary antibodies used in Western blot experiments.

Antibody	**Trading house**	**Reference**
anti-Rabbit IgG-Alexa488	Invitrogen (Molecular Probes, Eugene, OR, USA)	A11008
anti-Goat IgG-Alexa488	Invitrogen (Molecular Probes, Eugene, OR, USA)	A11055
anti-Mouse IgG-Alexa488	Invitrogen (Molecular Probes, Eugene, OR, USA)	A11001

2.2. Buffers and solutions

The composition of MLocke 25 buffer (pH 7.4 at 37° C) used in all experimental work with cerebellar granule cells is: 4 mM $NaHCO_3$, 10 mM N-[tris (hidroxymetil) metil] glicine, 5 mM glucose, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 134mM NaCl and 25 mM KCl.

The composition of sample buffer used for SDS-PAGE in this work is 26.6 mM Tris-HCl pH 6.8, 0.86% sodium dodecyl sulfate (SDS), 0.43% (v/v) β-mercaptoethanol, 0.014% bromophenol blue prepared in 20 mM Tris pH 7 and 37.3% glycerol.

The composition of the electrophoresis buffer used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) throughout the experimental work is: 25 mM Tris, 190 mM Glycine, 10% SDS (pH 8.3).

The transfer buffer composition used in Western blotting in all this work is: 25 mM Tris, 190 mM Glycine, 0.1% SDS, 20% methanol (pH 8.3).

The composition of phosphate buffered saline (PBS) is 4.3 mM Na, 0.4 mM KH2PO4, 137 mM NaCl and 27 mM KCl (pH 7).

2.3. Biological Material

Cultures of cerebellar granule cells were prepared from 7 days old Wistar rats, weighing 20-25 g. The rats were supplied and maintained by the Animal Service of the University of Extremadura, where they have been fed and maintained at a constant temperature of 22-23 º C and humidity ranging between 60 and 80 %.

2.4. Cell cultures

2.4.1. Cerebellar granule cells primary cultures

Cultures of CGN were obtained from dissociated cerebella of 7 day old Wistar rats as described previously (Samhan-Arias et al., 2004). Cells were plated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 5 mM glucose, 19.63 mM KCl, 3.7 ng/mL insulin, 7 µM para-aminobenzoic acid, 50 U/mL penicillin, 25 U/mL streptomycin, 0.91 mM pyruvate and 2 mM L-glutamine on 35 mm dishes coated with poly-D-lysine, at a density of $2.75x10^6$ cells/dish. Cultures were maintained at 37ºC in a humidified atmosphere of 95 % air/5 % CO_2. Cytosine arabinofuranoside (10 µM) was added to fresh culture medium 48 h after plating to prevent replication of nonneuronal cells. The culture medium was replaced with serum-free medium 7 days after plating. Cells were maintained afterward in serum-free F12 medium supplemented with 12.5 mM glucose, 20.82 mM KCl, 5 µg/ml insulin, 0.1 mg/ml apo-transferrin, 20 nM progesterone, 50 U/ml penicillin, 25 U/ml streptomycin, 0.1 mg/ml pyruvate, 2 mM L-glutamine. All experiments were performed using mature CGN at 9–10 days in vitro (DIV).

2.5. Viability assays

2.5.1. MTT assay

Neuronal viability was assessed by the 3-(4,5-dimethyl-thiazole-2yl)-2,5-diphenyltetrazolium bromide (MTT) reduction test as described in (Martin-Romero et al., 1996; Samhan-Arias et al., 2004). Viable cells reduce MTT to formazan which can be determined spectrophotometrically. In brief, the culture medium was replaced with 2 mL of MLocke 25 buffer and incubated for 15 min with 0.3 mg MTT. Thereafter,

formazan was dissolved in 1 mL of dimethyl sulfoxide (DMSO) and measured at 490 nm and 700 nm.

2.5.2. Treatment of CGN with Rotenone

To investigate the effect of rotenone on CGN viability, the treatment was performed at 9 DIV in CGN. To this end, the plates containing the CGN were incubated for 12 hours at 37 ° C with 0, 2, 5, 10, 15, 20, 30 and 50 nM of Rotenone prepared in DMSO. After the incubation period the plates were subjected to MTT assay described in section 2.5.1.

2.5.3. Treatment of CGN with 6-OHDA

To investigate the effect of 6-OHDA on CGN viability, cells at 9 DIV were incubated for 24 hours at 37 ° C with 0, 5, 10, 15, 20, 25, 30 and 50 µM of 6-OHDA prepared in DMSO. After the incubation period the viability was evaluated by MTT assay described in section 2.5.1.

2.5.4. Flavonoids protection against rotenone-induced cell death in CGN

To investigate the protective effect of the flavonoids: kaempferol and epicatechin, cells at 9 DIV were incubated for 1 hour with 0, 2, 5 and 10 µM of each. Thereafter, rotenone (IC_{50} concentration) was added to the culture medium and incubated for 12 hours. After the incubation period the cell viability was evaluated by MTT assay described in section 2.5.1.

2.5.5. Creatine protection against rotenone-induced cell death in CGN

To investigate the protective effect of creatine, cells at 9 DIV were incubated for 1 hour with 0, 5, 10, 15, 20 and 30 mM. Thereafter, rotenone (IC_{50} concentration) was added to the culture medium and incubated for 12 hours. After the incubation period the viability was evaluated by MTT assay described in section 2.5.1.

2.6. Protein quantification and determination

2.6.1. Preparation of cell lysates

CGN cultured for 9 DIV were washed with buffer MLocke 25. Then, the CGN were resuspended and centrifuged at 4 ° C for 2 min at 2000g in a refrigerated

Eppendorf microcentrifuge, pellets of lysed cells were re-suspended in 100 µL of buffer (50 mM HEPES, pH 7.4, 100 mM NaCl , 0.1% CHAPS, 1 mM DTT and 0.1 mM EDTA) for colorimetric measurements and in 200 µL of buffer (5 mM NaP, pH 7, 1 mM EDTA, 0.5% Tween 20 supplemented with protease inhibitor cocktail Roche Biochemicals (COMPLETE)) for Western blotting.

2.6.2. Protein concentration

Protein concentration was determined by the method of Bradford (1976) using the Bio-Rad (Hercules, CA, USA) protein assay reagent and bovine serum albumin as standard.

2.6.3. Western blotting

SDS-PAGE were run at a concentration of 15%, 12.5% and 7.5% acrylamide, depending on the molecular weight of the protein of interest, using 20 µg protein of CGN lysates per lane. Gels were transferred to nitrocellulose membranes of 0.2 µm or 0.4 µm average pore size (TransBloTTransfer Medium, BioRad). Nitrocellulose membranes were blocked by 1 h incubation at room temperature with 5% (w/v) non-fat dry milk in PBS. Then, nitrocellulose membranes were washed three times with PBST, e.g. PBS supplemented with 0.05% polyoxyethylenesorbitan monolaurate (Tween 20). Immunodetection of proteins was performed with its specific antibody at recommended dilutions. After incubation with the first antibody overnight, membranes were washed six times with PBST and incubated for 1 h at room temperature with the secondary antibody IgG conjugated with horseradish peroxidase (anti-rabbit, anti-mouse and anti-goat with a dilution of 1:8.000, 1:50.000 and 1:500.000, respectively), then washed six times with PBST, followed by incubation for 3 min with SuperSignal West Dura Substrate (Pierce). Western blots were revealed by exposure to an AmershamHyperfilm MPautoradiography film (GE Healthcare, UK).

2.7. Cell death pathways

2.7.1. Caspase-3 and Cathepsin D activation

Caspase-3 and cathepsin D activations were investigated by Western blotting after SDS-PAGE of lysates as described in 2.6.3. To detect caspase-3 was used a polyclonal rabbit anti-activated caspase-3 antibody (1:200; Calbiochem PC679) against

the 17 kDa cleaved (active) fragment of caspase-3. For positive control, activated recombinant human caspase-3 (Calbiochem) was also loaded to the gels. Cathepsin D was revealed using a goat polyclonal anti-cathepsin D antibody (1:100; sc-6994 from Santa Cruz Biotechnology).

2.7.2. Calpain activity

Calpain activity was measured using the fluorogenic substrate Suc-LY-AMC (Calbiochem-Merck KGaA,). An aliquot of lysate with 30 µg of protein was incubated with 2.2 mL of the reaction buffer containing 50 mM Tris–HCl pH 7.5, 50 mM NaCl, 5 mM β-mercaptoethanol, 5 mM CaCl and 50 µM Suc-LY-AMC. The release of 7-amino-4-methyl-coumarin (AMC) was monitored at an excitation wavelength of 380 nm and 460 nm of emission on a fluorescence spectrometer. To detect non-specific protease activity, the reaction rate was also measured in the presence of 10 µM Z-2-LLY-FMK (Calbiochem-Merck KGaA), a specific inhibitor of calpain, and the value was subtracted from that observed in the absence of the inhibitor. Fluorescence units were converted into moles of AMC released using a standard curve obtained with free AMC, and calpain activity was expressed in pmol of AMC cleaved per minute per milligram of protein.

2.7.3. Hoechst 33258 and propidium iodide staining

A direct estimation of disrupted plasma membrane CGN was obtained through a modified double-staining technique as described in (Soares et al., 2008) with modifications. Briefly, culture medium was discarded, attached cells were washed with MLocke 25 buffer, and a stock solution of bisbenzimide (Hoechts 33258 at 10 mg/mL) was added to dishes, yielding final concentration of 20 µg/mL, during 30 min at 37 º C. To the same dishes, a stock solution of propidium iodide (PI) (10 mg/mL) was added, yielding a final concentration of 20 µg/mL, during the final 5 min of staining. Bisbenzimide- (stains genetic material) and PI-stained (indicates disrupted plasma membrane cells) cells were examined and photographed, using a Nikon Diaphot 300 inverted microscope. Filters for PI fluorescence used a 550 nm wavelength, and for bisbenzimide, a 420–505 nm wavelength.

2.8. Measurement of CGN mitochondrial membrane potential.

Mitochondrial membrane potential (Δψm) was monitored in CGN with tetramethylrhodamine ethyl ester (TMRE). Cells were stained with TMRE (0.1 µM) and 0.01% pluronic-F127 at 37 °C. Cell fluorescence images were acquired each 15 seconds for 30 min using the Hamamatsu CCD camera with an excitation filter of 470 nm and a dichroic mirror DM510 plus an emission filter of 590 nm. Digital images were collected at fixed times of exposure with a Hamamatsu Hisca CCD camera, and subsequently analyzed with the Argus/Hisca software. At the end, 5 µM Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), a potent reversible inhibitor of mitochondrial oxidative phosphorylation which depolarises mitochondrial membrane potential and induces apoptosis, was added to get the intensity of fluorescence after complete depolarization of mitochondria.

2.9. Reactive oxygen and nitrogen species

2.9.1. Intracellular oxidative stress

2',7'-Dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes) staining has been used as a measure of intracellular oxidative stress and it was performed as described in (Samhan-Arias et al., 2004), with modifications. Briefly, at 9 DIV, CGCs were switched to MLocke 25 buffer and incubated with 10 µM H_2DCFDA. Thereafter, cell dishes were placed in the thermostatic plate (37 ºC) of a Nikon Diaphot epifluorescence microscope, and images were taken each 10 seconds for 20 minutes with an excitation filter of 470 nm and a dichroic mirror DM510 plus an emission filter of 510 nm. Digital images were collected at fixed times of exposure with a Hamamatsu Hisca CCD camera, and subsequently analyzed with the Argus/Hisca software.

2.9.2. Measurement of GSH levels

GSH measurements were determined using the compound monochlorobimane (MCB) as described in (Keelan et al., 2001) with modifications. MCB is a nonfluorescent bimane, which is freely permeable across the cell membrane. Within the cytosol, it is conjugated to GSH to form a fluorescent product. Briefly, cells at 9-10 DIV were switched to MLocke 25 buffer and incubated with 10 µM MCB. Cell dishes were placed in the thermostatic plate (37 ºC) of a Nikon Diaphot epifluorescence microscope, and images were taken each 10 seconds for 20 minutes with an excitation filter of 380

nm and a dichroic mirror DM510 plus an emission filter of 510 nm. Digital images were collected at fixed times of exposure with a Hamamatsu Hisca CCD camera, and subsequently analyzed with the Argus/Hisca software.

2.9.3. Nitrotyrosines

Protein nitrotyrosines were revealed by western blotting after SDS-PAGE of lysates as described in 2.6.3, using a polyclonal mouse anti-nitrotyrosine antibody (Calbiochem CC22.8C7.3, Calbiochem-Merck KGaA) diluted 1 : 1500 in PBST.

2.10. Determination of the intracellular free Ca^{2+} concentration in CGN and cell bioenergetics markers

2.10.1. Measurement of the cytosolic Ca^{2+} concentration

Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured basically as indicated in previous papers (Gutierrez-Martin et al., 2005; Samhan-Arias et al., 2004). CGC were loaded with fura-2 by incubation in DMEM-F12 for 45–60 min with 5 µM fura-2-acetoxymethyl ester (fura-2 AM) and 0.025% Pluronic-F127 at 37 ºC. Afterwards, CGC were washed with MLocke 25 buffer and the culture dish was placed in a thermostatic controlled plate (Warner Instrument Co., Hamden, CT, USA) of a Nikon Diaphot 300 inverted microscope, equipped with an epifluorescence attachment and excitation filter wheel. To measure the $[Ca^{2+}]_i$, ratio fluorescence images were obtained with excitation filters of 340 and 380 nm and a dichroic mirror DM510 and absorption filter (emission side) of 510 nm. Digital images were taken with a Hamamatsu ORCA R^2 CCD camera and Lamdba 10–2 filter wheel controller and subsequently analyzed with the HC image software.

$$[Ca^{2+}]_i = K_d \cdot \{(R - R_{min})/(R_{máx} - R)\} \cdot \beta$$

Where R is the average value calculated from the fluorescence ratio (340/380), and R_{max} and R_{min} are the values of ratios (340/380) for the probe fura-2 bound to Ca^{2+} and the free fura-2 in the CGN (not-bound to Ca^{2+}), respectively. The parameter β represents the average value of the ratio of fluorescence intensities between the free fura-2 and fura-2 bound to Ca^{2+} at a wavelength of 380 nm. The dissociation constant

(K_d) of this probe for Ca^{2+} is 225 nM, allowing this probe to determine values of $[Ca^{2+}]_i$, from 20-30 nm to 0.5-1 mM.

2.10.2. Measurements of the effect of rotenone on cytosolic Ca^{2+} homeostasis in CGN

To test the effect of rotenone in the cytosolic free Ca^{2+} concentration in CGN, the intracellular Ca^{2+} concentration has been measured as indicated above. The neurons were incubated with fura-2 AM as indicated in 2.10.1 and were subsequently washed with MLocke 25 buffer. Then, the ratio images of fluorescence intensities at 340/380 were obtained as described previously. The basic experimental protocol followed was as follows: images of the fluorescence intensity ratio 340/380 were obtained for several minutes, then 10 or 15 nM of rotenone was added to the plates and images were acquired with short intervals of time until stability signal of the fluorescence intensity ratio (340/380) in the soma of CGN.

2.10.3. Effect of flavonoids (kaempferol and epicatechin) and creatine in the response of intracellular Ca^{2+} to rotenone

To investigate the effect of each one of these compounds in the cytosolic Ca^{2+} response to rotenone; kaempferol, epicatechin or creatine were added at the concentrations of 10 μM, 10 μM and 30 mM respectively to the extracellular medium. Therafter, ratio images of fluorescence intensities at 340/380 were acquired for several minutes before addition of rotenone. To evaluate the possible synergistic effect of flavonoids with creatine, each of the flavonoid was incubated with creatine several minutes before addition of rotenone. Data acquisition was done as described in 2.10.2.

2.10.4. Treatment of CGN with nifedipine, MK-801, KB-27943 and cyclopiazonic acid (CPA) to study their effects in the cytosolic Ca^{2+} homeostasis alterations observed in response to rotenone

Treatment with various inhibitors/blockers of the Ca^{2+} transport systems of the plasma membrane was accomplished by adding these modulators in the plate after having a stable signal of ratio intensities of fluorescence (340/380) in CGN *in vitro* previously incubated with fura-2 AM and washed with MLocke 25. Thereafter, images

of fluorescence ratio intensity 340/380 were recorded until the signal reached a new stabilization value before addition of rotenone.

2.10.5. Measurements of the effect of rotenone on SOCE activity in CGN

To evaluate the effect of rotenone in store-operated Ca^{2+} entry (SOCE) following depletion of ER Ca^{2+} stores we have followed a standard protocol, as in (Gruszczynska-Biegala et al., 2011). Briefly, CGN were incubated with fura-2 AM, and subsequently switched to MLocke 25 buffer without Ca^{2+} and the plate was placed in a thermostated cell holder. After 5-10 min in the cell holder (to ensure proper temperature equilibration), 15 nM rotenone was added to the extracellular medium and images of fluorescence ratio intensity 340/380 were recorded during 45 min. Finished the incubation time with rotenone, 1 μM thapsigargin (TG) were added to the cell suspension and, after 10 min, 1 mM Ca^{2+} was added and the ratio fluorescence images were acquired every 10 seconds during 5 min.

2.10.6. Creatine kinase activity

CK activity in lysates has been measured as described earlier in (Lagoa et al., 2009). Briefly, a volume of lysate containing 15-25 μg of protein was added to the assay buffer composed of: 100 mM Tris–TES buffer pH 7.0, 20 mM glucose, 10 mM magnesium acetate, 1 mM adenosine diphosphate, 10 mM adenosine monophosphate, 0.6 mM oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$), 35 mM phosphocreatine, 10 μM diadenosine pentaphosphate, ≥ 1.5 U/mL hexokinase and ≥ 1.5 U/mL glucose-6-phosphate dehydrogenase. CK activity was calculated from the rate of formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH), monitored spectrophotometrically at 340 nm, at 37 ºC. CK activity is expressed in μmol of phosphocreatine hydrolysed per minute per milligram of protein.

2.10.7. Creatine kinase and phosphorylated AMP-kinase levels

CK and phosphorylated AMP-kinase levels were monitored by western blotting after SDS-PAGE of lysates as described in 2.6.3, using a goat polyclonal anti-CK-B antibody (1 : 1000; sc-15157 from Santa Cruz Biotechnology) and a rabbit polyclonal anti-phospho-AMPKα (Thr172) antibody (1:500; 2535 from Cell Signaling Technology®), respectively.

2.11. Statistical analysis

Results are expressed as means ± standard error (SE). Differences among treatments were assessed by analysis of variance (Popova et al., 2004) with Tukey HSD (Honest Significant Difference) multiple comparison test using SigmaStat 3.10 (Systat). Significant difference was accepted at the $p<0.05$ levels. All biochemical data were confirmed with duplicate measurements of at least experimental triplicates of each condition.

III. Results

3.1. Sustained exposure (12 h) of CGN to the neurotoxins rotenone and 6-OHDA

3.1.1. Rotenone and 6-OHDA induce cell death in CGN

The cytotoxicity of 2, 5, 10, 15, 20, 30 and 50 nM rotenone as well as the cytotoxic effect of 0, 5, 10, 15, 20, 25, 30, 50, 75 and 100 µM 6-OHDA were determined by the MTT assay.

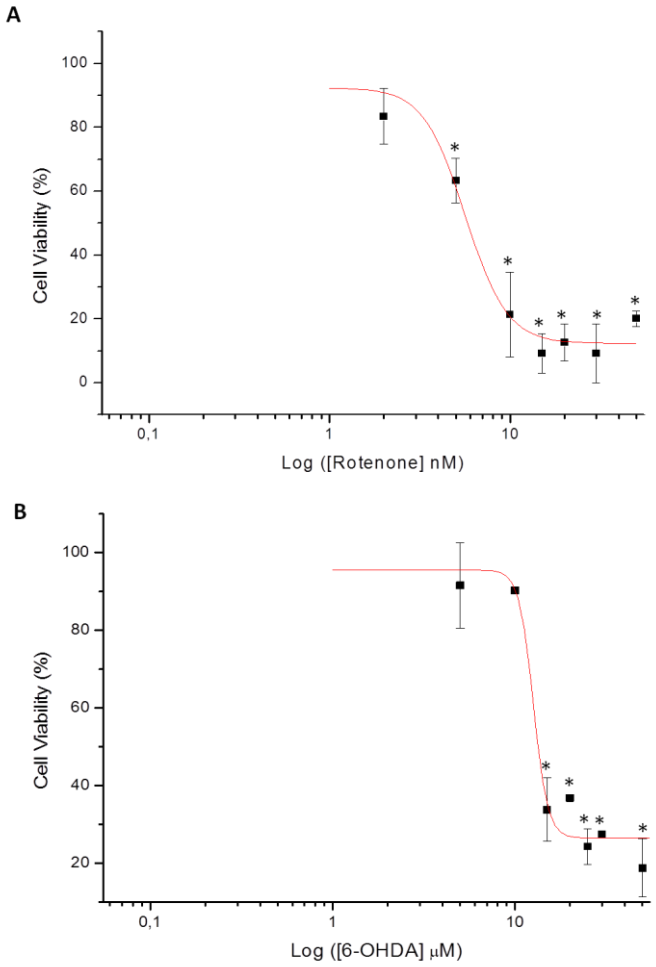

Figure 3.1: Effect of neurotoxins in CGN viability. (A) Dose-response curve obtained by MTT cell viability assay at 2, 5, 10, 15, 20, 30 and 50 nM rotenone for 12 h in CGN. **(B)** Dose-response curve obtained by MTT cell viability assay at 0, 5, 10, 15, 20, 25, 30, 50, 75 and 100 µM 6-OHDA for 24 h in CGN. Independent experiments were repeated three times. Values are presented as mean±SEM. *p<0.05 compared to non-treated cells.

Figure 3.1A shows that the cell viability decreased in a dose-dependent manner by the treatment with rotenone for 12 hours. Rotenone (5,65±0,51 nM) triggered 50% cell

death and the concentration of 5 nM was chosen for subsequent experiments. In Figure 3.1B is also observed that the cell viability was decreased in a dose-dependent manner by the treatment with 6-OHDA for 24 hours. 6-OHDA (12,61±1,05 µM) generated 50% of cell death.

Due to the specificity of 6-OHDA for catecholaminergic neurons and the higher toxicity of rotenone in CGN (nM), the latter was chosen for all the subsequent experiments.

3.1.2. Creatine, but not kaempferol or epicatechin, protects against rotenone-induced cell death in CGN

The effect of creatine, kaempferol and epicatechin on rotenone-induced cell death was evaluated.

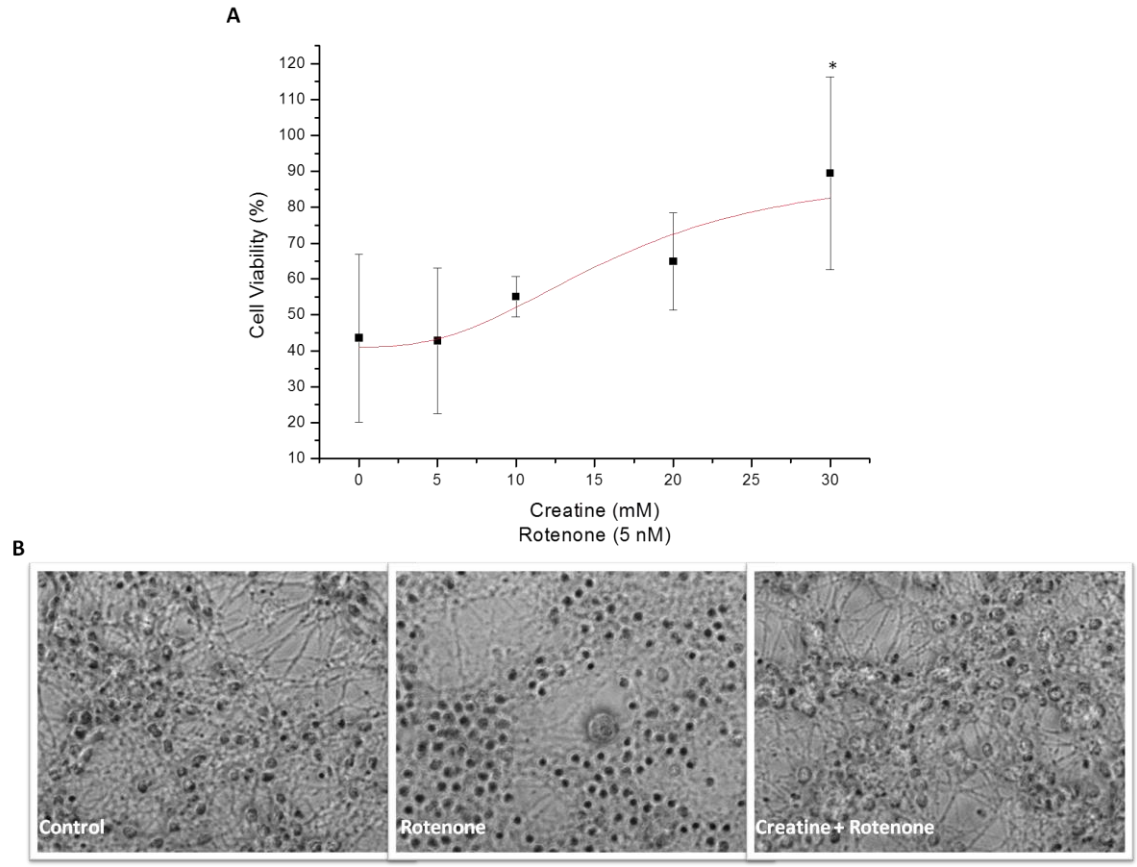

Figure 3.2: Effect of creatine on rotenone-induced cell death in CGN. (A) Creatine inhibits rotenone-induced cytotoxicity in CGN, treated with 5 nM rotenone for 12 h. Creatine (5, 10, 20 and 30 mM) was added 1 h before rotenone treatment. **(B)** Phase contrast images of CGN: 12 h with the vehicle (DMSO) is represented as *control*; 12 h of rotenone incubation is presented as *rotenone* and 12 h of rotenone incubation with a previous (1h) addition of creatine is shown as *creatine+rotenone*. Independent experiments were repeated three times. Values are presented as mean±SEM. *p<0.05 compared to cells treated with rotenone only (0 mM Creatine).

Figure 3.2 shows that pre-co-treatment with creatine significantly inhibited rotenone-induced cell death. Creatine (10-30 mM) increased the cell viability, and 30 mM creatine improved the cell viability up to the control level (around 100% of viability) ($p< 0.05$). On the other hand, kaempferol and epicatechin, in spite of showing no cytotoxicity in the concentrations range 2-20 µM (data not shown), failed to protect against rotenone-induced cell death (data not shown). In agreement with the MTT results, the morphological observations revealed that creatine significantly reversed the cellular damage triggered by rotenone, as shown in Figure 3.2B.

3.1.2.1. Creatine in combination with epicatechin present a weak synergistic effect against rotenone-induced cell death in CGN, while kaempferol antagonizes the protective effect of creatine

Despite the lack of protective effect of the tested flavonoids in rotenone-induced cell death, their putative capacity to increase/decrease the protective effect of creatine was assessed by MTT. Figure 3.3 shows that it is clear a negative effect of kaempferol, as the results pointed out that kaempferol antagonizes the effect of creatine. In the other hand, epicatechin, which have no effect by itself in the protection of rotenone-induced cell death, elicits a weak increase of the protection of creatine, revealing a weak synergistic effect between them on the protection against rotenone-induced neuronal death.

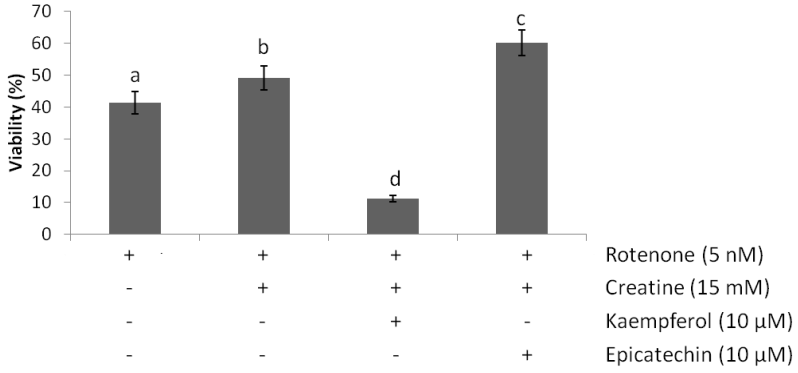

Figure 3.3: Effect of kaempferol and epicatechin on the protective effect of creatine on rotenone-induced cell death in CGN. Epicatechin increases the effect of creatine revealing a weak synergistic effect, while kaempferol decreased creatine´s effect against rotenone-induced cytotoxicity. Independent experiments were repeated three times. Values are presented as mean±SEM. All the values are statistically different one of each other ($p<0.05$).

3.1.3. Cathepsin D activation in rotenone-induced cell death in CGN

Next, we have experimentally assessed the putative pathway/s responsible for the neuronal death of CGN caused by rotenone.

A number of pro-apoptotic stimuli induce the activation of caspase-9, an initiator protease that activates execution caspases, such as caspase-3, leading to the development of apoptosis (Budihardjo et al., 1999). Many studies have also shown that activation of caspase-3 mediates neuronal apoptosis (Hartmann et al., 2000; Porter and Janicke, 1999). We investigated by western blotting the presence of active caspase-3 at 6, 8 and 12h of rotenone incubation, and as observed in Figure 3.4A no statistically significant differences were observed with respect to control non-treated CGN. These results indicate that death caused by rotenone in these cells does not occur through apoptosis, involving caspase-3. The evidence of a non-apoptotic cell death was confirmed by staining CGN with PI (PI-stained indicates cells with disrupted plasma membrane). As shown in Figure 3.4B, rotenone-induced cell death produced membrane disruption, which is verified by the staining with PI of 85.7 % of dyed cells.

Another cell death pathway observed in PD is necrosis. Proteolysis during necrosis does not rely on caspases but on calpains and cathepsins (Zong and Thompson, 2006). Since rapid cell death of CGN by necrosis mediated by activation of calpains is observed when cytosolic calcium concentration is kept steadily elevated (Choi, 1988; Franklin and Johnson, 1992; Garcia-Bereguiain et al., 2008; Gutierrez-Martin et al., 2005; Orrenius et al., 1989), calpains activity was measured. Calpain activity was measured using the fluorogenic substrate Suc-LY-AMC in lysates of 4, 6, 8 and 12h of rotenone incubation; however, no statistically significant differences were observed between rotenone-treated and control-untreated CGN (data not shown).

In this scenario and since autophagy has gained relevance in PD studies (Anglade et al., 1997; Schapira and Jenner, 2011) and others have shown that CGN death by autophagy is mediated by the activation of lysosomal proteases like cathepsin D (Maycotte et al.; Uchiyama, 2001), we decided to investigate the levels of the active fragment of cathepsin D. The levels of active cathepsin D were measured by western blotting of CGN lysates at 4, 6 and 8 h of rotenone incubation. As it is shown in Figure 3.4C activation of cathepsin D has been observed at 4h of rotenone incubation.

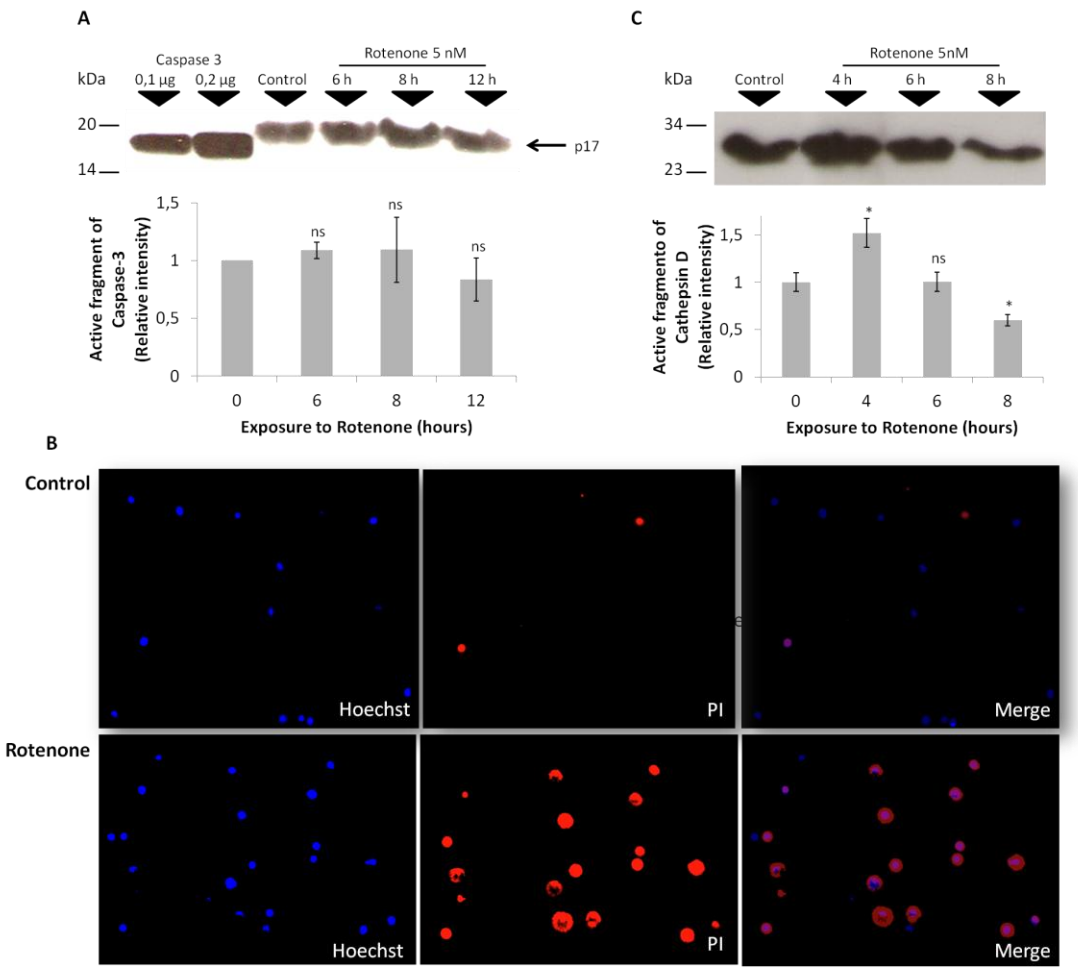

Figure 3.4: Evaluation of cell death pathways in CGN mediated by rotenone. (A) Western blot analysis of caspase-3 activated fragment at 6, 8 and 12h of incubation with rotenone; revealing no significant differences between control (0 h) and rotenone treated CGN. **(B)** Fluorescence microscopy of CGN; Hoechst dye staining genetic material (nuclei) and propidium iodide (PI) dye filling cells with disrupted plasma membrane. **(C)** Western blotting of cathepsin D at 4, 6 and 8 h of rotenone incubation showed an activation of cathepsin D at 4h of rotenone incubation. Independent experiments were repeated three times. Values are presented as mean±SEM. *p<0.05 compared to non-treated cells, ns: not significant.

3.1.4. Creatine completely attenuates rotenone-induced cathepsin D activation

The capacity of creatine and epicatechin to attenuate the activation of lysosomal cathepsin D in the cell death promoted by rotenone in CGN was also evaluated by western blotting. Lysates of 4 h of rotenone incubation with a pre-incubation of 1h of creatine or epicatechin were analyzed for experimental measurements of active fragment of cathepsin D. The results (Figure 3.5) indicated a complete attenuation of cell death by creatine (which restored the control levels of active cathepsin D) while epicatechin did not afford a significant reduction of the cathepsin D activation induced by rotenone.

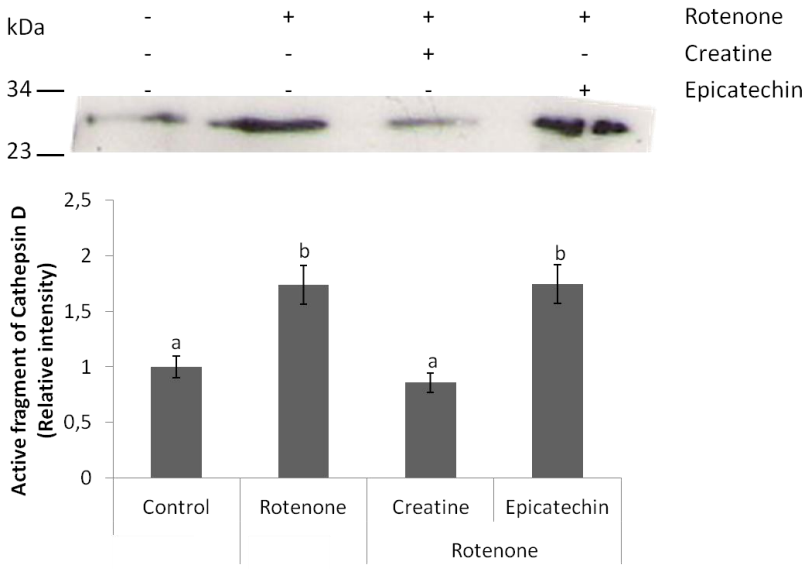

Figure 3.5: Effect of creatine and epicatechin on rotenone-induced increase of active cathepsin D levels in CGN. Independent experiments were repeated three times. Values are presented as mean±SEM. Control and creatine (a) as well as rotenone and epicatechin (b) are not significantly different between them.

These results are in good agreement with those previously obtained for cell viability with the MTT assay (*vide* 3.1.2). Therefore, the results suggest that autophagy may represent a potentially important cell death pathway for the death caused by rotenone in CGN, which deserves to be further explored.

3.1.5. Creatine protects against mitochondrial membrane depolarization caused by rotenone in CGN

Rotenone is a well-known blocker of the electron transport chain complex I. Thus, inhibition of mitochondrial complex I by rotenone may lead to mitochondrial dysfunction such as a decrease in ATP production and mitochondrial membrane depolarization (Barrientos and Moraes, 1999; Li et al., 2003; Sherer et al., 2003a).

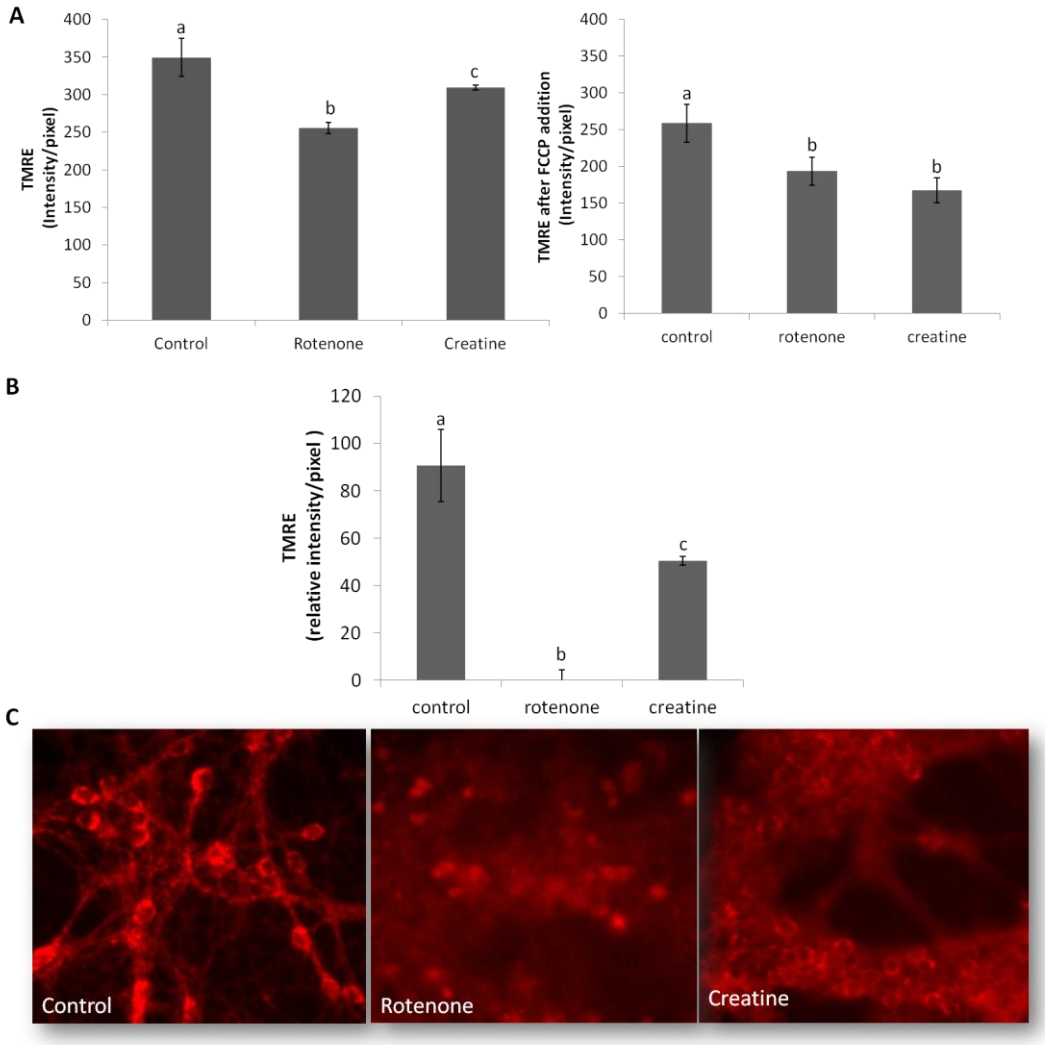

Figure 3.6: Effect of creatine on rotenone-induced mitochondrial depolarization. Neuronal cultures were pretreated with 30 mM of creatine for 1 h prior to rotenone treatment for 12 h. Mitochondrial membrane potential was monitored by TMRE fluorescence microscopy. **(A)** Average intensity per pixel obtained at a constant fixed time of 10 min or 5 min in fluorescence microscopy images acquired with CCD Hamamatsu ORCA R^2 and HImage software with the same light-flash duration (same exposure time) after TMRE addition (left) and after FCCP addition (right) respectively. **(B)** Relative intensity per pixel obtained by subtraction of the values of FCCP. Rotenone produced a decrease in TMRE fluorescence intensity (compared to control) indicating a significant mitochondrial membrane depolarization. Creatine was able to protect against the decrease caused by rotenone on the fluorescence intensity within CGN somas, indicating a protective effect against the mitochondrial membrane depolarization induced by rotenone. **(C)** Images of fluorescence microscopy of TMRE-loaded CGN acquired with a constant exposure time (flash-lamp time) of 0,2 s. Rotenone-treated neurons exhibited lower red fluorescence, revealing loss of mitochondrial membrane potential ($\Delta\psi m$) caused by the decrease in TMRE accumulation within these subcellular organelles. Pretreatment with creatine significantly protected neurons against rotenone-induced loss of $\Delta\psi m$, as demonstrated by the maintenance of red fluorescence intensity as well as by the distribution of TMRE fluorescence staining within CGN soma. Independent experiments were repeated three times. Values are presented as mean±SEM. All the values are statistically different one of each other ($p<0.05$).

Therefore, we experimentally assessed the effect of rotenone on $\Delta\psi m$ in the presence or absence of creatine, using TMRE. TMRE is a cell permeant, positively-charged, red-orange dye that readily accumulates in active mitochondria due to the large negative charge of their inner membranes. Indeed, it has been demonstrated that using a large number of cell types, depolarized or inactive mitochondria have decreased membrane potential and fail to sequester TMRE (Miyamoto et al., 2005; Narendra et al., 2010; Natoni et al., 2006). Most of CGN incubated with rotenone 5 nM by 12 h suffered a dramatic decrease in $\Delta\psi m$, as judged by a decrease in the accumulation of TMRE within the cells, shown by the loss of fluorescence intensity signal in microscopy images (Figure 3.6C). Pretreatment with creatine significantly protected cells against this loss of mitochondrial potential, as demonstrated by the maintenance of a high fluorescence intensity staining (Figure 3.6B) compared to that obtained for rotenone (at a constant fixed time of 10min, to minimize the incidence of other experimental variables like exposure to xenon-lamp irradiation and subsequently-induced photobleaching).

As a positive control FCCP was used. The protonophore FCCP collapses the proton gradient across the mitochondrial inner membrane, leading to abolition of the mitochondrial potential (Dispersyn et al., 1999). FCCP was added at the end of each assay and in cases where the membrane showed no depolarization (control and creatine), after FCCP addition red fluorescence intensity was decreased revealing a depolarization by FCCP (Figure 3.6A).

These observations imply that the protective effect of creatine against rotenone-induced cell death results, in part, from preventing mitochondrial depolarization.

3.1.6. Rotenone causes generalized oxidative stress but only a weak nitrosative stress in CGN

PD as well as rotenone-induced brain neurodegeneration has been widely related with oxidative stress. Indeed oxidative stress has been pointed as one of the central causes of sporadic PD.

In this study oxidative stress produced by rotenone in CGN was evaluated by three different and complementary methodologies: (1) H_2DCFDA staining has been used to monitor ROS levels causing a generalized intracellular oxidative stress; (2) protein nitrotyrosines have been measured in cellular lysates to ascertain the putative occurrence of nitrosative stress-mediated by peroxynitrite; and (3) GSH levels were

determined in whole CGN cells using the MCB to monitor the putative lowering of the intracellular reduction state, as this is the major water soluble antioxidant present in the neuronal cytosol.

H_2DCFDA is a cell-permeant dye used as an indicator for ROS in cells. Upon cleavage of the acetate groups by intracellular esterases and oxidation, the nonfluorescent H_2DCFDA is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) which can be detected by fluorescence microscopy with an excitation filter of 470 nm and an emission filter of 510 nm. Cells after 12 h of rotenone incubation as well as those with an additional pre-icubation by 1h with creatine or epicatechin were switched to MLocke 25 buffer and incubated with 10 µM H_2DCFDA. The intensity of fluorescence within the neuronal somas monitors the conversion of H_2DCFDA in DCF and can be used as a measurement of ROS produced within the cells. However, these measurements need to be done after a short time after H_2DCFDA addition to the extracellular medium because produced DCF diffuse from the cytosol to the extracellular medium with a half time in the minutes-scale range (Samhan-Arias et al., 2004). The results of measurements of fluorescence intensities in neuronal somas at a fixed time (1 min) after addition of H_2DCFDA to the CGN culture are presented in Figure 3.7A. The results pointed out a 6-fold increase in DCF compared to the control (treated with the vehicle DMSO), revealing a stimulated ROS production in CGN after 12h incubation with rotenone. Moreover, the results also showed a strong attenuation by creatine of the stimulation of ROS production induced by rotenone, noteworthy, higher than that afforded by the antioxidant flavonoid epicatechin.

Protein nitrotyrosines levels in CGN lysates have been used as indicator of the nitrosative stress and have been determined using Western blotting. As shown in Figure 3.7B, 12h incubation of CGN with rotenone elicits only a weak increase of protein nitrotyrosines with respect to control (treated with the vehicle DMSO only), remarkably, the treatment with (rotenone + creatine) or with (rotenone + epicatechin) decreased the levels of protein nitrotyrosines to values lower than those obtained with lysates of control CGN. Thus, these results allow concluding that rotenone induces a weak nitrosative stress in CGN, which is attenuated by creatine and, in this case, more strongly by epicatechin.

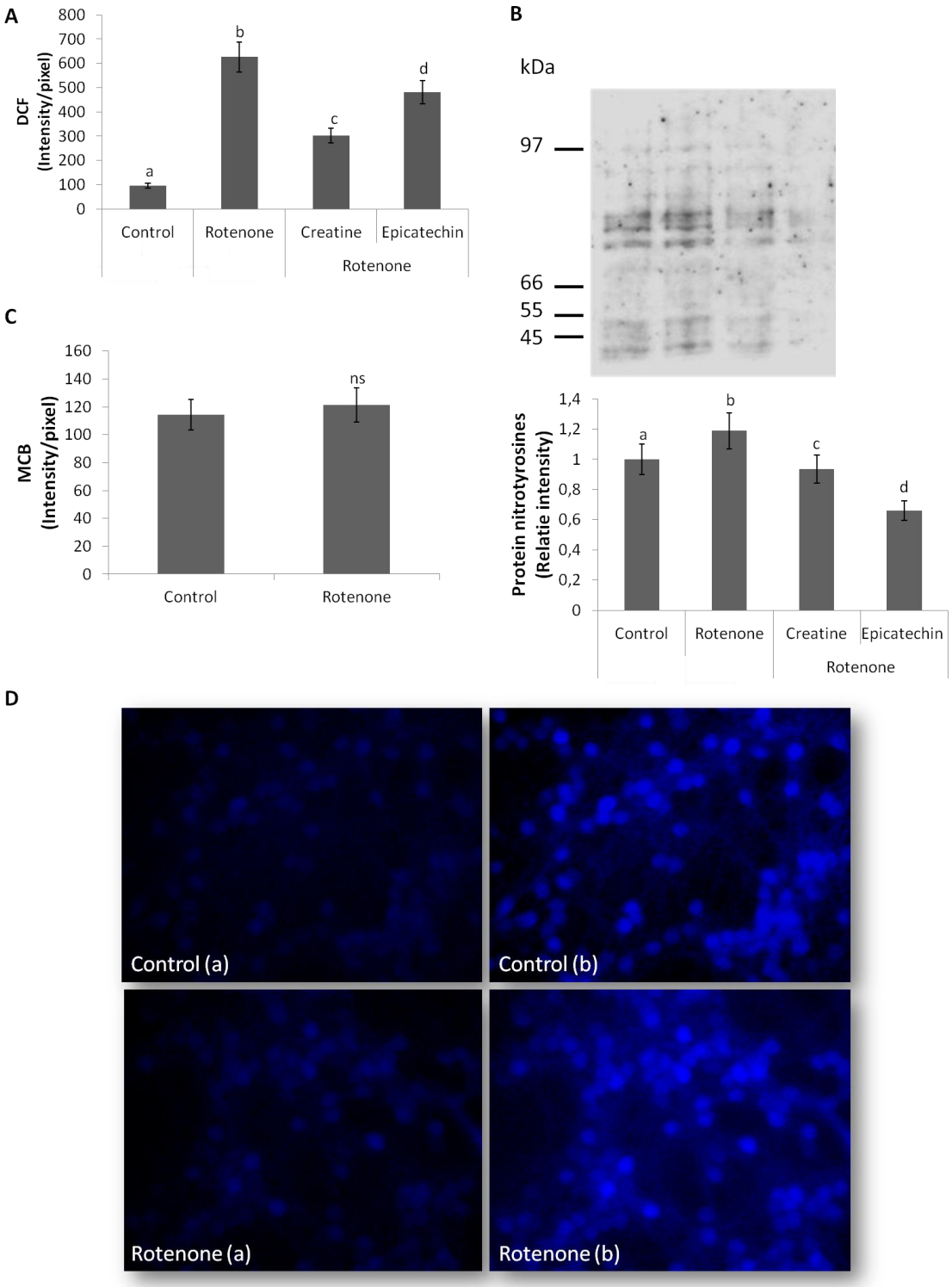

Figure 3.7: Oxidative stress induced by rotenone in CGN. (A) Intracellular ROS production detected by H_2DCFDA staining after 12 h of rotenone incubation. **(B)** Protein nitrotyrosines obtained by Western blotting of lysates of CGN treated during 12 h with rotenone. **(C)** Levels of GSH within CGN monitored with MCB after 12 h incubation with rotenone and in control cells. **(D)** Images of fluorescence microscopy of MCB-loaded CGN acquired with a constant exposure time (flash-lamp time) of 0,77 s; (a) 1 minute after MCB addition and (b) past, approximately, 10 minutes when the dye was already conjugated with GSH to form a fluorescent product. Independent experiments were repeated three times. Values are presented as mean±SEM. ns: not significant, a, b, c and d values are statistically different one of each other ($p<0.05$).

As briefly indicated above GSH is a major component of cellular antioxidant defenses. A decrease of GSH levels is currently observed in cells suffering strong oxidative stress conditions, due to increased GSH usage, and although part of the GSSG generated can be reduced back to GSH, the formation and export of GSH conjugates leads to GSH depletion under these conditions. The levels of GSH in CGN after 12h incubation with rotenone were determined using the cell permeable dye MCB. MCB is a nonfluorescent bimane, and within the cytosol it is rapidly conjugated with GSH to form a fluorescent product that can be detected by fluorescence microscopy with an excitation filter of 380 nm and an emission filter of 510 nm. The intensities of the fluorescence of the neuronal soma, at a stable and fixed time after addition of 10 μM MCB to the extracellular medium (10 min), are presented in Figures 3.7C and 3.7D, revealing that GSH levels are not altered by 12h incubation of CGN with rotenone.

3.1.7. Creatine protects against AMPK activation induced by rotenone in CGN

PD is a disease included within the large number of pathologies related to deregulated cellular bioenergetics, nowadays mainly attributed to a functional loss in mitochondrial complex I and consequently in the mitochondrial electron transport chain. Although it is assumed that this functional loss could compromise cell viability and lead to cell death, the functional impairment of cellular enzymes, which can attenuate this energetic depletion, namely CK and AMPK, has not been explored in detail. Each one of these enzymes is able to supply metabolic energy or to decrease metabolic energy waste in neurons suffering the bioenergetics deficit generated by mitochondrial functional loss as was mentioned in the Introduction.

CK levels were determined by western blotting of CGN lysates. Figure 3.8A shows that the levels of CK were not significantly altered after 12 h incubation of CGN with rotenone with respect to control (untreated) CGN, neither when incubation of CGN was done with rotenone after a pre-incubation of 1 h with creatine or epicatechin. As CK is an enzyme sensitive to modulation by ROS/RNS and in particular highly sensitive to peroxynitrite (Mihm and Bauer, 2002; Mihm et al., 2002), its activity in the CGN lysates cannot be predicted solely on the basis of the protein expression levels. Due to this, we also measured the CK activity of CGN lysates (Figure 3.8B), and the results showed that 12 h incubation with rotenone did not elicit a significant change of

the CK activity. Consistent with these results Western blotting of protein nitrotyrosines in CGN lysates did not reveal any significant increase of anti-nitrotyrosine labelling of the protein band corresponding to the molecular weight of CK (Figure 3.8A, right panel).

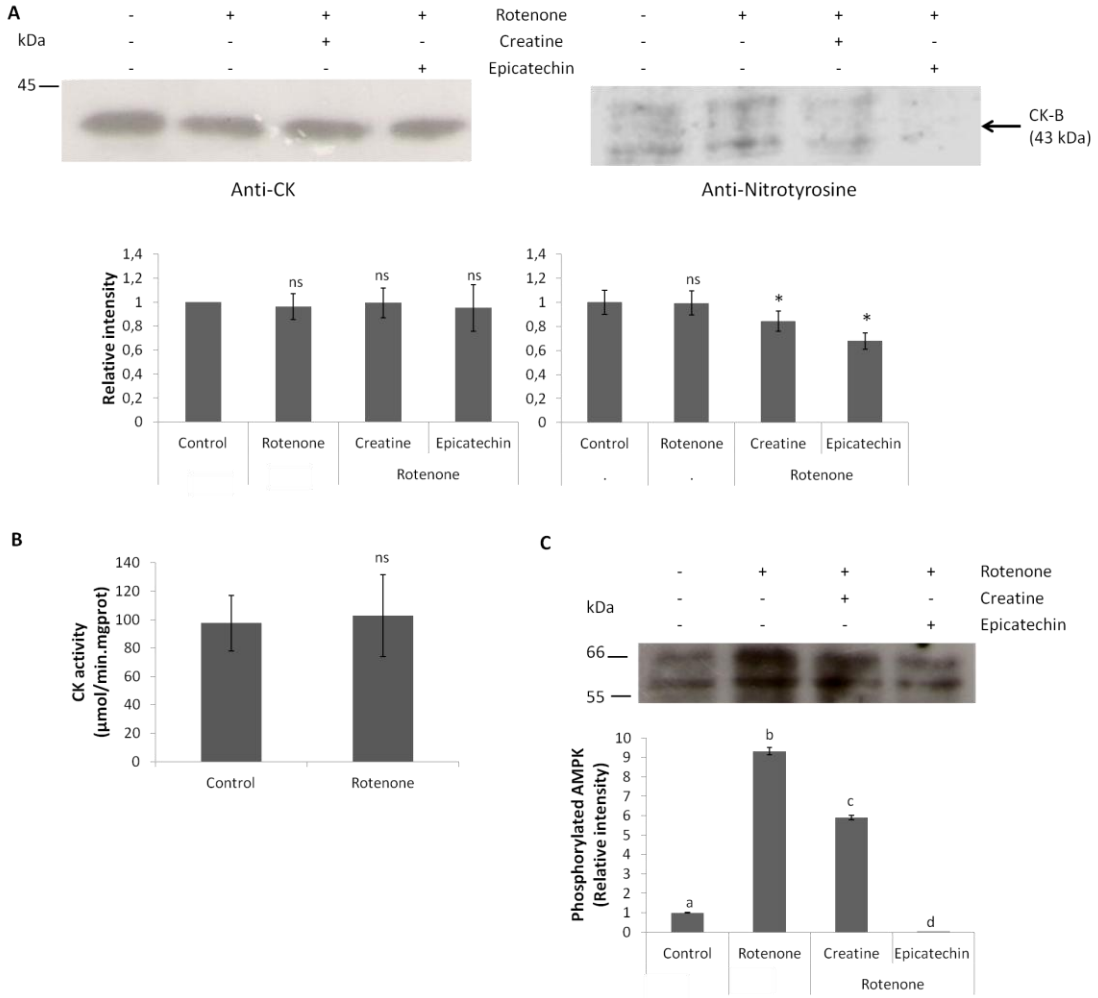

Figure 3.8: CK and AMPK activity in CGN after incubation with rotenone. (A) Western blot analysis of CGN lysates did not show significant differences in the level of CK as well as in anti-nitrotyrosine labelling of the protein band corresponding to the molecular weight of CK. **(B)** CK activity in CGN lysates after 12 h incubation with rotenone was measured by an enzymatic method based on the rate of formation of NADPH, and monitored spectrophotometrically at 340 nm, at 37 ºC. No statistically significant differences were observed between control CGN and after 12 h incubation with rotenone. **(C)** Phosphorylated AMPK levels detected by western blotting revealed a nearly 10-fold increase in CGN incubated 12 h with rotenone compared to control. Both, creatine and epicatechin significantly attenuated the increase of phosphorylated AMPK levels induced by rotenone. Independent experiments were repeated three times. Values are presented as mean±SEM. *$p<0.05$ compared to non-treated cells, ns: not significant, a, b, c and d values are statistically different one of each other ($p<0.05$).

In contrast, the phosphorylated AMPK levels, determined by Western blotting of lysates in the same conditions of CK, were increased nearly 10-fold by 12 h incubation with rotenone compared to control (incubated only with the vehicle DMSO) as shown in Figure 3.8C. The increase of the phosphorylated AMPK levels caused by rotenone was found to be strongly attenuated not only by creatine (approx. 40% reduction) but also by epicatechin, which decreased the phosphorylated AMPK levels to values even lower than those measured in control CGN lysates.

3.2. Acute exposure (30 min) of CGN to rotenone

3.2.1. Creatine attenuates Ca^{2+} homeostasis deregulation promoted by rotenone in CGN

The maintenance of a steady intracellular and cytosolic Ca^{2+} concentration, i.e. calcium homeostasis, can be regarded as a major bioenergetics marker for neuronal activity and neuronal survival, as briefly highlighted in the Introduction.

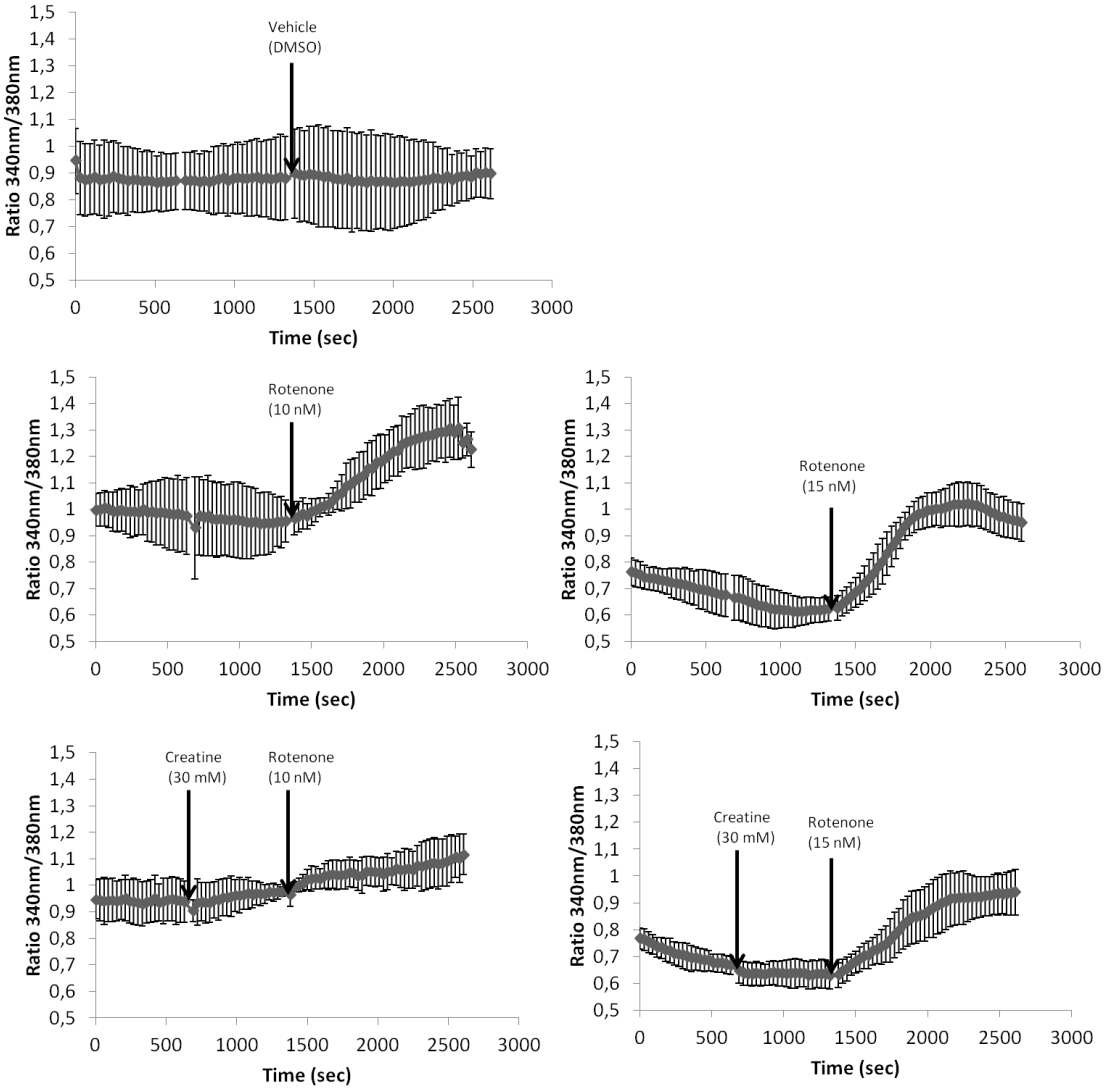

Figure 3.9: Creatine affords protection against deregulation of cytosolic Ca^{2+} by rotenone. Kinetic traces of the ratio 340/380 of fura 2-AM-loaded CGN after the additions indicated in the figure. After loading CGN with fura 2-AM the medium was changed to MLocke 25 and the ratio 340/380 measurements were initiated, creatine was added after ≈600 s (where indicated by an arrow) and rotenone after ≈1200 s. The results shown are the mean±SEM of experiments done by triplicate.

The ability of rotenone to alter cytosolic Ca^{2+} concentration was evaluated after an acute exposure to rotenone during 30 min, aiming to experimentally assess the possibility that impairment of intracellular Ca^{2+} homeostasis could be an early event in the neurotoxicity of rotenone. As this is a time much shorter than that used for cell viability assays we have increased the rotenone concentration in these assays up to values double (10 nM) and threefold (15 nM) of the IC_{50} obtained above for cell viability loss after 12 h incubation with rotenone.

After 45–60 min of incubation with 5 µM fura-2 AM and 0.025% Pluronic-F127 at 37 ºC in culture medium (DMEM-F12), CGN were washed with MLocke 25 buffer and the culture dish was placed in a thermostatic controlled plate. Fluorescence ratio images were obtained with excitation filters of 340 and 380 nm during 20 min until the signal of the fluorescence intensity ratio (340/380) in the soma of CGN was stabilized. Thereafter, rotenone was added and images were acquired during 30 min more at intervals of 30 seconds. As observed in Figure 3.9, both 10 and 15 nM rotenone concentrations elicit an increase of the ratio 340/380, i.e. of cytosolic Ca^{2+}, being steeper and larger at the highest rotenone concentration (Figure 3.10A).

The putative capacity of creatine to prevent the deregulation of cytosolic Ca^{2+} by rotenone was experimentally assessed for the two concentrations of rotenone. Creatine (30 mM) was added to the culture dish 10 min after stabilization and 10 min before rotenone addition. As depicted in Figure 3.9 and numerically represented in Figure 3.10 creatine significantly reduced the cytosolic Ca^{2+} elevation in both cases, revealing, as expected, more protective effect against 10 nM (≈80%) than against 15 nM of rotenone (≈40%). Nevertheless, taking into account that the larger the increase of the ratio 340/380 the better for ascertaining modulatory effects (higher signal to noise ratio), 15 nM of rotenone was chosen for all the subsequent studies.

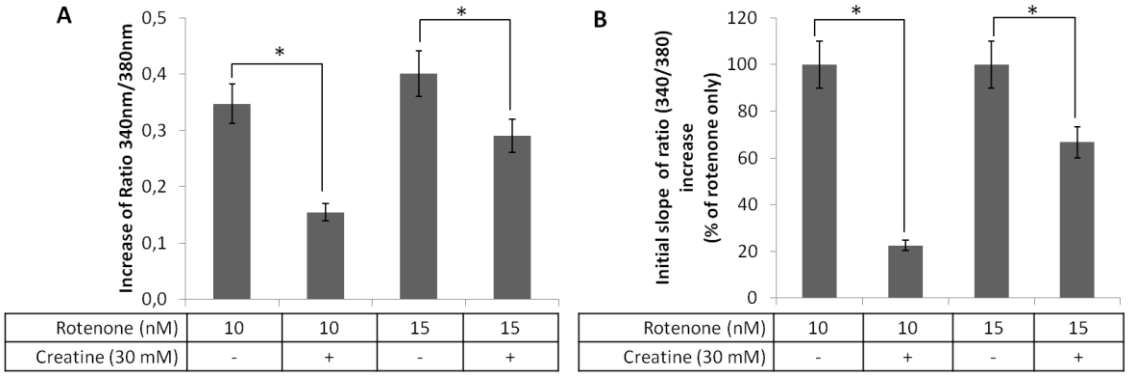

Figure 3.10: Creatine protection against deregulation of cytosolic Ca^{2+} by rotenone. (A) Ratio (340/380) increase after addition of rotenone (absolute values) obtained from the analysis of the kinetic traces shown in the Figure 3.9. **(B)** Initial slope of the ratio (340/380) increase after addition of rotenone expressed as percentage of the values obtained for the addition of only rotenone. *$p<0.05$ compared to cells treated only with rotenone.

3.2.2. Epicatechin, but not kaempferol, attenuated Ca^{2+} homeostasis deregulation promoted by rotenone, and, both flavonoids antagonized the protective effect of creatine

The possibility that the antioxidant flavonoids epicatechin and kaempferol could act as protectors against rotenone-induced Ca^{2+} homeostasis deregulation has also been evaluated, as well as the capacity of these two flavonoids to increase/decrease the effect of creatine.

The procedure was the same used for creatine, but in this case we have done co-incubation of creatine and each flavonoid to evaluate their putative synergistic/antagonistic effects. As it can be seen in Figure 3.12, like creatine, epicatechin was also able to significantly protect ($p<0.05$) against rotenone-induced Ca^{2+} homeostasis deregulation. However, when these two compounds (creatine and epicatechin) were added together there were no indications of synergistic effects, but on the contrary epicatechin showed an antagonistic effect with creatine, as the kinetic traces of the ratio 340/380 did not show a statistically significant reduction of the ratio increase with respect to those obtained after incubation with only rotenone (Figures 3.11 and 3.12). Similar results were found in the case of co-incubation of creatine with kaempferol (Figures 3.11 and 3.12).

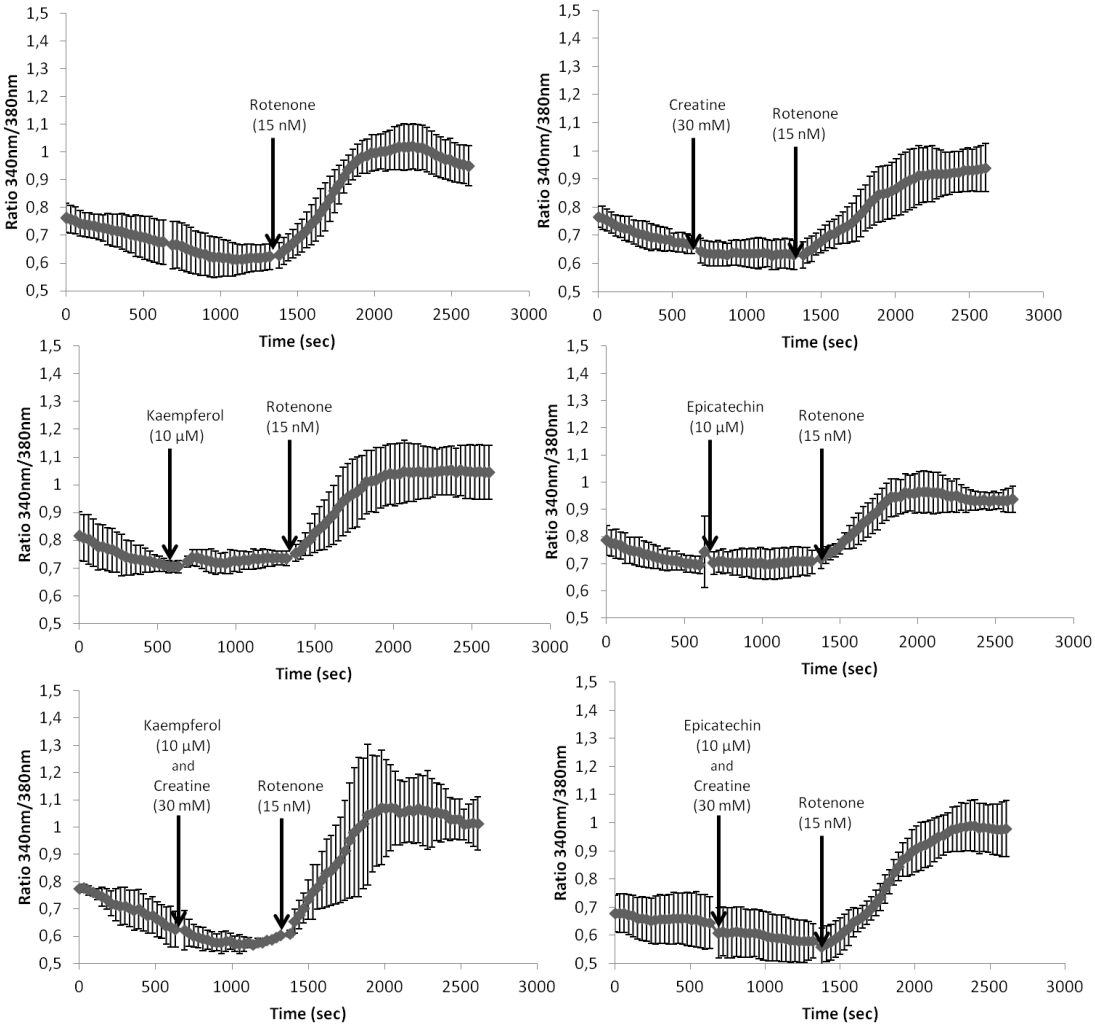

Figure 3.11: Effects of creatine, epicatechin and kaempferol (alone and in combination) on deregulation of cytosolic Ca^{2+} by rotenone. Kinetic traces of the ratio 340/380 of fura 2-AM-loaded CGN after the additions indicated in the figure. After loading CGN with fura 2-AM the medium was changed to MLocke 25 and the ratio 340/380 measurements were initiated. Creatine or epicatechin or kaempferol as well as creatine with epicatechin and creatine with kaempferol were added after ≈600 s (where indicated by an arrow) and rotenone after ≈1200 s. The results shown are the mean±SEM of experiments done by triplicate.

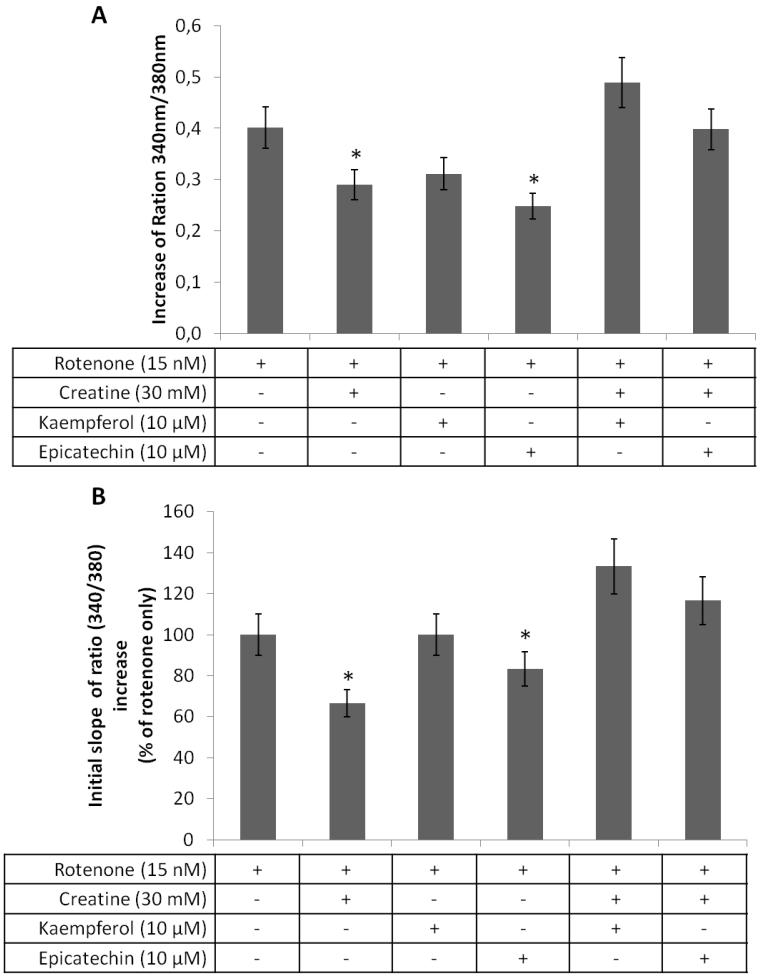

Figure 3.12: Creatine and epicatechin afford partial protection against deregulation of cytosolic Ca^{2+} by rotenone. (A) Ratio (340/380) increase after addition of rotenone (absolute values) obtained from the analysis of the kinetic traces shown in the Figure 3.11. **(B)** Initial slope of the ratio (340/380) increase after addition of rotenone expressed as percentage of the values obtained for the addition of only rotenone. *$p<0.05$ compared to cells treated only with rotenone.

3.2.3. Rotenone leads to Ca^{2+} homeostasis deregulation through functional alterations of several Ca^{2+} transport systems of the plasma membrane and ER

The deregulation of the steady cytosolic Ca^{2+} concentration by rotenone indicated above prompted us to develop experimental studies aiming to identify the putative impairment of the major Ca^{2+} transport systems involved in the control of CGN cytosolic Ca^{2+} homeostasis.

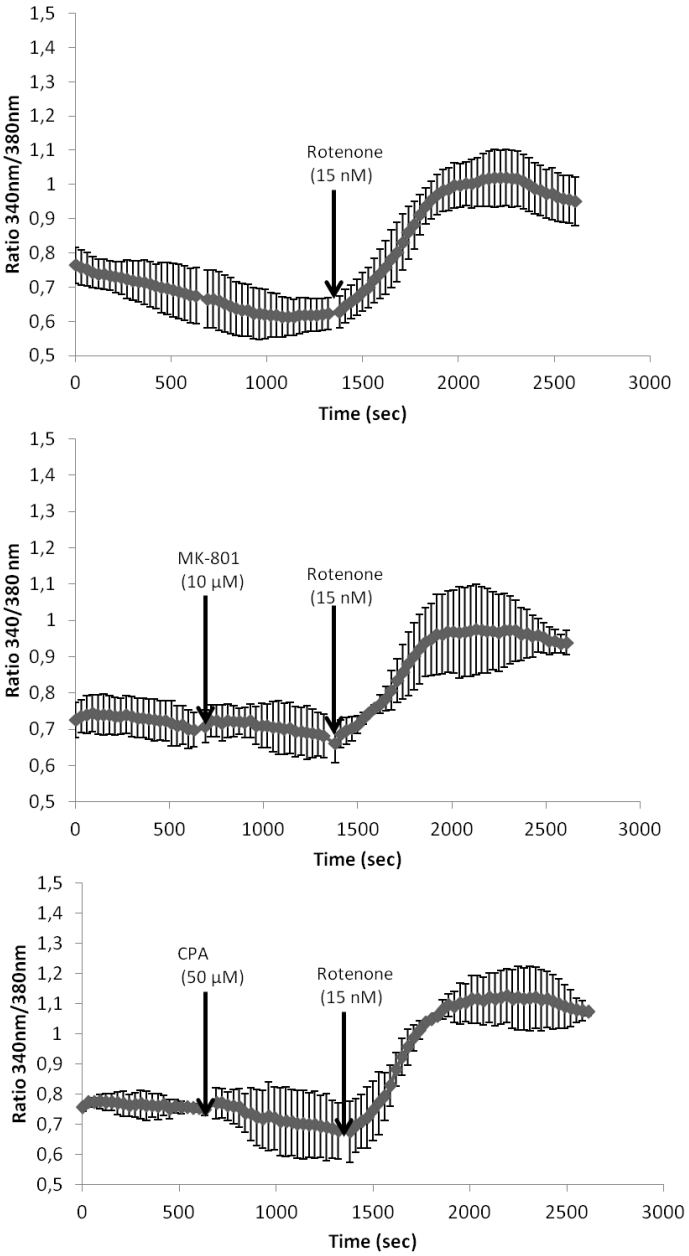

Figure 3.13: Effects of inhibitors of the major Ca^{2+} transport systems controlling the cytosolic Ca^{2+} homeostasis in CGN on Ca^{2+} deregulation caused by rotenone. Kinetic traces of the ratio 340/380 of fura 2-AM-loaded CGN after the additions indicated in the figure. After loading CGN with fura 2-AM the medium was changed to MLocke 25 and the ratio 340/380 measurements were initiated. MK-801 or CPA were added after ≈600 s and rotenone after ≈1200 s (where indicated by the arrows). The results shown are the mean±SEM of experiments done by triplicate. The kinetic traces obtained for nifedipine are almost identical to that of MK-801 and the kinetic trace obtained for cyclopiazonic acid (CPA) is almost identical to the averaged trace obtained for KB-R7943.

As briefly commented in the introduction, cytosolic free Ca^{2+} is approximately 10,000-fold lower than the free Ca^{2+} concentration in the extracellular space. Therefore, to maintain Ca^{2+} homeostasis, Ca^{2+} entering neurons must be pumped back across the

plasma membrane concentration gradient and/or rapidly sequestered by intracellular organelles (largely by ER and mitochondria). In mature CGN *in vitro* the plasma membrane Ca^{2+} transport systems have the highest impact in controlling the homeostasis of cytosolic Ca^{2+}, being in this regard L-VOCC and NMDA receptors the most relevant Ca^{2+} entry systems and PMCA and NCX the most relevant systems for extrusion of Ca^{2+} from the cytosol (Gutierrez-Merino, 2008). On these grounds, we started this study using inhibitors/blockers of these Ca^{2+} transport systems of the plasma membrane. More precisely, nifedipine (2 μM) has been used as L-VOCC blocker, MK-801 (10 μM) as a NMDA antagonist and KB-R7943 (5 μM) as inhibitor of NCX. In addition, we have also experimentally assessed the putative impairment of the Ca^{2+}-ATPase of sarco-endoplasmic reticulum (SERCA) using the specific inhibitor CPA (50 μM). These compounds were added to the culture dish and cytosolic Ca^{2+} measurements were performed as indicated previously. Each one of the tested inhibitors was added to the culture dish 10 min after stabilization of the ratio 340/380 measurements and 10 min before the addition of rotenone. Fluorescence ratio images were obtained with excitation filters of 340 and 380 nm with a time interval of 30 seconds. The results obtained demonstrated that nifedipine and MK-801 elicit a partial and statistically significant attenuation of the deregulation of calcium homeostasis caused by rotenone (Figure 3.14), therefore, indicating functional impairment of L-VOCC and NMDA by rotenone.

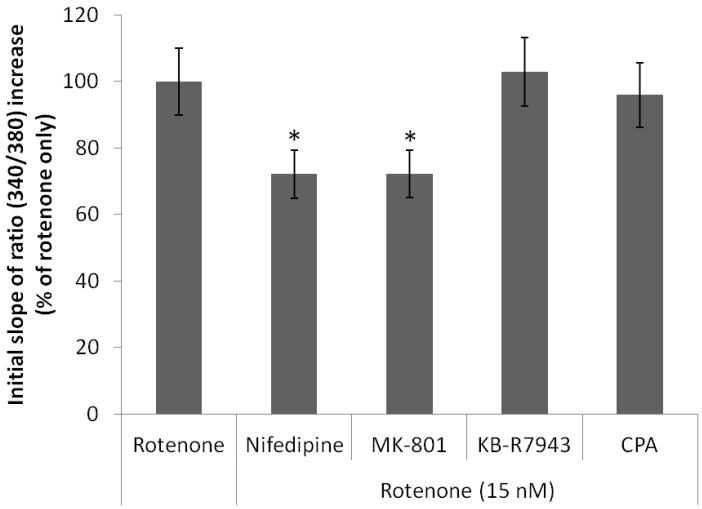

Figure 3.14: Effect of inhibitors of the major Ca^{2+} transport systems controlling the cytosolic Ca^{2+} homeostasis in CGN on Ca^{2+} deregulation caused by rotenone. Values presented are the initial slope of ratio (340/380) increase expressed as percentage of the values obtained for the addition of only rotenone. The results shown are the mean±SEM of experiments done by triplicate. *$p<0.05$ compared to CGN treated only with rotenone.

During last years many studies have pointed out the implication of SOCE in cellular responses to stress conditions mediated by Ca^{2+} deregulation, as briefly summarized in the introduction of this work. Therefore, we have assessed the putative functional impairment of SOCE on the cytosolic Ca^{2+} deregulation induced by rotenone in CGN.

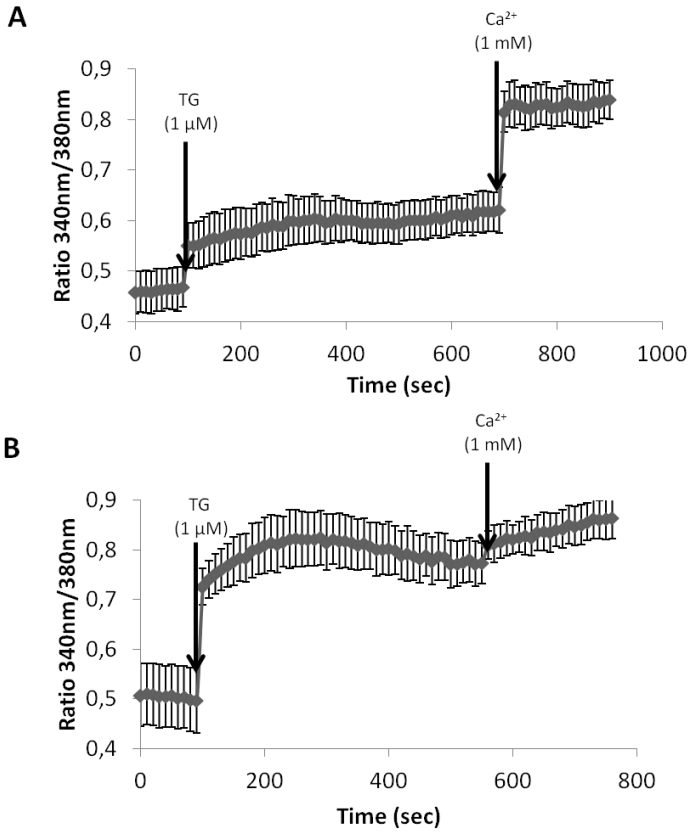

Figure 3.15: Rotenone induces functional impairment of SOCE in CGN. Kinetic traces of the ratio 340/380 of fura 2-AM-loaded CGN after the additions indicated in the figure. After loading CGN with fura 2-AM the medium was changed to MLocke 25 and the ratio 340/380 measurements were initiated. **(A)** CGN were treated with 1 µM thapsigargin (TG) in MLocke 25 without added Ca^{2+} in control conditions (45 min in MLocke 25 without added Ca^{2+} and in the absence of rotenone), and 600 s later 1 mM Ca^{2+} was added to the extracellular medium. **(B)** After 45 min of incubation with rotenone CGN were treated with 1 µM TG in MLocke 25 without added Ca^{2+}, and 600 s later 1 mM Ca^{2+} was added to the extracellular medium. The results shown are the mean±SEM of experiments done by triplicate.

After loading CGN with fura 2-AM (45–60 min of incubation with 5 µM fura-2 AM and 0.025% Pluronic-F127 at 37 ºC) in culture medium (DMEM-F12), CGN were changed to a MLocke 25 buffer without Ca^{2+} (0 mM Ca^{2+}). Fluorescence ratio images were obtained as indicated in the Materials and methods during 45 min. As shown by the results of the Figure 4.15, addition of 1 µM TG to CGN pre-incubated 45 min with

15 nM rotenone induced an increase of the ratio 340/380 which is approximately three-fold higher than the increase observed with control (untreated) CGN. This result point out that incubation with rotenone results in Ca^{2+} overload of ER. Later addition of 1 mM Ca^{2+} to the extracellular medium, after Ca^{2+} depletion of ER stores, should lead to opening SOCE inducing a further raise of the ratio 340/380 values as shown in the case of control (untreated) CGN (Figure 3.15A). Noteworthy, this response is largely attenuated in CGN incubated with rotenone (Figure 3.15B), pointing out that the treatment with rotenone has led to an almost complete blockade of the opening of plasma membrane Ca^{2+} channels responsible of SOCE. These results strongly support that functional impairment of SOCE also mediates rotenone-induced deregulation of cytosolic calcium homeostasis in CGN.

3.2.4. Acute (30 min) exposure of CGN to rotenone did not lead to oxidative stress neither to mitochondrial membrane depolarization

As we have shown that rotenone triggered an oxidative stress and a mitochondrial membrane depolarization in CGN after 12 h incubation, we wanted to ascertain whether these are early events or events taking place at a later stage than cytosolic Ca^{2+} deregulation. To this end, intracellular oxidative stress was measured using H_2DCFDA staining in living cells and protein nitrotyrosines levels in CGN lysates as indicated before in this work. The experiments were performed as described for the analysis of these experimental parameters after 12 h incubation with rotenone (see section 3.1.6), except that in this case the incubation with rotenone was only 30 min. As shown in Figure 3.16A, the oxidation of H_2DCFDA did not increase after incubation of 30 min with rotenone with respect to that measured for control (untreated) CGN because no increase of the fluorescence of DCF at a fixed time (1 min) is observed, on the contrary, it is significantly lowered. Thus, 30 min of incubation with rotenone are not enough to trigger an enhanced production of ROS in CGN. Regarding nitrosative stress, the results obtained by western blotting of lysates of CGN with 1h of incubation with rotenone showed no statistically significant differences in protein nitrotyrosine levels between rotenone-treated and control (untreated CGN lysates), indicating that an acute exposure to rotenone does not stimulate RNS production in CGN.

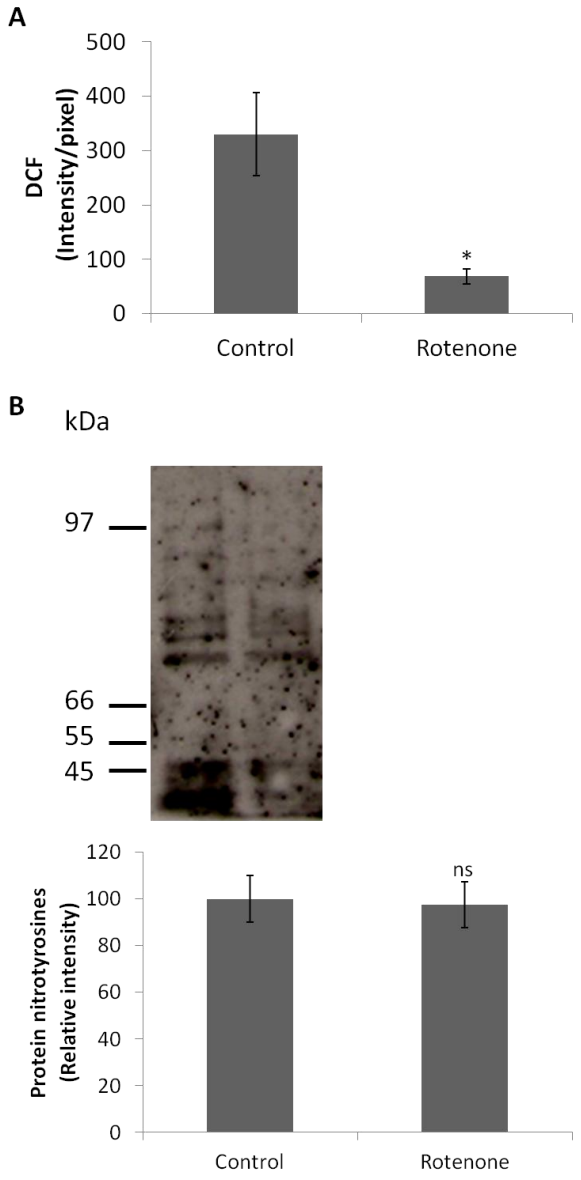

Figure 3.16: Incubation of CGN with rotenone for 30-60 min does not increase oxidative stress in CGN. (A) Intracellular ROS production detected by CGN stained with H_2DCFDA is not increased after 30 min incubation with rotenone. **(B)** Protein nitrotyrosines levels revealed by western blotting of CGN lysates do not increase after 1h incubation with rotenone. The results shown are the mean±SEM of experiments done by triplicate. *$p<0.05$ compared to non-treated cells, ns: not significant.

The effect of rotenone $\Delta\psi m$ was assessed using TMRE as in 3.1.5. The cells incubated with 15 nM of rotenone during 30 min do not seem to have a decrease in $\Delta\psi m$, as indicated by the maintenance of the accumulation of TMRE in cells (red fluorescence) (Figure 3.17B) and also by the preservation of intensity (Figure 3.17A) compared to that obtained for control. Thus, the results suggest that $\Delta\psi m$ is not significantly affected by an acute exposure of CGN to rotenone.

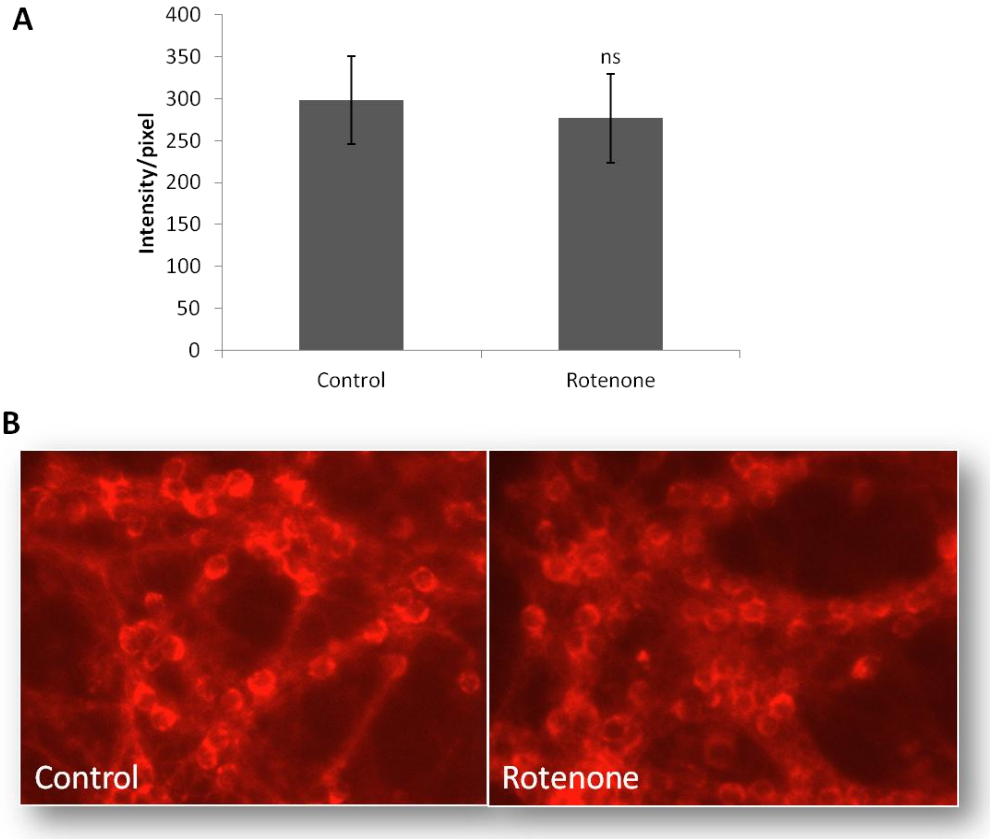

Figure 3.17: Effect of 30 min incubation with rotenone on the mitochondrial membrane potential (Δψm) of CGN. CGN were incubated with 15 nM of rotenone for 30 min. Δψm was monitored with the TMRE assay. **(A)** Average intensity per pixel obtained at a fixed time after addition of TMRE (10 min) from the analysis of quantitative fluorescence microscopy images acquired with the Hamamatsu ORCA R^2 CCD and HCImage software. Incubation with rotenone did not afford a statistically significant decrease in TMRE intensity (compared to control). **(B)** Images of fluorescence microscopy. Rotenone-treated neurons maintained the bright red fluorescence indicative of active mitochondria. Independent experiments were repeated three times. The results shown are the mean±SEM of experiments done by triplicate. ns: not significant.

IV. Discussion

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Although there is lack of certainty on the pathophysiology of neurodegenerative mechanisms underlying PD, it is well accepted that energy depletion, oxidative stress and mitochondrial dysfunctions are major factors associated with this disorder (Dexter et al., 1994; Jenner and Olanow, 1998; Schapira and Jenner, 2011). Furthermore, as have been highlighted in the Introduction, PD pathology is not restricted to DA degeneration. The application during the last years of medical techniques, such as TMS, SPECT, PET and fMRI has revealed functional anomalies of the cerebellum in PD and Parkinsonism patients (Brockmann et al., 2012; Cao et al., 2011; Kimura et al., 2011; Koch et al., 2009; Ni et al., 2010; Wu et al., 2009a; Wu et al., 2009b). Moreover, no single cellular and animal model to date has been able to recapitulate all the pathological features of PD, although the neurotoxic animal models of PD have contributed much to our understanding on human PD (Cannon and Greenamyre, 2010). Three neurotoxins, 6-OHDA, MPTP and rotenone, are the most successful agents so far to mimic Parkinsonism *in vitro* and *in vivo* (Beal, 2001).

Bearing this in mind, we have used a CGN/rotenone model which is a widely used model for the study of cellular and molecular correlates of cell death and neurodegeneration/neuroprotection (Contestabile, 2002).

Our results revealed a strong protection by the ergonenic molecule creatine against CGN death promoted by rotenone, indicating an energetic failure mediated by rotenone in these neurons, since creatine have been widely reported as an energetic compound (Andreassen et al., 2001a; Bender et al., 2008a; Pan and Takahashi, 2007; Wyss and Schulze, 2002). The mentioned energetic failure was further supported by the ability of creatine to prevent the mitochondrial depolarization triggered by rotenone, a target event of a cellular energetic unbalance.

Despite the many reports in the bibliography suggesting an involvement of oxidative stress as a primary event in PD, the flavonoid antioxidants kaempferol and epicatechin failed to afford protection of CGN against rotenone neurotoxicity. On the contrary, in this work we have found that kaempferol potentiates de cytotoxic effect exerted by rotenone. This potentiation of rotenone cytotoxicity by kaempferol could be accounted by the inhibition by kaempferol of mitochondrial complex I activity, a novel experimental finding reported in a previous work of this laboratory (Lagoa et al., 2011), because as shown in this publication 10 µM kaempferol produced approximately 30% inhibition of the activity of complex I. Indeed, as briefly summarized in the Introduction, inhibition by rotenone of mitochondrial complex I is accepted as the

primary cause of rotenone cytotoxicity. These results are also in good agreement with the ability of kaempferol to antagonize the protection afforded by creatine against rotenone. On the other hand, in the same study (Lagoa et al., 2011) 10 µM epicatechin showed a very weak effect on complex I activity. Consistent with this result, in this work we show that epicatechin did not antagonize the protection afforded by creatine against rotenone cytotoxicity, on the contrary, epicatechin has been found to elicit a weak increase of the protection effect of creatine. Thus, our results led to the conclusion that different flavonoids can be harmful or beneficial in PD therapy depending on their cellular actions as bioactive molecules, not only on their chemical antioxidant properties.

In addition, direct measurements of ROS production and protein nitrotyrosines levels point out that oxidative stress is not an early event in the rotenone cytotoxicity to CGN, because of the absence of oxidative and nitrosative stresses after an acute exposure (30 min) to rotenone. However, after 12 h an increased production of ROS was observed, as well as a weak nitrosative stress confirming that cellular oxidative stress is a delayed or later event in CGN death induced by rotenone. Noteworthy, creatine had a strongest effect against ROS overproduction induced by the treatment of CGN with rotenone than the flavonoid antioxidant epicatechin. This result is in good agreement with results reported by others showing that creatine have direct chemical antioxidant properties as it can act as a scavenger of free radical species (Lawler et al., 2002) and also indirect antioxidant capacities in cells and tissues (Sestili et al., 2011).

Our results show that rotenone promoted cellular death in CGN does not elicit a statistically significant activation of caspase-3, the major execution caspase in apoptotic neuronal death, nor of the calpains, the calcium-dependent proteases strongly activated in excitotoxicity-induced neuronal death. On the contrary, our results show that the levels of cathepsin D were elevated, a lysosomal protease that has been shown to degrade the excess of α-syn observed in DA cells during development of PD (Cullen et al., 2009), suggesting autophagy as the likely major pathway for rotenone-induced cellular death in CGN. Noteworthy, up-regulation of cathepsin D has been recently reported in the caudate nucleus of primates with experimental parkinsonism (Yelamanchili et al., 2011). Our results are also on line with those reported by others showing that rotenone induces autophagy neuronal death in SH-SY5Y cells (Filomeni et al., 2012). Let us note that a simple hypothesis would be that the energetic depletion induced by rotenone could drive to an energetic failure of the lysosomal proton pump,

which is in charge of maintaining an acidic environment within the lysosomal compartment, and subsequent lysosomal destabilization, i.e. autophagy. However, we are aware that further studies using other markers of lysosomal integrity and function are needed to gain a deeper knowledge of the leading molecular mechanisms involved in cell death pathway induced by rotenone, but due to the extent of these studies these are out of the scope of this Master thesis.

As our results clearly point out that a bioenergetics crisis is at the onset of the CGN death induced by rotenone, and this is on line with the major role of impaired bioenergetics in PD suggested by other authors (see above), we decided to experimentally assess whether the functional state of the main bioenergetics compensatory systems CK and AMPK is preserved or altered. As observed in Figure 3.8, the protein levels and activity of CK where unchanged. Moreover, it has been shown that CK is highly sensitive to an increase of nitroxidative stress in the brain and heart, which leads to a significant inhibition of this enzyme (Aksenov et al., 2000; Mihm and Bauer, 2002). Therefore, the maintenance of the activity of CK is fully consistent with other experimental observations done in this work, namely, that rotenone induced at most a weak nitroxidative environment in CGN and also the beneficial effect of creatine supplementation to revert the neurotoxicity promoted by rotenone. We wish to note that if CK was nitrosylated, the system should be irreversible inactivated and the supplementation with creatine would be expected to be ineffective.

The other bioenergetics compensatory system studied herein, AMPK, was strongly affected by rotenone, as shown by the approx. 10-fold increase of its phosphorylated active form compared to control. The simplest hypothesis to account for this fact is that AMPK is sensing the rise of AMP levels in parallel to falling ATP levels accompanying rotenone-induced bioenergetics depletion in CGN, but it is to be noted that a mild drop of the intracellular redox status (rise of the ratio NAD^+/NADH) may also contribute to the activation of AMPK (Rafaeloff-Phail et al., 2004). Despite the need for further experimental work to settle the molecular mechanisms underlying this activation, the reduction of AMPK active levels in the conditions that protect against rotenone-induced death, i.e. in the presence of creatine and epicatechin, indicates that activation of AMPK is a good marker of the rotenone-signaling pathway leading to CGN death. In a metaphorical sense we could say for CGN that "in bioenergetics crisis AMPK activity is largely stimulated (rotenone presence) while in time of bioenergetics prosperity AMPK remains largely inactive (creatine and epicatechin presence)". Indeed, it is well known

that AMPK can be seen as a 'fuel gauge' to monitor cellular energy status in other mammalian cells, see e.g. (Viollet et al., 2007).

In this scenario of energetic failure we look forward to understand the effect of rotenone in Ca^{2+} homeostasis, since cytosolic Ca^{2+} concentration can be regarded as a major bioenergetics marker for neuronal activity and survival.

The results revealed a sustained and early (observed within 30 min) increase in cytosolic Ca^{2+} concentration after acute rotenone treatment, which should lead to an increase in the activity of CGN in culture. This conclusion is in good agreement with recent studies, which indicate a correlation between PD and increased functional connectivity/activity in cerebellum (Cao et al., 2011; Wu et al., 2009a; Wu et al., 2009b). The experiments performed with TG, a drug used for emptying the Ca^{2+} store of the ER (Spohn et al., 2010), indicate that acute treatment with rotenone promotes a Ca^{2+} overloading of ER in mature CGN, as revealed by the larger increase of Ca^{2+} after TG addition in rotenone-treated cells than in control cells (Figure 3.15). This result is consistent with the expected result for cells working with a cytosolic Ca^{2+} concentration steadily elevated. In addition, the increased cytosolic Ca^{2+} concentration has been reported to contribute to the development of glutamate-induced excitotoxicity that in turn induces neurodegeneration (Frandsen and Schousboe, 1993; Li et al., 2004). The results obtained in this work unravel an effect of acute rotenone treatment in the function of NMDA receptor, namely, rotenone-induced a sustained stimulation of Ca^{2+} entry through NMDA receptors in CGN in culture. The simplest possibly is that rotenone stimulated basal glutamate release, a novel hypothesis that deserves to be experimentally assessed by direct measurements of glutamate secretion in our working conditions. In addition, the results obtained in this work have shown that acute rotenone treatment stimulated also Ca^{2+} entry through L-VOCC. Very recently, in a work of our laboratory it has been demonstrated that L-VOCC and NMDA receptors are vicinal proteins in the plasma membrane of mature CGN (Marques-da-Silva and Gutierrez-Merino, 2012). As noted in Marques-da-Silva (Marques-da-Silva, 2012) a functional consequence of this proximity is that activation of L-VOCC should evoke L-glutamate release near the NMDA receptors, thereby, potentiating its effect on the receptors due to the generated gradient of L-glutamate concentration. Experiments addressing the demonstration of these points are programmed in our laboratory, as these conclusions have been achieved near the end of this Master work. Noteworthy, one of the most relevant Ca^{2+} channels involved in the pacemaking activity of DA neurons is the L-

VOCC subtype $Ca_v1.3$ (Chan et al., 2007) and as L-VOCC are modulated by phosphorylation by protein kinase A and also by PK-CaM (Catterall, 2000; Grueter et al., 2006) an impaired bioenergetics should be expected to lead to an altered activity of these Ca^{2+} channels. Moreover, it is now accepted that Ca^{2+} entry through plasma membrane $Ca_v1.3$ Ca^{2+} channels during an excessive activity could be involved in PD pathology (Surmeier et al., 2011a).

Finally but not less important, CGN exposed to an acute treatment with 15 nM rotenone for 45 min has a strong downregulation of their SOCE response with respect to control (untreated) CGN. Indeed, once the ER stores are depleted in Ca^{2+} by addition of TG, rotenone-treated CGN have lost almost completely their ability to elevate cytosolic Ca^{2+} after Ca^{2+} addition to the experimental medium (without Ca^{2+} until that moment), in contrast to control CGN. These results are in line with those published by Selvaraj et al on April of this year (Selvaraj et al., 2012). For the first time, Selvaraj et al. demonstrated that in a mouse neurotoxin-based model of PD, reduced Ca^{2+} influx through transient receptor potential C1 (TRPC-1) channels in the plasma membrane of DA neurons triggers a cell death-inducing ER stress response. These results received a special editorial comment by Mattson MP (Mattson, 2012), aiming to highlight the relevance of Ca^{2+} homeostasis in PD, somehow overlooked until now. It is to be recalled here that these channels and in particular the isoforms TRPC-3 and -6 have been also involved in neuronal survival of CGN (Jia et al., 2007).

V. Conclusions and Future work

V. Conclusions and Future work

- ✓ Creatine protects against the loss of cellular viability triggered by rotenone in CGN, whereas the flavonoids kaempferol and epicatechin do not elicit a significant protection.

- ✓ The strong stimulation of AMP-kinase also supports that the toxicity triggered by rotenone in CGN is largely due to an energetic failure, being oxidative stress caused by the rise of ROS a later event. The molecular mechanism of rotenone-induced AMP-kinase activation needs further investigation to be established.

- ✓ Kaempferol antagonizes the neuroprotection afforded by creatine, suggesting avoiding the consumption of foods or infusions with high content in this flavonoid in the therapeutics of PD.

- ✓ The cellular death caused by rotenone in CGN correlated with the activation of the lysosomal protease cathepsin D, while caspase-3 and calpains activation are negligible, suggesting the prevalence of an autophagic pathway involving lysosomal destabilization. Further studies are needed to experimentally ascertain this hypothesis.

- ✓ Rotenone induces an early deregulation of cytosolic calcium homeostasis. Our results show that exposure to rotenone induced a sustained elevation of cytosolic calcium in CGN, mediated by calcium entry through L-VOCC channels and NMDA receptors. Rotenone also promoted calcium overloading of the ER and a down-regulated SOCE response in CGN.

- ✓ Up-regulation of cathepsin D, deregulation of calcium homeostasis and down-regulation of SOCE are recently recognized markers of PD neurodegeneration. Therefore, the results obtained in this work further validate the use of primary CGN in culture as a cellular model for studying the molecular mechanisms underlying the neurodegeneration of cerebellum elicited in PD.

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