MITOCHONDRIAL DNA IN NEURONS AND ITS MODULATION BY NEUROTOXINS

by

María Soledad Santos

Bachelor in Pharmacy, Universidad Complutense de Madrid, 1997

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School of Medicine

This dissertation was presented

by

María Soledad Santos

It was defended on

February 27th, 2006

and approved by

Donald B. DeFranco, Ph. D., Pharmacology

Elías Aizenman, Ph. D., Neurobiology

Qiming J. Wang, Ph. D., Pharmacology

Clayton E. Mathews, Ph. D. Pediatrics

Dissertation Advisor: Ian J. Reynolds, Ph. D., Pharmacology

Ian J. Reynolds, Ph. D.

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María Soledad Santos, PhD

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Mitochondria are essential for the function of all mammalian tissues, serving functions, such as ATP generation. Neurons are highly dependent on ATP production and consume more energy than other cells for their metabolism. Mitochondria are semi-autonomous organelles that contain their own DNA (mtDNA). Mutations and deletions in mtDNA lead to mitochondrial dysfunction that compromise neuronal viability. From the many approaches taken to investigate the role of mitochondria in neurodegeneration; however, few have focused on mtDNA dynamics.

First, I investigated whether mtDNA replication impairment plays a role in neurotoxicity. For this purpose, I tested two neurotoxins, glutamate and rotenone, which induce neuronal damage by different mechanisms. Our results show that mitochondrial dysfunction induced by different neurotoxins does not correlate with effects on mtDNA replication. Glutamate, at excitotoxic concentrations, does not affect mtDNA replication while rotenone induces a time and concentration dependent decrease of mtDNA replication. Also, rotenone effect on mtDNA replication seems to be independent of its acute toxic effect.

Several mechanisms have been proposed as responsible for rotenone's toxicity, such as complex I inhibition and increased ROS production. Our experiments ruled out the implication of these two mechanisms in rotenone-induced mtDNA replication decrease. Mitochondrial nucleotides are key regulators of mtDNA replication. However, our experiments show that rotenone effect on mtDNA replication does not correlate with mitochondrial nucleotide imbalances. Therefore, our results suggest that rotenone-induced mtDNA replication decrease is mediated by a yet to be described mechanism.

Mitochondrial function requires the coordination of all processes that take place at this organelle. I studied if a reduction in mtDNA replication could have an effect on mitochondrial membrane potential, movement and morphology. Experiments with rotenone treatments that reduce mtDNA replication have demonstrated that mtDNA replication decrease does not correlate with overall mitochondrial dysfunction at the time points used in this study.

In summary, this dissertation provides a first attempt to study the dynamics of mtDNA upon neurotoxin exposure. I conclude that rotenone decreases mtDNA replication in the absence of overt toxicity. This effect could play an important role in its long term effects as neurons could accumulate mitochondria with decreased mtDNA content.

FORWARD

"Caminante no hay camino
se hace camino al andar"
Antonio Machado (Spanish poet, 1875-1939)

Dedicated to all my families

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 INTRODUCTION

World populations are growing older (Kinsella and Phillips, 2005). This phenomenon is a consequence of better infectious disease control, medical advancements, and the improvement of worldwide public health. However, another consequence of aging populations is the increase in the incidence of age-related degenerative diseases especially in developed countries. Alzheimer's disease (AD) and Parkinson's disease (PD) incidences have steadily increased in the United States over the past 30 years (National_Parkinson's_Foundation, 2004; Alzheimer's_Association, 2005). Although some familial associations contribute to these diseases, most cases remain sporadic. Also, in these common neurodegenerative diseases, age is the most important risk factor.

The study of mitochondrial function in neurodegenerative diseases can bring further insight into the understanding of various neurodegenerative pathologies. From all the tissues in the human body, the effects of aging are more pronounced in those in which their cells cannot proliferate, such as myocytes, cardiomyocytes and neurons. Because mitochondria are the main site of cellular ATP generation, mitochondria in these cells are required to function properly for up to 80 years or more. Mitochondria are the only organelles in the cell that contain their own DNA, and the machinery required to transcribe and translate its encoded genes. Therefore, the coordination of nuclear (nDNA) and mitochondrial DNA (mtDNA) is a necessary requirement for the proper functioning of these semi-autonomous organelles. As we learn more about the functioning and the regulation of mtDNA in normal cell physiology, the relevance of proper mtDNA maintenance is becoming apparent in neurodegeneration.

Another developing field in the intersection of mitochondrial and neuronal functions is the study of mitochondrial morphology and trafficking. Due to their polarized shape and their

long neuronal processes, neuronal function depends on proper energy delivery by mitochondria. Recently, the study of mitochondrial trafficking under pathological conditions has opened new avenues for the study of the mechanisms behind neurodegeneration. Thus the main focus of this dissertation is the study of mtDNA under neurotoxic conditions and, to a lesser degree, the study of other mitochondrial functions including mitochondrial movement and morphology under the same neurotoxic scenarios.

The introduction begins with an overview of mitochondrial structure and functions required for normal mitochondrial function (Section 1.1). Because the main focus of this work is mtDNA, Section 1.2 provides a comprehensive review of mtDNA that includes its organization as nucleoids (Section 1.2.2), its replication (Section 1.2.4) and regulation of replication (Section 1.2.5). Among the best studied mtDNA-related pathologies are those that feature a depletion of mtDNA (Section 1.2.7). The implication of mtDNA in the normal aging process (Section 1.2.8) is addressed in the second part of this introduction which discusses the relationship between mitochondria and common neurodegenerative diseases (Section 1.3).

The introduction concludes with a discussion of the dissertation goals (Section 1.4). The overall hypothesis is that mitochondrial dysfunction leads to altered mtDNA dynamics, which can in turn accelerate neuronal degeneration. The specific aims concentrate on (1) determining the effects of neurotoxins on mtDNA replication and total mtDNA content, (2) the mechanism behind rotenone-induced mtDNA decrease observed in primary neurons, and (3) the possibility that a reduction in mtDNA replication could alter mitochondrial function.

1.1 OVERVIEW OF MITOCHONDRIAL STRUCTURE AND FUNCTION

Mitochondria are thought to derived from α-proteobacteria (Gray et al., 1999). It is believed that throughout their evolution mitochondria transferred most of their genes to the nucleus, and, in turn, received various “eukaryotic” genes of the host cell. It is now known that mitochondria contain approximately 700 different proteins and provide the cell with crucial functions such as energy production and apoptosis (Gaucher et al., 2004).

1.1.1 Mitochondria are double membrane organelles

Of the organelles in the cell, mitochondria are among the largest, and are the main sites of energy production during aerobic metabolism. As a result of their bacterial origin, mitochondria have a double membrane structure and their own DNA. The outer membrane is permeable to molecules of 10,000 Da in molecular weight and has equivalent composition of protein and lipid. The inner membrane composition is about 20% lipid and 80% protein, and has the highest protein fraction among other cell membranes. The inner membrane is also less permeable to most molecules and impermeable to charged molecules in particular. The mitochondrial inner membrane is organized into convoluted invaginations that project into the matrix called cristae (Lodish, 2000). Recent reports have demonstrated an organized distribution of proteins within the inner mitochondrial membrane. For example, the electron respiratory chain complexes are suggested to be located in the cristae inner membrane, while transporters for substrates and ions and protein translocators are restricted to the inner membrane facing the intermembrane space (Perotti et al., 1983; Gilkerson et al., 2000). There are contact sites between the outer and inner mitochondrial membranes; these sites are formed during the transport of nuclear-encoded precursor proteins into mitochondria.

1.1.2 Mitochondria depend on proper localization and assembly of their proteins

The majority of mitochondrial proteins (about 900) are encoded by nuclear nDNA, while only 13 proteins are mtDNA-encoded (Fernandez-Silva et al., 2003). Nuclear-encoded proteins need to be imported into mitochondria. These proteins are synthesized on cytoplasmic ribosomes in close proximity to the mitochondria. One or more targeting sequences direct proteins to their proper destination: the outer membrane, the intermembrane space, the inner membrane or the matrix (Lodish, 2000). With the help of chaperones in the cytosol and the mitochondrial matrix, proteins are transported through translocator import complexes at contact sites between the outer (TOMs) and inner (TIMs) membranes. The import process requires both the hydrolysis of ATP in cytoplasmic and matrix sites and depends on mitochondrial membrane potential ($\Delta\psi_m$). Proteins encoded by mtDNA are translated in the matrix and due to similar targeting sequences to those of nuclear-encoded proteins use the same import machinery to reach their proper location.

Mitochondrial function is the result of coordinated expression, localization and assembly of all the necessary nuclear- and mtDNA-encoded proteins. The assembly of all complexes in the electron transport chain is critical for mitochondrial ATP production. Of the five complexes that constitute the electron transport chain, only complex II is encoded exclusively by nDNA. The other four have at least 1 subunit encoded by mtDNA. Even though all the proteins that form the different complexes have been identified, the regulation of protein synthesis and the balance between the different complexes has recently been shown to be more intricate than previously thought. There is a structural dependence of the complexes; in particular, complex III assembly is required for complex I stability, while complex III stability is independent of complex I (Acin-Perez et al., 2004).

1.1.3 Mitochondria are dynamic organelles

Mitochondria are highly dynamic organelles that are continually moving in the cell. In addition, mitochondria undergo frequent fission and fusion. The relative rate of fusion and fission determines the overall morphology of the mitochondrial network at any given time. Even though some of the proteins implicated in fission and fussion have been described, their regulation and mechanism of action has not yet been elucidated (Youle and Karbowski, 2005). Mammalian proteins implicated in mitochondrial fusion are optic atrophy 1 (OPA1), mitofusin 1 (MFN1) and mitofusin 2 (MFN2). These three proteins are large GTPases that localize to different sites in the mitochondria. MFN1 and 2 are inserted into the outer membrane and form homo- and hetero-oligomers, while OPA1 is found at the intermembrane space partially anchored to the inner membrane. However, the specific function of these proteins in membrane fusion has not been elucidated yet (Youle and Karbowski, 2005). Recent studies of mitochondrial fusion *in vitro* have demonstrated that both outer and inner membrane fusion require GTP hydrolysis; $\Delta\psi_m$ is also required for inner membrane fusion (Meeusen et al., 2004). Mitochondrial fission is regulated by FIS1 and dynamin related protein 1 (Drp1) in mammalian cells. FIS1 resides at the outer membrane and the current hypothesis for its function in mitochondrial fission is to recruit Drp1 from the cytosol to the outer membrane, where Drp1 localizes at the foci of fission. However, the mechanism by which mitochondrial membranes break apart is not known yet (Youle and Karbowski, 2005).

One hypothesis for the requirement of an interconnected mitochondrial network is that it enables mitochondrial membrane potential to be distributed throughout the cell (Skulachev, 2001). This will allow proper energy delivery to areas of high energy demand in polarized cells, such as the neuronal synapse. Interestingly, mitochondria can travel distances that exceed one meter at velocities of 1μm/s (Goldstein and Yang, 2000). However this movement is pulsatile, with prolonged stationary periods. In fact, we have recently shown that mitochondria are actively recruited to sites of high energy demand such as the synapse. Moreover, changes in synaptic activity regulate the trafficking patterns of mitochondria (Chang, 2005). Mitochondrial movement in eukaryotic cells has been described to occur mainly along microtubules with a small portion associated to actin filaments (Ligon and Steward, 2000). Actin filaments have been suggested to tether mitochondria to specific regions, therefore regulating the microtubule-base movement (Krendel et al., 1998). The microtubule motors implicated in mitochondrial movement are dynein, for retrograde movement, and kinesin, for anterograde movement. The kinesin isoforms 1B and 5B have been shown to selectively interact with mitochondria (Tanaka et al., 1998; Terada and Hirokawa, 2000; Vale, 2003).

Mitochondrial fission and fusion events are important processes not only in the normal functioning of the cell but also in pathophysiological situations. For example, in pre-mitotic cells, mitochondrial fission is fundamental for the proper inheritance of mtDNA by daughter cells (Scott et al., 2003). Also, an increase in mitochondrial fission has been implicated in pathological situations, such as apoptosis in response to staurosporine treatment (Frank et al., 2001).

1.1.4 The multiple functions of mitochondria: from metabolism to apoptosis

Mitochondria are not only responsible for energy production in the form of ATP. Other functions such as the urea cycle, heme production and the synthesis of some steroids also take place in these organelles (Figure 1). The tricarboxylic acid cycle metabolizes substrates imported into the mitochondrial matrix, feeding reducing equivalents (NADH and $FADH_2$) to the respiratory chain

(Lodish, 2000). In transfering electrons to molecular oxygen, the respiratory chain also shuttles protons across the inner mitochondrial membrane. This process creates an inner mitochondrial membrane potential of approximately -180mV that provides the energy required to drive the phosphorylation of ADP to ATP.

Mitochondria are also essential for the regulation of neuronal calcium homeostasis. First, mitochondria produce the ATP that the Ca^{2+}-dependent ATPases need to pump Ca^{2+} out of the cell or into intracellular stores. Second, mitochondria take up Ca^{2+} through the Ca^{2+}-uniporter, which buffers intracellular [Ca^{2+}]. By sequestering any excess of cytoplasmic Ca^{2+}, mitochondria protect the cell from a toxic calcium overload. However, excessive Ca^{2+} uptake into mitochondria can also have a deleterious effect after an excitotoxic stimulus in neuronal cells (Stout et al., 1998).

Because the transfer of electrons along the respiratory chain is not 100% efficient, there is an "electron leakage" that can generate reactive oxygen species (ROS). The main sites of ROS production in the electron transport chain are complexes I and III. In fact, Boveris *et al.* (1973) suggested that mitochondria convert 2% of the oxygen they consume to superoxide. Because ROS can induce major damage to lipids, proteins and mtDNA, mitochondria have several mechanisms to eliminate ROS (Figure 1). Each individual ROS has its own reaction profile and removal mechanism. Superoxide is produced in or near complexes I and III and is scavenged by cytochrome c and superoxide dismutates (SODs). Hydrogen peroxide is generated spontaneously or by dismutation of superoxide and is removed by gluthatione peroxidase (reviewed by James and Murphy, 2002).

Mitochondria are also important in apoptosis. The first event in mitochondrial-mediated apoptosis is the release of specific proteins, such as cytochrome c and SMAC/Diablo, from the intermembrane space into the cytosol (Polster and Fiskum, 2004). This release occurs as a consequence of the pores formed in the outer mitochondrial membrane by the insertion of the Bcl-2 family members BAK and BAX. Most studies have focused on the opening of the permeability transition pore, however, the mechanism implicated in cytochrome c displacement from its binding to the inner mitochondrial membrane has not been studied extensively.

Cytochrome c has been demonstrated to be bound to the phospholipid cardiolipin. The disruption of the cytochrome c-cardiolipin interaction has been proposed as the triggering event for cytochrome c release (Iverson and Orrenius, 2004).

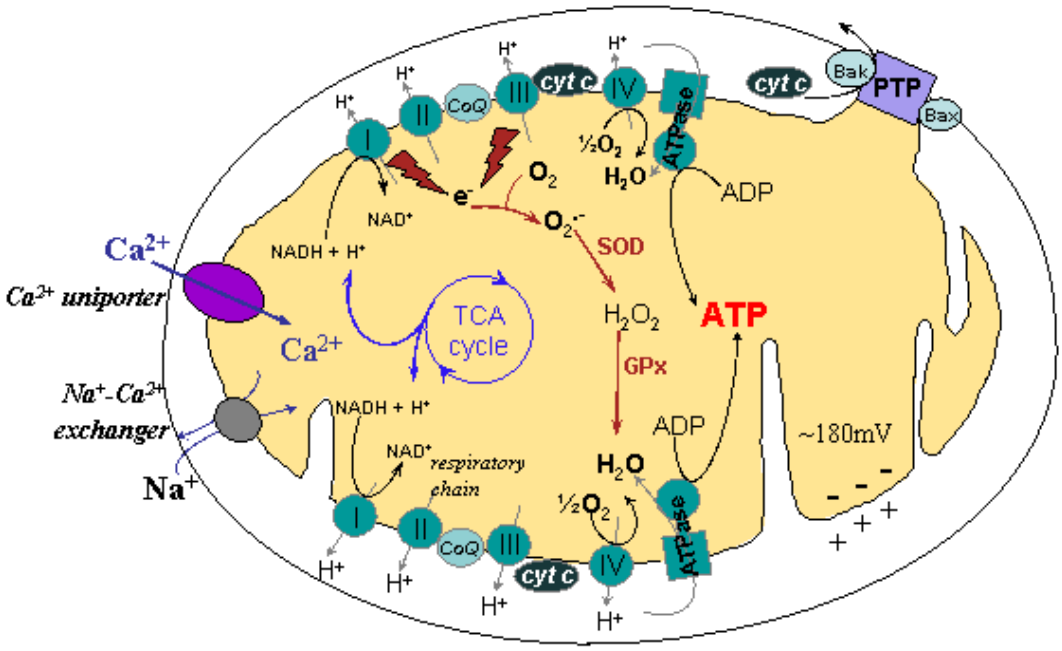

Figure 1. Mitochondrial functions.

Diagram of mitochondrial functions. I-IV: Complexes in the respiratory chain. TCA: Tricarboxylic acid. CoQ: Coenzyme Q. Cyt c: Cytochrome c. SOD: superoxide dismutase. GPx: Glutathione peroxidase. PTP: Permeability transition pore. Adapted from the Mitochondrial energetics Illustration from Mitomap (http://www.mitomap.org/mito_apop.pdf)

1.1.5 Mitochondrial turnover is a regulated process

Mitochondria turn over constantly, being replaced every 10-25 days. This constant turnover occurs even in tissues with low levels of cellular proliferation, such as brain, heart, liver and kidney (Menzies and Gold, 1971). Old mitochondria are recycled by autophagy. In this process, mitochondria are taken up by endoplasmic reticulum membranes and delivered to lysosomes for

degradation. Recently, the yeast protein, Uth1p, has been identified as necessary for mitochondrial autophagy (Kissova et al., 2004). Uth1p resides in the outer mitochondrial membrane and when mutated, mitochondrial autophagy is inhibited. This suggests that mitochondria are specifically targeted to be degraded. In some instances, mitochondria have been shown to be ubiquitin-tagged for autophagy. This is the case for sperm mitochondria in and mitochondria in insect flight muscles during apoptosis (Davis et al., 1994; Sutovsky et al., 2000). Although it has been suggested that mitochondria are selected for autophagy depending on their level of oxidative damage to their membranes, this hypothesis has not been conclusively demonstrated (Terman and Brunk, 2004).

1.2 MITOCHONDRIAL DNA

1.2.1 Structure and special features of mtDNA

Mitochondria are unique organelles because they require the coordination of two physically separated genomes. The mitochondrial genome only encodes 37 genes; 13 of these genes are polypeptides essential for oxidative phosphorylation (Figure 2). The other 24 genes encode the mitochondrial translation machinery: 2 ribosomal RNAs and 22 transfer RNAs. The mitochondrial genome is a closed-circular molecule of approximately 16.6 kb that accounts for only 0.5-1% of the total DNA content. mtDNA has a compact organization lacking introns and with some overlapping genes such as ND4 and ND4L subunits of complex I. The displacement loop or D-loop is one of two non-coding regions in mtDNA. The D-loop contains one of the origins of replication and three promoters for mtDNA transcription. Most of the identified regulatory elements in mtDNA replication bind to the D-loop region (reviewed by Garesse and Vallejo, 2001; and Fernandez-Silva et al., 2003).

Mitochondrial genetics differs from nuclear genetics in two respects. First, nuclear genes follow a Mendelian pattern of inheritance, whereas mitochondrial genes are maternally inherited. Second, the nuclear genome is either haploid or diploid while the mitochondrial genome is polyploid since there are thousands of mtDNA molecules per cell. When only one form of

mtDNA exists in a cell, the state is called homoplasmy; when two or more co-exist, the state is called heteroplasmy. In some cases, different individual mtDNA sequences, or haplotypes, have been shown to associate in what has been called haplogroups. Haplogroups of mtDNA have been linked to increase or decrease the risk for common neurodegenerative diseases (Wallace, 2005).

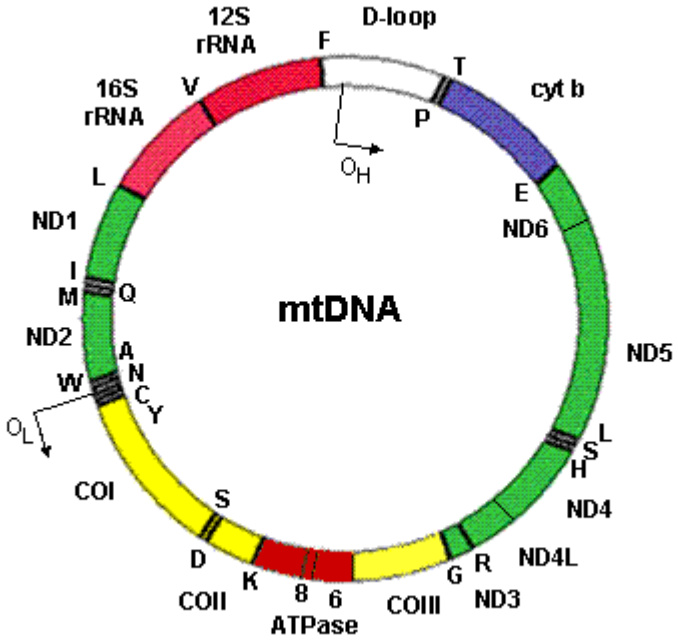

Figure 2. Diagram of mtDNA encoded genes

Genes encoded by the heavy strand are depicted at the outside perimeter, while light strand encoded genes are positioned at the inside of the mtDNA circle. All subunits from a single complex are colored the same. Blue: complex IV, green: complex I, yellow: complex III, red: ATPase, pink: ribosomal RNA (rRNA). Transfer RNAs are represented by the single letter code. D-loop: displacement loop. O_H: Replication origin for the heavy strand. O_L: Replication origin for the light strand. Adapted from Taylor RW and Turnbull DM. Mitochondrial DNA mutations in human disease. Nat Rev Genet. 2005 May;6(5):389-402.

1.2.2 Nucleoids are the structural units of mtDNA

In the yeast *Saccharomyces cerevisiae,* mtDNA was first described to have an organization of discrete foci within mitochondria called nucleoids (Miyakawa et al., 1984). These have been proposed to be composed of several copies of mtDNA molecules, and more importantly, to be the units of inheritance for mtDNA (Jacobs et al., 2000). Only recently has mammalian mtDNA been shown to be organized in nucleoid structures, with each nucleoid estimated to contain between 2-10 mtDNA molecules (Iborra et al., 2004; Legros et al., 2004). Nucleoids are stable complexes of mtDNA and several mitochondrial proteins. Among the proteins that form the nucleoids are some that participate in mtDNA replication, transcription and maintenance, such as the mitochondrial transcription factor A (TFAM), and the mitochondrial single-stranded DNA-binding protein (mtSSB) (Garrido et al., 2003). Studies aimed at elucidating the association of mtDNA and TFAM have suggested that TFAM has a "histone-like" function in packing mtDNA that is more important than its role in transcription (Alam et al., 2003). Another protein associated with nucleoids in mammalian cells is Twinkle. This protein has been demonstrated to have mitochondrial helicase activity, and a punctate distribution within mitochondria coincident with mtDNA (Spelbrink et al., 2001). Other proteins with functions seemingly unrelated to nucleoid maintenance, such as α–ketoglutarate dehydrogenase and aconitase, have also been described to associate with nucleoids (Kaufman et al., 2000). Interestingly, recent reports have demonstrated that the tricarboxilic acid cycle, aconitase, is essential for mtDNA maintenance in yeast (Chen et al., 2005). These results suggest an intimate relationship between the regulation of cellular metabolism and mitochondrial gene expression.

In terms of mtDNA and mitochondrial morphology, nucleoids have been shown to follow the movements of the mitochondrial network. mtDNA nucleoids have been shown to divide and distribute after mitochondrial division (Garrido et al., 2003). This distribution has been proposed as a requirement to ensure proper mtDNA transmission to daughter mitochondria. A possible association of Drp1 with mtDNA nucleoids has been suggested because nucleoids tend to localize at or near the tips or constriction sites on the mitochondria. However, conflicting reports have suggested both nucleoid participation and absence of it in the mitochondrial division machinery (Garrido et al., 2003; Iborra et al., 2004; Legros et al., 2004).

1.2.3 mtDNA kinetics

Initial studies of mtDNA in the brain suggested that mtDNA has a much longer half-life, about 25 days, when compared to other tissues such as kidney or lung (6 and 4 days, respectively) (Menzies and Gold, 1971). These results led to the hypothesis that mtDNA replicates at a very slow pace in post-mitotic tissues. In fact, Wang *et al.* (1997) demonstrated that mtDNA turnover is slower in cortical neurons than in peripheral neurons and other non-neuronal cell types. However, the techniques used at that time lacked the sensitivity of more modern techniques, such as quantitative real-time PCR (QPCR). Localization of mtDNA replication is another process of mtDNA kinetics that has been the focus of several studies. In 1996, Davis and Clayton proposed that mtDNA replication occurs in mitochondria near the nucleus and then newly replicated mtDNA is transported to the appropriate cellular locations. This hypothesis is in accordance with the requirement of nuclear factors for mtDNA replication. However, recent results in muscle cells, and neuronal and epithelial cell lines from Magnusson *et al.* (2003) and Iborra *et al.* (2004) suggest that mtDNA replication occurs throughout the cell.

1.2.4 Replication of mtDNA is asymmetric and dependent on nuclear factors

Mitochondrial DNA is replicated and transcribed within the mitochondria. In these semi-autonomous organelles, mtDNA replication takes place independently from the cell cycle and from nuclear DNA replication. Factors involved in mtDNA metabolism are nuclear-encoded, including mtRNA polymerase, mtDNA polymerase γ (Pol γ) and most of the potential factors that regulate mtDNA replication, mtDNA transcription and mtRNA processing (Moraes, 2001). The generally accepted model for mtDNA replication consists of an asymmetric synthesis. This means that the two mtDNA strands replicate in an asynchronous manner from two independent origins. The synthesis starts at one of the multiple origins of replication of the heavy strand (O_H) at the D-loop region using a short RNA primer (Figure 2). mtDNA synthesis continues until reaching the origin of replication of the light strand (O_L), which is situated approximately two-thirds away around the mtDNA molecule. At this time, the synthesis of the light strand starts (Garesse and Vallejo, 2001). Because Pol γ requires short mtRNA primers; replication depends on mitochondrial transcription. Studies with a recombinant human DNA polymerase have

estimated that one round of mtDNA replication takes about 1 h (Graves et al., 1998). Recently, Holt *et al.* (2000) have proposed a coupled leading- and lagging-strand mechanism of mtDNA synthesis in human mitochondria. These authors suggested that cells use different synthesis mechanism under specific physiological conditions. However, these results remain controversial (Bogenhagen and Clayton, 2003; Bowmaker et al., 2003; Holt and Jacobs, 2003; Brown et al., 2005).

Unlike mitochondrial proteins that freely diffuse within the mitochondrial matrix, mitochondrial genomes in mammalian cells have been suggested to be tethered to the inner mitochondrial membrane, restricting the diffusion of mtDNA (Iborra et al., 2004). Therefore, nucleoid distribution within the mitochondrial network is important in determining mtDNA inheritance by daughter cells. Recently, selective mtDNA replication was shown to be involved in mtDNA segregation in yeast (MacAlpine et al., 2001). From a mixed population of mtDNA molecules, those with a specific origin of replication were replicated more efficiently, and more importantly, were inherited preferentially. Therefore, in yeast, fidelity of mtDNA inheritance seems to be achieved by associating mtDNA replication with nucleoid segregation (Scott et al., 2003).

1.2.5 Polymerase γ and other nuclear factors control mtDNA replication

DNA polymerase γ (Pol γ) is the only enzyme described to replicate mtDNA (reviewed by Kaguni, 2004). Pol γ is composed of two subunits: one catalytic and one accessory. Crystallographic data and more recent *in vivo* studies demonstrate that this enzyme forms heterotrimers of one catalytic subunit and two accessory subunits in humans (Carrodeguas et al., 2001; Yakubovskaya et al., 2006). Interestingly, the stoichiometry is not conserved among species since Pol γ consists of a single catalytic subunit in yeast and a heterodimer in *Drosophila*. The catalytic subunit carries out three different activities: DNA polymerization, 3'5' exonucleolytic proofreading, and 5'-deoxyribose phosphatase (dRP) lyase activity, which is required for mtDNA repair. The accessory subunit assists with binding to double-stranded DNA that is necessary for the processivity of the enzyme (Kaguni, 2004). Accuracy of Pol γ is

essential for mtDNA replication, with mutations in the enzyme linked to myopathy and progressive external ophthalmoplegia syndromes characterized by deletions and increased mutations in the mtDNA (Longley et al., 2005).

The replication of mtDNA regulates mtDNA copy number; therefore the reduction or lack of any factor required for mtDNA replication can decrease mtDNA content. For instance, TFAM knockout mice are embryonic lethal due to mtDNA depletion (Larsson et al., 1998). Conversely, mtDNA copy number does not necessarily increase as a result of over expression of a protein involved in mtDNA replication (Spelbrink et al., 2000). Exceptions to these rule are some factors, such as TFAM, mitochondrial transcription factor B2 (mtTFB2) and the helicase Twinkle, which have been shown to be directly proportional to mtDNA copy number (Ekstrand et al., 2004; Matsushima et al., 2004; Tyynismaa et al., 2004). Because these three factors have also been demonstrated to associate with mtDNA nucleoids, it is not clear if they regulate mtDNA copy number through their role in mtDNA replication or as structural proteins of mtDNA nucleoids (Garrido et al., 2003).

1.2.6 mtDNA is more susceptible to oxidative damage that nDNA

Mitochondria are both the main sites of ROS production and the main targets of ROS-induced damage. Due to its close proximity to the electron transport chain, mtDNA shows levels of oxidized DNA 2-3 times greater than nDNA (Hudson et al., 1998; Raha and Robinson, 2000). 8-oxoGuanine and thymine glycol are the most frequent oxidation products of mtDNA (Ames, 1989). Studies over the last decade have confirmed that mitochondria lack a nucleotide excision repair mechanism. However, mitochondria from various organisms have been described to have base excision repair (BER), the main mechanism for oxidatively-damaged DNA removal (Van Houten et al., 2006). Nevertheless, cell types such as neurons and glia have less efficient mtDNA repair when compared to other cell types, such as vascular endothelial and smooth muscle cells (Mandavilli et al., 2002). There are two characteristics that make oxidative damage to mtDNA of relevance for overall mitochondrial function. First, mtDNA proximity to ROS production at the inner mitochondrial membrane makes it more susceptible to oxidative damage than nuclear DNA, regardless of mtDNA repair capacity. Second, oxidative lesions on mtDNA can impede

mitochondrial RNA polymerase activity, which results in the reduction of mtDNA transcription. Therefore, as a consequence of oxidized mtDNA damage, there could be a reduction in mtDNA encoded electron transport chain proteins that would lead to an overall reduction of mitochondrial function (Van Houten et al., 2006).

1.2.7 mtDNA depletion as a consequence of HIV treatment

Defective mtDNA synthesis and maintenance result from multiple deletions or depletion of the mitochondrial genome. Depletion of mtDNA has been observed in two instances. The first one is due to genetic mtDNA depletion syndromes. These syndromes present as autosomal recessive disorders due to mutations in the genes responsible for nucleotide metabolism. In particular, thymidine phosphorylase gene mutations alter the nucleoside and nucleotide pools in mitochondria leading to impaired mtDNA replication (Nishino et al., 1999; Van Houten et al., 2006). The other instance in which mtDNA depletion can be detected is in individuals treated with *N*ucleoside analogue *R*everse *T*ranscriptase *I*nhibitors (NRTIs) as part of the highly active antiretroviral therapy to treat individuals infected with HIV. NRTI therapy causes mitochondria-related side effects in 15-20% of the treated population. Defective mtDNA replication and subsequent mtDNA depletion due to NRTIs treatment can cause cardiac dysfunction, hepatic failure, skeletal myopathy, and lactic acidosis (reviewed by Lewis et al., 2003). NRTIs are pro-drugs that must be triphosphorylated to exert their inhibitory action. Thymidine kinases (TK) are responsible for these sequential phosphorylations. There are two TK isoforms, one cytoplasmic (TK1) and another mitochondrial (TK2). The toxicity of each NRTI depends upon two factors; (1) the specific affinity of each NRTI for either TK1 or TK2, (2) the expression level of each TK isoform in different tissues. In this regard, TK2 is expressed at higher levels in terminally differentiated tissues, such as striated and cardiac muscle. For example, although AZT is phosphorylated more efficiently by TK1, its toxicity is mainly seen in striated and cardiac muscle due to their high expression levels of TK2. Once inside the mitochondria, NRTI triphosphates and deoxynucleotide triphosphates compete to be incorporated into newly synthesized mtDNA. If a NRTI triphosphate is incorporated into mtDNA, the nascent chain is terminated due to the lack of 3'-OH group (Mitsuya et al., 1990). Other mechanisms of action for NRTI toxicity have been suggested such as direct inhibition and alteration of the fidelity of Pol γ.

Regardless of the mechanism for NRTI toxicity, this treatment decreases levels of mtDNA, which disrupts the adequate supply of respiratory chain proteins. As a consequence, there is a decrease in ATP production accompanied by a yet to be understood increase in ROS production and subsequent oxidative damage. In fact, oxidative damage in response to long-term AZT treatment has been shown in skeletal muscle in mice, rats and in cell culture (de la Asuncion et al., 1999; Szabados et al., 1999; Yamaguchi et al., 2002).

1.2.7.1 An *in vitro* mtDNA depletion: rho 0 cells

One model to study the effects of mtDNA depletion are rho0 cell lines which are devoid of mtDNA. A rho0 cell line can be created by simply treating the parental cell line with low doses of DNA intercalating agents. Mitochondrial DNA replication is affected by DNA intercalating agents at low doses that do not affect nuclear DNA significantly. Therefore, agents such as ethidium bromide are used to generate rho0 cells. Morais *et al.* (1980) showed that to keep ethidium bromide treated cells growing exponentially, cells had to be supplemented with pyrimidines. The *de novo* synthesis of pyrimidines requires the enzyme dihydroorotate dehydrogenase, which depends on a functional mitochondrial electron transport chain. Therefore, in the absence of mtDNA, *de novo* synthesis of pyrimidines can not take place, and rho0 cells need to be supplied with uridine (Gregoire et al., 1984).

Structurally, rho0 cells have been shown to have a dramatic reduction in the amount of inner mitochondrial membrane cristae and exhibit a more punctate morphology (Gilkerson et al., 2000). Also, rho0 cells have been demonstrated to have lower $\Delta\psi_m$ and mitochondrial movement (Garcia et al., 2000; Jazayeri et al., 2003). Recent results have suggested a slight increase in the abundance of mitochondria in these cells. Due to their reduced $\Delta\psi_m$, rho0 cells have decreased activity of the TIM complex, while TOM protein expression levels seem to be unchanged (Mercy et al., 2005). Rho0 cells have been extensively used for cybrid studies since they can be repopulated with foreign mtDNA by fusion with enucleated cells (contain foreign mtDNA but devoid of nDNA). For example, studies in which rho0 cells were repopulated with mtDNA from PD patients platelets have demonstrated decreased electron transport chain activities when compared to cybrid lines of control patients (Swerdlow et al., 1996).

1.2.8 mtDNA mutations play a role in normal aging

The main risk factor for common neurodegenerative diseases, such as AD or PD, is age. Therefore, when studying mtDNA and mitochondria in these diseases, it has to be taken into account that both the mtDNA and the mitochondria may already bear some signs of aging that could exacerbate the consequences of the neurodegenerative disease. The free radical theory of aging proposes that ROS generated during normal respiration oxidizes cellular macromolecules, inducing a functional decline of the organism that could in time lead to death (Harman, 1972). As mentioned before, mitochondria are the prime site of ROS generation and mtDNA has been demonstrated to have higher levels of oxidative damage than nDNA (Salazar and Van Houten, 1997). In fact, mtDNA has been demonstrated to acquire point mutations and deletions in various aging organisms (Corral-Debrinski et al., 1992; Schwarze et al., 1995; Khaidakov et al., 2003). These findings have led to the speculation that mtDNA mutations could be responsible for aging by causing defective energy production. To test this hypothesis, Trifunovic *et al*. (2004) and later Kujoth *et al.* (2005) generated mice that carry defective Pol γ. By a single residue substitution, Pol γ was impaired from its proofreading ability, while its catalytic activity was unchanged. This mutation generates an error-prone mtDNA Pol γ. Indeed, results from both groups show an increase in mtDNA mutations in these transgenic mice (mutation rate is 3-8 times higher than wild-type animals). The mutant also showed a decrease in the respiratory chain complexes encoded by mtDNA and in ATP production (Trifunovic et al., 2004). Also, the mutant showed symptoms consistent with human aging, such as weight loss, hair loss, reductions in fertility, and significantly shorter life span. These findings strongly support the concept that mtDNA mutation accumulation contributes to aging. At first, Trifunovic *et al* (2004) proposed that the aging effects observed in these transgenic mice could be due to increased ROS production, Kujoth *et al* (2005) did not observe an increase in markers of oxidative damage. Alternatively, they reported an increase in apoptotic markers, suggesting that apoptosis may be the main force driving mammalian aging. In a more recent report, Trifunovic and colleagues (2005) have reported the absence of any significant increase in oxidative damage in the form of protein carbonyls or elevated antioxidant enzymes in the mutant mice. These results go against the proposed vicious cycle theory by which increased ROS production could lead to accumulation of mtDNA mutations that, in turn, would lead to more ROS production. According to the authors, the sole

agent responsible for the premature aging of these mutant mice is the electron transport chain defect observed. Therefore, results from experiments in these mutant mice have opened the debate about the relationship between oxidative damage and mtDNA mutations and also about the role both phenomena play in normal aging.

1.3 NEURODEGENERATIVE DISEASES AND MITOCHONDRIA

All neurodegenerative diseases have common features regardless of their pathology. First, they usually start by affecting specific functions of the nervous system. Second, they begin silently progressing for many years, often more than a decade. Third, when clinical symptoms eventually present, the degree of neuronal loss no longer supports normal functioning. And, fourth, they are irreversible. Because of the slow progress, loss of neurons is not accompanied by an intense tissue reaction or cellular response, making the early diagnosis of these diseases difficult.

Dysfunction of mitochondrial energy metabolism leads to reduced ATP production, impaired calcium buffering, and generation of ROS. Mitochondria are both the sources and targets of ROS. Generation of ROS appears to be increased in damaged mitochondria and in cells with compromised mitochondrial function. Because neurons are highly dependent on oxidative phosphorylation, mitochondria have been suggested to play a key role in neuronal degeneration. Next, I will present recent data demonstrating the participation of mitochondria in the pathologies of the most common neurodegenerative diseases as well as obscure neurodegenerative diseases caused by mtDNA mutations.

1.3.1 Alzheimer's disease

AD is estimated to affect 4.5 million Americans, being the most common dementia in the elderly (Alzheimer's_Association, 2005). The presence of amyloid plaques and neurofibrillary tangles, and neuronal loss are the main histopathological findings of AD. Some forms of early-onset familial AD have been associated with mutations in the amyloid β (Aβ) precursor protein and in

the proteins presenilin 1 and 2 (Table 1). However, familial AD is responsible for only 5% of the AD cases (Schon and Manfredi, 2003). The association between impaired energy metabolism and the pathophysiology of AD has been recognized in the last several years. For example, postmortem brain tissue from AD patients was shown to have decreased activity of α-ketoglutarate dehydrogenase (Sheu et al., 1994). More recently, the mitochondrial protein Aβ-binding alcohol dehydrogenase (ABAD) was shown to interact with Aβ (Table 1). Lustbader *et al.* (2004) demonstrated that the direct interaction between Aβ and ABAD increased oxidative stress in cultured neurons. In another approach to demonstrate the association between mitochondria and AD, Li and colleagues (2004) crossed mice heterozygous for SOD2 with mice overexpressing mutant Aβ. These mice displayed increased oxidative damage and an eight to ninefold increase in amyloid plaque burden at 4 months of age.

In terms of mtDNA implication in AD, there has been no association between specific mtDNA mutations in AD until recently. Results have shown an increased number of mtDNA mutations in the D-loop region from AD brains (Coskun et al., 2004). Moreover, in AD brains, there is a 50% reduction in mtDNA content that is accompanied by a similar reduction in the levels of transcript for subunit ND6 of complex I. It has been suggested that these reductions could decrease oxidative phosphorylation, therefore being of relevance for the neurodegeneration process (Beal, 2005).

Another aspect of mitochondrial physiology implicated in AD is mitochondrial trafficking. When the microtubule associated protein, tau, is over expressed, mitochondrial transport to the peripheral cell compartments is impaired (Ebneth et al., 1998). The same effects were observed after introducing AD-related mutations in presenilin, a protein found in the endoplasmic reticulum and the growth cones (Pigino et al., 2003). A disturbance in mitochondrial trafficking could lead to improper ATP delivery to high energy demand compartments in the neuron and impaired Ca^{2+} homeostasis, therefore challenging neuronal survival.

1.3.2 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that consists of progressive wasting and paralysis of muscles of the limbs and trunk, which leads to death within 2-5 years after diagnosis (ALS_association, 2005). ALS incidence is 2 in 100,000 people, with estimates of 30,000 Americans suffering from this disease. The neurodegeneration in ALS targets the cortical motor neurons and the anterior horn cells in the spinal cord. As with other neurodegenerative diseases, most ALS cases are sporadic. However, mutations in the SOD1 protein have been associated with some familial ALS cases (Table 1). In fact, a transgenic mouse model of ALS has been generated by introducing a G93A mutation into the SOD1 gene. The most remarkable finding in the motor neurons of these mice is the presence of massive mitochondrial degeneration accompanied only by minimal neuronal cell death (Kong and Xu, 1998). Also, there was a significant decrease in mitochondrial respiration and ATP synthesis at the time of disease onset, which is associated with oxidation of mitochondrial lipids and proteins (Mattiazzi et al., 2002). Although, SOD1 was considered a cytoplasmic protein, Higgins *et al* (2003) have recently reported SOD1 presence at the mitochondrial intermembrane space. Another mitochondrial process that could be implicated in the pathogenesis of ALS is apoptosis. Recent results have demonstrated the binding of SOD1, in its wild-type and mutant forms, to Bcl-2 at the OMM (Vijayvergiya et al., 2005). Because mutant SOD1 has been shown to aggregate in mitochondria from spinal cord neurons, it was suggested that mutant SOD1 could facilitate apoptosis by sequestering Bcl-2.

1.3.3 Huntington's disease

Unlike the other common neurodegenerative diseases presented here, Huntington's disease (HD) is a chronic autosomal-dominant disease that affects one out of every 10,000 Americans (Huntington's_Disease_Society_of_America, 2005). This disease is caused by a CAG-repeat expansion in the huntingtin gene, which introduces polyglutamine residues at the N-terminus of the protein. HD symptoms include choreoatheotic movements, cognitive impairment, and emotional disturbances (Table1). The main neuronal system affected is the striatum; the mutated huntingtin protein aggregates in the nucleus of striatal neurons leading to their degeneration, as

reviewed by Beal (2005). The implication of mitochondria in HD comes from both *in vivo* and autopsy studies. HD patients have been shown to have high levels of lactate in the cortex and basal ganglia (Jenkins et al., 1993). In animal studies, treatment with complex II inhibitors induces striatal degeneration similar to HD. Electron microscopy studies showed the localization of the mutant protein to mitochondrial membranes in neurons. Also, mitochondria from HD patient lymphoblasts displayed lower $\Delta\psi_m$ and greater susceptibility to depolarization in response to calcium stimuli when compared to control mitochondria (Panov et al., 2002). Interestingly, the pathogenesis of HD has also been associated with defects in mitochondrial trafficking in motor neurons (Piccioni et al., 2002; Trushina et al., 2004). The presence of polyglutamine tract protein aggregates impaired trafficking of mitochondria along the axon. This effect is most likely due to disruptions of cytoskeletal interactions. Results from our laboratory have recently demonstrated that over expression of mutant huntingtin protein can create a physical "roadblock" in neuronal processes, which might prevent mitochondria from reaching cellular regions of high energy demand (Chang et al., 2005).

1.3.4 Parkinson's disease

After AD, PD is the second most common neurodegenerative disease in the elderly, affecting 1.5 million Americans (National_Parkinson's_Foundation, 2004). The onset of the symptoms usually occurs between the fifth and seventh decade, and include rigidigy, tremor and bradykinesia. The neurodegeneration in PD takes place at the substantia nigra, with the selective loss of dopaminergic neurons. In some cases, PD has also been associated with the presence of intracytoplasmic inclusions denominated by Lewy bodies (Schon and Manfredi, 2003). PD was the first neurodegenerative disease in which mitochondrial respiratory chain dysfunction was implicated. The discovery of a parkinsonian syndrome in response to the meperidine analog N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in humans and laboratory animals provided the first evidence of a deficiency in complex I in PD (Davis et al., 1979; Langston et al., 1983). Accordingly, studies in PD patients showed decreased complex I activity and oxidative stress in the substantia nigra (Schapira et al., 1989). Decreased activity of complex I can promote ROS production and may explain the increase in oxidative damage to DNA and lipids found in brains of PD patients (Dexter et al., 1994).

Several genes have been linked with familial PD. One of these genes is α-synuclein; where mutations or triplication of this gene is linked to autosomal dominant forms of PD (Shen and Cookson, 2004). Although α-synuclein protein is known to be a major component of Lewy bodies, its physiological function remains unclear (Table1). In terms of its possible association with mitochondrial function, Sherer and colleague(2003b)s showed that inhibition of complex I by rotenone leads to α-synuclein aggregation (Sherer et al., 2002). Evidence from other oxidative stress studies further support the notion that α-synuclein aggregates are a consequence of increased oxidative stress (Ischiropoulos and Beckman, 2003). Other genes implicated in PD are parkin, DJ-1 and PINK1. These genes are associated with autosomal recessive forms of PD, which are characterized by early onset and slow progression of the disease (Moore et al., 2005). Parkin has been described as an ubiquitin E3 ligase; however, its target proteins remain to be elucidated. Recent results have linked parkin to mitochondrial function (Palacino et al., 2004). Using a proteomic approach, the authors demonstrated a decrease in several electron transport chain proteins in the *parkin* knockout mice. Also, overexpression of parkin has been shown to protect against cytochrome c release (Darios et al., 2003). Another gene implicated in recessive PD is DJ-1 (Shen and Cookson, 2004). Recently, DJ-1 was described to localize to the outer mitochondrial membrane when over expressed and under oxidizing conditions. PINK1 contains a mitochondrial localization sequence that targets the protein to the mitochondria; this localization sequence is not disrupted by any of the PINK1 mutations implicated in PD. Wild-type PINK1 has been shown to protect cells from loss of $\Delta\psi_m$ and apoptosis induced by protease inhibitors. This protection was eliminated by the presence of PINK1 mutations (Valente et al., 2004).

Several mtDNA haplotypes have been associated with reduced PD incidence while a cluster of several haplotypes has been shown to increase the risk of PD and PD with dementia (Beal, 2005). Interestingly, this supercluster was associated with non-synonymous substitutions in complex I subunits encoded by mtDNA. Also, studies with cybrids derived from PD platelets demonstrated reduced activity in complex I (Swerdlow et al., 1996). Three mutations in mtDNA have been associated with parkinsonism, however patients had unusual forms of the disease, usually associated with other symptoms. For example, patients with mutations in the 12S mtRNA gene develop a syndrome that includes not only parkinsonism but also deafness and neuropathy (Finsterer, 2002).

1.3.4.1 Rotenone model of Parkinson's disease: Mechanism of toxicity

In 2000, Betarbet and colleagues developed an animal model of PD by chronic infusion of rotenone, a complex I inhibitor, in rats (Betarbet et al., 2000). Even though rotenone was shown to consistently inhibit complex I in the brain, there was a selective degeneration of the dopaminergic neurons in the striatal pathway. This degeneration was accompanied by oxidative damage to the striatum, and the presence of inclusions in nigral cells that were positive for ubiquitin and α-synuclein. Moreover, animals in this model were hypokinetic and exhibited shaking similar to the rest tremor observed in PD patients (Betarbet et al., 2000).

Since the development of the rotenone PD model (Betarbet et al., 2000), several groups have used rotenone in both animals and *in vitro* systems to further elucidate the molecular mechanisms implicated in the degeneration of nigral cells. Studies from Sherer *et al.* (2003b) suggested that rotenone neurotoxicity involves oxidative damage, and not ATP depletion. Additionally, treatment with 2-deoxyglucose to mimic the ATP depletion observed with rotenone did not have toxic effects *in vitro*. Moreover, antioxidant treatment of both SK-N-MC neuroblastoma cells and organotypic substantia nigra cultures reduced oxidative damage and rotenone-induced toxicity (Sherer et al., 2003b; Testa et al., 2005). Increased ROS production due to rotenone treatment has been suggested to induce cytochrome c release from mitochondria followed by activation of caspase-3, ultimately leading to neuronal death by apoptosis (Sherer et al., 2002; Pei et al., 2003). Interestingly, accumulation of α-synuclein seems to enhance rotenone toxicity (Orth et al., 2003). More recent results have suggested that rotenone induces microtubule depolymerization, which could explain the selective degeneration of dopaminergic neurons. According to Ren *et al* (2005), the effects of rotenone on microtubules disrupts vesicular transport that lead to the accumulation of dopamine vesicles at the soma. Dopamine leaking from vesicles into the cytosol leads to its oxidation and increased oxidative stress

1.3.5 Some mtDNA mutations are responsible for neurodegenerative diseases

Diseases associated with mutations in mtDNA have been traditionally described as mitochondrial diseases. In 1988, two groups described the first associations between a mtDNA mutation and a large-scale deletion with two different mitochondrial diseases (Holt et al., 1988;

Wallace et al., 1988). Since then more and more mutations, deletions and duplications have been linked to a myriad of diseases. On November 9, 2005, there were 257 disease associations to the mtDNA sequence variations in the Mitomap database (www. Mitomap.org). From these, 131 were in the coding regions or D-loop of mtDNA, while the other 126 affected either the ribosomal or transfer RNAs. Some diseases are associated with more than one mutation in mtDNA; at the same time, single mtDNA mutations can be associated with more than one disease (DiMauro and Schon, 2001). In addition, mtDNA diseases present with diverse symptoms that usually affect more than one tissue. Mutations in mtDNA result in impairment of oxidative phosphorylation. Some of the characteristic features of mitochondrial diseases are lactic acidosis, massive mitochondrial proliferation and cytochrome c oxidase deficiency. The tissues most affected by mtDNA mutations are usually heart, skeletal muscle, and brain since they are highly dependent on oxidative metabolism. Therefore, many mitochondrial disorders are labeled as encephalocardiomyopathies (Schon and Manfredi, 2003).

Table 1. Mitochondria related genes implicated in neurodegenerative diseases

Neurodegenerative disease due to:	**Mutated gene product**
Primary mutations in mtDNA:	
Leigh syndrome	CCOIII, ND5, $tRNA^{Trp}$, $tRNA^{Val}$
LHON	Complex I mtDNA encoded subunits
NARP	Comples V (ATPase) subunit 6
Parkinsonism	12S rRNA
Nuclear gene mutations in mitochondrial proteins:	
ALS	SOD1
AD	ABAD
Nuclear gene mutations in non-mitochondrial proteins:	
AD	Presenilin 1,2
HD	Huntingtin
PD	Parkin, α-synculein

LHON: Leber hereditary optic neuropathy NARP: Neuropathy, ataxia and retinitis pigmentosa CCOIII: cytochrome c oxidase (Adapted from Schon and Manfredi, 2003).

Mutations in mtDNA are also associated with selective neuronal degeneration (Table 1). Mutations in the mtDNA-encoded subunits of complexes I and V result in selective neurodegeneration (reviewed by Schon and Manfredi, 2003). Leber hereditary optic neuropathy (LHON) is a disease characterized by blindness due to optic nerve degeneration. LHON has been linked to mutations in complex I subunits. Even though most patients are homoplasmic for the mutation (all mtDNA molecules carry the mutation) and all tissues have decreased complex I activity, the optic nerve is the most affected by these mutations. Leigh syndrome, a necrotizing encephalomyelopathy, and neuropathy, ataxia and retinitis pigmentosa (NARP) syndrome have both been linked to mutations in the ATPase 6 subunit of complex V. These syndromes usually present with mutation loads of 70-90% in the brain. Defects in the ATPase complex lead to a reduction in mitochondrial ATP.

1.4 DISSERTATION GOALS

Mitochondria are semi-autonomous organelles crucial for the function of all mammalian tissues. They serve a number of essential functions, perhaps the most important is the generation of ATP. In particular for neurons, ATP production at local sites of energy demand is likely to be a critical process in maintaining cellular viability. If we consider ATP production the main character in mitochondrial function, we cannot forget about all the other "supporting" functions that contribute to the efficient performance of mitochondria in all tissues. I started this project with the hope of reaching a better understanding of the role of mitochondria in neurodegeneration by studying their DNA.

Mitochondrial DNA has been traditionally studied in two ways. A more basic approach is to study its structure, replication, and transmission in systems that allow for easy genetic manipulation such as yeast or transformed cell lines. Studies in these systems continue to bring further insight into the basic functioning of mtDNA. A more clinical approach focuses on mtDNA mutations and mitochondrial diseases. Studies arising from this approach linked several mtDNA mutations with rare diseases of maternal inheritance. At the intersection of these two fields is the study of mtDNA in a system that allows for modeling neurodegenerative conditions,

such as primary cortical neurons. From mtDNA studies, it has been demonstrated that mtDNA abnormalities can lead to mitochondrial dysfunction. Therefore, I hypothesized if the reverse was true that mitochondrial dysfunction leads to altered mtDNA dynamics, which can in turn accelerate neuronal degeneration.

Excessive amounts of neurotoxins, such as glutamate or rotenone, have been shown to alter mitochondrial function. Therefore my first specific aim was to determine the effects of neurotoxins on mtDNA replication and total mtDNA content. First, I adapted the BrdU incorporation method (Davis and Clayton, 1996) to our primary neurons in an attempt to study both the rate and the localization of mtDNA in primary neurons. Also, I developed a method to study mtDNA replication by [^{3}H]thymidine labeling of mtDNA in pooled cells. These two methods in combination with real-time PCR experiments have allowed us to assess the response of mtDNA upon treatment with glutamate or rotenone. Results from this first aim show that mitochondrial dysfunction induced by different neurotoxins does not necessarily correlate with effects on mtDNA replication. In particular, glutamate treatment for up to 6 hours does not affect mtDNA replication rate. However, rotenone induces a time- and concentration-dependent decrease in mtDNA replication. Interestingly, the rotenone effect on mtDNA replication seems to be independent of its acute toxic effect.

Intrigued by the effect of rotenone on mtDNA, I decided to study as a second specific aim the mechanism behind the rotenone-induced mtDNA decrease observed in primary neurons. Because rotenone is most effective as a complex I inhibitor, I hypothesized that the rotenone-induced mtDNA decrease was mediated by complex I. However, other complex I inhibitors did not reduce mtDNA replication. Others have shown an increased ROS production in response to rotenone; therefore, as a second possible mechanism of rotenone's effect on mtDNA replication, I aimed to study if increased ROS production was responsible for the mtDNA replication decrease. However, results from my experiments do not support a role for ROS in modulating mtDNA replication decrease, since neither H_2O_2 nor vitamin E pretreatment affected mtDNA replication rate. A third factor regulating mtDNA replication is the mitochondrial nucleotide pools (mtdNTPs) Control of the available mtdNTP pools is known to be important for replication fidelity. MtdNTP experiments confirmed that the decrease of mtDNA replication I observed in

response to rotenone is not mediated by a decrease of mtdNTP pools. Therefore, my results suggest that reduced mtDNA replication following rotenone treatment is mediated by a novel mechanism.

A third specific aim hypothesized that a reduction in mtDNA replication could alter mitochondrial function, movement and morphology. First, I studied how rotenone treatments that reduce mtDNA content affect $\Delta\psi_m$, as an indicator of overall mitochondrial function. Second, I studied the effects of the same rotenone treatments on mitochondrial morphology and movement. My results show that the higher rotenone concentration that decreases mtDNA has several effects on primary neurons reducing not only $\Delta\psi_m$, but also mitochondrial length and movement. Because a lower rotenone concentration that induces a similar response in mtDNA did not have the same effect on other mitochondrial parameters, my results demonstrate that mtDNA replication decrease does not correlate with an overall mitochondrial dysfunction. Taken together, these results suggest that rotenone can exert two distinct effects on mitochondrial function: a mtDNA replication effect that takes place at low doses, and a bioenergetic effect that occurs at higher doses.

2.0 MATERIALS AND METHODS

2.1 REAGENTS AND SOLUTIONS

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

2.2 CELL CULTURE

2.2.1 Primary neuronal and astrocyte cell culture

Primary cultures of rat forebrain neurons were prepared as described previously (Malaiyandi et al., 2005). All experiments were performed on neurons after 11-15 days in culture. Primary cultures of Type I astrocytes were prepared from cortices of postnatal day 1-4 rat pups as described previously (Kress et al., 2002). All astrocytes experiments were performed 2-6 days after plating.

2.2.2 Transfection of primary neuronal cortical cultures

Cortical neurons were transfected with a mitochondrially targeted enhanced-Yellow Fluorescent Protein (mt-eYFP) with or without cotransfection of a cytosolic enhanced-Cytosolic Fluorescent Protein (eCFP) plasmid. The cytosolic eCFP (cCFP) vector was recently prepared in our laboratory (Rintoul et al., 2003). After 10-12 days in culture, primary neuronal cultures were transfected in high-glucose Dulbecco's modified Eagle's media (DMEM) with 2 µg of mt-eYFP plasmid or 1 µg mt-eYFP and 1 µg cCFP plamids combined with 2.5 µl of lipofectamine 2000

(Invitrogen) for 6 h at 37° C. At this time, all media was replaced with conditioned media collected at 8 and 11DIV from other neuronal cultures. Cultures were then treated with rotenone for mitochondrial function experiments or label with 5-Bromo-2'-deoxy-Uridine (BrdU) 18h after transfection for mtDNA replication experiments.

2.2.3 Vascular smooth muscle derived A10 cell line

The A10 cell line derives from embryonic rat thoracic aorta and was kindly donated by Dr. A. F. Stewart (Endocrinology Division, University of Pittsburgh). Cells were cultured in high glucose DMEM with 10% fetal bovine serum, antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), and 2 mM L-glutamine. For mtDNA depletion experiments, cells were plated at a density of 1.5×10^5/well.

2.3 ROTENONE AND DRUG TREATMENTS

Neurons were treated with rotenone at 5, 50 and 500 nM for different time periods by preparing serial dilutions in B27 medium from a 10 mM stock solution (in dimethyl sufoxide, DMSO). Pyridaben and piericidin A treatments were prepared similarly starting from 2 mM (in ethanol 100%) and 2.5 mM (in DMSO) stock solutions, respectively. I also treated cells with 125 µM vitamin E (Alfa Aesar, Ward Hill, MA) by adding to each well 5 µl from a 25 mM stock solution in ethanol.

2.4 TOXICITY MEASUREMENTS

For assessment of drug toxicity, 24 hours before the end of the experiment, medium was replaced with either fresh neurobasal media with B27 supplement or with conditioned media. At the end of the experiment, supernatant was collected and assayed for lactate dehydrogenase (LDH) released into the medium using a Cytotoxicity Detection Kit (Roche, Indianapolis, IN).

LDH release was measured by and absorbance at 490 nm in a Victor3V plate reader (Perkin Elmer, Boston, MA). Results were normalized to 100% LDH release, determined by exposing cells to 30μM $ZnCl_2$ in the presence of 20 μM sodium pyrithione, (zinc ionophore) for 10 minutes and then returned to the incubator for 24 h more in the presence of conditioned media (Kress et al., 2002). Results from LDH assay were confirmed by exclusion of trypan blue assay. LDH results were normalized against total protein content in each well.

2.4.1 Validation of protein content as normalization factor

Protein content was determined by the micro-protein assay using the BCA protein assay kit according to manufacturer's instruction (Pierce Biotechnology, Rockford, IL). This method relies on the color formation after reaction with bicinchoninic acid (BCA) of four peptides (cysteine, cystine, tryptophan and tyorosine) (Wiechelman et al., 1988). One potential problem when using this assay as normalization factor for LDH measurements could be that highly toxic treatments do not only induce cell death but also cell deattachement from the culture plate. If protein content is used as normalization factor, cell deattachment could lead to underestimation of cell death rate. Our primary cortical cultures require poly-D-lysine to attach to the culture plate; therefore, cell deattachement is not a confounding factor in my experiments. Moreover, I have visually inspected cultures after all drug treatments and I have observed dead cells attached to the culture plate. To further confirm this observation, I analyzed the possible reduction in protein content after piericidin A treatment, the most toxic complex I inhibitor used in my experiments. Treatment with 0.5, 5, and 50 nM piericidin A for 48 h induce a cell viability decrease of 35, 85, and 95%, respectively; however, this is not accompanied by a significant reduction in protein content (data not shown). Therefore, I used total protein content as normalization factor for toxicity, [^{3}H]thymidine incorporation and mitochondrial dNTP assays.

2.5 mtDNA REPLICATION

2.5.1 *In situ* localization by BrdU incorporation

To detect mtDNA replication *in situ*, I used the previously described BrdU immunohistochemical method (Davis and Clayton, 1996). To inhibit any possible nuclear DNA replication from its small astrocytic fraction, in some cultures, neurons were pretreated for at least 1 h with 20 μM aphidicolin, which remained in the medium throughout the labeling period. Neurons were treated with 90 nM BrdU in the cultured medium for different time periods. Then, cultures were washed three times with phosphate buffer solution (PBS), and fixed with 4% formaldehyde for 10 minutes at 25°C. Neurons were permeabilized by 1 minute treatment with -20°C acetone. Following rehydration with PBS, DNA was denatured by 2 N HCl digestion for 1 h at room temperature. Then, two 5 min incubations with 100 mM borate buffer, pH 8.5 were used to neutralize the acid. Cells were then washed with PBS three times. Primary anti-BrdU antibody (monoclonal; Roche) was diluted 1:50 in PBS/0.1% BSA and incubated for 1 h at 25°C. Secondary antibodies Alexa 488 or 546 anti-goat IgG (Invitrogen) were diluted 1:500 in PBS/0.1% BSA and incubated for 30 min at 25°C. To counter stain the nuclei, neurons were incubated with Hoechst 33342 for 10 min at 25°C. Some cells were mounted onto glass slides with flouromount G (Southern Biotechnology Associates, Inc. Birmingham, AL). Alternatively, cultures were incubated with anti-BrdU antibodies and nucleases as described by Magnusson (2003) with modifications. In this case, cells were fixed and permeabilized as described above. Then, cells were blocked in 1% normal goat serum for 30 min at 37°C; followed by incubation with anti-BrdU solution for 3h at 37°C in a humidified chamber. An Alexa 546 mouse anti-goat IgG was used as secondary at 1:500 dilution for 30 min at room temperature.

2.5.2 Tritiated thymidine incorporation assay

Neurons were incubated with 2.5 μCi/ml methyltritiated thymidine ([^{3}H]thymidine) (50 Ci/mmol, MP Biomedicals, Irvine, CA) per well for different time periods. At the end of the labeling period, cells were washed twice with PBS and treated with ice-cold 5% trichloroacetic acid for 30 minutes at 4°C. Then, cells were rinsed with PBS at 25°C. To solubilize the

trichloroacetic acid precipitable materials, 1 ml 0.5% sodium dodecyl sulfate (SDS) in 0.5 N sodium hydroxide (SDS/NaOH) was added to each well and incubated for 1 h at 37°C or overnight at 4°C. The materials were then transferred to scintillation vials and mixed with 5 ml scintillant. Counting was performed using a liquid scintillation counter. Protein concentration after SDS/NaOH solubilization was determined by micro-protein assay using the BCA protein assay..

2.6 DEPLETION OF mtDNA IN A10 CELLS

Depletion of mtDNA in A10 cells was achieved by treatment with the DNA intercalating agent, ethidium bromide. After 24 h, cells were transfer to DMEM supplemented with 250 µg/ml ethidium bromide, 50 µg/ml uridine, and 1mM sodium pyruvate (EB-DMEM). Cells were cultured on EB-DMEM for 10-12 days, and were re-plated as necessary. After 10-12 days, total DNA was isolated as described in section 2.7.1. Depletion of mtDNA was confirmed using the PicoGreen (Invitrogen) staining for mtDNA as described by Ashley *et al.* (2005). In my experiments, A10 cells plated on coverslips were stained by incubating the cells for 1h at 37°C with 3µl/ml of PicoGreen solution in the culture medium. Then, cells were washed 3 times with PBS and visualized with a fluorescence microscope as described in section 2.8.

2.7 REAL-TIME PCR ESTIMATION OF mtDNA CONTENT

Total DNA samples were extracted with the Flexigene kit (Qiagen) according to manufacturer's protocol. The mtDNA content was estimated by amplifying a portion of the cytochrome b (cyt b) gene of mtDNA and comparing it to the amplification profile the beta actin (b-actin) nuclear gene (Gourlain et al., 2003). PCR primers and product sizes for both genes are indicated in Table 2.

Table 2: Primer sequences and product sizes for real-time PCR genes.

Gene	Forward primer	Reverse primer	Product size
cyt b	5'-CATCAGTCACCCACATCTGCCG-3'	5'-GGGCGGCGATAATGAATGGGAGG-3'	384 bp
b-actin	5'-AGC GGG AAA TCG TGC GTG-3'	5' CAG GGT ACA TGG TGG TGC C-3'	397bp

Copy number was estimated by using recombinant plasmids containing the target sequences. Both gene fragments were inserted into linearized vectors and cloned using the TA cloning system (TOPO TA Cloning, Invitrogen). Target sequences in the recombinant plasmids were confirmed by automatic sequencing at the DNA Sequencing Core Facility at the University of Pittsburgh. The concentrations of purified recombinant plasmids were determined at 260 nm and the corresponding copy numbers were calculated based on their molecular weight and size. Serial dilutions were used to establish standard curves for each plasmid. Copy numbers for each gene were extrapolated from their standard curves.

The 25-µL PCR reaction mixture contained 1x Brilliant SYBR Green QPCR Master Mix (Stratagene), 100 nM each primer, Rox dye, and 2µl of total genomic DNA extract. From every sample, duplicates of 3 different dilutions were tested for each gene: 1-0.01ng for cyt b and 50-0.5ng for b-actin. Real-time PCR conditions were denaturalization for 10 min at 95°C, followed by 40 cycles of 30 seconds at 95°C, 1 min at 60°C and 1 min at 72°C. The dissociation curve that followed the PCR consisted on denaturation segment for 30 s at 95°C and 81 cycles of annealing/extension for 30 seconds starting at 55°C. The fluorescent signal intensities were recorded and analyzed during PCR in an Mx300P (Stratagene) or MyIQ (Biorad) sequence detector systems. Dissociation curves were generated after each PCR reaction to determine if the fluorescent signals observed may have resulted from nonspecific structures, such as primer-dimers extensions.

2.8 MITOCHONDRIAL DEOXYNUCLEOTIDE SEPARATION AND THIN LAYER CHROMATOGRAPHY

2.8.1 Mitochondrial dNTPs separation

mtdNTPs were separated following the method described by Pontarin *et al.* (2003) with modifications. Briefly, 10mm diameter plates were incubated with 60µCi/plate [^{3}H]thymidine for 24h. Then, neurons were transferred to a cold room and washed 4 times with PBS. Cells were scrapped in 1.5ml of extraction buffer containing (in mM): 210 mannitol, 70 sucrose, 10 Tris-HCl, pH7.5, 0.2 EGTA and 0.5mg/ml BSA. Neurons were homogenized by aspiration and rapid expulsion through a 22-gauge 1½-inch needle into a 3 cc syringe. At this time, 20 µl samples were taken from the homogenate to measure the protein content. Then, homogenates were centrifuged for 20 min at 19,000 x g, the pellet suspended in 0.5ml of extraction buffer and centrifuged again. After the second centrifugation, 900 µl of ice-cold 60% methanol-HPLC grade was used to suspend the pellets. Then, samples were transferred to -20°C for at least 1 h. Pellets were centrifuged again at 19,000 x g for 20 min and supernatants were boiled for 3 min and evaporated in a speed-vac. Dry residues were dissolved in 50µl of water and dried again. The second dry residue was dissolved in 15 µl of water, centrifuged at 10,000 x g for 10 min.

2.8.2 Thin layer chromatography (TLC)

TLC analysis was used as described by Anderson *et al* for adenine (2004). TLC of mtdNTPs was performed using PEI-cellulose plates (EMD Chemicals). Samples (10µl) were spotted and allowed to dry. Plates were developed first with 100% methanol (HPLC grade). Plates were removed from the tank when methanol had reached the top of the plate and allowed to dry. Then they were transferred to a 0.1 M acetic acid and 0.9 M LiCl containing tank, letting the solvent front to reach the top of the plate. Plates were sprayed three times with EN3HANCE spray from Perkin Elmer and a radiographic plate exposed for 1 week at -80°C. Internal carriers were used to localize the different compounds. Values obtained after densitometric scanning were normalized against protein content in each plate.

2.9 FLUORESCENCE IMAGING

All experiments that involved fluorescence imaging were performed in a system consisting of a BX61WI Olympus Optical (Tokyo, Japan) light microscope fitted with Olympus Optical LUMPlanFI 60X or 40X water immersion quartz objectives. The light source was a 75 W Xenon lamp Lambda LS17 with a Lambda 10-2 filter controller (Sutter Instrument, Novato, CA). Light was detected using a CCD camera (Orca, Hamamtsu, Shizouka, Japan) and data acquisition was controlled using Simple PCI software (Compix, Cranberry, PA). Light with which cells were illuminated and the dichroic and emission filters use for the different techniques are listed on Table 3.

Table 3. Flourescence settings for the different techniques used

Dye or Protein	Excitation light	Dichroic mirror	Emission filter
Alexa 488	495±10	505	535±50
Alexa 546	540±25	565	610±75
Rh123	495±10	505	535±50
EYFP	495±10	505	535±50

2.10 MITOCHONDRIAL MEMBRANE POTENTIAL MEASUREMENTS

Rhodamine 123 (Rh123) (Invitrogen) was used in $\Delta\psi_m$ experiments as described previously (Malaiyandi et al., 2005). In these experiments, the fluorescence signal between cells varies because Rh123 is a single wavelength dye. Therefore, an excitation light exposure time was chosen to avoid saturation of the fluorescence signal. Data from Rh123 experiments were normalized in two ways. First, in experiments studying the effect of complex I inhibitors on $\Delta\psi_m$,

baseline fluorescence was assigned the zero value while the unity was assigned to the maximal increase in fluorescence observed after complete dissipation of $\Delta\psi_m$, in response to FCCP. Second, in experiments studying the long term effect of rotenone on $\Delta\psi_m$, values were normalized to the baseline fluorescence taken as the unity.

2.11 MITOCHONDRIAL MOVEMENT AND MORPHOLOGY

Neurons were transfected with mt-eYFP as described above. Control and rotenone treated coverslips were blinded before movie acquisition and remained blind until movement analysis was performed. For these experiments, I acquired 3 min movies from 3 different transfected cells per coverslip. Also, neurons were perfused for 1 minute with 1 μM propidium iodide to ensure the viability of the neurons selected to be recorded. Mitochondrial movement was analyzed using a macro-based analysis program as previously described (Rintoul et al., 2003). The results from each coverslip were averaged and combined. Mitochondrial length was determined with a masking function in the Simple PCI software.

2.12 STATISTICAL ANALYSIS

All experiments were performed in triplicates for each condition, and from at least three different cell culture preparations. Results are expressed as mean± S.E.M. Statistical analysis was performed by one-way ANOVA with Bonferroni post test to compare all conditions or by paired t-test using the Prism 4.01 software (Graph Pad Software, San Diego CA). p values < 0.05 were regarded as significant.

3.0 STUDY OF mtDNA DYNAMICS UNDER NEUROTOXIC CONDITIONS

3.1 INTRODUCTION

Mitochondria are the target of several neurotoxins, including rotenone, high doses of glutamate and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Over the last decade, attention has mainly focused in elucidating toxic mechanisms of these drugs, specifically in terms of apoptosis and mitochondrial function. However, replication and total content of mtDNA has not been extensively studied under these pathological conditions. MPTP has been described to induce Parkinsonism in its neurotoxic form, 1-methyl-4-phenylpyridinium ion (MPP^+), mainly due to inhibition of complex I of the electron transport chain (Davis et al., 1979; Langston et al., 1983). Interestingly, MPP^+ has also been shown to decrease mtDNA replication by a mechanism independent of complex I inhibition in SH-SY5Y cells and other non-neuronal cell types (Miyako et al., 1999). In a previous report from this group, rotenone was suggested to induce an increase on mtDNA content in HeLa cells (Miyako et al., 1997). To investigate if mtDNA replication and content are affected by mitochondrial dysfunction in primary cortical neurons, I used toxic doses of glutamate and rotenone. Both high doses of glutamate and rotenone affect mitochondrial function by different mechanisms. Rotenone, which also inhibits complex I activity, has been effectively used to produce a PD model in rodents (Betarbet et al., 2000). Moreover, brain and platelets of PD patients have consistently demonstrated reductions in complex I activity (Schapira et al., 1989). Therefore, complex I inhibition is suggested to be in part responsible for the pathophysiology of PD's. However, the suggested mechanism for rotenone toxicity does not involve mtDNA replication or content.

Glutamate, a major excitatory neurotransmitter, becomes a powerful neurotoxin under ischemia/reperfusion conditions. Excessive presynaptic release of glutamate overstimulates

NMDA receptors in the postsynaptic neuron leading to a sustained increase in intracellular calcium. Mitochondrial injury caused by neuronal calcium overload is essential in glutamate toxicity (Stout et al., 1998). Although the toxic mechanisms proposed for rotenone and glutamate are different, both have the same end point, neuronal death due to mitochondrial dysfunction. Replication of mtDNA has been suggested to be tightly regulated, however, it is also known that physiological conditions such as neuronal activity and several stress stimuli can induce a change in the total content of mtDNA of a given cell (Fernandez-Silva et al., 2003).

Here, I studied the effect of both neurotoxins on mtDNA replication and content. BrdU labeling followed by immunostaining and [^{3}H] thymidine incorporation were used to analyze mtDNA replication. mtDNA content was measured by QPCR, I found no correlation between neurotoxins effect on mtDNA replication versus neuronal toxicity. Thus, disruption on mtDNA replication and content induced by neurotoxins does not necessarily trigger cell death.

3.2 CHARACTERIZATION OF mtDNA REPLICATION IN CORTICAL NEURONS

To label newly synthesized mtDNA, I have used two methods. First, cortical cultures were treated with the thymidine analgog BrdU (90 nM) and incorporation of this label into newly synthesized mtDNA revealed by immunocytochemistry (Davis and Clayton, 1996). Because primary cortical neurons are post mitotic cells, BrdU was only incorporated into mtDNA as revealed by the absecence of BrdU staining of nuclei (Figure 3, B-D). No signal was observed in neurons that were not exposed to BrdU (Figure 3A). mtDNA replication was first detected after a 3 h incubation period. Replication of mtDNA has been proposed to take place at the perinuclear area (Davis and Clayton, 1996). At the 3 h time point, most of the staining accumulated in the perinuclear area, consistent with earlier studies. However there was also clear staining in the processes of some neurons, which suggests that mtDNA replication could also occur in the processes. However, this BrdU labeling of mtDNA replication does not allow discerning between mtDNA replication in the processes and newly replicated mtDNA rapidly transported to the processes. Labeling periods of 24 and 72 h show increased mtDNA replication in both the perinuclear area and processes. In order to quantify mtDNA replication with this method, 3 fields with similar numbers of neurons were selected from each coverslip using phase contrast. Then, fluorescence pictures were taken and the number of replicated mtDNA was analyzed with the imaging software Simple PCI. The results from each coverslip were averaged and combined. As seen in Figure 3 E, mtDNA replication peaked after 72 h BrdU labeling.

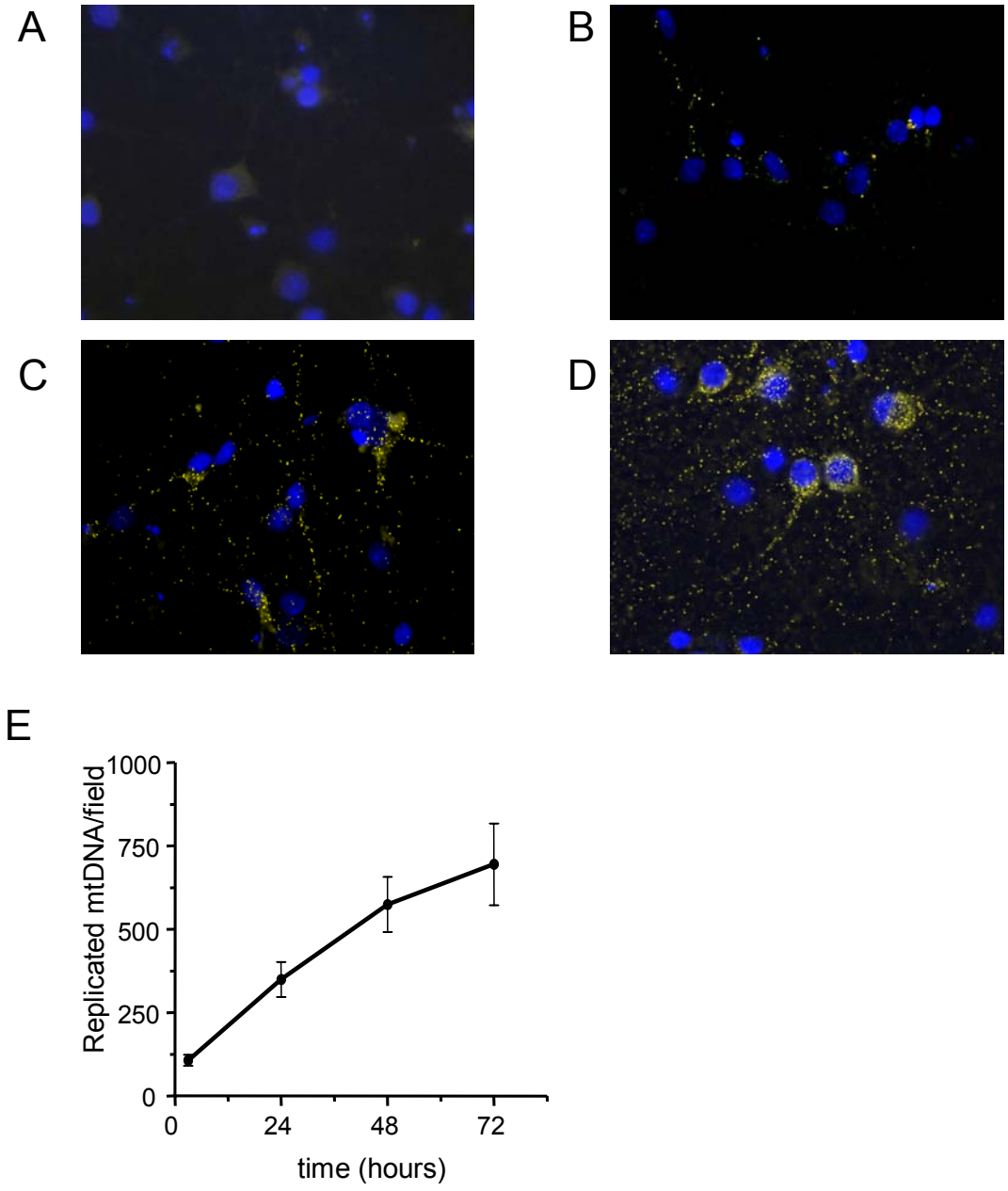

Figure 3. Characterization of mtDNA replication on primary cortical neurons by BrdU incorporation. Primary neurons were treated without (A) or with 90 nM BrdU for 3, 24 or 72 h (B-D). BrdU incorporated into mtDNA was detected by immunocytochemistry with a monoclonal anti-BrdU antibody followed by a secondary antibody coupled to either Alexa 488 or 546. Nuclei were counterstained with Hoechst 33342. (E) Time course of mtDNA replication detected by BrdU incorporation. For quantification, 3 fields with similar numbers of neurons were selected from each coverslip using phase contrast. Then, fluorescence pictures were taken and the number of replicated mtDNA analyzed with the imaging software Simple PCI. The results from each coverslip were averaged and combined. Each data point is representative of at least three experiments (mean ± SEM).

As a control to ensure the specificity of the BrdU antibody, fixed cortical neurons labeled with BrdU for 24 h were digested with DNase to reveal mtDNA as described by Magnusson (Magnusson et al., 2003). Unlike the previously described BrdU immunostaining method, this DNase method does not require acidic treatment of the samples, which means that the structure of fluorescent proteins is maintained; allowing for co-localization of fluorescent proteins and BrdU staining. I was able to co-localize mtDNA positive staining and mitochondria in neurons transfected with the mt-eYFP (Figure 4). Similar to results from Figure 3, mtDNA colocalizes with mitochondria not only in the cell body but also along the processes.

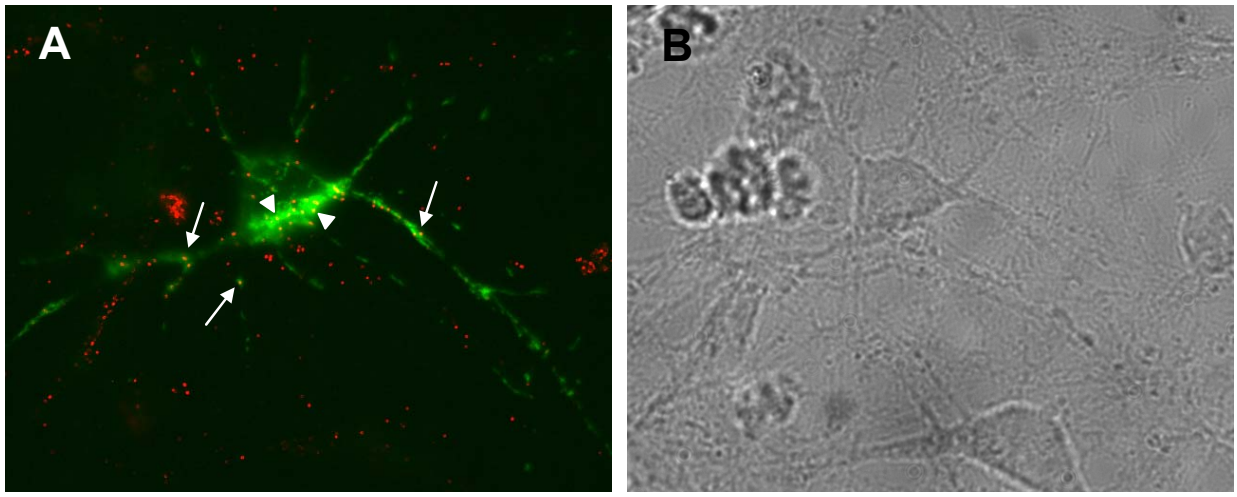

Figure 4. Immunocytochemical localization of mtDNA

Primary cortical neurons were transfected with mt-eYFP and 24-48 h later labeled with BrdU for 24h. (A) BrdU incorporated into mtDNA was detected by immunocytochemistry with a monoclonal anti-BrdU antibody. BrdU incorporated into mtDNA (red) co-localizes with mitochondria (mt-eYFP, in green) in the cell body (arrowheads) and in the processes (arrows). BrdU staining outside green mitochondria comes from untransfected neurons in the same field, as it can be seen in the phase contrast picture (B).

In a second method, mtDNA was metabolically labeled by incubating cortical cultures with [^{3}H]thymidine and assessing the incorporation of radioactivity into mtDNA (Figure 5A). As for the BrdU method, mtDNA replication was first observed after a 3 h incubation with [^{3}H]thymidine. A peak in mtDNA replication occurred following a 24 to 48 h labeling period. Because our cortical cultures have some small glial fraction, I ruled out the possibility that [^{3}H]thymidine was incorporated into glial nuclei by incubating cultures with aphidicolin 1 h prior labeling with [^{3}H]thymidine. Aphidicolin inhibits nuclear DNA polymerase α but not mitochondrial Pol γ. The incorporation rate of [^{3}H]thymidine was not affected by aphidicolin (data not shown). I confirmed the ability of the [^{3}H]thymidine incorporation method to detect mtDNA replication in dividing cells by labelling primary cultured astrocytes with [^{3}H]thymidine in the presence or absence of aphidicolin (Figure 5 B). Thus, I was able to differentiate between nuclear and mtDNA replication (without aphidicolin) from only mtDNA replication (with aphidicolin).

In summary, my results show that mtDNA replication is a dynamic process that takes place in both dividing and non-dividing cells. mtDNA replication can be measured by ^{3}H-thymidine incorporation and localized by BrdU staining.

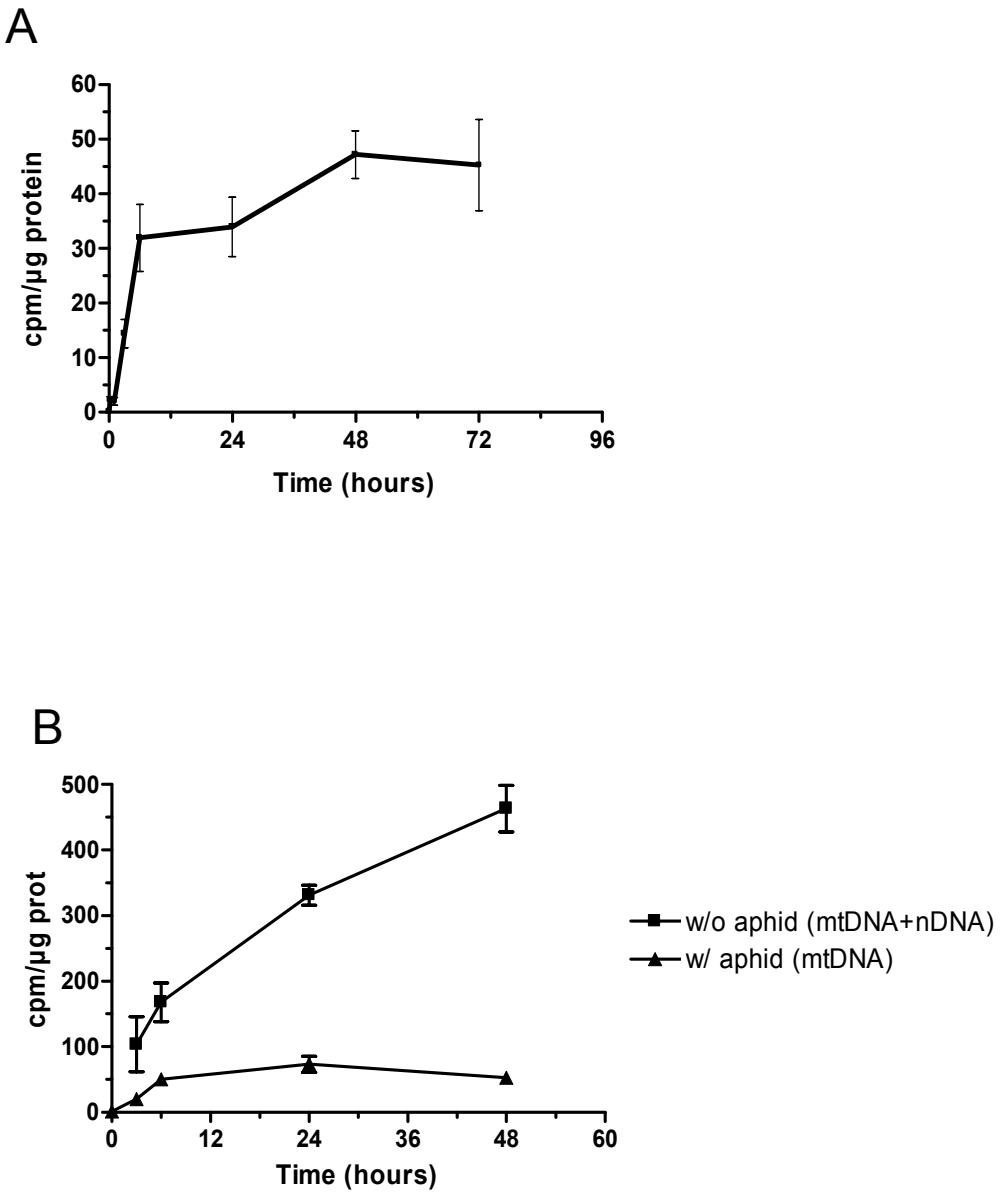

Figure 5. mtDNA replication on primary cortical neurons by [^{3}H]thymidine incorporation.

(A) Time course of mtDNA replication detected by [^{3}H]thymidine incorporation in primary neurons. Each data point is representative of at least three experiments (mean ± SEM). (B) Representative time course of [^{3}H]thymidine incorporation into nuclear and mt DNA (without aphidicolin) and only mtDNA (with aphidicolin) in primary cultured astrocytes.

3.3 EXCITOTOXICITY AND mtDNA REPLICATION

In this study, glutamate was used at conditions that induce excitotoxicity and mitochondrial dysfunction. Cortical cultures were treated with 30 μM glutamate/ 1 μM glycine for 10 minutes at 37°C. In our neuronal cultures, this treatment induces approximately 40% death after 24 hours as determined by LDH release (Figure 6 A). Because I wanted to examine replication in live neurons, I used relatively short labeling periods for both BrdU and [^{3}H]thymidine. Neurons were labeled for 3 h with BrdU immediately after glutamate treatment. Figures 6 B and C show no difference in the distribution of BrdU staining in control and glutamate treated cells after 3 h labeling period. Also, two blinded observers did not report any differences in the distribution of mtDNA replication when asked to compare glutamate treated neurons to controls. Quantification of replicated mtDNA/ field revealed no effect of glutamate treatment (Figure 7 A). Because the [^{3}H]thymidine incorporation method to study mtDNA replication appears to be more sensitive at short time periods, I labeled control and glutamate treated neurons with [^{3}H]thymidine for 3 and 6 h after glutamate treatment (Figure 7 B). As for the BrdU labeling, no mtDNA replication difference was observed after glutamate treatment. Therefore, the extent of mtDNA replication and the distribution of mitochondria with replicated mtDNA do not change 3 h after toxic glutamate treatment.

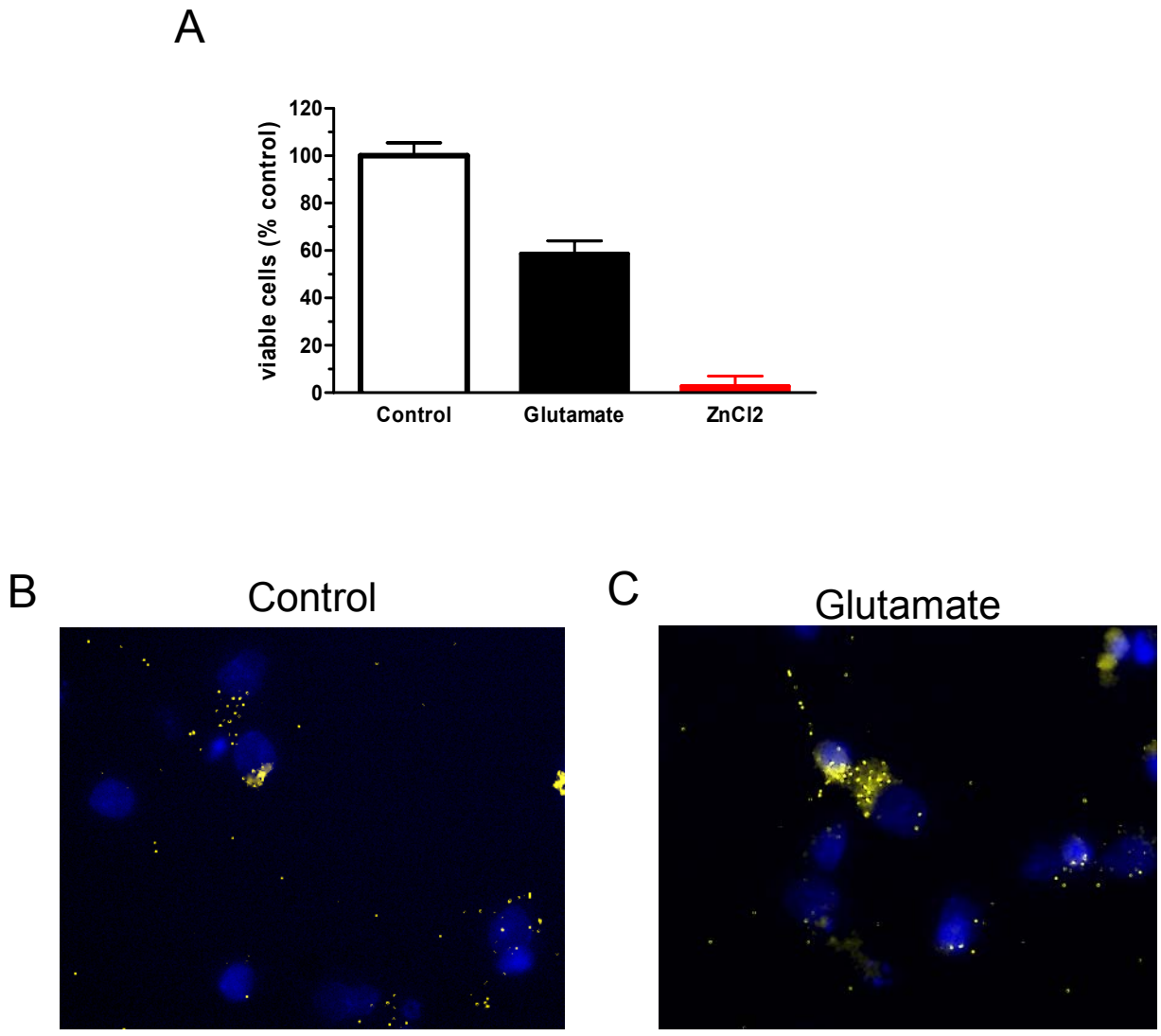

Figure 6. Glutamate does not affect mtDNA replication localization.

(A) Toxicity in primary cortical neurons 24 h after a 30 μM glutamate/ 1 μM glycine treatment for 10 min at 37°C as measured by LDH release. Same treatment but with $ZnCl_2$ instead of glutamate/ glycine was used a positive control of LDH release. Results were normalized as describe in the methods. Each data point is representative of at least three experiments (mean ± SEM). After 3 h labeling treatment, replicated mtDNA does not distribute differently in control neurons (B) compared to glutamate treated ones (C).

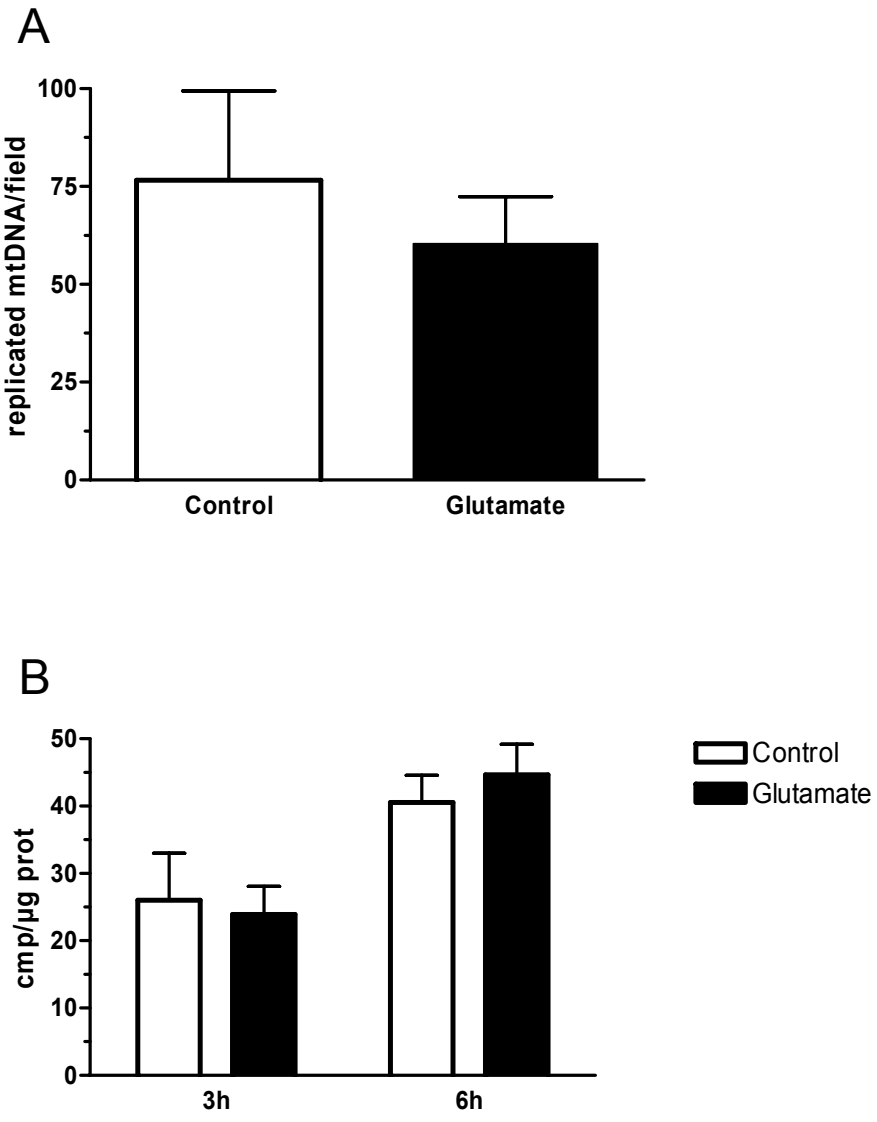

Figure 7. Glutamate treatment has no effect on mtDNA replication rate.

(A) BrdU incorporation measurement after glutamate treatment in cortical neurons. Newly replicated mtDNA was quantified as described in the methods. (B) ^{3}[H]thymidine incorporation into mtDNA 3 and 6 h after glutamate treatment. Each data point is representative of at least three experiments (mean ± SEM) No significant differences ($p<0.05$) were observed.

3.4 mtDNA REPLICATION IN RESPONSE TO ROTENONE TREATMENT

To study mtDNA dynamics in a chronic neurotoxic model, I treated cortical cultures with rotenone for extended time periods. As in the glutamate experiments, I examined live neurons treated with neurotoxin prior to any appearance of damaged cells. Other reports in primary neurons have shown that 50 nM rotenone over 24 h led to a 40% decrease in viability when measured by trypan blue exclusion (Pei et al., 2003). Because rotenone had not been used in our cortical cultures, I first determined a treatment window that would allow us to measure mtDNA replication prior to cell death. Neurons were treated with increasing rotenone doses (from 5 nM to 500 nM) over a 72 h period (Figure 8). Cellular viability was studied by measuring LDH released in the cultured medium which allowed us to measure toxicity and [^{3}H]thymidine incorporation from the same well. Cellular viability results were confirmed by trypan blue exclusion in a separate set of experiments. Also, I always inspected cultures for damage cells (neurite beading and shrunken cell bodies) before collecting conditioned media. To measure mtDNA replication, neurons were labeled with [^{3}H] thymidine in the last 24 h of the experiment. When treated with 5 nM rotenone for 72 h, cellular viability decreased only by 15% over 24 and 72 h periods (* $p<0.01$ *vs.* control, $n\geq3$). However, at the same condition, mtDNA replication decreased by 40% when compared to control (* $p<0.01$ *vs.* control, $n\geq3$). Also, treatment with 50 nM for 24 h or 48 h had a larger effect on mtDNA replication (35% and 53%, respectively) than on neuronal viability (25% and 15%, respectively). Treatment with 50nM for a longer time period (72 h) or higher doses (500 nM) did not further reduce mtDNA replication, but significantly reduced neuronal viability.

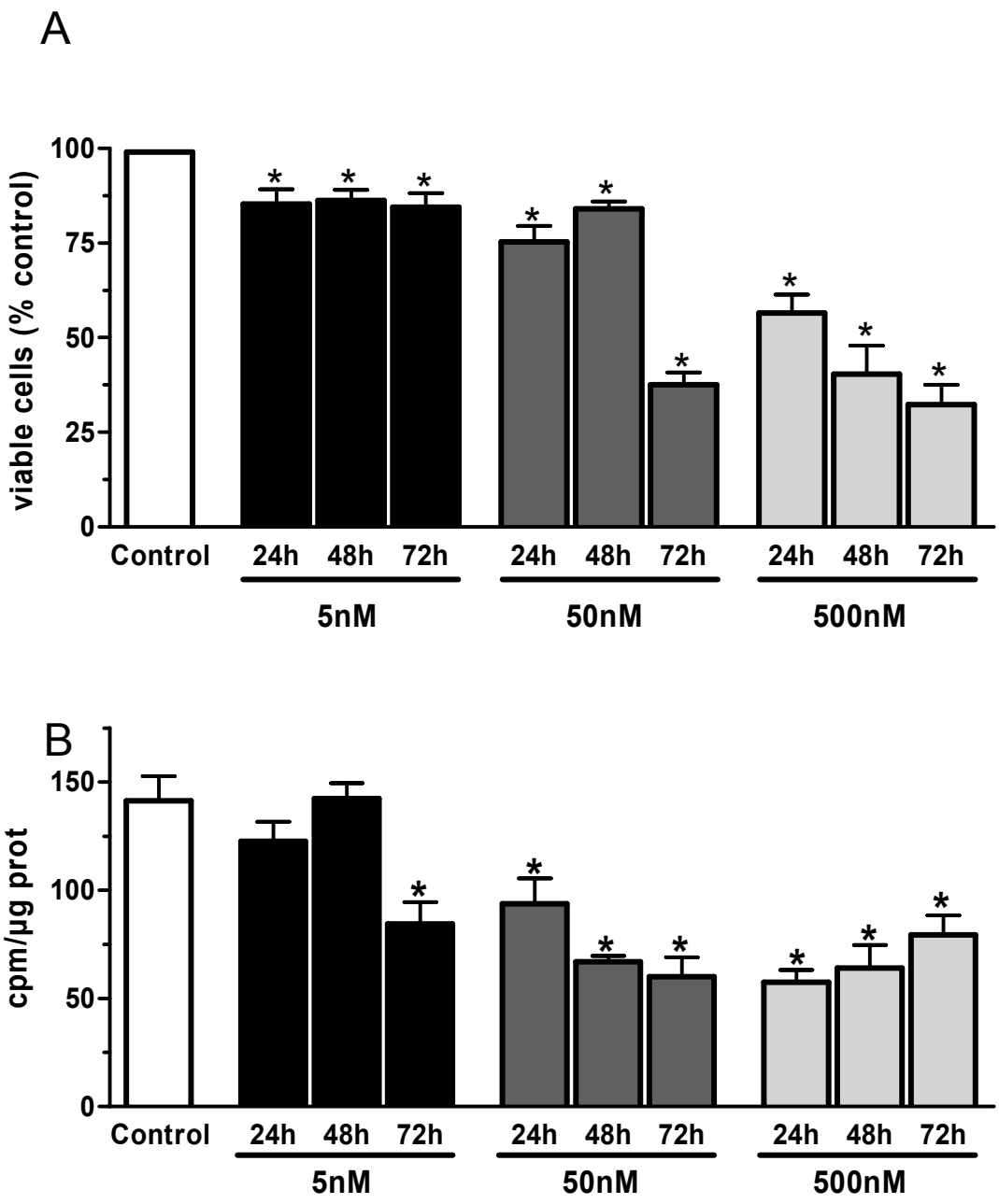

Figure 8. Low doses of rotenone inhibit mtDNA replication on primary cortical neurons.

Rotenone treatment with 5 nM for 72 h or 50 nM for 48 h induced only a 15% decrease on cellular viability when measured by LDH release (A). However, these same rotenone treatments induced 50% decrease on mtDNA replication when measured by [^{3}H]thymidine incorporation (B). Each point is representative of at least three experiments (mean ± SEM, *,# $p < 0.01$ *vs.* control).

Then I treated cells with 50 nM rotenone for 24 h followed by a washout period of either 24 or 48 h. As for previous experiments, neurons were labeled with [^{3}H]thymidine for the last 24 h of the experiment. Toxicity and mtDNA replication were measured as described before. Interestingly, results from these experiments indicate that rotenone effects on mtDNA replication do not directly correlate with its toxic effect. As seen in Figure 9, this treatment followed by a 48 h washout induced less toxicity than 72 h 50 nM rotenone treatment, while the decrease in mtDNA replication observed at 24 h persisted through the 48 h washout. Thus, the inhibition of mtDNA replication by rotenone is irreversible over the time course I studied.

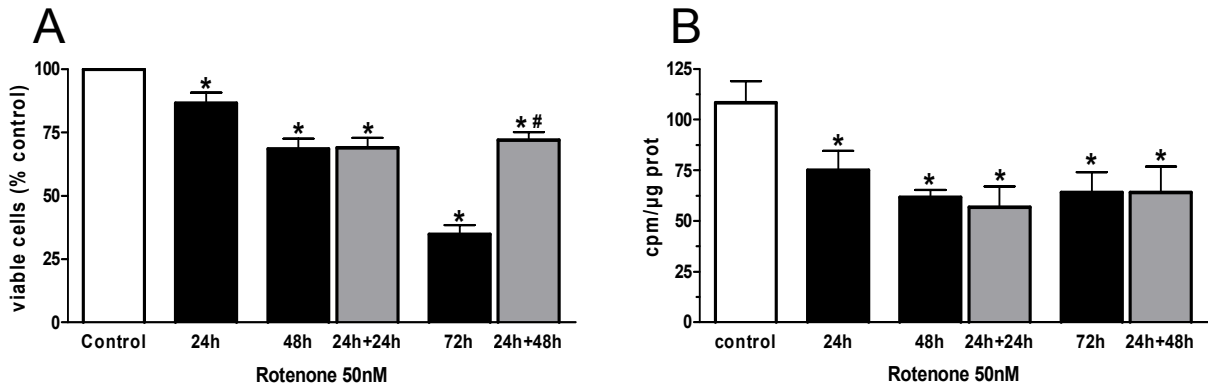

Figure 9. Rotenone effect on mtDNA replication does not reverse after rotenone removal.

Toxicity (A) and mtDNA replication (B) measurements in cortical neurons with different 50 nM rotenone treatments. Neurons were treated with rotenone for 24, 48 and 72 h. Also, some neurons received a 24 h rotenone pulse followed by either 24 h (24+24) or 48 h (24+48) removal of rotenone. mtDNA replication decrease persists even after 48 h rotenone removal while the toxicity observed at this time point is similar to that observed after 24 h rotenone. Each point is representative of at least three experiments (mean ± SEM, (A) * $p < 0.001$ *vs.* control, # $p<0.01$ 72 h *vs.* 24h + 48 h, (B) * $p < 0.05$ *vs.* control).

3.5 RESPONSE TO ROTENONE IN mtDNA CONTENT

Decreased mtDNA replication in rotenone treated cells could translate into a decrease in total mtDNA content in the cell. To study mtDNA content I choose treatments that induced a large decrease in mtDNA replication and very low neuronal toxicity (i.e., 48 h with 50 nM rotenone and 72 h with 5 nM rotenone). To measure mtDNA content, I used the QPCR plasmid standard curve approach described by Gourlain *et al.* (2003) adapted for the SYBR Green technique. Briefly, this method used QPCR to measure both the mitochondrial cytochrome b gene and the nuclear b-actin gene using total cellular DNA as starting material. Copy numbers for cyt b and b-actin were extrapolated from standard curves generated with recombinant plasmids containing the target sequences. As shown in Figure 10 A, rotenone induces a significant decrease in mtDNA content following 50 nM rotenone for 48 h and 5 nM for 72 h treatments in cortical neurons. Also, the rotenone effect in mtDNA content was reproduced on A10 smooth muscle derived cell line when treated with 50 nM for 48 h (Figure 10 B). As a positive control for mtDNA depletion, these cells were transiently depleted of their mtDNA by treatment with 250 μg/ml ethidium bromide (EB) for 10-12 days. EB treatment induced a ~80% reduction on mtDNA content on A10 cells. These QPCR results were confirmed by PicoGreen staining for mtDNA as described by Ashley *et al.* (2005). As mentioned previously, PicoGreen is a fluorescent DNA-specific dye mainly used for DNA quantification that has recently been adapted for mtDNA visualization. In my experiments, A10 cells plated on coverslips were stained with PicoGreen and visualized by fluorescence microscopy. As seen in Figure 11, treatment with EB for 10 days clearly decreases mtDNA signal.

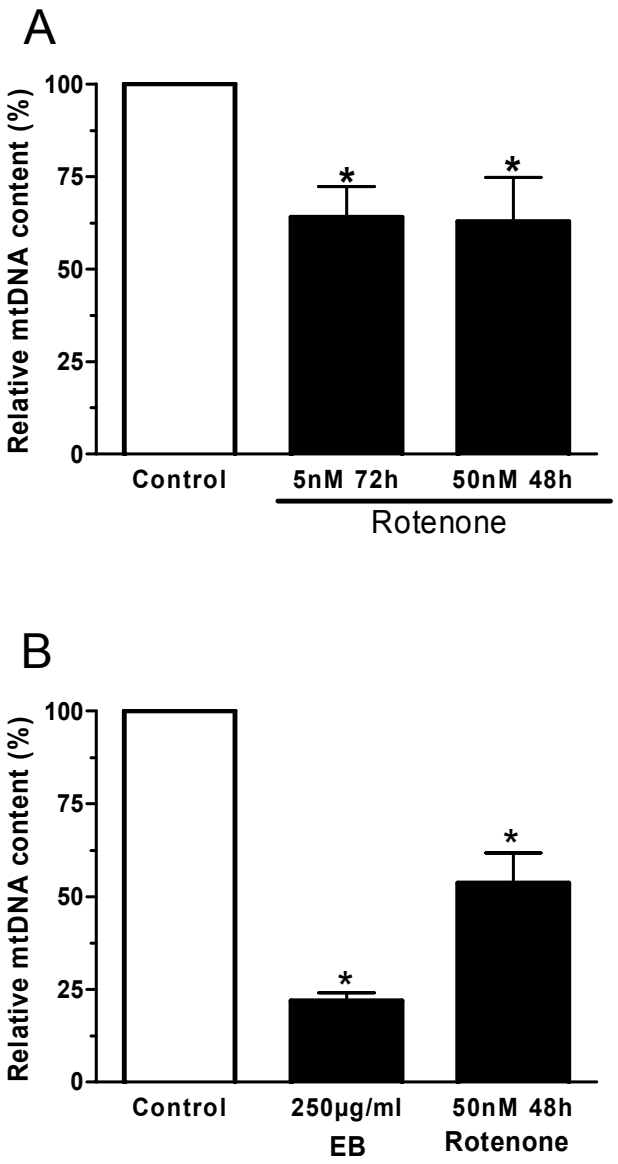

Figure 10. Rotenone decreases mtDNA content in primary neurons and in A10 smooth muscle derived cells.

(A) Rotenone treatment with 5nM for 72h and 50nM for 48h significantly reduce mtDNA content in cortical neurons. (B) Rotenone effect on mtDNA content was reproduced on A10 cells when treated with 50nM for 48h. As control, these cells were transiently depleted of their mtDNA by treatment with 250µg/ml ethidium bromide (EB) for several days. Each data point is representative of at least three experiments (mean ± SEM, * $p < 0.01$ *vs.* control).

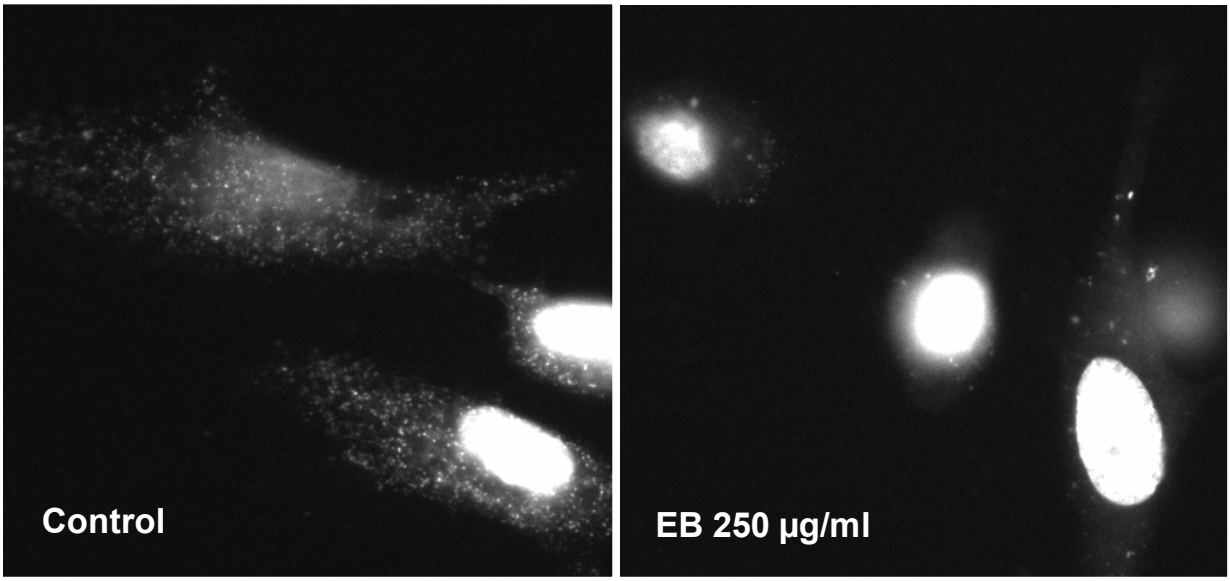

Figure 11. Ethidium bromide treatment depletes mtDNA.

A10 cells were treated with EB 250µl/ml for 10-12 days and stained with PicoGreen. mtDNA is visible as punctuate staining in the cytoplasm.

3.6 DISCUSSION

This study is a first attempt to analyze mtDNA dynamics in central neurons. I have shown that mtDNA replication can be effectively studied by BrdU incorporation into individual mitochondria and [^{3}H]thymidine labeling of mtDNA in pooled cells. I also showed that mitochondrial dysfunction induced by different neurotoxins does not necessarily correlate with effects on mtDNA replication. In particular, glutamate treatment for up to 6h did not affect mtDNA replication rate. However, rotenone induced a time and concentration dependent decrease of mtDNA replication. Furthermore, the effect of rotenone on mtDNA replication is independent of its acute toxic effect.

Data from two separate reports lead to the hypothesis that mtDNA replicates infrequently in post-mitotic tissues. First, results from Menzies and Gold (1971) suggested that mtDNA has a much longer half-life, about 25 days, in the brain than in other tissues such as kidney or lung (6 and 4 days, respectively). Second, cortical neurons were showed to have a slower mtDNA turnover when compared to peripheral neurons and other non-neuronal cell types (Wang et al., 1997). However, my results in cortical neurons clearly show incorporation into mtDNA when measured with even a 3 h labeling period with both BrdU and [^{3}H]thymidine (Figures 3 and 5). Other studies in replicating cells have shown BrdU incorporation into mtDNA as early as 30 min after addition of BrdU (Magnusson et al., 2003). As suggested by the authors, one explanation for the longer labeling time necessary to detect replication in cultured primary neurons when compared to replicating cells could be that pre-mitotic cells must duplicate their mtDNA before each mitosis.

Another interesting point about mtDNA replication is where this replication occurs. Neuronal cultures are the ideal system to study the localization of mtDNA replication thanks to their distinct shape that allows differentiation between cell body and processes. Because mitochondria depend on nuclear-encoded factors for mtDNA maintenance, Davis and Clayton proposed in 1996 that mtDNA replication occurs in mitochondria near the nucleus. Newly

replicated mtDNA would then be transported in mitochondria to the appropriate cellular locations (Davis and Clayton, 1996). However, recent results from Magnusson *et al.* (2003) suggest that mtDNA replication occurs throughout the cell in multinucleate myotubes and in the neuronal cell line, SH-SY5Y. Moreover, my results in primary neurons show BrdU incorporation into mtDNA along the processes following 3 h incubation. However, 3 h could be plenty of time for mtDNA to be transported from the soma into the processes given that I have estimated that mitochondria in cortical neurons travel at velocities between 0.4-0.6 μm/s (Rintoul et al., 2003 and unpublished observations) and the microscopic field studied is approximately 140μm across. One major drawback of these incorporation assays is that they do not distinguish between analog incorporation due to mtDNA replication and DNA repair. MtDNA represents only 0.5-1% of total cellular DNA content, which suggests that the majority of DNA repair takes place in the nucleus. However, I did not detect any nuclear BrdU incorporation in my post-mitotic neurons, suggesting DNA repair may not be detected with this technique. Although some BrdU spots were seen in the cell body, careful observation of the mt-eYFP and BrdU co localization pictures clearly demonstrates that the observed BrdU incorporation comes from mitochondria (Figure 4).

Excessive pre synaptic release of glutamate activates NMDA receptors in the postsynaptic neuron and elevates intracellular calcium. Consequently, there is substantial mitochondrial calcium accumulation, mitochondrial membrane depolarization, ROS generation and, ultimately, cell death (Reynolds, 1999). Recently, Lee and colleagues showed that CREB, a transcription factor activated in response to elevation of intracellular calcium, not only localizes to the mitochondrial matrix, but also regulates the expression of several mitochondrial genes, like subunits ND2, ND4 and ND5 from complex I (Lee et al., 2005). Also, studies from another group have shown that in response to glutamate, cerebellar neurons increase mt-mRNA expression that is followed by a substantial decrease in the 6h following glutamate stimulus (Mehrabian et al., 2005). However, my results show no change in mtDNA replication in response to glutamate when measured by either BrdU or [^{3}H]thymidine incorporation at 3 or 6 h after glutamate insult (Figure 6 and 7). To explain this discrepancy, one has to take into account that I studied mtDNA replication while the reports mentioned above have studied expression of mtDNA genes. Even though mtDNA replication and transcription are similarly regulated, the only one report in which mtDNA copy number and mt-mRNA changed in response to decreased

neuronal activity occurred after 3 and 7 days treatment with tetradotoxin (Hevner and Wong-Riley, 1993).Therefore, mtDNA replication and mtDNA gene expression may not be affected similarly in response to short term treatments with glutamate.

Labeling of newly synthesized mtDNA with BrdU has allowed us to show that localization of mtDNA replication does not change following glutamate treatment (Figure 6). Because previous results from our laboratory showed that glutamate induced a fast and significant remodeling of mitochondrial morphology accompanied by a reduction in mitochondrial movement (Rintoul et al., 2003), I proposed to study if glutamate treatment would alter mtDNA replication distribution. In studying the recovery after glutamate treatment, I have shown that mitochondrial movement and morphology do not recover within the first hour after glutamate treatment; however, within the next hour, mitochondria recover both their normal shape and movement (Rintoul et al., 2003). My results suggest that mtDNA replication occurs throughout the neuron, because I see no change in distribution of newly replicated mtDNA after glutamate treatment, when mitochondria are immobilized. For BrdU to be incorporated into mtDNA, it needs to be first phosphorylated by thymidine kinase 2 (TK 2) in the mitochondrial matrix, and then be further phosphorylated by nucleotide kinases to dBrdUTP. Since the earliest time at which I was able to detect BrdU incorporation was after 3 h labeling, one could speculate that the phosporylation of BrdU and incorporation into mtDNA takes about 3 h. If mtDNA were to occur only in the perinuclear area, glutamate treated neurons would synthesize mtDNA in this region during the 2 h period untill mitochondrial regain normal movement and shape, and then distribute the newly synthesized mtDNA throughout all neuronal processes. However, experiments in which mitochondrial movement is halted for the 3 h labeling period would give a more definitive answer to the question where does mtDNA replication occur.

Unlike glutamate, rotenone induces a significant decrease in mtDNA replication and content. The mtDNA replication decrease was observed in response to treatments that induced minimal neuronal toxicity (Figure 8). Nevertheless, these doses (5-50nM) were in the same range as the free rotenone concentration in the brain calculated for the animal rotenone model (20-30 nM) (Betarbet et al., 2000). These concentrations are known to induce an incomplete inhibition of complex I activity (Davey and Clark, 1996). Because the observed effects occur at very low

toxicities, they could be implicated in the long term effects of rotenone. A 24 h rotenone pulse induces a decrease on mtDNA replication that persists for up to 48 h after rotenone removal, while toxicity decreases during this period (Figure 9). These results show that rotenone effects on mtDNA replication do not correlate of its toxicity and that prolonged decrease of mtDNA replication and content could be responsible for the long-term effects of rotenone. Because primary neuronal cultures can not be studied for long periods of time, experiments in systems that support long-term studies, such as the rotenone animal model, will be necessary to further explore if long-term treatment with rotenone could lead to mtDNA depletion.

The pharmacological agents that are most often associated with mtDNA depletion syndromes are either nucleoside analogs or DNA intercalating agents (Lewis et al., 2003; Okamaoto et al., 2003). However, the complex I inhibitor, MPP^+, inhibits the replication of mtDNA by a mechanism that does not involve any of these processes. The effects of MPP^+ on mtDNA replication were suggested to be independent from its complex I inhibition. In addition, an increase in mtDNA content was found to occur in response to rotenone doses ranging from 50-150 nM in HeLa cells (Miyako et al., 1997). However, the analysis of mtDNA in these studies was crude relying on southern blot analysis with no inclusion of positive or negative control for mtDNA content. I have observed decreased mtDNA in response to rotenone not only in primary cortical neurons but also in the vascular smooth muscle A10 cell line. Importantly, my results were obtained by the more sensitive QPCR technique and as positive control I used ethidium bromide, one agent used to completely deplete cells from their mtDNA when preparing rho0 cells (Figure 10).

In conclusion, my results show that rotenone decreases mtDNA replication and content. Moreover, this rotenone effect on mtDNA does not directly correlate with its toxicity. However, my results suggest that decrease of mtDNA replication or content is not a universal response to neurotoxins since glutamate did not affect mtDNA dynamics. Nevertheless, rotenone effect on mtDNA replication and content could play an important role in its long term effects as neurons could accumulate mitochondria with decreased mtDNA content in the absence of overt toxicity.

4.0 MECHANISM UNDERLYING ROTENONE INDUCED DECREASE IN mtDNA REPLICATION AND CONTENT

4.1 INTRODUCTION

From all the complexes in the mitochondrial electron transport chain, complex I (NADH-ubiquinone oxidoreductase) is the largest among them. Complex I comprises 7 mtDNA-encoded subunits plus about 39 subunits encoded by nDNA (Chomyn et al., 1985; Fearnley et al., 2001). The overall structure of this complex is L-shaped, with one arm embedded in the IMM. This arm contains all the mtDNA-encoded subunits, including ND1 (Earley et al., 1987). The catalytic center of the enzyme is located in the other arm, which faces the mitochondrial matrix (Grigorieff, 1998). Rotenone has been described as a semiquinone antagonist of complex I inhibitor and has become its classical inhibitor. The rotenone binding site has been suggested to directly involve subunit ND1. Other inhibitors of complex I include pyridaben and pieridin A, which are both semiquinone antagonist of complex I. In terms of binding sites of all three inhibitors, rotenone and pyridaben seem to bind complex I at one site, while piericidin A can bind at two different sites. These inhibitors also differ in their kinetic behaviors. Finally, in terms of potency, pyridaben is twice as potent as rotenone while piericidin A is four times as potent (Degli Esposti, 1998). To investigate whether the rotenone effect on mtDNA is mediated by inhibition of complex I, I compared the effects of pyridaben and piericidin A on mtDNA replication.

Another consequence of complex I inhibition is increased ROS production. Because complex I has a site of electron leakage upstream of the rotenone-binding site; partial inhibition of complex I can enhance ROS production (Kushnareva et al., 2002). In fact, rotenone has been proposed to induce its toxicity through this ROS production rather than other downstream

effects, such as ATP depletion (Sherer et al., 2003b). Interestingly, Pol γ can be the target of oxidative damage, which may result in reduced mtDNA replication (Graziewicz et al., 2002). In a model of myocardial infarction, Ide *et al.* (2001) have shown that mtDNA copy number decreases in response to increased ROS production. Also, in a yeast model of Freidreich's ataxia, mtDNA is reduced as a consequence of oxidative stress (Karthikeyan et al., 2003). Therefore my goal was to determine if increased ROS production is implicated in the mtDNA replication and content reduction that I observed in response to rotenone treatment.

A third factor regulating mtDNA replication is the mitochondrial nucleotide pools (mtdNTPs). Due to the IMM impermeability to charged molecules, the pool of mitochondrial dNTPs is separated from the cytosolic pool. To maintain their nucleotide pool, mitochondria use two different pathways: (1) import of cytosolic dNTPs and (2) synthesis of dNTPs by salvaging deoxynucleosides within mitochondria. In post mitotic cells, *de novo* dNTP synthesis enzymes are down regulated which forces these cells to rely entirely on the deoxynucleoside salvaging pathway (Chabes and Thelander, 2000). Control of the available dNTP pools inside mitochondria is known to be important for replication fidelity (Wernette et al., 1988; Martomo and Mathews, 2002). Moreover, imbalances or depletion of mtdNTPs pool have been proposed to play a role in mtDNA depletion syndromes (Nishino et al., 1999). Therefore, I examined the possibility that the mtDNA decrease induced by rotenone could be the consequence of a decrease in the mtdNTP pool.

In chapter 3, I have shown that rotenone decreases mtDNA replication and content. However, the mechanism underlying this effect remains to be elucidated. In this chapter, I have studied three possible mechanisms implicated in rotenone effect on mtDNA replication. First, I studied the effect of other complex I inhibitors on mtDNA replication. Second, I studied the possibility that increased ROS production is responsible for mtDNA decrease. And third, I checked the possibility that a decrease on mtdNTP pools induced by rotenone could be responsible for reduced mtDNA replication and content decrease.

4.2 ROTENONE EFFECT ON mtDNA REPLICATION IS INDEPENDENT FROM COMPLEX I INHIBITION

In order to reveal whether the effect of rotenone on mtDNA replication is mediated by its complex I inhibition, I performed parallel studies of mtDNA replication using two other complex I inhibitors, pyridaben and piericidin A. One consequence of complex I inhibition is a reduction in $\Delta\psi_m$. Because these complex I inhibitors have not been used in primary cortical neurons, I first examined the effect of both pyridaben and piericidin A on $\Delta\psi_m$ as a readout of their complex I inhibition and then compared it to rotenone effect. Rh123 was used at quenching concentrations. Therefore, in response to mitochondrial depolarization, Rh123 is released from mitochondria to the cytosol resulting in an increase in Rh123 fluorescence. The responses of complex I inhibitors were compared to the complete depolarization induced by 750 nM FCCP. Figure 12 A shows that both pyridaben and piericidin A induce $\Delta\psi_m$ loss comparable to that seen with rotenone. To elucidate the effect of these inhibitors on mtDNA replication, I used complex I inhibitor treatments similar to rotenone treatments that induced mtDNA decrease. Cortical neurons were treated for 48 h at concentrations ranging from 5 to 500 nM for pyridaben and 0.5 to 50 nM for piericidin A. Both complex I inhibitors showed significant toxicity in my cultures at these doses (Figure 12 B and C). As for rotenone experiments, neurons were labeled with [^{3}H]thymidine for the last 24 h of the experiment. Surprisingly, mtDNA replication did not decrease after 48 h treatments with either inhibitor, even at doses that greatly reduce neuronal viability (Figure 12 D and E). Therefore, my results suggest that reduced neuronal cell viability induced by complex I inhibition does not necessarily generate defects in mtDNA replication. This further implies that the rotenone effect on mtDNA replication might not be mediated through its inhibition of complex I.

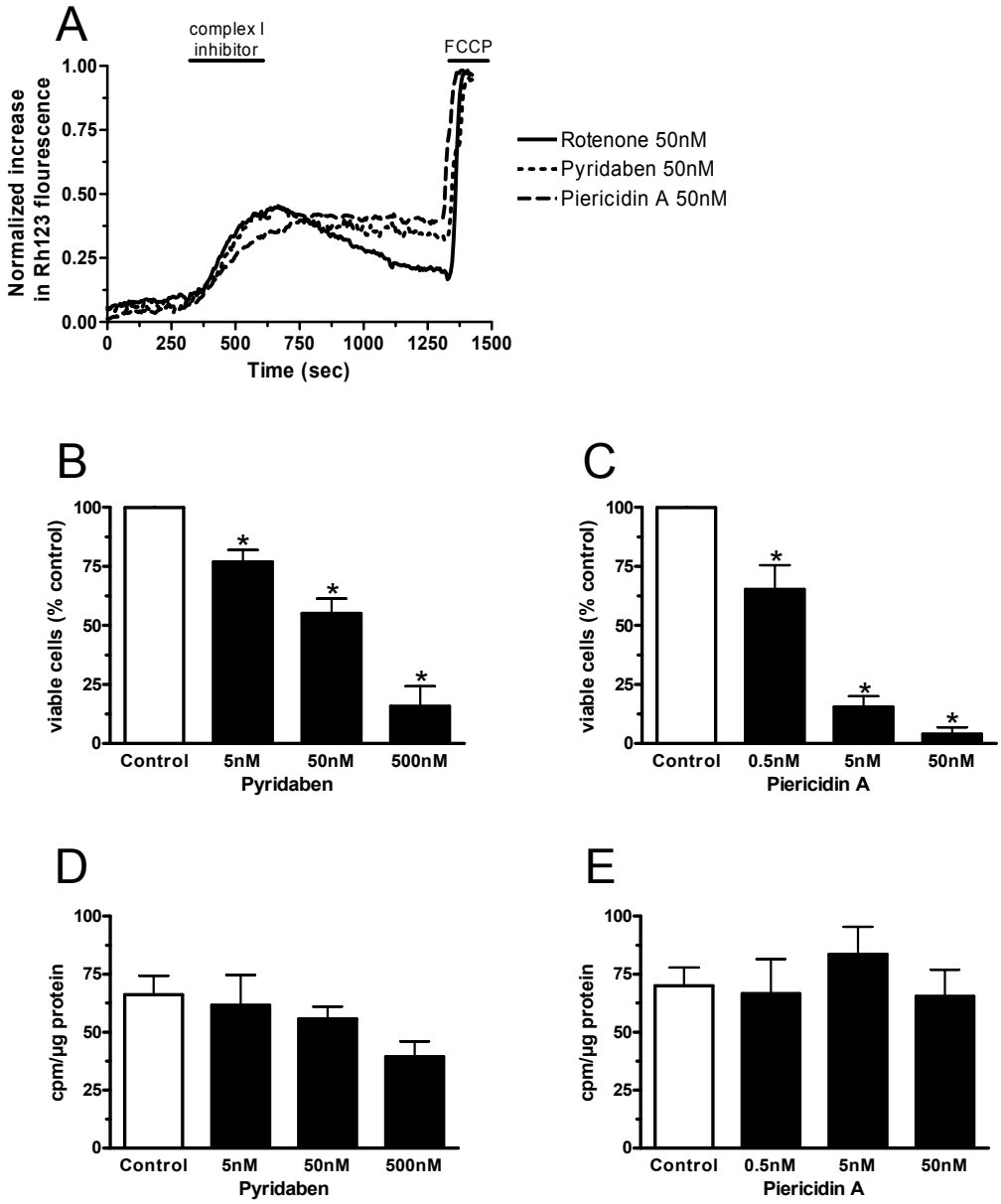

Figure 12. Complex I inhibitors other than rotenone do not decrease mtDNA replication.

(A) Representative experiment showing that complex I inhibitors pyridaben and piericidin A induce mitochondrial membrane depolarization comparable to that of rotenone when measured by Rh123 flourescence. Pyridaben (B and D) and Piericidin A (C and E) do not decrease mtDNA replication (D and E) even though they have a clear toxic effect (B and C) on primary cortical cultures. Each point is representative of at least three experiments (mean ± SEM, (B) * $p < 0.05$ vs. control, (C) * $p < 0.001$ vs. control).

4.3 ROS PRODUCTION DOES NOT PARTICIPATE IN ROTENONE INDUCED MTDNA DECREASE

To investigate whether an increase of ROS production is responsible for the alterations on mtDNA replication and content observed in response to rotenone, I used two paradigms. First, I increased ROS in cultured neurons by treating them with hydrogen peroxide (H_2O_2). Second, I protected my neurons from increased ROS production with the antioxidant vitamin E.

4.3.1 Effect of oxidative stress on mtDNA replication

Oxidative stress was produced by treating neurons with H_2O_2. This paradigm has been used as a model of oxidative stress in neuronal cultures by other laboratories (Dargusch and Schubert, 2002). Neurons were treated with H_2O_2 for 10 min, returned to regular culture medium and examined 24 h later. Then, I measured both toxicity and mtDNA replication as described above. Treatment for 10 min with 50 and 100 μM H_2O_2 induced significant decreases in neuronal viability of 50 and 70%, respectively ($p<0.01$, n=3) (Figure 13 A). Surprisingly, mtDNA replication was not greatly reduced when taking into account the degree of toxicity induced by H_2O_2 treatment; I observed only 51 and 63% mtDNA replication decrease with 50 and 100 μM H_2O_2, respectively ($p<0.01$, n=3) (Figure 13 B). Moreover, to better illustrate the relationship between mtDNA replication and toxicity, I calculated the ratio cpm/ % viable cells. As seen in Figure 13 C, there were no significant differences in the ratios between control and 50 and 100 μM H_2O_2 treatments, which argues against a preferential inhibition of mtDNA replication by ROS.

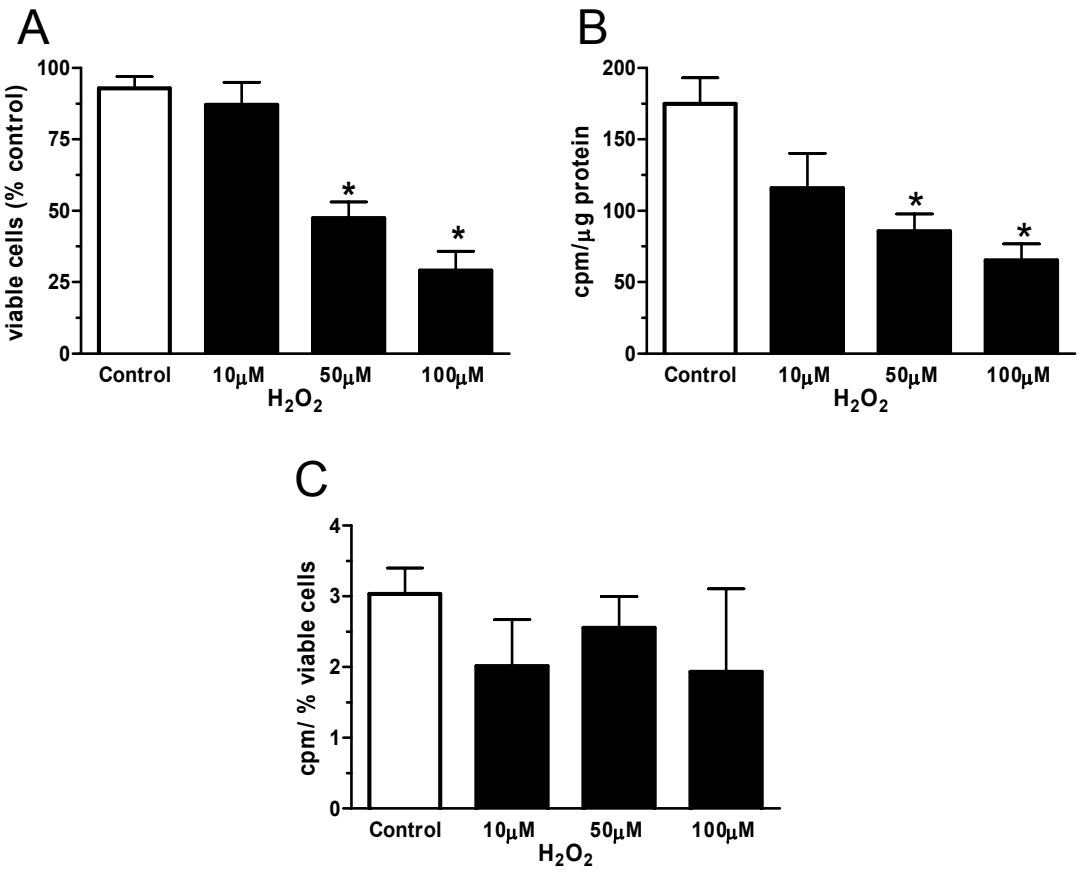

Figure 13. Increased ROS does not decrease mtDNA replication.

Neurons were treated for 10 min at 37°C with 10 to 100 μM H_2O_2. Toxicity (A) and mtDNA replication (B) were measured 24 h later. (C) mtDNA replication, as measured with [^{3}H]thymidine, normalize per number of viable cells. Each point is representative of at least three experiments (mean ± SEM, * $p < 0.01$ *vs.* control).

4.3.2 Prevention of ROS production by vitamin E treatment

Rotenone induced ROS production can be reduced with the antioxidant vitamin E. Vitamin E was demonstrated to reduce rotenone toxicity in SK-N-MC neuroblastoma cells (Sherer et al., 2003b). In a first set of experiments, I verified that vitamin E at the concentration used by Sherer *et al.* (2003) protects my cortical neurons from oxidative stress induced by H_2O_2 treatment. Neurons were treated with 50 µM H_2O_2 for 10 min and 24 h later assayed for toxicity as described above. As shown in experiments described above, this treatment induces a 50% decrease in neuronal viability (Figure 13 and 14). Some neurons were pre treated for 24 h with 125 µM vitamin E, which remained in the culture medium for the duration of the experiment. Vitamin E effectively protected neurons from H_2O_2 injury. Neurons treated with vitamin E and H_2O_2 showed a 25% increase in viability when compared to H_2O_2 treated neurons ($p<0.05$, n=3) (Figure 14). Therefore, 125 µM vitamin E protects primary neurons from ROS injury.

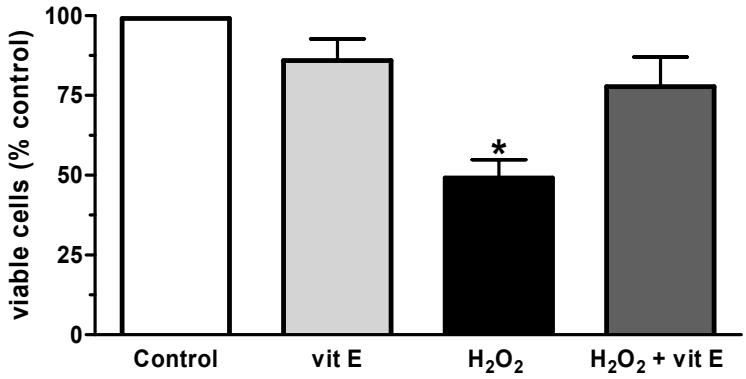

Figure 14. Antioxidant treatment with vitamin E protects primary neurons from H_2O_2 toxicity.

Neurons were treated with 50 µM H_2O_2 for 10 min at 37 °C and returned to regular medium for 24 h, time at which toxicity was assayed. Neurons were pre treated with 125 µM vitamin E 24 h before H_2O_2 treatment, vitamin E remained in the medium throughout the experiment. Control cells were treated with vehicle. Each point is representative of at least three experiments (mean ± SEM, * $p< 0.001$ *vs.* control).

To further explore if oxidative stress is implicated in mtDNA replication decrease, neurons were treated for 48 and 72 h with 50 nM rotenone and 125 μM vitamin E. Rotenone treatments of 50 nM for 48 and 72 h decreased neuronal viability by 15% and 60%, respectively; while mtDNA replication shows a similar reduction (53% and 56%). For the 48 h time point, neurons were pretreated with vitamin E for 24h; for the 72 h treatment, neurons received rotenone and vitamin E simultaneously. Interestingly, I did not observe any vitamin E protection from rotenone toxicity at the 48 h time point. However, vitamin E, indeed, protected neurons from rotenone toxicity after 72 h treatment (33% viable cells with rotenone vs. 58% viable cells with vitamin E and rotenone, * $p< 0.05$ vs. control, # $p< 0.05$ vs. rotenone treatment, n=3). Surprisingly, vitamin E did not reverse rotenone effect on mtDNA replication at either 48 or 72 h time points (Figure 15).

Results from my experiments do not support a role for ROS in modulating mtDNA replication decrease, since neither H_2O_2 nor vitamin E pretreatment affected mtDNA replication rate. Taken together, my results suggest that the reduced mtDNA replication and content observed in response to rotenone may not be mediated by an increase in ROS production.

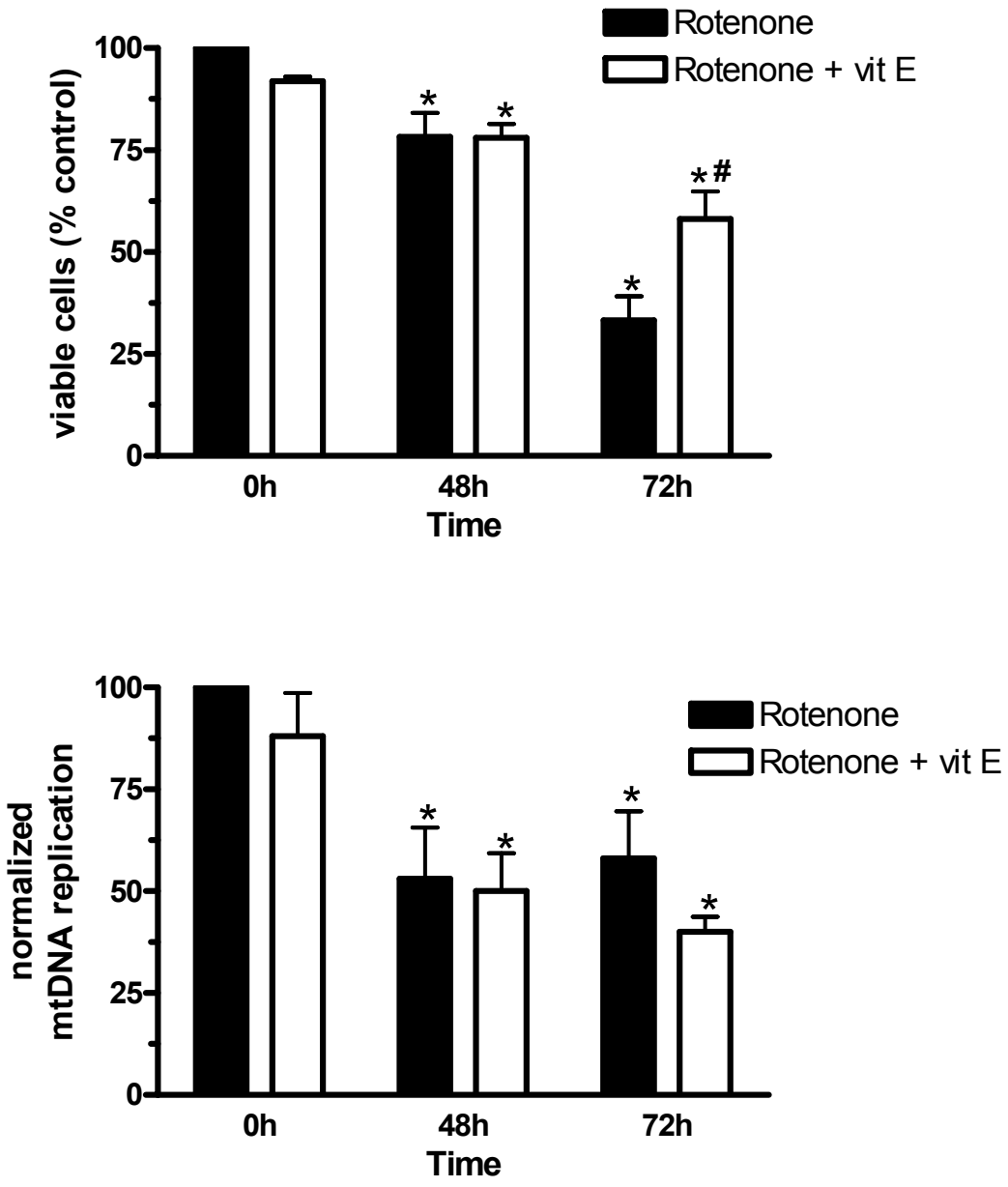

Figure 15. Antioxidant treatment with vitamin E does not prevent from rotenone effect on mtDNA replication.

Neurons were treated with 125 μM vitamin E coinciding with 48 and 72 h 50 nM rotenone treatment. Although vitamin E significantly prevented rotenone induced toxicity at 72 h, it had no effect on rotenone induced mtDNA replication decrease. mtDNA replication data was normalized to 100% as control values. Each data point is representative of at least three experiments (mean ± SEM, * $p < 0.01$ *vs.* control, # $p < 0.001$ *vs.* rotenone treatment alone).

4.4 DEPLETION OF THE mtDNTP POOL IS NOT RESPONSIBLE FOR THE ROTENONE INDUCED mtDNA REPLICATION DECREASE

The aim of the next experiments was to test the hypothesis that depletion of the mtdNTP pool was the cause for the rotenone-induced decrease in mtDNA replication. To measure mtdNTP pool I followed the extraction method described by Pontarin *et al.* (2003) using TLC separation of the nucleotides. In this method, one centrifugation and one washing step are used to isolate mitochondria and nuclei from the cytosolic fraction. Because of their impermeability to charged molecules, mitochondrial membranes retain nucleotides. On the contrary, nuclei are permeable for nucleotides, therefore, nuclear nucleotides rapidly reach equilibrium with the cytosol and are removed from the pellet in the washing step (Pontarin et al., 2003). For these experiments I chose conditions that had the largest effect on mtDNA replication and content: 48 h 50 nM rotenone and 72 h 5 nM rotenone. Neurons were treated as in previous experiments and were labeled in the last 24 h of the experiment with 60 μCi/plate of [^{3}H]thymidine. At this time mtdNTPs were isolated and separated by TLC as described in the methods. As seen in Figure 16, I localized dTTP and thymidine in the thin layer chromatograms from the position of internal standards. The fact that I observed dTTP in samples from all conditions demonstrates that [^{3}H]thymidine was effectively incorporated into dTTP in the mitochondrial fraction. In response to rotenone treatment, mtdTTP decreased only at the 50 nM dose by 63% ($p<0.01$ $n=4$). However, 5 nM rotenone treatment for as long as 72 h did not induce any significant change on mtdNTP pools. Therefore, my results suggest that the mtDNA replication decrease in response to rotenone is not due to a decrease in mtdNTP pools.

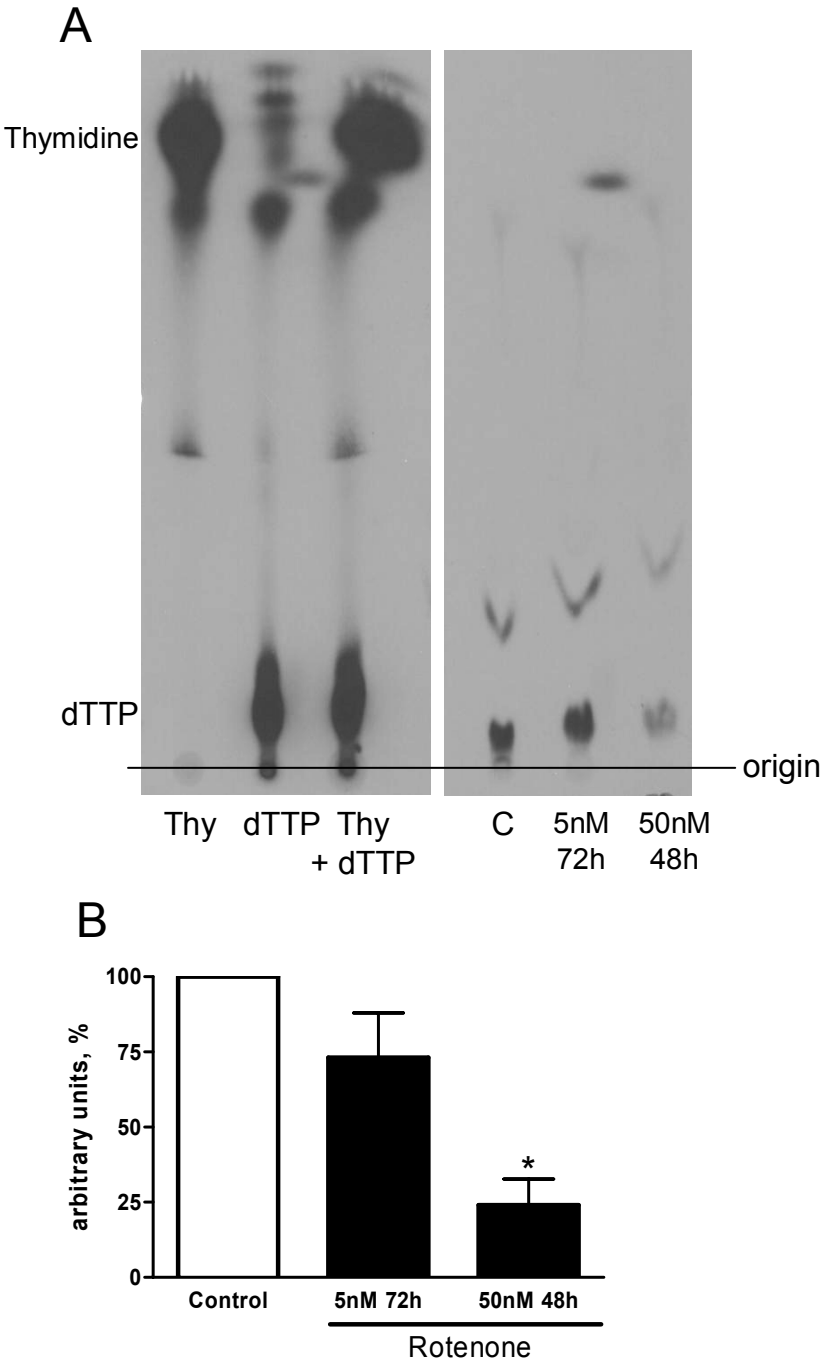

Figure 16. Rotenone decreases mtdTTP at 50 nM but not at 5 nM doses.

mtdNTPs were isolated and separted using TLC. (A) Representative TLC plate from control, 5 nM 72 h, and 50 nM 48 h rotenone treated primary neurons. (B) TLC results quantification shows significant decrease in the mtdTTP after 48 h 50 nM rotenone treatment but not at the 72 h 5 nM treatment. Each point is representative of at least three experiments (mean ± SEM, * $p < 0.01$ *vs.* control).

4.5 DISCUSSION

In this chapter, I studied the mechanism behind the rotenone induced mtDNA decrease observed in primary neurons. I have shown that inhibition of complex I induced by rotenone and other inhibitors is not correlated with the mtDNA decrease. Also, my results do not support a role for ROS in modulating the decrease of mtDNA replication. Finally, mtdNTP experiments confirmed that the decrease of mtDNA replication I have observed in response to rotenone is not mediated by a decrease in mtdNTP pools.

In studying the possibility that complex I inhibition is responsible for rotenone induced mtDNA decrease, I initially focused upon mtDNA replication. All three complex I inhibitors studied here, rotenone, piericidin A and pyridaben, have been suggested to act as semiquinone antagonist of complex I. However, recent studies suggested different binding properties for these three inhibitors (Ino et al., 2003). Nevertheless, all three compounds have been shown to effectively inhibit complex I activity in mitochondria isolated from different tissues (Degli Esposti, 1998). Results from my experiments show that all three inhibitors effectively dissipate $\Delta\psi_m$. The fact that rotenone withdrawal results in $\Delta\psi_m$ recovery is in accordance with previous results in type one cells from carotid body from Duchen *et al.* (Duchen and Biscoe, 1992). Neither pyridaben nor piericidin A withdrawal resulted in $\Delta\psi_m$ recovery. This result could be explained by the different binding kinetics of these inhibitors to Complex I. These inhibitors have been used mostly in isolated mitochondrial in suspension which does not allow for drug withdrawal experiments, therefore, the reversibility of these inhibitors has is not very well characterized. In my experiments, the range of neuronal toxicity was different for all three inhibitors, rotenone and pyridaben exhibiting less toxicity than piericidin A. These differences could be explained by the different potencies of each inhibitor, their different inhibitory capabilities, or by undescribed actions of these compounds on other mitochondrial sites or even in the cytosol. Nonetheless, the fact that all three inhibitors

depolarize $\Delta\psi_m$ in a similar manner reassures us that I was indeed inhibiting complex I. Despite their complex I inhibition, neither piericidin A nor pyridaben decreased mtDNA replication. These results suggest that rotenone is exerting its effect on mtDNA replication by a different mechanism than complex I inhibition. As mentioned in Chapter 3, another inhibitor of complex I, MPP^+ does inhibit mtDNA replication in a manner suggested to be independent from its complex I inhibition (Miyako et al., 1997). In subsequent reports, the authors demonstrated that MPP^+ inhibits mtDNA replication by directly interacting with the D-loop and other branched structures of mtDNA. The same study also showed that rotenone at 1 μM doses did not alter the D-loop in their *in vitro* assay (Umeda et al., 2000; Iwaasa et al., 2002). The discrepancy between their results and ours in terms of the mechanism for rotenone inhibition of mtDNA replication could be explained by structural differences between rotenone and MPP^+.

One of the consequences of complex I inhibition is an increase in ROS production (Sherer et al., 2003b). Therefore, I hypothesized that ROS over production can impair mtDNA replication. Evidence of oxidative stress in the forms of low levels of reduced gluthatione and oxidized proteins, DNA, and lipids have been found in PD patients brains (Dexter et al., 1989; Alam et al., 1997; Pearce et al., 1997; Floor and Wetzel, 1998). Oxidative stress has also been suggested to be responsible for rotenone toxicity because treatment with antioxidants like vitamin E and coenzyme Q protected the SK-N-MC cell line and organotypic substantia nigra cultures from rotenone toxicity (Sherer et al., 2003b; Testa et al., 2005). Accordingly, my results show that vitamin E protects from rotenone toxicity following 72 h of treatment. The absence of vitamin E protection from rotenone toxicity after 48 h is probably due to the lack of sensitivity of my LDH assay to detect small changes in toxicity at this length of rotenone exposure. Rotenone treatment at 50 nM for 48 h induced only a 15% decrease on neuronal viability; while at 72 h, neuronal viability decreased by 60%, increasing the range for vitamin E to exert its protection. Oxidative damage has been recently shown to induce a decrease on mtDNA copy number in failing hearts after myocardial infarction (Ide et al., 2001; Ikeuchi et al., 2005). Pol γ can also be the target for oxidative damage, which may result in reduced mtDNA replication. In a similar paradigm to the one that I used, oxidative damage to Pol γ was induced by H_2O_2 treatment in human fibroblast (Graziewicz et al., 2002). However, 2 h treatments with 250 and 400 μM H_2O_2 induced only a 15% decrease on Pol γ activity. Therefore, my H_2O_2 concentrations may be too

low to have an effect on Pol γ activity. In summary, increased ROS production does not seem to be involved in mtDNA replication decrease, because neither H_2O_2 nor vitamin E pre treatment affected mtDNA replication rate.

All together my results suggest several mechanisms for the diverse effect of rotenone effect on primary neurons. First, complex I inhibition, likely through increased ROS production, might be responsible for its toxic effects. Second, while decreased mtDNA replication may not be related with rotenone toxicity in response to 48 and 72 h exposures, it could be related to its long term effects that may occur in whole animal exposures to this and related environmental toxins.

The control of mtdNTP pools has been shown to be of relevance for the fidelity of mtDNA replication (Wernette et al., 1988; Martomo and Mathews, 2002). Moreover, some mtDNA depletion syndromes have been suggested to results from imbalances or depletion of mtdNTPs pool (Nishino et al., 1999). Recent reports have demonstrated that asymmetries in mtdNTPs reduce mtDNA polymerase fidelity (Song et al., 2005). Because dNTPs are DNA precursors, I hypothesized that rotenone may induced a decrease on mtdNTP pools and will be responsible for a decreased mtDNA replication. However, my results do not support this hypothesis because treatment with 5 nM rotenone for 72 h has no significant effect on mtdTTP levels even though this treatment induces a significant reduction in mtDNA replication and content. Interestingly, higher rotenone concentrations (50nM) did decrease mtdTTP levels. These results could be explained by different mechanisms in which rotenone interacts with mitochondrial function, such as $\Delta\psi_m$ dissipation. Most studies regarding mtdNTPs and their involvement in mtDNA metabolism have utilized healthy pre- or post-mitotic cells. There is no evidence for mtdNTP imbalances in mitochondrial dysfunction scenarios. Mitochondrial dysfunction usually results in a reduction in $\Delta\psi_m$, because most of the mitochondrial matrix import mechanisms depend on $\Delta\psi_m$, there is the possibility that a reduced $\Delta\psi_m$ could result in a reduction of mtdNTP pools.

In conclusion, my results suggest that reduced mtDNA replication following rotenone treatment is mediated by an unknown mechanism. Complex I inhibition and subsequent ROS production have been implicated with rotenone toxicity (Sherer et al., 2003b). My results from this chapter and Chapter 3 suggest that in fact rotenone toxicity is mediated by its complex I inhibition and increased ROS production in primary cortical neurons. However, its acute toxic effect does not correlate with its effect on mtDNA replication. Because mtDNA replication decrease occurs in the absence of overt cellular toxicity and the rotenone doses used in this study are similar to the estimated rotenone concentration in the brain of the rotenone PD model (Betarbet et al., 2000), the mtDNA decrease that I observed in these studies could be an important player in the the long-term degeneration induced by rotenone.

5.0 MITOCHONDRIAL HOMEOSTASIS AFTER ROTENONE TREATMENT

5.1 INTRODUCTION

Several neurodegenerative diseases have been associated with abnormal mitochondrial morphology and trafficking. For example in AD, over expression of tau and pathological mutations in presenilin have been shown to block mitochondrial transport to neuronal processes (Ebneth et al., 1998; Pigino et al., 2003). Huntington's disease is also associated with altered mitochondrial trafficking in motor neurons (Piccioni et al., 2002; Trushina et al., 2004). I have recently shown that glutamate, through NMDA receptor activation, impairs mitochondrial movement and alters morphology leading to the change from an elongated to a punctuate structure. Moreover, other toxic insults like elevated $[Zn^{2+}]_i$ or NO inhibit mitochondrial movement without affecting mitochondrial morphology (Malaiyandi et al., 2005; Rintoul et al., 2006). However, in most cases mitochondrial morphology was studied in response to acute treatments with neurotoxins and not prolonged treatments, which may more accurately reflect conditions in chronic neurodegenerative diseases.

In elucidating a mechanism for the neurotoxin effect on mitochondrial movement and morphology, our laboratory established that glutamate induces motility and morphological changes, while NO halts mitochondrial movement without changing mitochondrial morphology. The protonophore FCCP, which induces complete dissipation of $\Delta\psi_m$, decreases mitochondrial movement in primary neurons (Rintoul et al., 2003). In addition, oligomycin, which inhibits ATP synthesis, reduces mitochondrial movement without changing mitochondrial morphology. All together, results from our laboratory suggest that mitochondrial movement is associated with mitochondria holding $\Delta\psi_m$. It is important to note that most studies of mitochondrial dynamics involved only acute applications of neurotoxic stimuli. Therefore, I do not know how

mitochondrial movement will respond to a prolonged $\Delta\psi_m$ depolarization. Interestingly, cells depleted of mtDNA maintain $\Delta\psi_m$, although at lower levels than wild type cells. mtDNA depletion also leads to changes in mitochondrial morphology from a normal reticular network to a more punctuate pattern (Garcia et al., 2000; Gilkerson et al., 2000). It has also been reported that upon glutamate stimulation, there is an increase in $[Ca^{2+}]_i$, and results from Rintoul *et al* (2003) suggest that this $[Ca^{2+}]_i$ elevation is responsible for the mitochondrial morphology changes observed in response to glutamate.

In order to study if a reduction in mtDNA replication could alter other mitochondrial functions, I studied the effect of rotenone treatments that reduce mtDNA content on $\Delta\psi_m$, mitochondrial movement and morphology. First, to study $\Delta\psi_m$, I used Rh123 in neurons treated with 50 nM rotenone for 48 h and 5 nM for 72 h. The response to complete dissipation of $\Delta\psi_m$ by FCCP was used as an indicator of overall mitochondrial function. Second, I studied the effects of rotenone treatments on mitochondrial morphology and movement by transfecting primary neurons with mt-eYFP, which allows for tracking of mitochondrial dynamics by fluorescence microscopy. My results show that 50 nM rotenone has a bioenergetic effect on primary neurons reducing not only $\Delta\psi_m$, but also mitochondrial length and movement. Because 5 nM rotenone treatment did not have the same effect, my results demonstrate that mtDNA replication decrease does not correlate with an overall mitochondrial dysfunction.

5.2 ROTENONE EFFECT ON $\Delta\Psi_m$

Acute complex I inhibition with rotenone, pyridaben or piericidin A induces a decrease on $\Delta\psi_m$ (Figure 12). Because neurons need to hold some $\Delta\psi_m$ to maintain ATP production, among other functions, I studied the degree of depolarization induced by rotenone following long term treatment. Primary neurons were treated with 5 nM rotenone for 24 and 72 h and with 50 nM rotenone for 24 and 48 h. In all these conditions, the increase in Rh123 fluorescence was studied in response to FCCP as a measurement of the $\Delta\psi_m$. Figure 17 A shows two representative traces of control and 48 h 50 nM rotenone treated neurons in response to complete $\Delta\psi_m$ dissipation by FCCP. Analysis of responses to FCCP upon different treatments shows that 5 nM rotenone did not induce a significant change in $\Delta\psi_m$ at either 24 or 72 h. However, 50 nM rotenone treatment did induce a significant decrease of 45% in $\Delta\psi_m$ as early as 24 h into the treatment. Interestingly, a 48 h treatment did not induce a further decrease $\Delta\psi_m$ (49% reduction) (Figure 17 B).

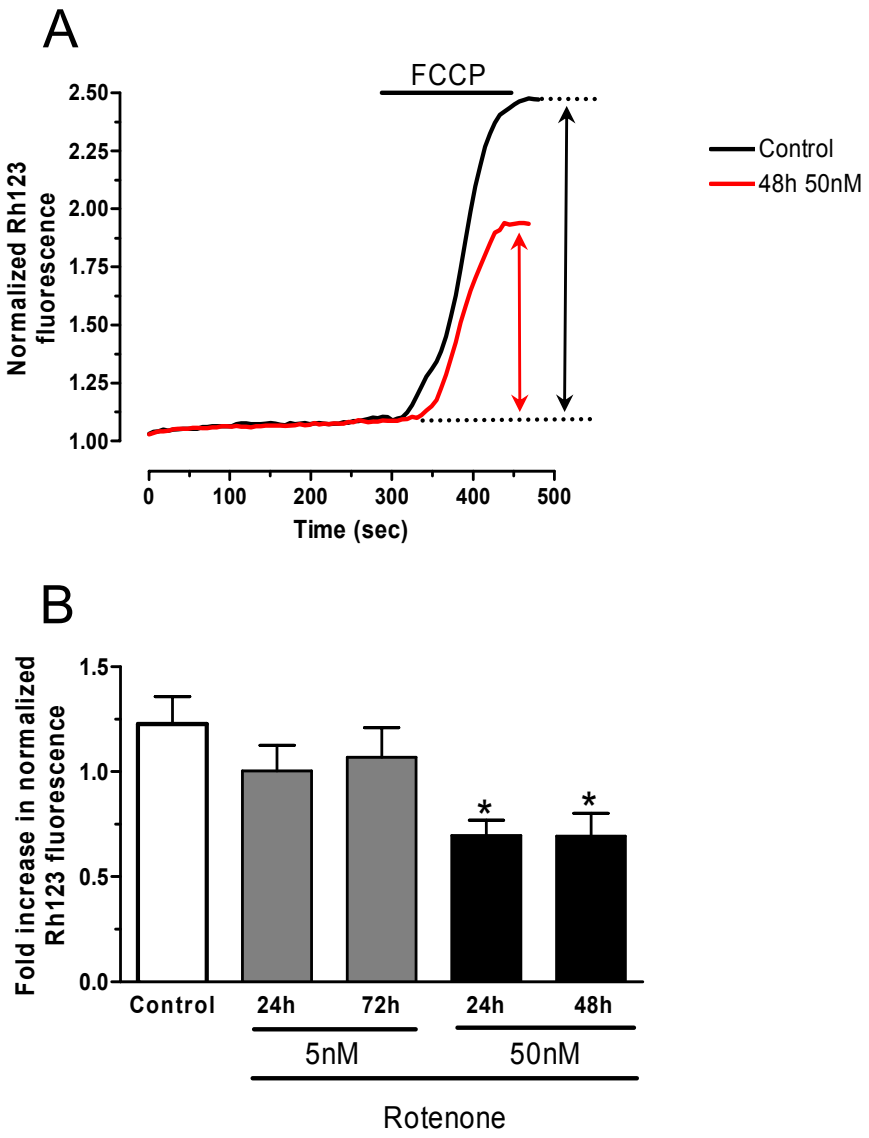

Figure 17. Long term treatment with rotenone decreases mitochondrial membrane potential.

(A) Representative traces of $\Delta\Psi_m$ changes in control and 48 h 50 nM rotenone treated neurons using Rh123. (B) Summary graph of Rh123 fluorescence increases in response to FCCP in neurons treated with 5 and 50 nM rotenone for different time periods. Values were normalized to the unity as the baseline fluorescence. Each point is representative of at least three experiments (mean ± SEM, * $p < 0.01$ *vs.* control).

5.3 ROTENONE EFFECT ON MITOCHONDRIAL MORPHOLOGY

In order to study rotenone effects on mitochondrial morphology, I transfected primary neurons with the mitochondria-targeted eYFP (mt-eYFP) protein. Neurons were then treated with 5 nM rotenone for 72 h or with 50 nM rotenone for 24 and 48 h. To ensure the objectivity of my results, control and rotenone treated coverslips were randomized and analysis was carried out by an observer blinded to the treatment conditions. In previous studies with mt-eYFP transfected neurons, I noted a small fraction of transfected neurons that do not survive this procedure. These dead neurons display a very distinct punctuate mitochondrial morphology, which resembles the morphology induced by acute glutamate treatment (Rintoul et al., 2003). In order to exclude dead cells from analysis, neurons were perfused with the vital dye propidium iodide for 1 min prior to imaging. Only transfected neurons that did not take up the dye were selected for imaging. Figure 18 B and C show two representative micrographs of the mitochondrial morphologies observed in response to 50 nM rotenone for 48 h. Although most treated neurons presented mostly short, almost punctuate mitochondria, some of them had a mixed population of short and long mitochondria. I determined mitochondrial length with a masking function in the SimplePCI software. Changes in mitochondrial morphology were only significant for the 50 nM rotenone treatment over 48 h. Treatments with 5 nM rotenone at 72 h or 50 nM rotenone for 24 h did not induce a change on mitochondrial morphology. (Figure 19).

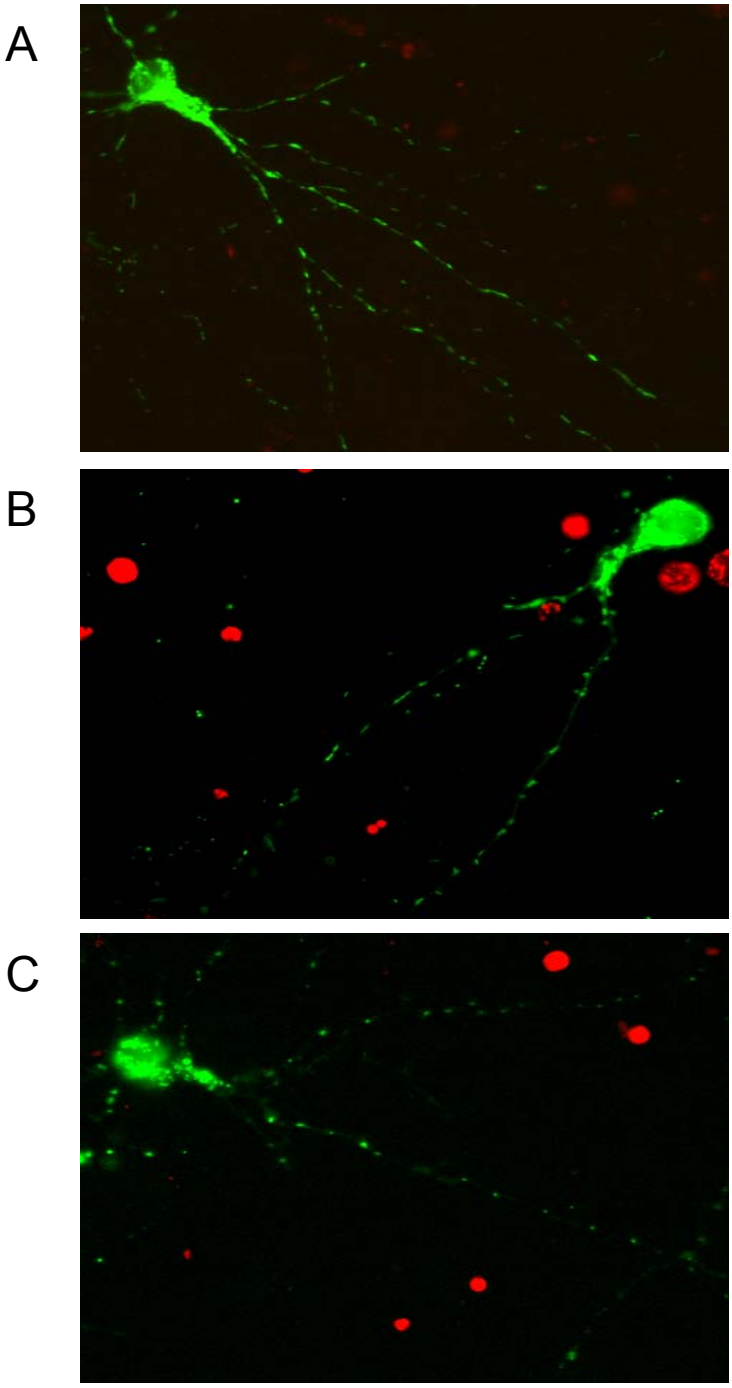

Figure 18. Decrease on mitochondrial length after 48 h of 50 nM rotenone treatment.

Representative micrographs of mt-eYFP transfected neurons. (A) Control neuron, (B-C) 50 nM rotenone treated neurons for 48 h. Neurons were stained with propidium idodide (in red) to assure all neurons selected for imaging were alive.

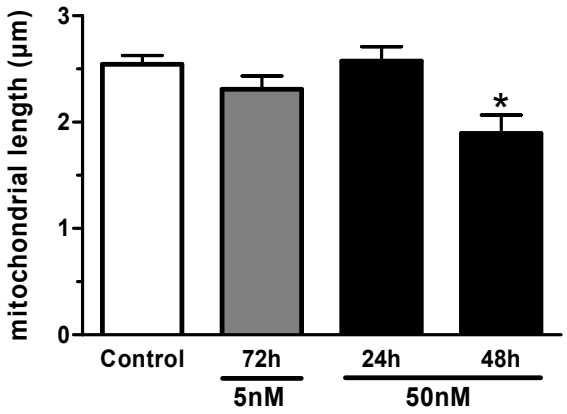

Figure 19. Rotenone reduces mitochondrial length at 50 nM 48 h treatment.

Mitochondrial length measurement with different rotenone treatments. Pictures from 3 mt-eYFP transfected neurons per coverslip were taken and mitochondrial length was determined with a masking function in the Simple PCI software. Each point is representative of at least three experiments from different neuronal preparations (mean ± SEM, * $p < 0.01$ *vs.* control).

5.4 ROTENONE DECREASES MITOCHONDRIAL MOVEMENT

While many compounds have been examined for their effects on mitochondrial morphology, only glutamate and rotenone significantly decreased mitochondrial length. However, many other compounds have been shown to decrease mitochondrial movement albeit they posses different mechanisms of action (Reynolds et al., unpublished observations). For example, compounds that induce $\Delta\psi_m$ dissipation have been associated with decreased mitochondrial movement. Because prolonged rotenone treatment decreases $\Delta\psi_m$, I examined mitochondrial movement upon these treatments. As for the mitochondrial morphology experiments, control and rotenone treated coverslips were blinded before movie acquisition and remained blind until movement analysis was performed. For these experiments, 3 min movies from 3 different transfected cells per coverslip were acquired, then analysis was performed using a macro-based analysis program as previously described (Rintoul et al., 2003). Results from these experiments show that rotenone decreases mitochondrial movement at 50 nM, while 5 nM rotenone treatment for 72 h has no

effect on movement. Interestingly, 50 nM rotenone treatment at both 24 and 48 h decrease mitochondrial movement by 40 and 49%, respectively (Figure 20). Because 50 nM rotenone treatment also induced a reduction in $\Delta\psi_m$, my results suggest a correlation between rotenone effect on $\Delta\psi_m$ and mitochondrial movement.

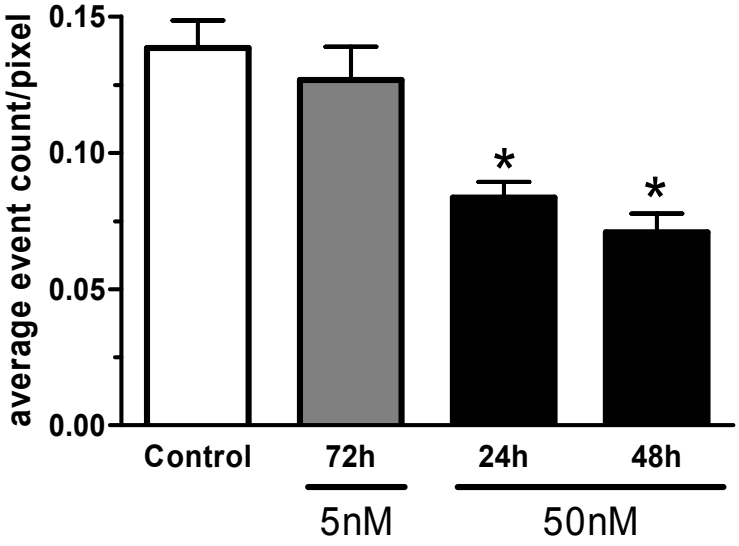

Figure 20. Mitochondrial movement decreases with 50 nM rotenone treatment.

Mitochondrial movement was studied in neurons expressing mito-eYFP after 5 and 50 nM rotenone treatments for different time periods. Control and rotenone treated coverslips were blinded before movie acquisition and remained blind until movement analysis was performed. I acquired 3 min movies from 3 different transfected cells per coverslip. Each point is representative of at least three experiments from different neuronal preparations (mean ± SEM, * $p < 0.01$ *vs.* control).

5.5 DISCUSSION

Here, I have studied mitochondrial function upon treatments that induce a decrease in mtDNA replication. I have shown that treatment of primary neurons with 50 nM rotenone induces a decrease in $\Delta\psi_m$ that is sustained for 48 h. However, treatments with 5 nM rotenone had no effect in $\Delta\psi_m$ even at 72 h treatments. Similarly, mitochondrial movement decreased only in response to 50 nM treatments with rotenone. Mitochondrial length was only decreased after a 48 h treatment with 50 nM rotenone. Interestingly, in Chapter 3, I observed a decrease in mtDNA replication with both 5 and 50 nM doses. Taken together, these results suggest that rotenone can exert two distinct effects on mitochondrial function; a mtDNA replication effect that takes place at doses as low as 5 nM, while a bioenergetic effect occurs at higher doses (i.e. 50 nM).

Among other effects, inhibition of complex I results in a dissipation of $\Delta\psi_m$, which translates to decreased ATP production. Studies in mesencephalic neurons have shown that rotenone doses of 20 nM for 24 h induce a 25% decrease in $\Delta\psi_m$, accompanied by a 40% decrease in neuronal survival over 48 h (Nakamura et al., 2000). In our primary cortical cultures, rotenone treatments with 5 nM for 72 h and 50 nM for 48 h induced 15% decrease in neuronal survival (Figure 8, Chapter 3). My results from $\Delta\psi_m$ experiments show that only 50 nM doses were effective in significantly reducing $\Delta\psi_m$ (45% reduction), while the 5 nM doses had no significant effect. Because my measurements were obtained with the fluorescent dye Rh123, I do not know how the decrease on $\Delta\psi_m$ translates into changes in the milivolts of membrane potential in the inner mitochondrial membrane. Also, I do not know if these changes translate to a reduction in the cellular ATP levels. Recent results have shown that 100 nM and 10 nM rotenone treatment for 6-8 h induce 32 and 15% decrease in cellular ATP, respectively (Sherer et al., 2003b). The differences I have observed between the 5 and 50 nM doses in terms of $\Delta\psi_m$ could be explained by the different degrees of complex I inhibition and the contribution of other complexes to $\Delta\psi_m$. Complex I inhibition by rotenone has a threshold effect by which changes in respiration and ATP synthesis occur only after 72% inhibition of its activity (Davey and Clark, 1996). Also, the other complexes in the electron transport chain contribute to $\Delta\psi_m$ by moving H^+ into the intermembrane space. It is possible that the dissipation

effect in response to 5 nM rotenone can be compensated by the other complexes but not in response to 50 nM. All together, results from my $\Delta\psi_m$ experiments support the hypothesis that only 50 nM rotenone has a bioenergetic effect on primary neurons.

Mitochondrial movement has been linked to changes in $\Delta\psi_m$ by our laboratory and others (Rintoul et al., 2003; Miller and Sheetz, 2004). In these reports, mitochondrial movement in axons and dendrites was reduced in response to complete dissipation of $\Delta\psi_m$ by either FCCP or CCCP. Because of the long term toxicity of these uncouplers, a relationship between mitochondrial movement and $\Delta\psi_m$ after prolonged treatments could not be established. My results show that rotenone over 24 and 48 h induces a reduction in mitochondrial movement that is associated with a reduction in $\Delta\psi_m$. One consequence of lower $\Delta\psi_m$ is reduced ATP production. Results from other studies in the laboratory have led us to hypothesize that movement inhibition results from local loss of ATP (Rintoul et al., 2003; Reynolds et al., 2004; Malaiyandi et al., 2005). Although I do not know to what extent rotenone decreases ATP production in my primary neurons, this decrease could be enough as to impede mitochondrial movement in parallel with the changes in $\Delta\psi_m$ I observed. A decrease on mitochondrial movement could result in a disruption of ATP delivery to areas of high energy demand, such as synapses. Therefore, the long-term decrease on mitochondrial movement I have observed could contribute to rotenone toxicity. In fact, for the 50 nM rotenone treatment I observe a 15% decrease on neuronal viability after 48 h; however, 24 h later, after 72 h of 50 nM rotenone treatment, a 50% decrease in viability was observed. Therefore the increased toxicity after 72h rotenone treatment could be related to the prolonged decrease on mitochondrial movement I observed in the first 48 h of treatment.

Another consequence of 50 nM rotenone treatment is a reduction in mitochondrial length observed only after 48 h of treatment but not at earlier time points. Previous results from glutamate experiments led to the conclusion that morphological changes result from $[Ca^{2+}]_i$ generated by glutamate activation of NMDA receptors. In view of my results, it could be suggested that prolonged $\Delta\psi_m$ loss accompanied by decreased mitochondrial movement have consequences for the ionic homeostasis of the neuron that induce a reduction in mitochondrial length. Another explanation for the reduction in the measured mitochondrial length could be an

increase in mitochondrial fission. Frank *et al.* (2001) have recently reported that mitochondrial fission is associated with apoptotic cell injury and that prevention of mitochondrial fission reduced $\Delta\psi_m$ loss and release of cytochrome c.

One drawback from all our movement and length experiments is that the effects observed are restricted by our recording methodology. To ensure that all processes analyzed belong to the same neuron, all recorded fields included the cell body; however, mitochondria in the processes of transfected neurons can extend over 4 to 5 fields in distance far from the cell body. Therefore, I could be under representing the effects of rotenone by studying a mitochondrial subpopulation proximal to the cell body.

The results from my experiments of rotenone effects on mitochondrial function lead us to reach two conclusions. First, increasing doses of rotenone exert increasing levels of mitochondrial dysfunction. Rotenone at 5 nM induces a decrease on mtDNA replication but has no effect on $\Delta\psi_m$ or mitochondrial movement and morphology. Higher doses, like treatment of primary neurons with 50nM rotenone, have both a bioenergetic and a decreased mtDNA replication effect. Second, the bioenergetic effect of rotenone includes $\Delta\psi_m$ loss, reduction in mitochondrial movement and length. All these effects may contribute to the toxic effect observed after 72 h of 50 nM rotenone treatment. The mitochondrial effects observed in this study could be of relevance for the understanding of neurodegenerative diseases where exposure to even low levels of toxins could exist for prolonged periods of time.

6.0 DISCUSSION

This discussion focuses on mtDNA and its role in neurodegeneration. In the fist part, I describe various aspects of mtDNA biology, such as replication and turnover. I use the comparison between mtDNA in pre- and post-mitotic cells to place into perspective my results from several neurotoxins within the growing field of mtDNA biology. Second, I focus on the novel effect of rotenone on mtDNA replication I observed and its significance in the pathogenesis of PD. In an attempt to better understand all the effects of rotenone on mitochondrial function, the third part of this discussion describes other rotenone effects on $\Delta\psi_m$ and mitochondrial movement. This part describes the growing body of literature regarding the relevance of mitochondrial trafficking in neuronal function. The discussion finishes with an overall conclusion about the significance of my results in terms of both mtDNA biology and neurodegeneration.

6.1 mtDNA DYNAMICS IN POST- AND PRE- MITOTIC CELLS

Mitochondria and its DNA have been the focus of intense studies over the last two decades. While mtDNA biology has been studied in pre-mitotic and cancer cells, the fact that most pathogenic mtDNA mutations present as encephalocardiomyophathies makes the study of mtDNA dynamics in post-mitotic cells of special interest.

6.1.1 "A recipe for mitochondrial youth: divide daily" (Hofhaus et al., 2003).

Mitochondrial diseases are rare in tissues with high proliferation rates such as the bone marrow. In these tissues, deleterious mtDNA mutations are purged by selective elimination of cells with the less efficient mitochondria (Hofhaus et al., 2003). The blood of mtDNA disease patients eliminates cells carrying mtDNA deletions and point mutations over time (Lee et al., 1994). For example, large mtDNA deletions can cause a multisystem disease called Pearson's syndrome. Children with this syndrome are transfusion dependent; however, this dependency disappears spontaneously if the child survives other symptoms of the disease.

Several tissues with terminally differentiated cells, such as skin or small intestine, do not show accumulation of mtDNA mutations. Although these tissues are populated by post-mitotic cells, they show a very high cell replacement rate. In this case, cells harboring spontaneous pathogenic mtDNA mutations will simply be replaced by new ones as part of the regeneration process of these tissues. Therefore the study of mtDNA diseases has focused on tissues with terminally differentiated cells that are not replaced, and are highly energy dependent, such as muscle, heart, and brain, in which mtDNA pathogenic mutations are more apparent.

6.1.2 Hurdles in the study of mtDNA : discerning mtDNA from nDNA.

mtDNA constitutes only 0.5 to 1% of total cellular DNA, therefore, studies of mtDNA content or mtDNA replication require approaches than can circumvent its low abundance relative to nDNA.

Using imaging techniques

Mammalian mtDNA is about 17kb in size, which makes *in situ* visualization with common fluorescent DNA dyes such as ethidium bromide and 4',6'-diamidino-2-phenylindole (DAPI) difficult. These dyes show a weak, diffuse staining for mtDNA, and also bind to mtRNA which interferes considerably with the mtDNA signal (Dellinger and Geze, 2001; Garrido et al., 2003). The very intense fluorescence from nDNA staining can also hinder the observation of mtDNA localized near the nucleus. Recently, a fluorescent DNA-specific dye primarily used for

quantification of double-stranded DNA in solution, PicoGreen (Singer et al., 1997), has been adapted to visualize mtDNA in both live and fixed cells (Ashley et al., 2005). In my studies, I have used PicoGreen stain to detect mtDNA depletion in smooth muscle A10 cells as a positive control for the QPCR experiments (Figure 11). Experiments using PicoGreen in live neurons could bring further insight into mtDNA dynamics in situations such as neuronal development or neurotoxic scenarios. However, these experiments could be difficult to perform in mature neurons due to the high density of processes in these cultures.

Taking advantage of PCR amplification

Thanks to the development of the real-time quantitative PCR technique (QPCR), the study of mtDNA content can now be achieved in a highly sensitive manner. QPCR does not require large quantities of starting material; therefore mtDNA can be studied even at the single-cell level (Cantuti-Castelvetri et al., 2005). Two intrinsic features of both the eukaryotic cell and the PCR technique have facilitated an accurate determination of the number of mtDNA copies per cell. First, nDNA has a fixed number of gene copies throughout the somatic cell's life, while the number of mtDNA copies per cell can vary under different physiological and pathological situations. Therefore, any nDNA gene can be use as internal control to which mtDNA can be compared. Second, the PCR reaction is very specific for short DNA sequences, therefore plasmids encoding target sequences can be used to generate standard curves that allow for the precise determination of the number of mtDNA copies per cell. QPCR results from Legros *et al.* (2004) have confirmed the previous estimations of mtDNA molecules per cell: 1600-2000 copies/cell in primary fibroblasts and 2600-4100 copies/cell in immortalized cell lines. Moreover, the QPCR technique has allowed for faster and more precise detection of mtDNA depletion in response to NRTIs therapy (Cote et al., 2002).

Using thymidine analogs incorporation methods

Newly replicated mtDNA can be studied by labeling with either [^{3}H]thymidine or its analog BrdU. These approaches have the disadvantage that both nuclear DNA and mtDNA will incorporate [^{3}H]thymidine and BrdU. If quantification is required, [^{3}H]thymidine can only be used in quiescent pre-mitotic cells or in post-mitotic cells. In my study, I have quantified mtDNA replication in primary astrocytes as a model of pre-mitotic cells. Astrocytes were treated with

aphidicolin to inhibit nDNA replication (Figure 5). Interestingly, both astrocytes and neurons demonstrated similar incorporation kinetics in their mtDNAs, suggesting that mtDNA replicates at similar rates in these pre- and post-mitotic cells.

Another approach is to study cells deficient in the enzyme thymidine kinase 1 to avoid nuclear incorporation of BrdU or [^{3}H]thymidine (Davis and Clayton, 1996; Karbowski et al., 2001). The results described in Chapter 3 demonstrate that mtDNA dynamics can be succesfully studied in primary cortical neurons. Previously, primary central neurons were thought to replicate their mtDNA at an extremely low rate (Wang et al., 1997). However, my studies demonstrated active replication of mtDNA in cultured primary neurons. These reults provide new impetus to study the dynamics of mtDNA in the CNS in both developmental and disease states. However, one drawback of both the BrdU and [^{3}H]thymidine assays is that the mtDNA replication rate measured is only a relative one because this methodology does not allow for the quantification of total cellular mtDNA content. In other words, these techniques can not determine the percentage of the total mtDNA replicates in a given time period.

One approach to obtain a more precise measurement of the mtDNA replication rate would be the combination of both BrdU labeling and PicoGreen staining. BrdU would measure newly replicated mtDNA while total mtDNA would be measured by PicoGreen staining, leading to a more precise estimate of the extent of mtDNA replication. Decreased mtDNA replication not accompanied by a decrease in mtDNA content could be readily detectable by this method. One possible technical problem of BrdU/Pico Green experiments could come from the different sensitivity of the BrdU antibodies and the PicoGreen stain as suggested by Ashley *et al* (2005). However, careful titration of both reagents could overcome this problem.

6.1.3 Replication and dynamics of mtDNA nucleoids

Nucleoids have been described as the organizational structures of mtDNA within mitochondria. Since the discovery of mammalian nucleoids (Garrido et al., 2003), previous knowledge about mtDNA have to be reexamined. mtDNA molecules are neither free to diffuse in the

mitochondrial matrix nor independent from each other, but are arranged in organized structures. This brings several restrictions with respect to mtDNA distribution, replication and most importantly propagation.

Replication of mtDNA within nucleoids

Replication of mtDNA molecules within a single cell is important for the propagation of mutations that could affect the level of heteroplasmy, specifically in post-mitotic cells. In this regard, mtDNA nucleoids have been suggested to be the units of mtDNA inheritance. Studies in yeast have shown that the number of segregating units is similar to that of mtDNA nucleoids, and lower than the number of mtDNA molecules (Jacobs et al., 2000). This makes mtDNA nucleoids likely candidates for being the mtDNA segregating units. However, how replication takes place within a single mtDNA nucleoid has not yet been elucidated in either yeast or mammalian cells. Several models for mtDNA replication have been proposed (Jacobs et al., 2000; Iborra et al., 2004). It has been suggested that all mitochondrial genomes replicate independently (Bogenhagen and Clayton, 1977; Iborra et al., 2004). This would imply that some molecules could replicate more than once during a cell cycle.

As mentioned in the Chapter 1, Davis and Clayton proposed that mtDNA replication occurs in mitochondria localized near the nucleus with newly-replicated mtDNA subsequently transported to appropriate cellular locations. However, recent results with improved labeling techniques for BrdU have suggested that mtDNA replication occurs throughout the cell in several neuronal and non-neuronal cell lines (Garrido et al., 2003; Magnusson et al., 2003; Iborra et al., 2004; Legros et al., 2004). The higher fluorescence signal from the perinuclear area observed in all these studies could be explained by the higher number of mitochondria in this region. However, no study has yet demonstrated unequivocally that mtDNA replication occurs either in the perinuclear area or in the periphery. Neurons provide an ideal cell system to analyze the localization of mtDNA replication because their distinct shape allows for the differentiation between perinuclear area (the cell body) and the periphery (the processes). Similar to mtDNA replication, protein synthesis was thought to occur in ribosomes localized near the nucleus. According to this, once synthesized, mature proteins were transported in vesicles to the cell periphery. However, recent results have demonstrated that protein synthesis from specific

mRNAs can occur in the pre-synaptic terminals (Job and Eberwine, 2001). In these experiments, neuronal cell bodies were mechanically transected from their dendrites and protein synthesis was assessed 1 to 5 h after transection. Similar transection studies with BrdU in primary neurons would demonstrate if mtDNA replication could take place at mitochondria far away from the nucleus.

Dynamics of mtDNA nucleoids

mtDNA nucleoid distribution within the mitochondrial network is another process important for the propagation of mtDNA mutations and the heteroplasmy level. For example, the mechanism of mtDNA segregation before and during mitosis determines the genetic identity of daughter cells. However, information about how mtDNA is distributed in mammalian mitochondria during fission and fusion events is very limited.

In studies in pre-mitotic cells, Margineantu *et al.* (2002) have described different mitochondrial morphologies during the cell cycle. During the S phase, mitochondria appeared mostly fragmented; but recovered their reticular morphology as cells transitioned from mitosis to G1. *In situ* hybridization revealed that essentially all the fragmented mitochondria contained mtDNA molecules. Recently, nucleoids have been shown to move within the mitochondrial network and to distribute such that proper mtDNA distribution is ensured upon mitochondrial fission and fusion, with each mitochondrion having one or more nucleoids (Garrido et al., 2003). Interestingly, nucleoids seem to be uniformly spaced within a single mitochondrion suggesting that they are not free to diffuse in the mitochondrial matrix (Iborra et al., 2004). In yeast, nucleoids have been suggested to be tethered to the inner mitochondrial membrane by a yet to be described protein or complex of proteins (Chen and Butow, 2005). In fact, mtDNA nucleoids have lower motility than mitochondrial matrix proteins (Legros et al., 2004). Since nucleoids are frequently localized at or near the tips of mitochondria, it has been suggested that mitochondria divide at positions close to nucleoids as a mechanism to ensure proper mtDNA inheritance (Iborra et al., 2004; Legros et al., 2004). An association of nucleoids with the fission protein, Drp1, has been suggested but not demonstrated (Garrido et al., 2003). Further experiments to elucidate the association between Drp1 and nucleoids could reveal a very interesting relationship between the mitochondrial fission and replication machinery.

Alterations in mitochondrial distribution and morphology have been associated with various neurodegenerative diseases. Changes in mitochondrial fission and fusion could have a deleterious effect on nucleoid distribution in mitochondria within neurons. For example, excessive mitochondrial fission could result in mitochondria without nucleoids that would compromise the electron transport chain activity. Previous results from our laboratory have shown that glutamate induced significant remodeling of mitochondrial morphology accompanied by a reduction in mitochondrial movement (Rintoul et al., 2003). In Chapter 3, I studied mtDNA replication rate and distribution 3 h after a glutamate insult. However, no changes in either mtDNA replication or distribution were observed. Also, BrdU staining of primary neurons with rotenone treatments that induce a decrease on mtDNA replication did not show any aberrant distribution of the mtDNA (data not shown), albeit mitochondrial movement and length decrease. This normal mtDNA distribution under neurotoxin treatments could be explained by the low motility of the mitochondrial nucleoids.

6.1.4 mtDNA replication *vs.* mitochondrial turnover

When considering mtDNA content, two factors contribute to the number of mtDNA molecules in a cell at any given time: mtDNA replication rate and mitochondrial turnover. In principle, replication of mtDNA can occur independently of other mitochondrial functions since it requires its own set of enzymes and precursors. However, recent results from studies in yeast have suggested an intimate relationship between metabolic and mtDNA replication regulation (Chen et al., 2005).

In recent years, several studies have aimed to elucidate how mitochondria are degraded. It is known that damaged mitochondrial proteins are degraded by the ATP-dependent matrix enzyme, Lon protease (Bakala et al., 2003). However, a similar specific degradation pathway has not been described for the mtDNA, suggesting that mtDNA is either repaired or degraded with the entire organelle by autophagy (Terman and Brunk, 2004). Autophagy is a normal process that takes place in the cell in the absence of any external stress. However, in pre-mitotic cells, autophagy can be stimulated by nutrient deprivation and hormones released during fasting. Also,

in sympathetic neurons, autophagy is responsible for the removal of mitochondria in conditions of trophic factor withdrawal in the presence of caspase inhibitiors (Tolkovsky et al., 2002). Studies from Alzheimer's patients brains have suggested increased autophagy that led to accumulation of cellular debris (Hirai et al., 2001). Ultrastructural studies in human samples from Parkinson's disease have also localized autophagocytosed mitochondria in degenerating neurons (Chu et al., 2005).

According to the mitochondrial theory of aging (Kopsidas et al., 2000), ROS generated in the mitochondria can cause mtDNA mutations that progressively accumulate. These mtDNA mutations can impair the transcription of mtDNA encoded mRNA that could lead to a decrease in energy production and ultimately to cell death. In addition, de Grey (2002) proposed that mitochondria carrying mutated DNA are less susceptible to be degraded by autophagy since their membranes only minimally oxidized. Because of their mutated mtDNA, these mitochondria would have decreased respiration and ROS production. However, this hypothesis has yet to be confirmed. Moreover, it has not been proven that oxidative damage to mitochondrial membranes targets mitochondria for autophagy (Terman and Brunk, 2004).

My studies suggest that the observed effect of rotenone on mtDNA replication and content is due to a decrease in mtDNA replication and not to an increase in mitochondrial turnover. The reduction observed in mtDNA replication (50%) and content (40%) were similar which suggests that rotenone is decreasing mtDNA content by decreasing mtDNA replication. If rotenone would be decreasing the total mtDNA content by affecting mainly mitochondrial turnover, I would observe little or no change in the [^{3}H]thymidine incorporation experiments. However, further studies to elucidate changes in mitochondrial turnover in response to rotenone treatment will bring further insight into this hypothesis. These studies will be of special interest because as mentioned previously, the mitochondrial theory of aging proposes that one of the signs of mitochondrial aging is accumulation of damaged mitochondria due to decreased removal by autophagy.

6.2 NOVEL ROTENONE EFFECT ON mtDNA

6.2.1 Environmental toxins in the pathogenesis of PD

Several epidemiological studies have suggested that certain pesticides and environmental toxins could be implicated in PD pathogenesis (Seidler et al., 1996; Gorell et al., 1998). However, data coming from these studies are not very conclusive because they are taken from self-reporting of household or occupational exposures (Mayeux, 2003). In a study by Engel (2001), orchard workers in Washington State were questioned about their long-term exposures to insecticides, herbicides, and fungicides. Although a slight increase in risk for PD was observed in this population, no specific pesticide could be identified that may have been responsible. Nevertheless, when administered systemically, some of these pesticides, such as paraquat, maneb and rotenone, can reproduce specific features of PD in rodents (Takahashi et al., 1989; Brooks et al., 1999; Betarbet et al., 2000). The combination of two of these pesticides, maneb and paraquat, induced greater dopaminergic degeneration than either of them alone (Thiruchelvam et al., 2000), suggesting a synergistic effect of these toxins in the development of PD. Annonacin, the major acetogenin of the tropical plant *Anona muricata*, is a natural complex I inhibitor. Consumption of fruit and medicinal preparations of the leaves from this plant has been proposed to be the cause of an atypical form of PD in Guadalupe (Caparros-Lefebvre and Elbaz, 1999; Lannuzel et al., 2003). Recently, intravenous administration of annonacin to rats was shown to induce a significant loss of dopaminergic cells in the substantia nigra (Champy et al., 2004). In summary, epidemiological and experimental data suggest that environmental toxins and pesticides that target the mitochondrial respiratory chain might have a role in PD pathogenesis.

6.2.2 Complex I inhibition in PD

Most of the environmental toxins suggested to participate in PD pathogenesis act as complex I inhibitors, which implicates mitochondrial dysfunction and its subsequent oxidative stress as one mechanism responsible for this disease. Studies with PD brain and platelets have shown a moderate but consistent decrease in complex I activity (Schapira et al., 1989). Also, there have been reports demonstrating increased oxidative stress in the forms of GSH depletion and

oxidized DNA, lipids and proteins in the brains of PD patients (Dexter et al., 1989; Alam et al., 1997; Pearce et al., 1997; Floor and Wetzel, 1998). Because complex I is one of the main sites of ROS production, a decrease in complex I activity could explain the oxidative stress signs found in PD brains (Dexter et al., 1994). In the case of PD animal models, experimental data suggest that rotenone induces its toxicity through ROS production rather than other downstream effects of complex I inhibition, such as ATP depletion (Sherer et al., 2003b). However, conflicting reports have suggested that energy deprivation (through ATP depletion) in response to rotenone treatment is reponsible for its toxic effect in dopaminergic neurons and *in vivo* (Tieu et al., 2003; Kweon et al., 2004a; Moon et al., 2005). In these *in vivo* and *in vitro* studies, rotenone toxicity was partially reduced by treatment with the ketone body, D-β-hydroxybutyrate, to increase electron transport chain activity. Nevertheless, other PD animal models with complex I inhibitors such as MPTP and paraquat have also shown increased oxidative damage evidenced by increased lipid peroxidation (Rios and Tapia, 1987; Fukushima et al., 1995).

6.2.3 Are primary cortical cultures a good model for the study of PD?

Chronic infusion of rotenone has been used as an animal model of PD (Betarbet et al., 2000). This model is characterized by selective dopaminergic degeneration despite uniform inhibition of complex I throughout the brain. Animals infused with rotenone develop some of the behavioral symptoms characteristic of patients with PD, such as rigidity and resting tremor. However, the use of this animal model for the study of the molecular mechanisms implicated in the pathogenesis of PD has several disadvantages. First, only one third of treated animals develop striatal lesions, which points out the variability of the model (Sherer et al., 2003a; Zhu et al., 2004). Also, animal studies are limited in the number of techniques and treatments that can be implemented, and experiments do not clearly allow for the study of early events in PD pathogenesis.

As a more simplify method to study PD pathogenesis, several groups have studied the effects of rotenone in mesencephalic cultures (Gao et al., 2002; Moon et al., 2005). These studies have demonstrated a greater susceptibility of dopaminergic neurons to rotenone when compared

to non-dopaminergic neurons. However, these mesencephalic cultures have not been extensively used because (1) they are difficult to obtain, maintain, and genetically manipulate and (2) the low fraction of dopaminergic neurons within the culture requires their selection by a dopaminergic marker, such as tyrosine hydroxylase expression during or after the experiment. Most of the data elucidating the mechanism behind rotenone toxicity comes from experiments in neuronal cell lines with phenoptypes reminiscent of dopaminergic neurons, such as SK-N-MC or the MN9D cell lines (Sherer et al., 2003b; Kweon et al., 2004b). Although these cell lines have the advantage of being easy to study biochemically and easy to manipulate genetically, they do not always share with primary neurons their distinct shape and synaptically interconnected network, which imposes into mitochondria the requirement of properly distribute energy to support their synaptic activity. This is of special relevance when studying a mitochondrial toxin such as rotenone.

In my study, I have used primary cortical neurons to study the effects of glutamate and rotenone on mtDNA replication. Although these cultures are a mixed population of neurons, their maturity and connectivity are similar to those of primary dopaminergic neurons, allowing for the results obtained from these cultures to be readily translated into dopaminergic neurons. In the case of the study of mtDNA replication, primary cortical cultures have also the advantage of being post-mitotic which allows for the labeling of mtDNA replication without the requirement of drug treatments that will inhibit the nuclear DNA polymerases as it would be the case if using a neuronal cell line. Therefore, in my studies, I decided to use primary cortical cultures because the cellular environment of these neurons is the most similar to that of dopaminergic neurons regarding mtDNA and mitochondrial morphology which are the main targets of my study. Also, primary cortical neurons are easier and more consistently obtained than primary dopaminergic cultures, which facilitated the reliability of the data.

6.2.4 Mechanism of acute rotenone toxicity

Based on my experiments with primary cortical neurons, I suggest that the association between complex I inhibition and the toxicity exerted by rotenone is likely mediated by an increase in

ROS production. I have used rotenone doses (5-50 nM) in the same range as the free rotenone concentration in the brain calculated for the animal rotenone model (20-30 nM) (Betarbet et al., 2000). This concentration was determined to moderately inhibit complex I activity in isolated rat brain mitochondria (Davey and Clark, 1996). In my experiments in cortical neurons, these rotenone doses induced a time and concentration dependent toxic effect. Moreover, experiments with other complex I inhibitors such as pyridaben and piericidin A clearly demonstrated the toxic effect of complex I inhibition in neurons, therefore suggesting a link between complex I activity and neuronal survival. The protective effect of the antioxidants vitamin E and coenzyme Q against rotenone toxicity observed by Sherer *et al.* (2003b) lead to the conclusion that ROS overproduction plays a major role in rotenone toxicity. My results are consistent with other studies where treatment with either vitamin E or coenzyme Q protected the SK-N-MC cell line and organotypic substantia nigra cultures from rotenone toxicity (Sherer et al., 2003b; Testa et al., 2005). Accordingly, my results show that neurons treated with vitamin E can be protected from rotenone toxicity over a 72 h period. Both pyridaben and piericidin A have been suggested to be more potent than rotenone in their complex I inhibition (Degli Esposti, 1998). Also, both inhibitors induced larger toxic effects than rotenone in my experiments. However, the increase in ROS production in response to these inhibitors has not been studied in primary neurons. It would be interesting to study if the most toxic of all three complex I inhibitors, piericidin A, is also the one inducing the largest increase in ROS production. In summary, my results support the hypothesis that rotenone's acute toxicity is mediated to some extent by an increase in ROS production due to complex I inhibition.

6.2.5 mtDNA and oxidative damage

As described above, ROS generation at complex I has been linked to PD pathogenesis in both *in vivo* and *in vitro* studies (Schapira et al., 1989; Sherer et al., 2003b). Several studies have demonstrated mtDNA damage in response to oxidative stress. The fact that increased ROS can damage mtDNA and subsequently decrease the activity of the electron transport chain is of special relevance for neuronal survival since neurons are highly dependent on oxidative phosphorylation for their ATP production. Neurons have two mechanisms to maintain redox homeostasis: (1) high levels of antioxidants, such as SOD and GSH and (2) efficient repair of

oxidative mtDNA damage to maintain a low mutation rate in their mtDNA (Harrison et al., 2005). Few studies have aimed to elucidate the level of mtDNA damage in response to oxidative stress in neurons or brain. Harrison and colleages (2005) compared the response of primary cerebellar neurons and astrocytes to similar oxidative stress levels. Neurons were shown to have higher levels of antioxidants, such as GSH, when compared to astrocytes. In spite of this, mtDNA in neurons showed a higher degree of oxidative damage accompanied by lower mtDNA repair rates than astrocytes. The authors attributed this increase in mtDNA oxidative damage to a decrease in Pol γ expression and activity. As described in the introduction, Pol γ participates in the BER pathway; therefore, a decrease in its activity could have a deleterious effect not only for mtDNA replication but also for its repair of oxidative damage.

Other *in vivo* studies have examined the effect of caloric restriction (a treatment known to increase resistance to oxidative stress) on both the rate of ROS production and the level of oxidative damage to mtDNA (Stuart et al., 2004; Sanz et al., 2005). Both studies reported decreased levels of oxidized mtDNA in response to reduced ROS production induced by the caloric restriction treatment. Also, Schriner *et al.* (2005) have shown that in mice over expressing the antioxidant enzyme catalase in mitochondria the level of mtDNA deletions in skeletal muscle and heart was significantly lower than wild-type animals. Therefore, there is some evidence in the literature to support the hypothesis that an increase in ROS production can lead to a decrease in mtDNA replication and content.

These studies led me to suggest that oxidative damage induced by rotenone could be responsible for decreasing mtDNA replication. Experiments in which I have induced oxidative stress by direct application of H_2O_2 did not show a correlation between increased ROS and mtDNA replication. Furthermore, rotenone significantly decreases mtDNA replication not only at high (50 nM) but also at very low (5 nM) concentrations. However, we know from studies in isolated brain mitochondria that 20 nM rotenone doses induce only a minimal increase in the release of H_2O_2, and that at 50 nM rotenone the Vm of ROS generation is only half-maximal, although electron transport chain activity is reduced by 50% and 90%, respectively (Votyakova and Reynolds, 2001). Even if my studies were performed in neuronal cultures and results obtained from isolated brain mitochondria are not readily translated in my system, it could be

presumed that at 5 nM rotenone treatment the ROS production increase in only minimal. This would not be the presumption for the 50 nM rotenone dose, in which ROS production probably increases significantly. This is further supported by my experiments in which vitamin E significantly reduced 50nM rotenone-induced toxicity. The fact that I observed decreased mtDNA replication in the absence (5 nM rotenone) and presence (50 nM rotenone) of significant oxidative damage suggest that another mechanism may be implicated in the rotenone effect on mtDNA replication. However, I have not directly measured ROS production in response to rotenone in my neuronal cultures; therefore, future experiments studying ROS production in response to my rotenone treatments could bring further insight into these questions. Also, I have observed only a partial protective effect of vitamin E on rotenone induced toxicity. Because vitamin E is not a very strong oxidant scavenger, I cannot exclude the possibility that decreased mtDNA replication could be caused by very low levels of oxidative damage. Further studies with this rotenone paradigm should investigate oxidative damage to mtDNA and to Pol γ.

6.2.6 mtDNTPs in the regulation of mtDNA replication

The replication and maintenance of mtDNA requires not only a large number of nuclear enconded enzymes but also balanced nucleotides pools. One important feature of Pol γ is its high fidelity in mtDNA replication (Kunkel and Mosbaugh, 1989; Johnson and Johnson, 2001; Longley et al., 2001). This accuracy in replication is due in part to the high nucleotide selectivity of its active site. However, this process depends on adequate dNTP concentrations, because the probability that Pol γ will insert the incorrect nucleotide depends to some extent on the ratio correct/ incorrect dNTP. Therefore, changes in the relative and/or the absolute concentrations of the dNTP can reduce Pol γ replication fidelity (Kunz et al., 1994). In fact, dNTP pool assymetries in heart mitochondria have been shown to reduce the fidelity of Pol γ (Song et al., 2005).

As mentioned in Chapter 1, mtdNTP pool imbalances have been shown to contribute to the pathogenesis of several human diseases. For example, mutations in the thymidine phosphorylase gene cause a neurogastrointestinal encephalomyopathy syndrome mainly characterized by mtDNA depletion. In this syndrome, the mitochondrial accumulation of

thymidine disrupts the mitochondrial dTTP pool, which affects Pol γ activity and leads to mtDNA depletion. Also mtdNTP pools can be affected by treatment with nucleoside analogs, such as those used for HIV therapy, which also leads to mtDNA depletion.

In my experiments, I studied the possibility that rotenone could be decreasing mtDNA content by reducing the mtdNTPs available for mtDNA synthesis. Using rotenone treatments that reduced mtDNA replication, I studied a possible mtdNTP imbalance by assessing the incorporation of [^{3}H]thymidine into dTTP. I only observed decreased dTTP levels in response to 50 nM but not to 5 nM rotenone. As shown in Chapter 5, this 50 nM treatment not only induces decreased mtDNA replication but also reduces $\Delta\psi_m$, mitochondrial movement and morphology. Rotenone 5 nM, which also induces a decrease on mtDNA, did not reduce dTTP. Therefore, I concluded that the mechanism by which rotenone decreases mtDNA does not necessarily involve a decrease in mtdNTPs. To explain dTTP reduced levels observed in response to 50 nM rotenone, one has to consider that protein import into the mitochondria is $\Delta\psi_m$ dependent. The decrease in dTTP levels observed in response to 50 nM rotenone could be due to decreased import of the enzymes implicated in the salvaging pathway that controls the synthesis of mtdNTPs in post-mitotic cells. Also, it is not known if import of mtdNTPs into mitochondrial matrix depends on $\Delta\psi_m$. Further studies examining the level of the enzymes implicated in the metabolism of mtdNTPs will test this hypothesis.

6.2.7 Novel effect of rotenone effect on mtDNA

In this study, I have observed a significant decrease in mtDNA replication and content upon rotenone treatment. These effects on mtDNA replication and content were time and concentration dependent. Additionally, decreased mtDNA replication and content were detected in response to treatments that induced minimal neuronal toxicity, which suggests that the effect of rotenone on mtDNA may not be related to its acute toxicity. As mentioned previously, the doses used here (5-50nM) were in a similar range to those used in the PD animal model (5-20nM) (Betarbet et al., 2000). In my experiments, the fact that rotenone also reduced mtDNA content in another cell type, the A10 smooth muscle cell line, suggests that the effects of rotenone on mtDNA are not restricted to neuronal cell types. However, it is important to note

that the consequences of reduced mtDNA content could have more deleterious effects in neurons due to their oxidative phosphorylation dependency. To gain further insight into the rotenone effect, it would be interesting to know the time course for the observed decreases in mtDNA replication versus mtDNA content.

Is rotenone effect on mtDNA replication irreversible?

Experiments with 24 h rotenone treatments showed a decrease in mtDNA replication that persists for up to 48h following rotenone withdrawal, despite the decreased toxicity during this period. These results further support the notion that rotenone effects on mtDNA replication do not correlate with its acute toxicity. One conclusion from these experiments is that rotenone effect on mtDNA replication is irreversible in the 48 h following rotenone withdrawal. Very little is known about the reversibility of complex I inhibition in cells. Most of the experiments aimed to characterize rotenone kinetics of complex I inhibition have been performed in cuvette-based experiments or complex I membrane-bound particles that do not allow for withdrawal of rotenone. Binding experiments have demonstrated a non-competitive binding mode of rotenone to complex I. However, how these kinetics affect the overall function of the electron transport chain in cells has not been thoroughly studied. Experiments by Barrientos *et al.* (1999) showed that rotenone treatment of the osteosacoma-derived cell line 143B with 10nM rotenone induces a 60% decrease on complex I activity whereas the inhibition of overall mitochondrial respiration is reduced only by 35%. In experiments from chapter 5, I have shown that the effect of 50nM rotenone for 5 min on $\Delta\psi_m$ is readily reversible upon rotenone withdrawal. Also experiments in single isolated mitochondria with rotenone doses between 5-50nM induce a reversible $\Delta\psi_m$ depolarization (Dr. Vergun, personal communication). According to Barrientos *et al.* (1999) data, in my experiments, 50nM rotenone treatment for 24h should significantly inhibit complex I and to a lesser degree mitochondrial respiration. After withdrawal of rotenone, mitochondrial respiration could return to its normal levels, due to the proper functioning of the other three complexes in the electron transport chain. This would support neuronal viability as seen after 48 h rotenone withdrawal. Therefore, rotenone effect on neuronal toxicity can be considered reversible while its effect on mtDNA replication is long-lasting and persists even 48 h after rotenone withdrawal. One possibility to explain this observation is that rotenone could be inhibiting one or more of the regulatory pathways of mtDNA replication. The transcription

factors TFAM, mtSSB1, and 2 are not only essential for mtDNA replication but also components of mtDNA nucleoids (Garrido et al., 2003). The expression of these mitochondrial transcription factors is regulated by nuclear transcription factors, such as the Nuclear Respiration Factor 1 (NRF-1) and NRF-2, which are also implicated in the regulation of genes from other metabolic pathways (Scarpulla, 2006). NRF-1 and 2 are expressed in response to changes in the cellular energy demands through peroxisome proliferators activator receptors (PPARs) activation. Based on their structure and supported by experimental data, synthetic ligands of PPARs have been suggested to inhibit complex I in the electron transport chain in a manner similar to that of rotenone (Jove et al., 2004; Scatena et al., 2004). Conversely, rotenone, at the low doses used in my study, could interact with PPARs inhibiting the transcription of TFAM and mtSSB1, and therefore, reducing the rate of mtDNA replication over periods of time of 48h. As metioned previously, TFAM, mtSSB1, and 2 are structural components of the mitochondrial nucleoids. An additional mechanism by which rotenone could affect mtDNA replication without inducing an acute toxic effect would be by disrupting the structure of nucleoids in a manner that would impede Pol γ activity. This effect could be detected in the first 24h of rotenone treatment and could persist in the absence of rotenone because it would require the assembly of new nucleoids before mtDNA replication could resume.

In future experiments, it would be important to study if the mtDNA replication defect reverses during longer periods of rotenone withdrawal. Because the effect of rotenone on mtDNA replication also induces a decrease on mtDNA content, it is conceivable that the recovery of normal mtDNA levels would take a significant amount of time since it would require the synthesis of new mtDNA molecules starting from an already reduced mtDNA pool. If, indeed, mtDNA replication defects in response to short rotenone exposures do not return to normal levels even after longer washout periods; then it is likely that function of the electron transport chain would be affected by the lack of subunits for 3 out of its 4 complexes. This could severely compromise neuronal survival. If all these speculations are true, then it is tempting to suggest that the consequences of short term exposures to environmental toxins, such as rotenone, have more deleterious and long-lasting effects than previously thought.

Does rotenone affect Pol γ activity?

Results from my experiments strongly suggest that the mtDNA decrease observed in response to rotenone is not a consequence of complex I inhibition *per se*, increased ROS production, or decreased levels of mtdNTP pools. Other mechanisms that have been implicated in the control of mtDNA replication and its content are mitochondrial transcription factors, such as TFAM (Larsson et al., 1998), the helicase Twinkle (Tyynismaa et al., 2004), and Pol γ (Hance et al., 2005). Unfortunately, there is no data suggesting a relationship between these factors and rotenone. Pol γ consists of two subunits, a catalytic subunit that is constitutively expressed at low levels and an accessory subunit which expression in *Drosophila* is regulated by the transcription factor DREF, that also regulates other genes such as mtSSB (Kaguni, 2004). The accessory subunit has been shown to increase the catalytic and processivity activities of the enzyme complex (Lim et al., 1999). Perhaps, rotenone could be interfering with the association of both subunits. This would translate in an immediate reduction in mtDNA replication, since the catalytic subunit can replicate mtDNA in the absence of the accessory subunit albeit at a much slower pace. Also, this effect could be long-lasting requiring more than 48h after rotenone withdrawal for the enzymatic complexes to regain their normal activity. Experiments aim to elucidate the activity of the Pol γ complex in the presence of rotenone would help to elucidate this hypothesis.

Another hypothetic mechanism to explain the effect of rotenone on mtDNA would be an increase in mtDNA mutations, especially in the control region of mtDNA. One recent report has suggested that increased mutations in the D-loop region of mtDNA impair binding of transcription factors and Pol γ (Coskun et al., 2004). The most common cause for mtDNA mutations are oxidative lesions in response to increased ROS levels. Recent experiments in the colon cancer cell line HTC116 have demonstrated an increase in mtDNA oxidation in response to 300 nM rotenone treatment for 24 h (Achanta et al., 2005). Although in my experiments, I could not establish a direct relationship between ROS production and decreased mtDNA replication. As mentioned previously, vitamin E is not a very strong oxidant scavenger; therefore, there could be very low levels ROS production, but enough to induce oxidative damage to mtDNA. Future experiments utilizing a newly developed QPCR approach to detect

mtDNA mutations (Van Houten et al., 2005) could study the mtDNA mutation rate in the control region as a possible mechanism for the novel effect of rotenone in mtDNA. Because mtDNA mutations have been shown to impair Pol γ from binding to mtDNA, this effect would immediately decrease mtDNA replication and content. Also, this effect could lead to mtDNA depletion by restricting the polymerizing activity of Pol γ.

Consequences of mtDNA replication and content decrease

My results suggest that long term rotenone treatments could lead to a prolonged reduction in mtDNA replication that could ultimately lead to mtDNA depletion. One of the consequences of the reduction in mtDNA content is a reduction in the mtDNA encoded transcripts and proteins. Based on my experiments, it is not clear whether the mtDNA decrease observed leads to reduced expression of mitochondrial proteins. If mtDNA encoded proteins decrease, then the electron transport chain activity may decrease due to improper synthesis and assembly of subunits within its complexes. Such a reduction would lead to a drop in $\Delta\psi_m$, as was observed in response to oxidative stress (Santos et al., 2003). Although I have observed a significant decrease in $\Delta\psi_m$ in response to 50 nM rotenone treatment, there were no effects on $\Delta\psi_m$ with other doses (5 nM) that also decreased mtDNA replication and content. Because the degree of decreased mtDNA replication and content was similar with both rotenone doses, my results do not lead us to conclude that the reduced $\Delta\psi_m$ observed is a consequence of mtDNA reduction. However, the reduction in $\Delta\psi_m$ as a consequence of decreased mtDNA content could be an effect that would become apparent after longer treatments when complexes present at the beginning of rotenone treatment would be degraded. A better approach to elucidate if rotenone, through its effect on mtDNA, is inducing a decrease in the synthesis of mtDNA peptides would be to label with ^{35}S-methionine newly synthesized peptides in the mitochondrial matrix. These experiments can be performed by inhibiting nuclear protein synthesis with specific inhibitors that bind to the nuclear 40S ribosomal subunit, such as emetine, while leaving mitochondrial protein synthesis intact (Takeuchi and Ueda, 2003). Because mtDNA also encodes for the ribosomal RNAs required to synthesize all peptides, one would expect a decrease in all 13 peptides encoded by mtDNA rather than in a specific peptide.

6.2.8 Study of mtDNA replication in degenerative diseases

Evidence for the role of mtDNA mutations in normal aging and in neurodegenerative diseases comes from both autopsy and animal studies. As mentioned previously, mice carrying a Pol γ defective in proof-reading showed an accelerated aging phenotype (Trifunovic et al., 2004; Kujoth et al., 2005). There is also another study of the relationship between mtDNA depletion, electron transport chain activity and neurodegeneration. In this report from Sorensen (2001b), *MI*tochondrial *L*ate-*O*nset *N*eurodegeneration (MILON) mice were generated by conditionally knocking-out TFAM in hippocampal and cortical neurons. Because *Tfam* knock-outs are embryonic lethal, *Tfam* expression was disrupted in the MILON mice starting at postnatal day 14. These mice developed normally until 5-6 months of age. At this point, their physical condition deteriorated rapidly with the mice dying within 1-2 weeks. Interestingly, these mice had a significant decrease in mtDNA copy number and mtRNA levels accompanied by a decrease in all complexes that required mtDNA encoded proteins. The most interesting feature of these mice was that they showed no sign of neurodegeneration at 4 months of age, despite their significant decrease in mitochondrial function. However, a rapid and profound neurodegeneration took place in the following month leading to neuronal apoptosis and the death of these mice. Another important observation from this report is the time lag between mitochondrial dysfunction and neuronal death. This time lag has also been observed in patients with mitochondrial neurodegenerative syndromes (Oldfors et al., 1995). To explain this observation in patients, the authors suggested that the mtDNA heteroplasmy levels could increase with age. Results from the MILON mice and from the Pol γ mutant mice strongly suggest a role for mtDNA depletion and mtDNA mutations in normal aging that could affect the progression of neurodegenerative diseases, such as PD and AD.

In fact, aging in the brain has been associated with decreased electron transport chain activity (Bowling et al., 1993). Also, aging is associated with an increasing decline in the number of dopaminergic neurons of the substantia nigra (Fearnley and Lees, 1991; Ma et al., 1999). In postmortem human substantia nigra, both neurons and glia have high aggregate levels of mtDNA mutations (Cantuti-Castelvetri et al., 2005). Although the level of each mtDNA mutation is not high, the combined aggregate burden of all the mutations in a single cell is significant. In terms

of mtDNA mutations in neurodegenerative diseases, brains from AD patients have recently been shown to have both increased levels of mtDNA mutations and a decrease in the ratio mtDNA/ nDNA (Coskun et al., 2004). Although mtDNA mutations have been proposed to be responsible for mitochondrial dysfunction in PD, there is no evidence suggesting that these mtDNA mutations lead to a decrease in mtDNA content as has been demonstrated for AD patients. Therefore, studies in postmortem brains from PD patients would help to elucidate if, indeed, there is a decrease in mtDNA content in this disease. However, if mtDNA decrease is an early event in the pathogenesis of PD, as my data suggest, neurons with decreased mtDNA content may be the first ones to degenerate and die and therefore would not be detectable in postmortem tissues. Studies aimed to establish the course of events in the development of mitochondrial dysfunction will be useful to more clearly elucidate the role of mtDNA mutations in the pathogenesis of PD.

6.2.9 Does rotenone's effect on mtDNA replication contribute to its toxicity *in vivo*?

In this study, I have demonstrated that low doses of rotenone induce a decrease on mtDNA replication in the absence of overt toxicity. Also, I have demonstrated that this decrease on mtDNA replication is accompanied by a decrease on mtDNA content. As mentioned above, a prolongued decrease on mtDNA replication could ultimately lead to mtDNA depletion. My experiments were performed in primary cortical cultures for the reasons explained perivously. One disadvantage of these cultures is that they do not support long-term studies; therefore, we have not been able to study the effect of rotenone on mtDNA replication at times longer than 72h. Experiments to prove the hypothesis that long term rotenone treatment causes mtDNA depletion should be performed in systems suitable for these long term experiments, such as an animal model or organotypic cultures.

Because rotenone effect on mtDNA replication takes place at lower doses and shorter times than rotenone-induced acute toxicity, it could be suggested that mtDNA replication decrease is an early-event in the pathogenesis of the rotenone PD model. Results from animal studies treated with rotenone have suggested increased oxidative stress as responsible for its toxicity (Sherer et al., 2003b). However, these data comes from animals sacrified after they

develop PD symptoms and not at early stages of the disease. To this date, no study has attempted the study of mtDNA content on this animal model. The study of mtDNA content in the substantia nigra and other brain regions from rotenone treated animals would help to corroborate the effect on mtDNA replication I have observed with *in vitro* experiments. One drawback from the rotenone animal model is its variability in the response to the subcutaneous treatment (Sherer et al., 2003a; Zhu et al., 2004). An alternative method to study the molecular mechanisms implicated in the progressive neurodegeneration observed in PD could be organotypic cultures. These cultures consist on slices of substantia nigra and surrounding tissue (Testa et al., 2005) or the full nigrostriatal pathway (substantia nigra, striatum and cortex) (Kress and Reynolds, 2005). Organotypic cultures have two advantages: (1) they allow for long-term studies since they survive up to 60 days in vitro (Plenz and Kitai, 1998) and (2) they allow for time-course studies aim to elucidate early events in the pathogenesis of PD. Their main drawback is that they require technical expertise with organotypic cultures to maintain a reliable supply.

What role plays mtDNA replication and content decrease in dopaminergic neuronal toxicity *in vivo* could be inferred from results in the MILON mice (Sorensen et al., 2001a). As mentioned previously, these animals have reduced levels of mtDNA due to the lack of TFAM. The reduction of mtDNA in the hippocampus and cortex not only leads to neurodegeneration but also makes neurons more susceptible to other injuries. In the case of rotenone, exposure to low levels of this toxin that induce an irreversible decrease on mtDNA content would translate into a reduction of the mitochondrial electron transport chain activity. This reduction would render neurons less able to cope with the high energy demants for their normal functioning and would predispose them to other kinds of neuronal injuries. As for the MILON mice, rotenone-induced mtDNA decrease could grow unnoticed for some time with neurons not showing signs of toxicity. Then a rapid and profound degeneration could take place leading to neuronal death and the appearance of PD symptoms. In fact, this proposed mechanism would be reminiscent of the progression of PD observed in patients, with PD symptoms presenting with 60% of the dopaminergic neurons have already disappeared (Dauer and Przedborski, 2003). Although rotenone exposure may or may not be implicated in a fraction of sporadic cases of PD, a slow but progressive decrease of mtDNA could be implicated in the neurodegenerative process that takes place in PD patients.

6.3 EFFECTS OF ROTENONE ON MITCHONDRIAL FUNCTION

6.3.1 Relationship between mtDNA reduction and mitochondrial functions

Because mtDNA encodes 13 peptides of the electron transport chain, its maintenance and proper translation are essential for the production of ATP. Complete depletion of mtDNA, as seen in rho0 cells, leads to decreased $\Delta\psi_m$, and induces changes in mitochondrial morphology (Garcia et al., 2000; Gilkerson et al., 2000). Experiments from Chapter 3 show decreased mtDNA upon rotenone treatment. This mtDNA decrease should be followed by decreased synthesis of mtDNA encoded proteins of the electron transport chain that would ultimately lead to a drop in $\Delta\psi_m$. As mention previously, decreased $\Delta\psi_m$ not only has consequences for the energy production functions of the mitochondria but also for other functions, such as protein import that could further compromise mitochondria's ability to produce ATP. Results from Chapter 5 have shown a reduction in $\Delta\psi_m$ upon 50 nM rotenone treatment for 48 h. However, treatment with 5 nM rotenone for 72 h did not significantly decrease $\Delta\psi_m$, yet both treatments induce similar reductions in mtDNA content. Therefore, my results suggest that the $\Delta\psi_m$ effect observed is due to complex I inhibition by rotenone and not to a decrease in mtDNA content. Interestingly, results from experiments with an inducible dominant negative Pol γ show a parallel decrease in mtDNA content and cytochrome oxidase subunit 2 protein that was accompanied by a decrease in $\Delta\psi_m$ after 10 days (Jazayeri et al., 2003). Also, results from Santos J. *et al.* (2003) suggested an intimate link between $\Delta\psi_m$ and the presence of mtDNA lesions because only cells with low $\Delta\psi_m$ also showed mtDNA damage. Further studies to investigate the time course mtDNA, protein and $\Delta\psi_m$ decrease will bring further insight into the relationship between mtDNA content and other mitochondrial functions.

6.3.2 Mitochondrial trafficking

6.3.2.1 Why do mitochondria move in neurons?

Neurons are elongated cells with multiple compartments, such as dendrites and axons, which can extend far from the cell body. Due to their distinct shape, synaptic transmission and plasticity functions occur at distant sub-cellular domains that require proper energy delivery for efficient

function. Energy delivery to these sub-cellular domains cannot be supported by the slow rate of ATP diffusion (Blerkom, 1991). Also, mitochondria participate in other cellular functions such as buffering of cytosolic calcium that also require the presence of these organelles at the synapses. Therefore, in neurons, mitochondria are found in areas of high metabolic demand, such as synapses and active growth cones (Hollenbeck and Saxton, 2005). The regulation of mitochondrial trafficking is not only important for the normal physiological conditions but also in neurodegenerative diseases in which proper mitochondrial function become essential for neuronal survival. In fact, alterations in mitochondrial distribution and morphology have been associated with various neurodegenerative diseases, such as AD (Ebneth et al., 1998), HD (Trushina et al., 2004), and stroke (Rintoul et al., 2003).

6.3.2.2 How do mitochondria move in neurons?

In recent years, several studies have established that mitochondria movement is saltatory and bidirectional (Hollenbeck and Saxton, 2005). Mitochondria moving along the axon have been shown to switch from anterograde to retrograde movement (Morris and Hollenbeck, 1993). Compared to other organelles that have a fast and smooth movement, individual mitochondria are characterized by their "movement-less" periods or stationary time (Hollenbeck and Saxton, 2005). Physiological and intracellular signals regulate mitochondrial movement and their stationary time. For example, young cortical neurons have shorter and more mobile mitochondria, which has been suggested to allow for the transient energy distribution required during synaptogenesis (Chang, 2005). Conversely, mitochondria in synaptically mature neurons are longer and less mobile (Chang, 2005).

The most accepted model for mitochondrial movement is that mitochondria move along microtubules with actin filaments acting as modulators that control the rate of movement along microtubules and the tethering of mitochondrial to specific regions. As mentioned in Chapter 1, mitochondria use motor proteins for their bidirectional movement along the microtubules, with anterograde organelle transport being mediated by proteins of the kinesin superfamily (isoforms 1 and 5) and retrograde transport by the dynein family (Hollenbeck, 1996). Recent studies have started to elucidate the intracellular signaling that regulates these motor proteins. For example,

cyclin-dependent kinase 5 inhibition and glycogen synthase kinase 3 activation have been shown to induce kinesin-1 detachment from the mitochondrial surface (Ratner et al., 1998; Morfini et al., 2002).

In terms of trafficking, mitochondria have been proposed to be assembled in the cell body and then be delivered to the appropriate neuronal location. Interestingly, in a recent study in guinea pig myenteric neurites, mitochondrial transport was not affected by other structures such as varicosities or stationary mitochondria that moving mitochondria encounter in their travel along the axons (Berghe et al., 2004). Not only are mitochondria delivered to distant compartments of the cells, but they are also retrieved from the axon. One hypothesis is that this retrograde transport serves to remove mitochondria that will undergo autophagy. One study supporting this hypothesis has described that mitochondrial movement along the axon is linked to $\Delta\psi_m$, with ~90% of high $\Delta\psi_m$ mitochondria moving towards the growth cone and ~80% of the low $\Delta\psi_m$ ones moving towards the cell body (Miller and Sheetz, 2004).

Recent studies have begun to elucidate the intracellular signals that govern mitochondrial trafficking. For example, local stimulation with nerve growth factor (NGF) has been shown to initiate a signaling cascade that results in mitochondria clustering adjacent to the stimulus (Chada and Hollenbeck, 2004). Therefore, NGF is the first factor to be described as a "docking" signal for mitochondria. Through the phosphorylation and subsequent actin modification in response to NGF, mitochondria have been suggested to dislodge from microtubules tracts to establish a more static interaction with actin (Reynolds and Rintoul, 2004). Other studies have suggested that synaptic activity and intracellular Ca^{2+} signalling also modulate mitochondrial movement. In myenteric neurites, mitochondrial trafficking was blocked by TTX and inhibited by depletion of intracellular Ca^{2+} stores but not by removal of extracellular Ca^{2+} (Berghe et al., 2004). Also, results from our laboratory have demonstrated that the level of synaptic activity modulates mitochondrial trafficking patterns in axons and dendrites differently (Chang, 2005). In fact, during periods of synaptic overactivity, mitochondria were actively recruited to post-synaptic sites in dendrites, while in axons the opposite was true. In summary, it has become apparent that mitochondrial movement and trafficking are highly regulated processes that contribute to the overall neuronal function.

6.3.3 Neuronal injury and mitochondrial movement

Many neurodegenerative diseases such as ALS, HD, and AD have been linked to defects in either axonal transport in general or to disabled mitochondrial function and transport. In models of AD, overexpression of tau protein (Ebneth et al., 1998) or mutations in the presinilin protein (Pigino et al., 2003) impair mitochondrial transport to peripheral cell compartments. Recent studies with a cybrid line carrying mtDNA from AD patients have shown reduced mitochondrial movement accompanied by a lower fraction of moving mitochondria (Trimmer and Borland, 2005). The pathogenesis of HD has also been associated with defects in mitochondrial trafficking in motor neurons (Piccioni et al., 2002; Trushina et al., 2004). In these studies, aggregates of polyglutamine tract-containing proteins confounded mitochondrial axonal trafficking likely due to disruptions of cytoskeletal interactions. Results from our laboratory have recently demonstrated that over expression of mutant huntingtin protein can create a physical "roadblock" in neuronal processes, which could prevent mitochondria from reaching cellular regions where they are needed (Chang et al., 2005).

In our laboratory, we have extensively studied how mitochondrial movement is altered upon different neurotoxic scenarios in cortical neurons (Rintoul et al., 2003; Malaiyandi et al., 2005; Rintoul et al., 2006). Neurotoxic doses of glutamate, intracellular [Zn^{2+}] elevations, and NO at pathophysiological concentrations halt mitochondrial movement. However, the mechanisms by which these agents inhibit mitochondrial movement seem to be somewhat different. For example, glutamate and NO decrease mitochondrial movement at concentrations that also dissipate $\Delta\psi_m$. Moreover, FCCP reproduces the effect of glutamate on mitochondrial movement. Therefore, our results suggest that energized mitochondria are essential to drive mitochondrial movement. Interestingly, elevation of intracellular [Zn^{2+}] exerts its effect on mitochondrial movement by initiating a signaling cascade that requires PI3K activity and does not involve $\Delta\psi_m$ depolarization (Malaiyandi et al., 2005). All these results were obtained by short-term treatment (10-30 min) of cortical neurons. My experiments described in Chapter 5 are the first ones to describe a mitochondrial movement decrease that lasts for more than 24 h. As for experiments with glutamate and NO, rotenone treatments that induce $\Delta\psi_m$ depolarization also decrease mitochondrial movement. These results suggest that mitochondrial movement can be

reduced for long periods of time. As mentioned previously, this reduction in mitochondrial movement could be related to the neuronal toxicity observed later following rotenone treatment. It is important to note, that although mitochondria have been implicated in the pathogenesis of PD, my study is the first attempt to study the consequences of rotenone in a PD model in mitochondrial trafficking. In a recent report, rotenone has been shown to induce microtubule depolarization that impairs dopamine vesicle transport (Ren et al., 2005). Therefore, this cytoskeletal effect of rotenone could also be implicated in the reduction of mitochondrial movement I observed. Interestingly, it was proposed that the degeneration of dopaminergic neurons starts at the processes, with the cell body the last part in the neuron to degenerate (Testa et al., 2005). This hypothesis could be explained by the lack of proper energy delivery to the processes due to reduced mitochondrial movement. Further studies on mitochondrial movement in other models of PD will confirm this hypothesis.

6.3.4 Neuronal injury and mitochondrial morphology

Mitochondrial morphology is regulated by the rate of fission and fusion. Both events are essential for the maintenance of the mitochondrial network, which allows for optimal mitochondrial function. However, the mechanism of mitochondrial fission and fusion are relatively unknown pathways, and their implication in normal and pathological conditions have just started to be elucidated.

Mitochondrial fusion proteins linked to diseases in humans are OPA1 and Mfn2. Mutations in OPA1 cause autosomic dominant optic atrophy (ADOA). ADOA is the most prevalent inherited optic neuropathy. In this neuropathy, the patient progressively loses vision due to degeneration of retinal ganglion cells (Delettre et al., 2002). Although the pathological mechanism leading to loss of retinal ganglion cells due to OPA1 mutations is not very well established, it is interesting to point out that ADOA shares some pathophysiological symptoms with another optic neuropathy, LHON (Okamaoto et al., 2003). This mitochondrial disease is characterized by mtDNA mutations in complex I genes that reduce mitochondrial ATP generation (Carelli et al., 2004). The common symptomatology of these otherwise unrelated pathologies suggests that both mtDNA mutations and mitochondrial fission defects share a

common outcome, the depletion of ATP that leads to retinal ganglion cell degeneration. Also, mutations in the MFN2 gene have been linked to another peripheral neuropathy, Charcot-Marie-Tooth neuropathy type 2A (CMT2A). Mutations in the MFN2 gene were predicted to impair the GTPase activity due in part to impairment of Mfn2 mitochondrial localization (Rojo et al., 2002). Experiments in cell culture have demonstrated that mutations in the GTPase domain of Mfn2 affect its normal functioning. However, it has yet to be confirmed if the same is true for this neuropathy.

As mentioned previously, Drp-1 and Fis-1 are implicated in mitochondrial fission in mammalian cells. The current hypothesis is that Fis1 recruits Drp-1 to mitochondrial foci of fission. However, the signal transduction pathways that initiate mitochondrial fission have not yet been elucidated. Drp-1 has been shown to translocate from the outer mitochondrial membrane in response to Ca^{2+} release from the endoplasmic reticulum, suggesting that Ca^{2+} could be the second messenger implicated in mitochondrial fission. Further support for this hypothesis comes from experiments in which calcium ionophore A23187 treatment induced mitochondrial fission in myoblasts and astrocytes (Duncan et al., 1980). Also, Drp-1 has been implicated in apoptotic mitochondrial fission (Frank et al., 2001), because it translocates from the cytosol to defined foci on the mitochondrial outer membrane at the onset of apoptosis. Importantly, dominant-negative Drp-1 blocks staurosporine-induced mitochondrial fission, loss of $\Delta\psi_m$ and cytochrome c release and cell death (Frank et al., 2001).

The study of mitochondrial morphology in neurodegenerative diseases has not been the focus of as many studies as mitochondrial trafficking. Results from rho0 cell lines that were repopulated with mtDNA from PD and AD patients suggest abnormal mitochondrial morphologies such as swelling and disruption of the cristae in these cells when compared to cybrid lines from control subjects (Trimmer et al., 2000). However, the authors used $\Delta\psi_m$-sensitive dyes to assess mitochondrial morphology. If mutations in the mtDNA used to create the cybrid lines induce a decrease in mitochondrial function that is accompanied by a $\Delta\psi_m$ reduction, these would have an effect on the signal obtained from the dye, which could confound the morphology results. In addition, these experiments were performed the human neuroblastoma SH-SY5Y cell line that is susceptible to be differentiated into a neuronal phenotype. However,

the experiments were performed in undifferentiated cells which do not allow for good mitochondrial morphology evaluation. Because the mitochondrial network is not as spread out in undifferentiated cells as it is in neurons, the measurement of mitochondrial morphology in not as accurate as in differentiated neuronal cell lines or primary neurons.

Previous studies from our laboratory have demonstrated changes in mitochondrial morphology under two circumstances: glutamate and calcium ionophore treatments (Rintoul et al., 2003). In both cases, mitochondria responded by decreasing its length. Because in the absence of extracellular Ca^{2+}, glutamate did not reduce mitochondrial length, we concluded that an elevation in intracellular Ca^{2+} is implicated in the change of mitochondrial morphology. In experiments presented in Chapter 5, I have shown that rotenone treatments that decreased $\Delta\psi_m$ and movement also decreased mitochondrial length after 48 h treatment. However, it is not clear if the decrease in $\Delta\psi_m$ and movement is related to changes in mitochondrial length. Because mitochondrial depolarization could impair mitochondrial Ca^{2+} uptake throughout the neuron, it is tempting to speculate that the disruption of Ca^{2+} due to decreased $\Delta\psi_m$ have a deleterious effect on mitochondrial morphology. However, preliminary data has shown no striking differences in mitochondrial Ca^{2+} handling in rotenone treated and untreated neurons (data not shown). Since Drp-1 has been suggested to respond to changes in intracellular Ca^{2+} (Breckenridge et al., 2003), there is the possibility that long term treatment with rotenone induces subtle changes in intracellular Ca^{2+} that promote the translocation of Drp-1 to the outer mitochondrial membrane where it mediates mitochondrial fission. Further studies examining the localization of the Drp1 protein in rotenone treated cells could bring further insight into the remodeling process of mitochondria observed in rotenone treated cells.

Another possibility to explain my results concerns mitochondrial fission and its participation in apoptotic cell death. As mentioned previously, the mitochondrial network has been shown to adopt a fragmented morphology in response to apoptotic stimuli, such as staurosporine (Frank et al., 2001). The pro-apoptotic factros BAK and BAX have been shown to associate in large foci at the outer mitochondrial membrane at early stages of apoptosis (Youle and Karbowski, 2005). Also, loss of $\Delta\psi_m$ induced by several mitochondrial inhibitors, including rotenone, has been shown to induce the translocation of Bax to mitochondria (Smaili et al.,

2001). Although this data comes from experiments in a non-neuronal cell line, it is possible that a sustained decrease on $\Delta\psi_m$, as the one observed with 50nM rotenone, would induce the translocation of Bax to the outher mitochondrial membrane to initiate mitochondrial fission leading to apoptosis. However, if this is true in my experiments; the translocation of Bax would only take place after more than 24 h of decreased $\Delta\psi_m$. As shown in Chapter 3, neurons treated for 24 h with 50nM rotenone followed by 48 h of rotenone withdrawal survived more than cells treated for 72 h with rotenone, suggesting that apoptosis is not initiated in the first 24 h of rotenone treatment. Therefore, it might be suggested that prolongued inhibition of mitochondrial respiration by rotenone first induces a decrease on $\Delta\psi_m$ accompanied by a reduction in movement that ultimately leads to apoptosis. The apoptotic process would be mediated by translocation of Bax to the outer mitochondrial membrane, followed by mitochondrial fission and release of cytochrome c.

6.4 CONCLUDING REMARKS

6.4.1 Neurons, mitochondria and mtDNA

Thanks to the development of techniques to visualize mitochondria in living cells, mitochondrial morphology in response to physiological and pathological stresses has started to be examined. However, the study of mammalian mtDNA in living cells has proven to be more difficult. The adaptation of nucleic acid stains for visualization of mtDNA in living cells (Ashley et al., 2005), together with a better understanding of nucleoid structure, has opened the possibility to study mitochondrial movement, morphology, and mtDNA dynamics within a single cell. Given the limited information about how mtDNA is distributed in mammalian mitochondria during fission and fusion events, the study of mtDNA distribution within mitochondria is of special interest to determine the inheritance of mtDNA to daughter cells. A better understanding of mtDNA distribution within mitochondria will help to answer questions such as why the mtDNA depletion induced by NRTI treatment is not uniform in all cells in the affected tissue, resulting in a mosaic pattern of mitochondrial dysfunction.

In the last several years, it has become apparent that many processes that take place in mitochondria are intimately related. Studies in yeast have recently revealed a link between energy metabolism regulation and mtDNA replication (Chen et al., 2005). Also, studies from our laboratory have demonstrated the need of $\Delta\psi_m$ for mitochondrial movement (Rintoul et al., 2003). Results from this dissertation and previous results from our laboratory have shown decreased mitochondrial movement and length upon exposure to several neurotoxins. Although I have not observed changes on mtDNA distribution, the question remains as how do reduced mitochondrial movement and length affect mtDNA metabolism in terms not only of replication but also of transcription and translation of the genome. There is the possibility that reduced movement could affect the distribution of proteins in the electron transport chain within the mitochondria, affecting the overall ATP production. These questions are even more relevant in the context of mitochondria in neurons. As mentioned previously, several mtDNA diseases result in selective degeneration of neuronal cell types, albeit the presence of mtDNA mutations in all other cell types (Schon and Manfredi, 2003). Why certain mtDNA mutations only compromise the survival of retinal ganglion cells remains unknown. The elucidation of how mtDNA dynamics interact with other mitochondrial functions in the specific environment of neuron could bring new light to the understanding of common neurodegenerative diseases. An interdisciplinary approach that combines basic studies in lower organisms with results from animal models of neurodegenerative diseases could help to unveil the complexity of these organelles and their role in neuronal function.

6.4.2 Multiple actions of rotenone in mitochondrial function

Results from this dissertation demonstrate multiple actions of rotenone in mitochondrial function (Figure 21). Rotenone effects on mitochondria could be classified by their relationship to complex I. Rotenone effect on mtDNA replication would be complex I-independent, while its acute toxicity, increased ROS production, mitochondrial depolarization, and decreased mitochondrial movement and length could be considered complex I-dependent.

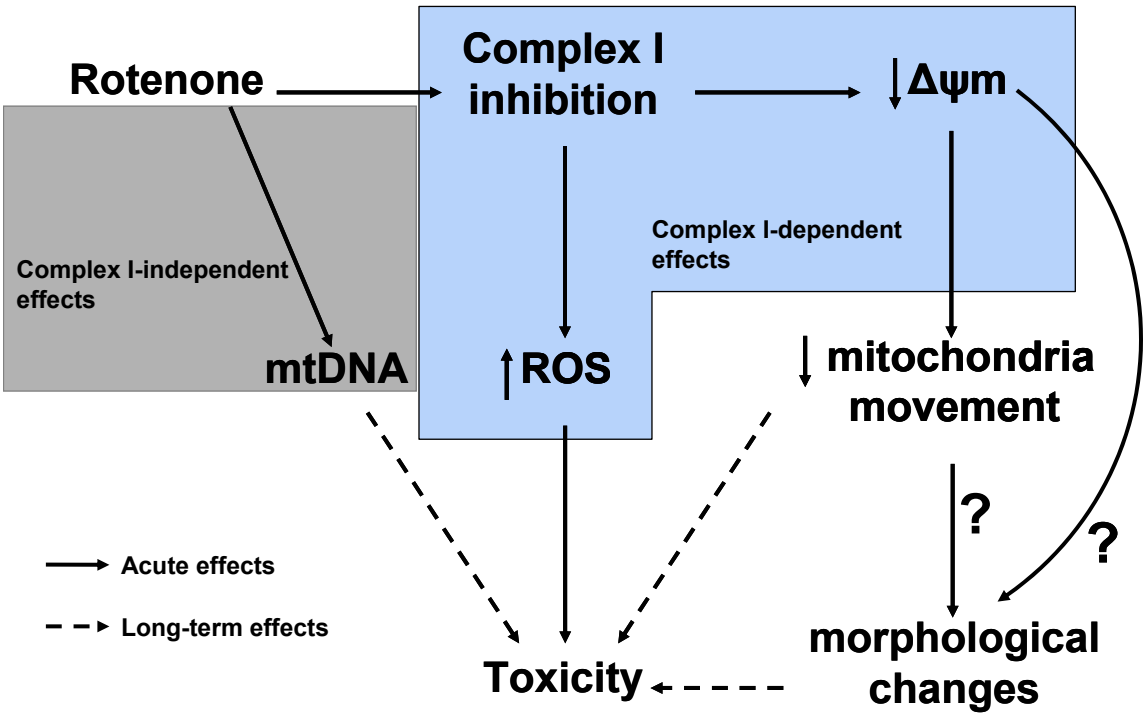

Figure 21. Diagram of rotenone effects on mitochondria

I have demonstrated that rotenone effect on mtDNA replication is not mediated by any of the following mechanisms: (1) complex I inhibition, (2) increased ROS production and (3) decreased mtdNTP pools. Although my results cannot pinpoint the mechanism by which rotenone exerts its mtDNA replication and content decrease, I have demonstrated that this effect is independent from its acute toxicity and appears to be long-lasting. Also, this effect takes place at lower doses than the complex I-dependent effects. Therefore, this rotenone-induced mtDNA replication decrease could play an important role in its long-term effects because neurons could accumulate mitochondria with decreased mtDNA content in the absence of overt toxicity.

With respect to the complex I-dependent effects of rotenone, my experiments with other complex I inhibitors reinforce the link between inhibition of complex I and neuronal toxicity. Also, experiments from this dissertation together with previous experiments from our laboratory strongly suggest the $\Delta\psi_m$ requirement for mitochondrial movement. However, how complex I inhibition, perhaps through decreased $\Delta\psi_m$ or decreased mitochondrial movement, lead to a decreased in mitochondrial length is still a matter of speculation.

An interesting question that arises from my results is how the different effects of rotenone interact with each other in the context of neurons. I have demonstrated that low doses of rotenone (5nM) induce a complex I-independent effect on mtDNA. However, it is not clear if prolonged exposure to low doses of rotenone, through mtDNA decrease and reduced electron transport chain expression could lead to decreased $\Delta\psi_m$ that would affect mitochondrial movement and morphology. Another consequence from decreased electron transport chain expression is a reduction in ROS levels (as seen in the MILON mice), which could be positive for neuronal survival. However, it is not clear if under circumstances of decreased complex I expression, low doses of rotenone could inhibit complex I to a degree that would trigger the complex I-dependent effects of rotenone.

In summary, my results indicate that increasing doses of rotenone exert increasing levels of mitochondrial dysfunction. Future studies of the effects of rotenone on mtDNA in systems that support long-term studies, such as organotypic cultures and the rotenone animal model, could help to elucidate the relationship of all the effects of rotenone on mitochondrial function and their contribution to neuronal degeneration.

6.4.3 mtDNA, rotenone, and neurodegeneration

In this study, I have demonstrated that rotenone, the environmental toxin implicated in the pathogenesis of PD, induces a decrease in mtDNA replication in the absence of overt neuronal toxicity. Also, this decrease on mtDNA replication persisted even after rotenone withdrawal, which suggests that short-time exposures to rotenone could have more deleterious effects than previously thought. MPP^+, another complex I inhibitor related to PD pathogenesis has also been shown to decrease mtDNA replication in a complex I-independent manner (Miyako et al., 1999). The relationship between mtDNA and other environmental toxins implicated in PD, such as maneb or paraquat, remains to be clarified. In summary, decreased mtDNA content could be implicated in the long term effects of rotenone, which could be relevant for occupational exposures to this pesticide.

Although mtDNA was discovered 40 years ago, it is only now that the role mtDNA plays in common neurodegenerative diseases is becoming apparent. Mutations in mtDNA were suggested to participate in neurodegeneration. However, the aggregate burden of mtDNA mutations, particularly point mutations, was challenging to demonstrate because the rate of each individual mtDNA mutation in a given cell is very low (Lin et al., 2002). The implication of mtDNA mutations in aging phenotypes and the observation in AD patients of increased mtDNA mutations accompanied by a decrease on mtDNA content support an essential role for mtDNA in normal and pathological neurodegeneration processes. Because the degeneration observed in common neurodegenerative diseases goes on for decades, it is difficult to replicate these processes *in vitro* or in animal model studies. Animal models developed to study common neurodegenerative diseases rely on mutations associated with familial cases of these pathologies or on toxins linked to that particular disease. However, most studies are performed in adult animals. Therefore, the rate of mtDNA mutation accumulation and the mitochondrial function observed in these models are those of an adult, but not old, animal. The combination of accelerated-phenotypes with established animal models of neurodegenerative diseases could reveal the extent of mtDNA implication in these degeneration processes.

Because the effects on mtDNA replication observed with low doses of rotenone occur in the absence of acute toxicity, neurons with decreased mtDNA could accumulate for long periods of time. Therefore, rotenone-induced mtDNA decrease could be an early event in the dopaminergic degeneration observed in the rotenone animal model of PD. Several questions remain unknown. First, it is not clear what are the consequences of this level of mtDNA decrease for the overall mitochondrial function. Also, a decrease in mtDNA and its subsequent mitochondrial dysfunction could increase neuronal vulnerability to otherwise non-injurious stresses. One unresolved question of the rotenone model is why only the dopaminergic neurons are the most affected by this toxin when complex I inhibition is uniform throughout the brain. It could be that early events in the pathogenesis of this model, as the proposed mtDNA decrease, could increase the susceptibility of specifically these dopaminergic neurons to other external stresses. Finally, postmortem studies in PD brains to elucidate the presence of mtDNA decrease would further demonstrate the implication of mtDNA in PD pathogenesis.

Neurons depend on mitochondria for most of their ATP generation. This crucial ATP generation and its distribution throughout the neurons require the coordination of other mitochondrial functions as diverse as mtDNA metabolism or fission and fusion events. As part of the inexorable process of neurodegeneration, one or more of these mitochondrial processes are affected in a manner that can compromise neuronal survival. Therefore, the study of the role of mitochondria in neurodegeneration can bring a better understanding of the mechanisms behind common neurodegenerative diseases. From this understanding, new strategies could be developed for the prevention and treatment of these devastating diseases.

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