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NEUROTOXICITY OF METHYLENEDIOXYMETHAMPHETAMINE (MDMA; "ECSTASY") ON RAT BRAIN MITOCHONDRIA

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“Para ser grande, sê inteiro: nada
teu exagera ou exclui.

Sê todo em cada coisa. Põe quanto és
no mínimo que fazes.

Assim em cada lago a lua toda
brilha, porque alta vive.”

Ricardo Reis

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O Decreto de Lei 288/70, art. 8, § 2,refere:

" É admitido na elaboração da dissertação o aproveitamento, total ou parcial, do resultado de trabalhos já publicados, mesmo em colaboração, devendo, neste caso, o candidato esclarecer qual a sua contribuição pessoal"

Neste sentido, integram esta dissertação resultados publicados nos trabalhos seguidamente enumerados, para os quais contribuiu pessoalmente, preparando todo o material experimental, procedendo às determinações analíticas, organização e análise dos resultados e redacção do texto, em colaboração com os restantes autores.

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Abstract

Repeated administration of 3,4 methylenedioxymethamphetamine (MDMA; “ecstasy”) results in long-lasting decreases of serotonin (5-HT) levels in most serotonergic nerve terminals. This effect may result in permanent neurological deficits, like sleep disorders, depressed mood, persistent elevation of anxiety, impulsiveness and hostility, and selective impairment of episodic memory, working memory and attention. Although several factors may contribute to MDMA-induced neurotoxicity, namely MDMA metabolism, sustained receptor stimulation, hyperthermia, enzymatic and non-enzymatic oxidation of neurotransmitters, inhibition of neurotransmitters synthesis, inflammation, and oxidative stress, the relative contribution of these factors for the toxicological outcome is still to be clarified.

Despite MDMA associated neurotoxicity is well documented, the majority of the scientific approaches are performed mainly in adult animal models, using chronic or short-term acute exposure. However, the greatest percentage of consumers are adolescents whose nervous system is still under development and may be more susceptible to neurotoxic insults. This reality renders important the use of an appropriate animal model that closely simulates what happens in humans. The present work aims to contribute for a better understanding of the consequences of MDMA exposure in adolescents, in what concerns to their brain mitochondrial integrity and function.

In the present dissertation we have tested the following main hypothesis: A possible contribution for the long-lasting neurotoxicity of MDMA on the serotonergic system may result from the fact that MDMA uses the 5-HT transporter (SERT) to enter the nerve terminals and release the vesicular 5-HT into the cell cytoplasm by reverting the functioning of the vesicular monoamine transporters (VMAT). Part of the released serotonin is metabolized by monoamine oxidase (MAO), with the consequent production of (hydrogen peroxide) H_2O_2. Since MAO is bounded to the external membrane of mitochondria, H_2O_2 may diffuse into this organelle and lead to formation of other reactive oxygen species (ROS) that could attack mitochondrial lipids, proteins and DNA. To test the formulated hypothesis, an adolescent animal model of exposure to MDMA was used. Adolescent male Wistar rats, aged 45 days, were divided in three series of four experimental groups. MDMA group of animals was administered four doses of 10 mg/Kg intraperitoneally (i.p.), two hours apart of each other and controls were given isovolumetric doses of saline. The contribution of MAO-B for MDMA-induced damage was assessed by including two experimental groups that consisted of the MAO-B selective inhibitior selegiline, plus MDMA (2mg/Kg of selegiline i.p. 30 min before exposure to MDMA) and selegiline (2mg/Kg i.p) administered animals. The

contribution of MAO-A for MDMA-induced damage was accessed by including two groups consisting of the selective MAO-A inhibitor clorgyline, plus MDMA (1mg/Kg of clorgyline i.p. 30 min before exposure to MDMA) and clorgyline (1mg/Kg i.p.). The putative protective effect of the drug acetyl-L-carnitine (ALC) against MDMA-induced damage was also tested. For this purpose, additional groups of animals treated with ALC plus MDMA (100mg/Kg ALC i.p. 30 min before exposure to MDMA) and ALC (100mg/Kg i.p.) were used.

One of the most prominent acute physiological effects of MDMA is the elevation of body temperature. Rat's body temperature was monitored every 15 minutes on the day of injections for a period of nine hours and then every day until the day of sacrifice (PND 59). Special attention was given to mitochondria and the biochemical evaluations were made either by the analysis of isolated whole brain mitochondria or dissection of specific brain areas. Whole brain mitochondria were analysed for lipid peroxidation, protein carbonylation and western blot analysis of subunit II of NADH dehydrogenase (NDII) and subunit I of cytochrome c oxidase (COXI). Dissected brain areas were analysed to detect a mitochondrial DNA (mtDNA) deletion comprising subunit I of NADH dehydrogenase (NDI), NDII and COXI mitochondrial genes by polymerase chain reaction (PCR). Specific brain slices were stained with 2,3,5-triphenyltetrazolium chloride (TTC) in order to determine the mitochondrial metabolic activity of tissues.

The results obtained in this dissertation confirmed the raised hypothesis, since the exposure of adolescent rats to a binge neurotoxic dose of MDMA resulted, in fact, in alterations at the brain mitochondrial level that could be prevented at a great extent by either MAO-B inhibition or ALC supplementation. In particular, the following alterations were observed:

- MDMA administration induced increased oxidation of lipids and proteins of brain mitochondria, observable at long-term after exposure. This effect was extensively prevented by MAO-B but not by MAO-A inhibition. ALC supplementation prevented this effect only at the level of protein oxidation.
- MDMA administration induced a deletion of the mitochondrial genome in a region that encompasses NDI, NDII and COXI mitochondrial genes and decreased the expression of the correspondent protein subunits NDII and COXI in several areas of the rat brain. MAO-B inhibition prevented this effect. However, MAO-A inhibition decreased even more the expression of COXI. ALC supplementation was able to diminish the effects but its protective activity is less pronounced than that observed with MAO-B inhibition. This was the first time that the deletion of mitochondrial genes, induced by MDMA, were described.
- MDMA administration induced dual effects in the mitochondrial metabolic performance, as evaluated by the triphenyltetrazolium cholride (TTC) assay, depending

on the studied brain regions. Brain regions like caudate putamens (CPU), nucleus accumbens (NAccb), ventral tegmental area (VTA), hippocampus (hip) and cerebellum (cereb) had its mitochondrial metabolism diminished while others, namely substancia nigra (SN) and raphe nuclei (RN), presented increased metabolic activity. MAO-B inhibition afforded protection against this effect in the majority of the brain regions analysed. The effect of ALC supplementation was less clear. In some of the regions, namely CPU, Naccb and cereb a protective effect was achieved, but in other (VTA, RN, SN and hip), an increased metabolic dysfunction was observed.

- MDMA is capable of inducing a hyperthermic response on the day of exposure and abnormal thermoregulation thereafter. This effect is not prevented by MAO inhibition or ALC supplementation;

MDMA administration was followed by a loss of body weight gain throughout the period of experiment. MAO-B inhibition was not able to significantly prevent. ALC administration, conversely, aggravated this effect, although in a non significant way.

In conclusion, MDMA-induced neurotoxic effects are associated with oxidative damage to brain mitochondria and these effects are dependent on MAO-B activity. MAO-A is also related with MDMA inflicted damage, but, its inhibition is not able to prevent the toxic effects. ALC administration prevented a great part of MDMA-induced damage, emphasising the central role played by ROS in the overall process and evidencing the importance of antioxidant neuroprotection for the prevention of the MDMA neurotoxic effects.

Resumo

A administração repetida de 3,4 metilenedioximetamfetamina (MDMA, “ecstasy”) resulta em decréscimos prolongados dos níveis de serotonina (5-HT) na maioria dos terminais serotonergicos. Este efeito pode resultar em deficits neurológicos permanentes, tais como alterações dos padrões de sono, alterações na fala, níveis de ansiedade elevados e persistentes, impulsividade, hostilidade e descoordenação selectiva da memória episódica, memória de trabalho e atenção. Embora vários factores possam contribuir para a toxicidade induzida pela MDMA, nomeadamente o seu metabolismo, a estimulação prolongada dos receptores, a hipertermia, a oxidação enzimática e não enzimática de neurotransmissores, a inibição da síntese de neurotransmissores, a ocorrência de processos inflamatórios e stress oxidativo, a contribuição relativa destes factores para os efeitos toxicológicos carece de clarificação.

Apesar da neurotoxicidade associada à MDMA estar bem documentada, a maioria dos estudos científicos são levados a cabo em modelos animais adultos, usando exposições crónicas ou curtas e agudas. Contudo, parte relevante dos consumidores encontra-se na adolescência, altura em que o sistema nervoso continua em desenvolvimento, podendo, desta forma, estar mais susceptivel a insultos neurotóxicos. Esta realidade torna importante o uso de um modelo animal apropriado que simule proximamente aquilo que se passa nos humanos. O trabalho aqui apresentado pretende contribuir para uma melhor compreensão das consequências da exposição de adolescentes a MDMA no que diz respeito à função e integridade das mitocondrias cerebrais.

Na presente dissertação foi testada a seguinte hipótese: Uma possível contribuição para a neurotoxicidade a longo prazo da MDMA no sistema serotonérgico pode ser explicada pelo facto da MDMA usar o transportador de 5-HT (SERT) para entrar nos terminais nervosos e induzir a libertação da 5-HT vesicular no citoplasma por reversão do funcionamento dos transportadores vesiculares de monoaminas (VMAT). Parte da serotonina libertada é metabolizada pela monoamina oxidase (MAO), com a consequente formação de peroxido de hidrogénio (H_2O_2). Uma vez que a MAO está ancorada à membrana externa da mitocondria, o H_2O_2 pode difundir para o interior deste organelo e originar outras espécies reactivas de oxigénio (ROS), as quais podem atacar lipidos, proteinas e DNA mitocondrial. Para testar a hipótese formulada usou-se um modelo animal adolescente de exposição a MDMA. Ratos Wistar adolescentes, com 45 dias, foram distribuidos por 3 séries de quatro grupos experimentais. Ao grupo de animais tratados com MDMA foram administradas intraperitonealmente (i.p.) quatro doses de 10mg/Kg em intervalos de duas horas, aos

animais controlo foram administradas doses isovolumétricas de solução salina. A contribuição da MAO-B para os danos induzidos pela MDMA foi estudada através da inclusão de mais dois grupos experimentais: um grupo previamente tratado com selegilina (um inibidor selectivo da MAO-B), numa dose de 2mg/Kg de selegilina administrados i.p., 30 min antes da exposição à MDMA e um grupo de animais tratados apenas com selegilina (2mg/Kg i.p.). A contribuição da MAO-A para os danos induzidos pela MDMA foi explorada através do uso de um inibidor selectivo da MAO-A, clorigilina, administrado também previamente numa dose de 1mg/Kg de clorigilina i.p., 30 minutos antes da exposição à MDMA. O possível efeito protector da acetil-L-carnitina (ALC) contra os danos induzidos pela MDMA foi também testado. Para este propósito, grupos adicionais de animais tratados com ALC e MDMA (100mg/Kg ALC i.p. 30 min antes da exposição à MDMA) e ALC (100mg/Kg i.p.) foram usados.

Um dos mais proeminentes efeitos fisiológicos da exposição aguda a MDMA é o aumento da temperatura corporal. A temperatura corporal dos ratos foi monitorizada a cada 15 minutos no dia de exposição, por um período de nove horas, e depois diáriamente até ao dia do sacrificio (PND 59). Foi dada especial atenção à mitocondria e as avaliações bioquimícas foram feitas por análise das mitocondrias isoladas do cérebro total ou por dissecção de áreas cerebrais específicas. As mitocondrias do cérebro total foram analisadas para determinação da peroxidação lipidica, carbonilação proteica e análise da subunidade II da NADH desidrogenase (NDII) e da subunidade I da citocromo c oxidase (COXI) por western blot. As áreas cerebrais dissecadas foram analisadas para detecção de uma delecção no DNA mitocondrial (DNAmt) que compreende os genes mitocondriais correspondentes à subunidade I da NADH desidrogenase (NDI), NDII e COXI por reacção de polimerase em cadeia (PCR).

A coloração de fatias epecíficas do cérebro com cloreto de 2,3,5-trifeniltetrazólio (TTC) foi feita para determinar a actividade metabolica mitocondrial dos tecidos.

Os resultados obtidos nesta dissertação confirmaram a hipótese formulada, uma vez que a exposição de ratos adolescentes a uma dose neurotoxica de MDMA resultou, de facto, em alterações a nivel das mitocondrias do cérebro sendo que este efeito foi prevenido em grande extensão tanto pela inibição da MAO-B como por administração de ALC. Em particular, as seguintes alterações foram observadas:

- A administração de MDMA provocou, a longo prazo, um aumento da oxidação de lipidos e proteinas nas mitocondrias cerebrais. Este efeito foi extensivamente prevenido pela inibiçao da MAO-B mas não da MAO-A. O suplemento de ALC preveniu este efeito a nível da oxidação proteica.
- A administração da MDMA induziu uma delecção no genoma mitocondrial numa região que compreende os genes mitocondriais NDI, NDII e COXI e levou à diminuição

da expressão das subunidades proteicas correspondentes NDII e COXI em diversas áreas do cérebro de rato. A inibição da MAO-B preveniu este efeito. Contudo, a inibição pela MAO-A acentuou o decrécsimo na expressão de COXI. A administração de ALC foi capaz de diminuir os efeitos mas a sua actividade protectora é menos pronunciada do que aquela que foi observada pela inibição da MAO-B. Esta foi a primeira vez que se decreveram delecções de genes mitocondriais provocados pela exposição a MDMA.

- A administração de MDMA induziu um efeito bilateral na performance metabolica mitocondrial que, de acordo com os ensaios realizados com o cloreto de trifeniltetrazólio (TTC), sofreu variações dependendo das regiões cerebrais estudadas. Áreas cerebrais como o caudado putamens, núcleo acumbens, area tegmental ventral, hipocampo e cerebelo revelaram um metabolismo mitocondrial diminuido, enquanto que outras, nomeadamente a substancia negra e o núcleo da rafe, apresentaram um aumento na actividade metabólica. A inibição da MAO-B conferiu protecção contra o efeito na maioria das regiões analisadas. A administração de ALC foi menos clara. Em algumas regiões, nomeadamente no caudado putamens, nucleo acumbens e cerebelo, alcançou-se um efeito protector, mas noutras (area tegmental ventral, nucleo da rafe, substancia negra e hipocampo) foi observado um aumento da disfunção metabólica..

-A MDMA é capaz de induzir uma resposta hipertérmica no dia de exposição e termoregulação anormal nos dias subsequentes. Este efeito não é prevenido por inibição da MAO-B nem por administração de ALC;

A administração de MDMA resultou numa diminuição do ganho de peso corporal ao longo do período das experiências. A inibição da MAO-B não foi capaz de prevenir este efeito de forma significativa. A administração de ALC, de forma oposta, agravou o efeito, embora de forma não significativa.

Em conclusão, os efeitos neurotóxicos induzidos pela MDMA estão associados a danos oxidativos nas mitocondrias do cérebro e estes efeitos são dependentes da actividade da MAO-B. A MAO-A também está relacionada com o dano induzido pela MDMA, mas, de forma contrária, a sua inibição não é capaz de previnir os efeitos tóxicos indicando, desta forma, uma acção específica da droga nas estruturas ricas em MAO-B (terminais serotonergicos) e descartando a inibição da MAO-A como uma estratégia protectora. A actividade preventiva da ALC sobre grande parte do dano induzido pela MDMA dá enfase ao papel central das espécies reactivas de oxigénio no processo global da toxicidade induzida por esta droga e evidencia a importância da neuroprotecção antioxidante para a prevenção dos danos oxidativos observados a longo prazo depois de uma administração aguda de MDMA.

Abbreviations list

AD	Alzheimer disease
ADH	Aldehyde dehydrogenase
ALC	Acetyl-L-carnitine
ALS	Amyotrophic lateral sclerosis
bp	Base pairs
Cereb	Cerebellum
CNS	Central Nervous System
COXI	Subunit I of mitochondrial compex IV (cytochrome c oxidase)
CPU	Caudate putamens
DA	Dopamine
DNPH	Dinitrophenylhydrazine
DOPAC	3,4-Dihydroxy-phenylacetic acid
eNOS	endothelial nitric oxide synthase
ETC	Electron Transport Chain
FCx	Frontal cortex
FFA	Free fatty acid
GABA	Gamma-aminobutyric acid
GSH	Reduced gutathione
GPx	Glutathione Peroxidase
5-HIAA	5-Hydroxyindole acetic acid
5-HIAL	5-Hydroxyindole acetaldehyde
Hip	Hippocampus
HSP-70	Heat shock protein-70
5-HT	Serotonin
iNOS	Inducible nitric oxide synthase
$HO^{\cdot}$	Hydroxil radical
H_2O_2	Hydrogen peroxide
8-OHdG	8-Hydroxy-2'-deoxyguanosine
i.p.	Intraperitonially
LC	L-Carnitine
L-DOPA	L-Dihydroxyphenyllamine
LHON	Leber's hereditary optic neuropathy
MAO	Monoamine oxidase
MAOi	Monoamine oxidase inhibitors
MDA	3,4-Methylenedioxyamphetamine
MDMA	3,4- Methylenedyoximethamphetamine
MERF	Myoclonic epilepsy associated with ragged-red muscle fibers

METH	Methamphetamine
MPT	Mitochondrial Permeability Transition
mtDNA	Mitochondrial DNA
NAccb	Nucleus accumbens
NDI	Subunit I of mitochondrial complex I (NADH dehydrogenase)
NDII	Subunit II of mitochondrial complex I (NADH dehydrogenase)
NA	Noradrenaline
NMDA	N-methyl-D-aspartate
N-ME-α-MeDa	N-methyl-α-methyldopamine
NO	Nitric Oxide
nNOS	neuronal nitric oxide synthase
NOS	Nitric Oxide Synthase
OXPHOS	oxidative phosphorylation
O_3	Ozone
PD	Parkinson's disease
$ONOO^-$	Peroxinitrite
PND	Postnatal day
PCR	Polymerase Chain Reaction
RN	Raphe nuclei
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SS	Serotonin syndrome
SERT	Serotonin transporter
SN	Substancia nigra
SOD	Superoxide dismutase
$O_2^{\cdot-}$	Superoxide radical
TH	Tyrosine hydroxilase
TNF	Tumor necrosis factor
TPH	Tryptophan hydroxilase
TRAIL	TNF-related apoptosis inducing ligand receptors
rRNA	Ribossomal RNA
tRNA	Transference RNA
TBA	Tiobarbituric acid
TTC	Triphenyltetrazolium cholride
UCP3	Mitochondrial uncoupling protein 3
VMAT	Vesicular monoamine transporters
VTA	Ventral tegmental area
α-MeDa	α-methyldopamine

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Outline of the dissertation

The present thesis is structured in four main chapters:

Chapter I

Section 1: General Introduction
In this section, a general overview on the contribution of mitochondrial damage for neurodegenerative diseases and xenobiotic toxicity is given. The most relevant insights on mitochondrial related disorders like Parkinson and Alzheimer are presented and discussed. The implications of prolonged and acute exposure to xenobiotics on mitochondrial function and integrity is also reviewed, with particular emphasis on MDMA.

Section 2: The animal model
In this section, an introduction to the animal model used is presented.

Section 3: General and specific objectives of the dissertation
The general and specific objectives of the dissertation are provided.

Chapter II
This chapter is divided in 3 sections, corresponding to the original manuscripts, and presents the experimental work, results obtained and specific discussions to answer the questions that derived from the general and specific objectives of this thesis.

Chapter III
In this chapter the unpublished results are presented.

Chapter IV
Section 1. Integrated overview of the performed studies.
The performed studies are integrated in a harmonized form.

Section 2. Conclusions
The conclusions that can be drawn from the present dissertation are summarized.

Chapter V
The references cited in the present dissertation are listed.

Overview

Mitochondria are gaining an important significance for the understanding of the mechanisms involved in several brain disorders. Coupling of respiration with oxidative phosphorilation (OXPHOS) performed in these organelles constitutes the major source of energy to brain cells and implies a correct mitochondrial performance that when disrupted can result in severe damage. Age-associated mechanisms and several related brain disorders, particularly the neurodegenerative ones, like Parkinson and Alzheimer are associated with severe mitochondrial dysfunction.

From what is at present known about MDMA-induced neurotoxicity, two main aspects have to be retained in order to understand the overall results here presented: (i) MDMA binds to the 5-HT re-uptake transporter and, inside axon terminals causes an acute and powerful release of neurotransmitters; (ii) excess of serotonin in the presynaptic nerve endings is metabolized by MAO flavoenzymes bounded to the external mitochondrial membranes.

The first results obtained in the present work lead us to conclude that the administration of a neurotoxic binge dose of MDMA to rats results in mitochondrial oxidative damage in the central nervous system (CNS), namely lipid and protein oxidation and mtDNA deletions, with subsequent diminished expression of the correspondent proteic subunits that are important constitutive elements of the electron transport chain (ETC).

When obtaining these results a theoretical hypothesis was designed. Increased H_2O_2 formation as result of increased MAO function inside presynaptic nerve endings lead to an overall increase of ROS inside mitochondria. Damage to several mitochondrial constituents could therefore have resulted, compromising this way the correct mitochondrial function and adequate energy supply.

To test the above mentioned hypothesis, additional experiments with MAO inhibitors (MAOi) were performed. Two isoforms of MAO exist: MAO-A and MAO-B. In brain, MAO-A is expressed predominantly in catecholaminergic neurons, whereas MAO-B is expressed in serotonergic neurons, astrocytes and glia. Although metabolism by MAO-B is only residual in the presence of MAO-A, it is fully effective in the absence of the later, as it happens inside serotonergic nerves.

The first experiments with MAOi, performed with selegiline, a MAO-B inhibitor, revealed, in accordance with the formulated hypothesis, a significant protection against the overall effects on mitochondria.

With the first hypothesis proven, a second question arised. If MAO-B inhibition was able to significantly protect against MDMA-induced mitochondrial neurotoxicity,

what happens to rat brain mitochondria if MAO-A inhibition is performed before MDMA administration? To answer this question, experiments with clorgyline, a specific MAO-A inhibitor, were performed. In this case, the previous administration of clorgyline revealed no protection against MDMA-induced neurotoxic effects. Moreover, toxicity was increased and the majority of animals exposed died during the experiments.

With the two monoamine oxidases tested, we decided to examine the effects of ALC supplementation on the MDMA-induced toxic effects at the brain mitochondrial level. ALC was chosen because of its antioxidant properties and its active role in mitochondria mainly by acting as a carrier of fatty acids across mitochondrial membranes for energy production through β-oxidation. As expected, ALC exposure protected at a great extent the mitochondrial damage associated with drug exposure.

Besides the new insights obtained from the above studies, further investigation in order to confirm previous findings, or complete the new studies here presented, was also performed. Measurement of body temperature of animals in the day of drug administration confirmed previous reports of several authors that included hyperthermia in the most pronounced effects of the drug. Failure of selegiline, clorgyline and ALC in protecting against this effect states mitochondrial damage, at the levels here studied, as unrelated with the characteristic deregulation of core body temperature.

ATP measurements on specific brain areas was also assessed in order to infer about the extent of drug-induced mitochondrial damage on ATP production. The results obtained confirmed a decrease of brain energy levels after exposure to MDMA and lead us to hypothesize about a possible involvement of the deletion of mitochondrial genes and deficient expression of the correspondent protein subunits with the uncoupling of oxidative phosphorilation.

TTC staining of brain slices was performed in order to macroscopically confirm the hypothesized mitochondrial metabolic dysfunction. The results achieved were in accordance with the expected although no direct correlation with the results concerning ATP quantification was observable.

Chapter I

1. General Introduction

2. Animal Model

3. General and specific objectives of the dissertation

1. General Introduction

1.1 An overview of mitochondrial structure and functions

Mitochondria are double membrane cytoplasmic organelles found in eukaryotic cells, structurally composed by two internal compartments, confined by an inner and an outer membrane. The mitochondrial matrix is contained within the inner membrane and the intermembrane space is comprised between the two membranes. The mitochondrial matrix encloses the mtDNA, RNA, protein synthesizing and destoxifying systems, constitutive mitochondrial proteins and various soluble enzymes involved in the tricarboxylic acid cycle and β-oxidation pathways. The external mitochondrial membrane is permeable to ions and solutes up to 14 KDa and has bounded enzymes that interface with the cellular cytoplasm. The inner membrane contains the ETC which is composed of five major complexes (I-V), each one with internal subunits. The intermembrane space contains proteins encoded by the nuclear DNA that include some that function as components of the ETC, apoptotic factors, transporters of polypeptides, metal ions and hydrophobic precursor proteins and enzymes responsible for metabolic processes (Koehler et al., 1998; Mesecke et al., 2005).

Coupling of OXPHOS with respiration through the ETC gives rise to energy production (Cooper, 2000) (Fig. 1) that, together with regulation of apoptosis and redox state, heme and iron sulphur center biosynthesis, amino acid and nitrogen metabolism, and calcium homeostasis modulation, constitute the most important functions of mitochondria (Murphy and Smith, 2000; Green and Kroemer, 2004).

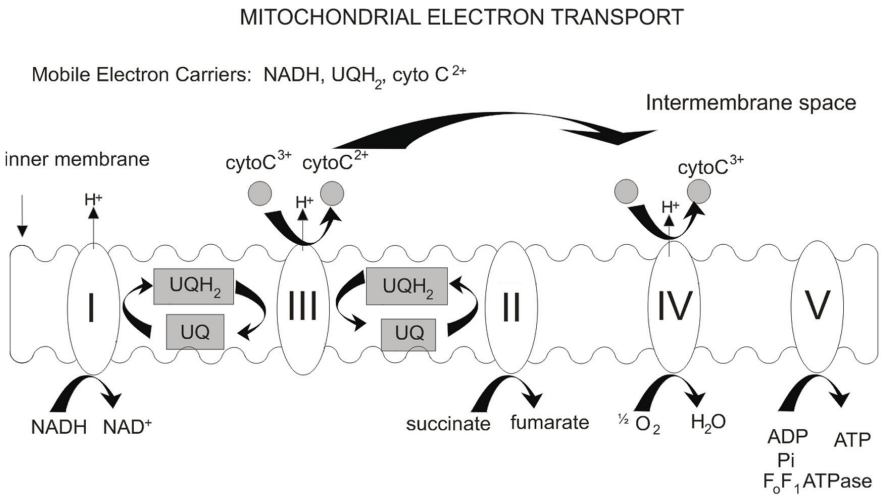

Figure 1.1: Coupling of oxidative phosphorylation with respiration throughout the mitochondrial electron transport chain. The mitochondrial complex I (NADH:ubiquinone reductase) is responsible for the oxidation of NADH and the energy produced in this step is further used to reduce ubiquinone (UQ). The mitochondrial complex II (succinate-ubiquinone reductase) is responsible for catalysing the oxidation of

succinate to fumarate (Hagerhall, 1997). The ubiquinol:cytochrome c reductase complex (complex III) is responsible for the oxidation of the reduced ubiquinol (UQH_2) and for the reduction of cytochrome c. Complex IV is finally responsible for the reduction of molecular oxygen to water. Throughout the entire process, proton pumping is always coupled to electron flow, thus ensuring the efficiency of energy production by the phosphorylation system, i.e., complex V, ADP/ATP and inorganic phosphate transporters.

1.2 The interaction of xenobiotics with the ETC

The disruption of the electrochemical proton gradient may be attained by the use of specific inhibitors of the ETC or by inhibiting the supply of reducing substrates to the respiratory chain. Chemical substances with potential to disrupt the correct mitochondrial performance include agents that increase membrane permeability and/or induce the permeability transition or behave as alternate electron acceptors. In any case, the correct mitochondrial function is compromised and severe mitochondrial damage could result. These conditions would disable the mitochondrial capacity to utilize the molecular oxygen and therein produce ATP.

1.2.1 ETC inhibitors

The mitochondrial complex I is the most vulnerable to chemical-induced damage. Three types of inhibition are recognized, namely NADH-flavin inhibitors, quinole antagonists and specific inhibitors (Table 1.1) and a large number of compounds with capability to block the function of this important structure (Degli Esposti, 1998) (see table1.1). Complex II inhibitors are the less potent and specific inhibitors of the ETC. Nevertheless, there is a great diversity of compounds with ability to reduce the enzymatic activity of this complex (Hagerhall, 1997; Miyadera et al., 2003). Complex III inhibitors present a great variability of effect among species (Degli Esposti et al., 1990; Degli Esposti et al., 1992; Ghelli et al., 1992; Vaidya et al., 1993; Kraiczy et al., 1996). There are four different groups of compounds with specific characteristics of inhibition, namely quinole antagonists, inhibitors of electron transfer between rieske Fe_2S_2 center and cytochrome c1, inhibitors of the Qi center and inhibitors of the bc_1 complex (Table 1.1). Finally, the inhibitors of complex IV, similarly to complex III are also divided in four categories according to the specific targets of action, namely heme-binding inhibitors (non-competitive with oxygen and cytochrome c), competitive inhibitors with oxygen, competitive inhibitors with cytochrome c, and non-competitive inhibitors with cytochrome c (Table 1.1). The inhibitors of complex V (ATPsynthase) act mainly by blocking proton conduction through Fo fraction of complex V (ATPsynthase), although the mycotoxins are the most potent ones, several other chemicals exist that share the same properties and similar characteristics of inhibition (Table 1.1).

Table 1.1: ETC inhibitors

	Mechanism of inhibition	Examples	References
Complex I inhibitors	NADH-flavin inhibitiors Quinole antagonists Specific inibitors	Rhein Myxothiazol, quinole aurachins Rotenone	(Degli Esposti, 1998)
Complex II inhibitors	Blockade of electron transfer between the enzyme and ubiquinone	Malonate, harzianopyridone, atpenins	(Hagerhall, 1997; Miyadera et al., 2003; Fernandez-Gomez et al., 2005)
Complex III inhibitors	Quinole antagonists (blockade of ubiquinol oxidation) Inhibitiors of electron transfer between rieske Fe_2S_2center and cytochrome c1 Inhibitors of the Qi center Inhibitors of bc_1 complex	Myxothiazol, oudemansins UHDBT, UHNQ Antimycin A, funiculosin, Quinolones Substituted phenols, metal cations, zinc ions	(Thierbach and Reichenbach, 1981; von Jagow and Link, 1986; Miyoshi et al., 1993; Palmeira et al., 1994; Link and von Jagow, 1995)
Complex IV inhibitors	Heme-binding inhibitiors (non-competitive with oxygen and cytochrome c) Competitive inhibitiors with oxygen Competitive inhibitors with cytochrome c Non competitive inhibitors with cytochrome c (non interfering with heme group)	Azide, cyanide, sulphide Carbon monoxide, nitric oxide Polycations Phosphate ions, alkaline pH	(Nicholls and Chance, 1974)
ATP-synthase (Complex V) inhibitors	Binding to the F1 or F0 subunits of the enzyme (blockade of proton conduction)	Mycotoxins, flavonoids, propanolol, paraquat, dicyclohexylcarbodiimide, oligomycin, aurovertin B	(Lardy et al., 1975; Linnett and Beechey, 1979; Lardy, 1980; Penefsky, 1985; Ueno, 1985; Wei et al., 1985; Bohmont et al., 1987; Palmeira et al., 1995; van Raaij et al., 1996; Shchepina et al., 2002)

Footnotes: UHDBT-Undecylhydroxydioxobenzothiazole; UHNQ- undecylhydroxynaphtoquinone.

1.2.2 OXPHOS uncouplers

Uncoupling of OXPHOS leads necessarily to a decrease of ATP production. In the table below (Table 1.2), a summary of the most common mitochondrial uncouplers is presented.

Table 1.2: OXPHOS uncouplers

OXPHOS uncouplers	Properties	Examples	References
Proton carriers	Mobilization of protons across lipid bilayers	Carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP), carbonylcyanide m-chlorophenylhydrazone (CCCP), dichlorophenol	(Crane, 1977; Terada, 1981; Sun and Mauzerall, 1996; Beauvoit et al., 1999)
Liophilic weak acids	Increased permeability of lipid membranes to protons with dissipation of the electrochemical proton gradient by the cycling movement of an uncoupler molecule	Substituted phenols, salicylanides, carbonyl cyanide	(McLaughlin and Dilger, 1980; Terada, 1981)
Free fatty acids	Protein mediated uncoupling by FFA (wasting of energy and inhibition of respiration by excessive FFA accumulation)	Sulfuramide, perfluorodecanoic acid	(Langley, 1990; Schnellmann and Manning, 1990; Wojtczak and Schonfeld, 1993; Hermesh et al., 1998)
Chanel-type ionophores	Chanels in the lipid membrane (increase of permeability with collapse of the proton electrochemical gradient)	Gramicidins	(Katsu et al., 1987; Luvisetto and Azzone, 1989)
Carrier-type ionophores	Formation of lipid-soluble complexes with ions (collapse of the electrochemical proton gradient)	Nigericin, valinomycin	(Toro et al., 1976; Felber and Brand, 1982; Kovac et al., 1982)
Cationic uncouplers	Increase in membrane permeability to ions (Interference with the physical integrity of the membrane-induction of the MPT)	Tris-S-C_4 , pentamidine	(Degli Esposti, 1998; Shinohara et al., 1998)
Membrane active compounds	Formation of chanells permeable to alkaline ions and/or protons or induction of the formation of large pores that lead to mitochondrial swelling	Alamethicin tamoxifen, cyclosporine A, nafoxidine	(Takaishi et al., 1980; Mathew et al., 1981; Hoyt et al., 2000; Simpson et al., 2002)
Alternate electron acceptors	Competition with the electron natural acceptors of the carrier-dissipation of proton electrochemical potential	Adriamycin, paraquat, substituted naphtoquinones,	(Doroshow and Davies, 1986; Bironaite et al., 1991; Henry and Wallace, 1995; Wallace, 1999)

Footnotes: OXPHOS-oxidative phosphorylation; FFA-free fatty acid; MPT-mitochondrial permeability transition

In this thesis, a particular attention will be given to brain mitochondria specifically focusing some aspects related with the ETC integrity and function, mtDNA mutations and oxidative stress. Within this context, the important role of MAO'S, enzymes responsible for brain monoamine neurotransmitter metabolism, bounded to the mitochondrial external membrane, will be described with detail.

1.3 The contribution of oxidative stress to mitochondrial dysfunction

Oxidative stress is a disturbance in the pro-oxidant-antioxidant balance in favour of the former, leading to potential damage. Both reactive nitrogen species (RNS) (Spanos and Yamamoto, 1989b) and ROS (Jacobson and Duchen, 2002; Ott et al., 2007) contribute to the development of oxidative stress conditions within cellular organisms. A sustained oxidative stress status may ultimately affect cellular function and, within the cell, mitochondria are particularly involved in this process, by being themselves potential generators of these reactive species that although being essential to some cellular functions could also contribute to cellular damage specially if produced in high amounts. Both ROS and RNS are by-products of ATP production (Wiseman and Halliwell, 1996) and their increased production generally arise from defects in mitochondrial respiratory chain structure and function. These species usually include oxygen radicals [(e.g. superoxide radical ($O_2^{\cdot -}$) and hydroxyl radical ($HO^{\cdot}$)] and certain non radicals [(e.g. hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and ozone (O_3)] (Wiseman and Halliwell, 1996) that when overproduced constitute potential damaging agents to mitochondria, its constituents and to the overall cellular function.

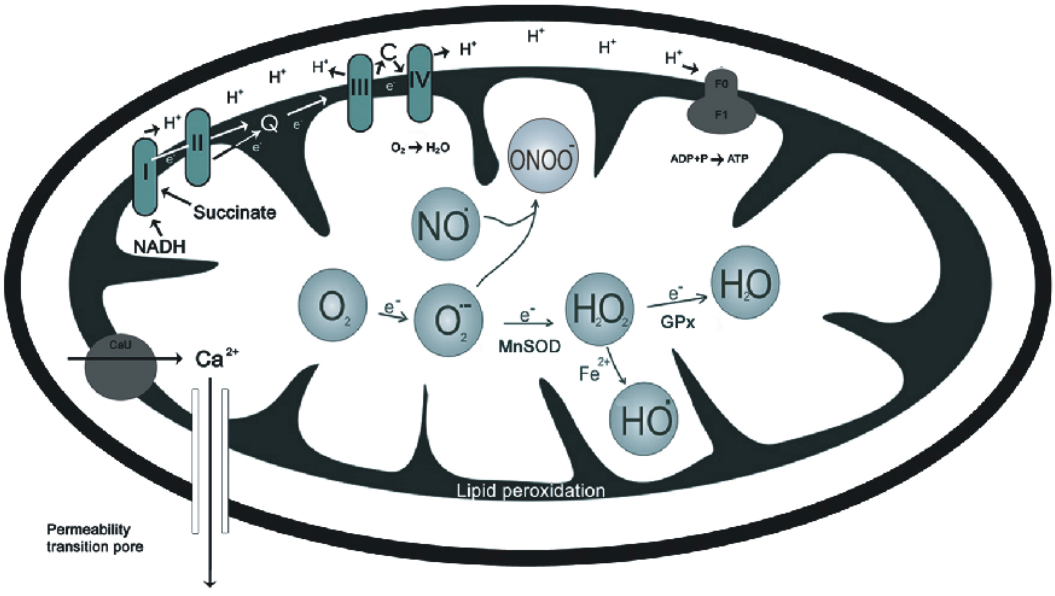

Figure 1.2: Formation of ROS and RNS inside mitochondria and mitochondrial antioxidant system.

In the ETC, the main producers of ROS are complexes I and III (Balaban et al., 2005), whose primary product of oxidation is $O_2^{\cdot -}$. This radical alone is not very toxic but further reaction with nitric oxide (NO) gives rise to the formation of the very reactive specie $ONOO^-$ that is also quickly converted to H_2O_2, spontaneously or through the catalysis of the enzyme superoxide dismutase (SOD). H_2O_2 may then interact with

transition metal ions generating $HO^{\bullet}$. Importantly, $HO^{\bullet}$ is one of the strongest oxidizing agents, producing severe damage in its vicinity, leading to oxidative damage of lipids, proteins and DNA (Halliwell, 1996; Valko et al., 2004). Additional important endogenous sources of oxidative stress are MAOs, flavoenzymes bounded to the external mitochondrial membrane. The oxidation of monoamine neurotransmitters by MAOs to its aldehyde derivatives gives rise to the production of H_2O_2 (Sandri et al., 1990; Giorgio et al., 2005), which may subsequently be transformed into $HO^{\bullet}$, as mentioned before.

1.3.1 Nitric oxide: damaging or protector?

NO is synthesized mainly by three enzyme isoforms of the enzyme nitric oxide synthase (NOS) that include the neuronal NOS (nNOS; typeI), inducible NOS (iNOS; typeII) and endothelial NOS (eNOS, typeIII) (Knowles and Moncada, 1994; Yun et al., 1996; Emerit et al., 2004). This RNS has a dual action in neuronal cells because although playing essential roles in the modulation of several physiological activities like the modulation of vascular tone (Palmer et al., 1987), neurotransmission (Garthwaite et al., 2005) and immune system (Hibbs et al., 1988; Stuehr and Nathan, 1989), it is also capable to induce cellular damage. NO binds to the mitochondrial cytochrome c oxidase (Bolanos et al., 1994) and decreases its affinity for O_2 thus affecting the basal mitochondrial performance and decreasing the levels of energy production (Almeida and Bolanos, 2001). Moreover, due to the increase in intracellular Ca^{2+} concentration that follows N-methyl-D-aspartate (NMDA) activation, nNOS is activated (Knowles and Moncada, 1994), resulting in the overproduction of NO, which is also able to cause the opening of the mitochondrial permeability transition (MTP) structures located in the mitochondrial membranes (Tatton and Olanow, 1999). This ultimate action has two main consequences: the loss of membrane potential and the activation of mechanisms involved in apoptotic cellular death by inducing the release of cytochrome c and other intramembrane apoptotic factors (Tatton and Olanow, 1999).

1.3.2 Main targets of oxidative modifications inside mitochondria

The mitochondrial genome is responsible for coding 13 proteins absolutely essential for respiration and oxidative phosphorilation (Anderson et al., 1981). These proteins include seven subunits of NADH dehydrogenase (NDI, II, III, IV, IVL, V and VI), three subunits of cytochrome c oxidase (COXI, II and III) and two subunits of ATP synthase (ATPase 6, 8). Any alteration of the mtDNA structure, resulting in abnormal expression

of these proteins, might imply altered mitochondrial activity thus representing a potential source of cellular dysfunction.

The mtDNA is one of the mitochondrial structures more prone to oxidative modifications, the presence of free radical generating enzymes together with poor repair mechanisms and the absence of protective histones, all converge to a high sensitivity of mtDNA to the occurrence of mutations (Carew and Huang, 2002; Taylor and Turnbull, 2005).

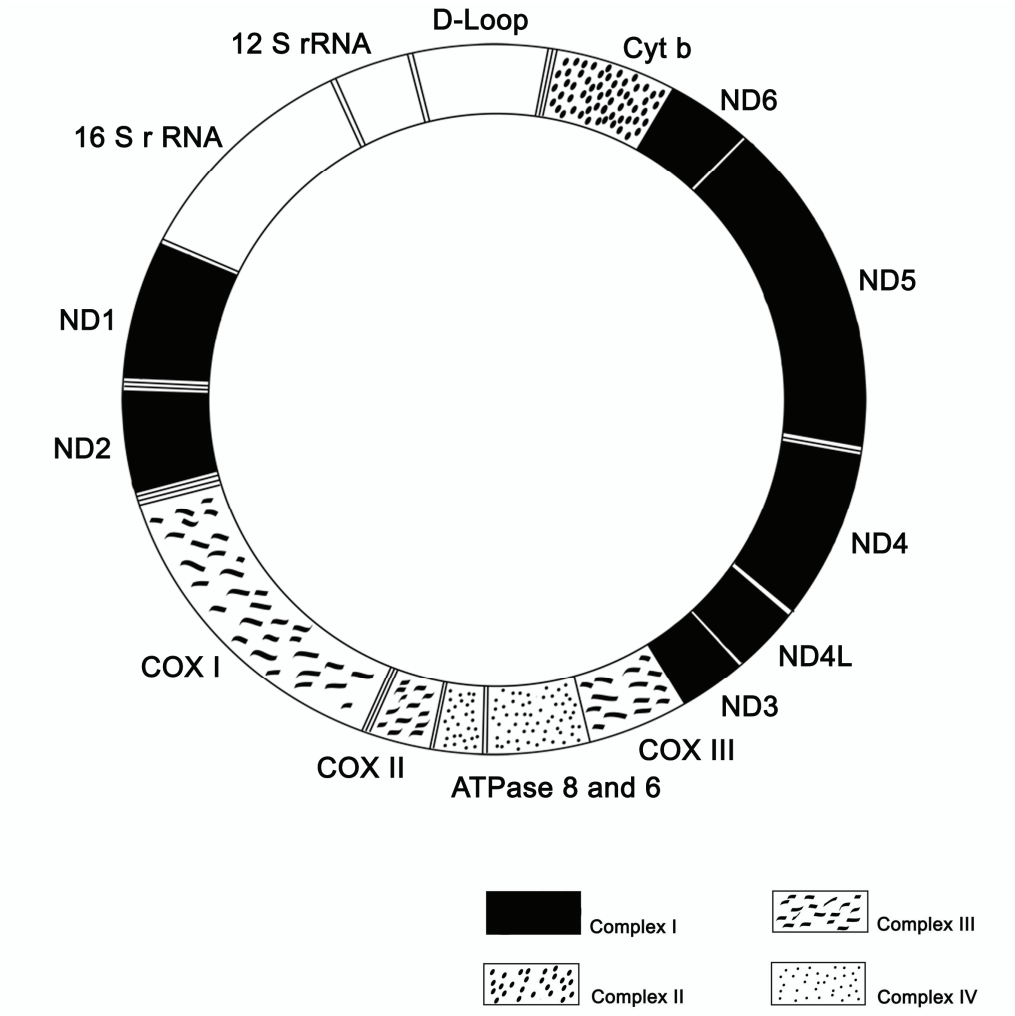

Figure 1.3: Mammalian mtDNA genome. mtDNA codes for two rRNA ((12S and 16S)-coding regions), 22tRNA (not signalled in the figure), and 13 subunits of the OXPHOS (oxidative phosphorylation) pathway (13mRNA-coding regions). Adapted from (Suliman et al., 2003).

Mitochondrial proteins and lipids are also prone to oxidation. The high content in polyunsaturated fatty acid residues in mitochondrial phospholipids gives rise to an extended formation of lipid peroxides upon exposure to pro-oxidant conditions (Esterbauer et al., 1991) and the direct oxidation of amino acids in the proteins of mitochondria could easily lead to the formation of protein carbonyls (Beckman and

Ames, 1998; Sohal, 2002). Moreover, cross-linking reactions of oxidized lipids with oxidized proteins or lipid-lipid and protein-protein interactions could lead to the complete loss of its normal functions with enhanced propensity to proteolytic degradation. Either primary or secondary mechanisms could converge to a full disruption of activity with obvious deleterious consequences to the overall cellular function.

A vicious circle of oxidative insults and loss of function could therefore, in extreme conditions, result in cellular death (Scarlett and Murphy, 1997; Petit et al., 1998).

1.3.3 Supplementation of antioxidants to mitochondria

Mitochondria are endowed with effective endogenous antioxidant defences from which the reduced glutathione (GSH)-linked enzymatic systems (Soderdahl et al., 2003) the glutathione peroxidases (Gpx) (Antunes et al., 1995; Pushpa-Rekha et al., 1995) and the thioredoxin systems (Arner and Holmgren, 2000) are important representative elements. Several conditions, however, disable endogenous protection against the oxidative insult produced. Prevention of mitochondrial oxidative damage could be further achieved by antioxidant administration but the difficulty to target specific antioxidants to mitochondria constitutes an obstacle to obtain satisfactory results. The development of new strategies for this purpose is emerging and the use of lipophilic cations, such as the triphenylphosphonium cation (TPP^+) for the co-transport of antioxidants, is actually viewed as a satisfactory and efficient alternative. The TPP^+ cation co-transports compounds with antioxidant properties into mitochondria by easily moving through phospholipid bilayers and being largely uptaken by mitochondria owing to the large membrane potential (Liberman et al., 1969; Ross et al., 2005). $Mito_{Q10}$ and $MitoE_2$ were the first mitochondria-targeted antioxidants explored and experimentally tested. The $MitoE_2$ consists of an agglomerate of α-tocopherol conjugated to TPP^+ (Smith et al., 1999). This conjugate is effective in preventing lipid peroxidation in mitochondria and the α-tocopherol radical formed is recycled by the endogenous mitochondrial coenzyme Q pool (Maguire et al., 1989; James et al., 2004). $MitoQ_{10}$ is constituted by a moiety of ubiquinol linked to TPP^+ (Kelso et al., 2001). With a high effectiveness against lipid peroxidation, Mito Q_{10} is also valuable for the detoxification of $ONOO^-$ and $O_2^{\cdot-}$(Kelso et al., 2001; James et al., 2005). In the table below (table 1.3), some of the most common mitochondria-targeted antioxidants are briefly presented.

Table 1.3: Mitochondria-targeted antioxidants

Mitochondrial targeted antioxidants	Protective action against	References
$MitoE_2$	Lipid peroxidation	(Maguire et al., 1989; Smith et al., 1999; James et al., 2004)
$MitoQ_{10}$	Lipid peroxidation; $ONOO^-$, superoxide	(Kelso et al., 2001)
MitoSOD	$O_2^{\cdot-}$	(Salvemini et al., 1999)
MitoPeroxidase	H_2O_2	(Filipovska et al., 2005)
MitoTEMPOL (a mitochondria-targeted verion of the nitroxide TEMPOL)	$O_2^{\cdot-}$; Fe^{2+}	(Murphy and Smith, 2007)
MitoPBN (a mitochondria targeted version of the spin trap phenylbutylnitrone)	Carbon-centered radicals	(Murphy et al., 2003)

Footnotes: SOD-superoxide dismutase, ONOO –peroxinitrite, $O_2^{\cdot-}$ -superoxide radical, H_2O_2 -hydrogen peroxide.

Alternatives to the use of TPP^+ as active intermediaries to target antioxidants into mitochondria could be achieved by the use of compounds that specifically act on the organelle, such as ALC and selegiline whose actions will be following described.

1.3.4 Measurement of oxidative damage in mitochondria

ROS and RNS produced in mitochondria during basal metabolism or as result of a cellular insult are capable of damaging several mitochondrial macromolecular constituents, such as lipids, DNA and proteins. Thus, the measurement of the oxidative state of mitochondrial lipids, proteins and DNA constitutes an important tool as biomarkers of oxidative damage (Richter, 1988; Laganiere and Yu, 1993; Garibaldi et al., 1994) and that is several times used to assess the extension of injury produced in a wide range of disease states. Increased oxidative damage to lipids of membranes (Zoratti and Szabo, 1995; Hagen et al., 1997) and proteins (Symonyan and Nalbandyan, 1972; Bowling and Beal, 1995) results, respectively, in decreased membrane fluidity and impaired ETC functioning. MtDNA oxidation can derive from both these events.

A summary of some of the methodologies commonly used to assess the oxidation of mitochondrial constituents is tabled below (table 1.4).

Table 1.4: Methods commonly used to assess oxidative damage in mitochondria.

Methodologies frequently used to measure oxidative damage in mitochondria	References
Lipid peroxidation measured by the TBA assay; hydroperoxide measurement by oxidation of Fe^{2+} in the presence of xylenol orange, gas chromatography/mass spectrometry to measure F2 isoprostanes.	(Jiang et al., 1991; Halliwell and Chirico, 1993; Rohn et al., 1993a; Jiang et al., 1999; Idris et al., 2005)
Protein oxidation measured by reaction of protein carbonyls with DNPH or through immunochemical detection of protein carbonyls; measurement of sulfhydril groups; MALDI-TOF mass spectrometry.	(Ellman, 1959; Levine et al., 1994; Yan and Sohal, 1998; Bailey et al., 2005)
DNA oxidative damage measured by the levels of 8-OHdG by gas chromatography/mass spectrometry with a selective ion monitoring analysis.	(Halliwell and Dizdaroglu, 1992; Mecocci et al., 1994; Herbert et al., 1996; Gabbita et al., 1998; Dizdaroglu et al., 2002; Wang et al., 2006)

Footnotes: TBA-thiobarbituric acid; DNPH- 2,4-dinitrophenylhydrazine; MALDI-TOF-matrix-assisted laser desorption ionization time-of-flight; 8-OHdG-8-hydroxy-2'deoxyguanosine.

1.4 Mitochondria and cell death

Mitochondrial damage may constitute an important trigering factor for neuronal cell death. A brief description about the contribution of mitochondrial damage cell death mechanisms is made below.

1.4.1 Apoptosis vs necrosis

There are two known mechanisms of cell death: apoptosis and necrosis. Both mechanisms might occur either isolated, in combination, or as sequential events (Davidson et al., 2001) and both are essential for normal cellular function.

Apoptotic cell death may occur through intrinsic or extrinsic molecular mechanisms (Fiers et al., 1999; Finucane et al., 1999; Nomura et al., 1999). The intrinsic pathway is the one most frequent in vertebrates (Green and Kroemer, 2004), but both the intrinsic or extrinsic mechanisms can stimulate apoptosis together or independently of one another.

The extrinsic apoptotic pathway involves the tumor necrosis factor (TNF-α) family receptors, the receptor of CD95L/FasL and TNF-related apoptosis inducing ligand receptors (TRAIL) (Kroemer et al., 2007). The intrinsic pathway involves mitochondria (Takahashi et al., 2004). In the intrinsic pathway, also known as the "The mitochondrial pathway", the mitochondrial permeability plays a crucial role (Scaffidi et al., 1998) and the mitochondrial permeability transition (MPT) is directly involved. MPT is very sensitive to conditions associated with apoptotic events such as increased ROS formation and increase in Ca^{2+} concentration with resultant caspase activation,

membrane voltage changes, mitochondrial oxidation status (Cassarino and Bennett, 1999) and the presence of pro-apoptotic Bcl-2 family members (Kroemer et al., 2007). This big family of proteins is localized at the mitochondrial membrane (Krajewski et al., 1993; Hsu et al., 1997; Wolter et al., 1997) and is composed of elements with death supressive and promoting activities (anti and pro apoptotic elements), respectively Bcl-2, Bcl-X_L and Bax, Bak, Bik, Bcl-X_s (Montal, 1998). Bcl-2 is also closely correlated with cytochrome c release (essential cofactor of mitochondrial complex IV of the ETC) modulation following oxidative stress and with the acidification that follows initial alkalinization in apoptotic processes (Takahashi et al., 2004). Caspases are additional important apoptotic controlling elements with proteolitic activity by cleaving after aspartate residues found to be intimately related with most of the forms of apoptotic cell death. This family of proteins work in a concerted manner by action of the initiator (caspases 3, 6 and 7) and effector (caspase 8, 9 and 10) caspases (Fuentes-Prior and Salvesen, 2004; Kroemer et al., 2007).
Additional important events related with the activation of apoptotic pathways include alterations in the morphology of cellular nucleus, chromatin condensation and nuclear fragmentation (Reed et al., 1998; Eckert et al., 2003).

Necrosis, another form of cell death, is favoured when severe oxidative stress occurs because oxidation might inactivate caspase enzymatic activity (Hampton et al., 1998). The main characteristics of this pathway are swelling of cytoplasm and mitochondrial matrix, followed by cell membrane rupture with associated critical reduction of ATP reserves (Davidson et al., 2001).

Oxidative stress induced by the formation of ROS and/or RNS lead commonly to a combination of apoptosis and necrosis (Kroemer et al., 1998). High levels of oxidative stress lead to necrosis, while moderate levels have an apoptotic response and mitochondrial membrane permeabilization is a common event of both death pathways (Kroemer and Reed, 2000).

Mitochondrial malfunction disrupts the function of cells, tissues, and organs and contributes to the development and emergence of a wide range of pathologic states (Leonard and Schapira, 2000a, b; Murphy and Smith, 2000; Smeitink et al., 2001).

The purpose of the following brief revision is to explain the association between mitochondrial brain dysfunction and some neurotoxic processes. In particular, neurodegenerative disorders, aging mechanisms and neurotoxicity induced by some drugs of abuse are revised.

1.5 Neurodegenerative diseases and mitochondria

Several lines of evidence point to the increase in oxidative damage to mitochondrial constituents as being closely implied in the pathogenesis of many neurodegenerative disorders.

Mitochondrial associated neurodegenerative diseases are the combined result of defects in OXPHOS with decreased ATP production and oxidative stress induced by increased formation of ROS (Esposito et al., 1999). The progression of such disorders is, in part, determined by mitochondrial integrity and function and its prevention is linked to an adequate mitochondrial performance.

A brief revision on the most common disorders associated with mitochondrial dysfunctions is following presented.

1.5.1 Alzheimer disease (AD)

One of the best characterized neurodegenerative disorders is the AD. AD genetic predisposition is based on genes encoding the amyloid percursor protein, presenilin-1 and presenilin-2 (Selkoe, 2001) but age is the main risk factor, a great part of the cases being sporadic (SAD), with little genetic links and late onset (Goedert et al., 1994).

AD is progressive, irreversible and associated with cell loss and mitochondrial abnormalities. Formation of extracellular plaques with associated proliferation of activated microglia and astrocytes are proeminent characteristics of the disease (Schipper, 1996; Sheng et al., 1997) but oxidative damage and defective mitochondria are refered as the earliest events (Nunomura et al., 2001; Castellani et al., 2002) and particullary the neurons from Hip and cerebral cortex are selectively lost (Fig. 1.4) (Bossy-Wetzel et al., 2004).

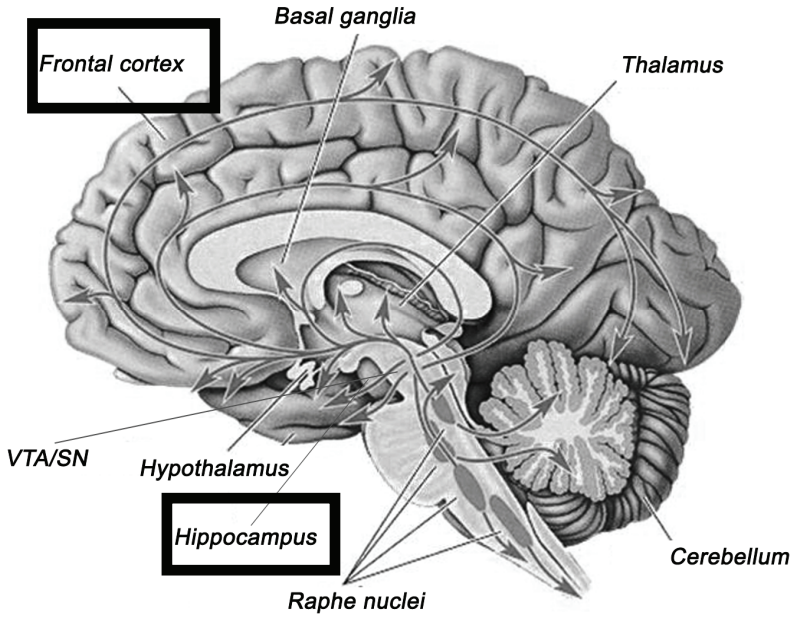

Figure 1.4: Injured brain areas in AD. Adapted from (Deutch and Roth, 1999)

The mitochondrial characteristics associated with AD are described in table 1.5.

Table 1.5: Common AD related mitochondrial alterations.

AD related mitochondrial alterations	References
Mitochondrial ROS production with associated brain oxidative damage	(Miyata and Smith, 1996; Ramassamy et al., 2000; Ramassamy et al., 2001; Lustbader et al., 2004; Crouch et al., 2005)
MtDNA oxidative damage	(Mecocci et al., 1994)
MtDNA deletions and rearrangements	(Corral-Debrinski et al., 1994)
Neuronal apoptosis, increased activated caspase activity	(Castellani et al., 2002; Gibson and Huang, 2002; Pratico, 2002)
OXPHOS deffects	(Mutisya et al., 1994; Verwer et al., 2000; Bosetti et al., 2002; Cardoso et al., 2004; Trimmer et al., 2004)
Increased mtDNA fragmentation	(Jossan et al., 1991; Saura et al., 1994)
Reduced mtDNA content	(Jossan et al., 1991; Saura et al., 1994)
Increased MAO-B brain levels	(Jossan et al., 1991; Saura et al., 1994)
Mutations in cytochrome c oxidase mitochondrial genes	(Parker et al., 1990; Parker et al., 1994)
Reduction in the ND6/ND2 ratio	(Coskun et al., 2003)
Defective mitochondria	(Nunomura et al., 2001; Castellani et al., 2002)
Decreased activity of cytochrome c oxidase	(Mutisya et al., 1994; Davis et al., 1997)
Increased expression of NOS2	(Heneka et al., 2001)
Disruption of energy metabolism	(Mattson, 2004)

Footnotes: ROS-reactive oxygen species; MtDNA-mitochondrial DNA; OXPHOS-oxidative phosphorylation; MAO-B-monoamine oxidase-B; ND6-subunit 6 of mitochondrial complex I; ND2-subunit 2 of mitochondrial complex I, NOS-nitric oxide synthase.

Sporadic AD (SAD) cases are often characterized by an increased ratio of SOD/catalase activity that may account for increased production of H_2O_2 (Gsell et al., 1995; Cassarino and Bennett, 1999) and consequently increased oxidative damage.

AD associated oxidative damage to cellular constituents as mitochondria, lipids, proteins and DNA are well documented in literature (Lyras et al., 1997; Munch et al., 1997; Wong et al., 2001; Dei et al., 2002). Increased oxidation in neuronal cells is frequently traduced in high levels of oxidized DNA bases (Mecocci et al., 1994; Gabbita et al., 1998) lipids and proteins (Lovell et al., 1995; Markesbery and Lovell, 1998; Butterfield et al., 2003). Lipid peroxidation found in the brain of AD patients has been associated with increased GSH and Gpx activities (Lovell et al., 1995) and oxidation of docosahexaenoic is one of the common characteristic products. One of the typical products of protein oxidation in AD, include creatinine kinase BB (Aksenova et al., 1999; Aksenov et al., 2000), that is associated with the characteristic senile plaques of this pathology (Frenkel et al., 2000; Selkoe, 2001), as well as nitrotyrosine, formed by reaction of $ONOO^-$ with protein tyrosine residues (Beckman et al., 1993).

1.5.2 Parkinson‘s disease (PD)

PD is a progressive neurodegenerative disease, for which major characteristic symptoms are depression, dementia, tremors and rigidity (Trimmer et al., 2004). As in AD, the great majority of PD cases are of sporadic nature.

Some of the most common characteristics of the disease include eosinophilic cytoplasmatic inclusions (Lewy bodies) in neurons of some brain areas (Forno and Norville, 1976; Pollanen et al., 1993), increased dopamine (DA) turnover by MAO-B with associated H_2O_2 production (Cohen and Spina, 1989; Spina and Cohen, 1989), a relative selective loss of dopaminergic neurons from the SN and other brainstem nuclei and S-nitrosylation of Parkin proteins (E3 ubiquitin-ligases that ubiquinate proteins involved in the survival of dopaminergic neurons), though several other factors are also involved.

Oxidative phenomena are main contributors to the evolution of this neurodegenerative disorder, with increased oxidative damage to DNA, lipids and proteins being well-reported characteristics of the pathology (Dexter et al., 1989; Sian et al., 1994; Alam et al., 1997; Pearce et al., 1997; Floor and Wetzel, 1998). PD-asociated DNA and protein oxidation are particularly well described. Studies on DNA oxidation in PD patients reported increased levels of 8OHdG in mitochondrial and total DNA (Alam et al., 1997; Shimura-Miura et al., 1999; Zhang et al., 1999). Neuronal cells of SN (Cortopassi et al., 1992; Soong et al., 1992; Cortopassi and Wang, 1995) are particularly affected (Fig. 1.5) as observed by the characteristic pattern of nigral cell death (Cassarino and

Bennett, 1999). It has been observed that complex I dysfunctionality is quite more related with increased protein oxidation by means of increased electron leakage (Turrens and Boveris, 1980; Hasegawa et al., 1990; Cassarino et al., 1997; Votyakova and Reynolds, 2001). Complex I is the major source of superoxide production in the ETC (Kudin et al., 2004; Lambert and Brand, 2004) and studies on its functionality in PD patients reported an increase of 47% more protein carbonyls (traducing considerable levels of oxidative stress) together with a significant loss of complex I 8 KDa subunit (Keeney et al., 2006).

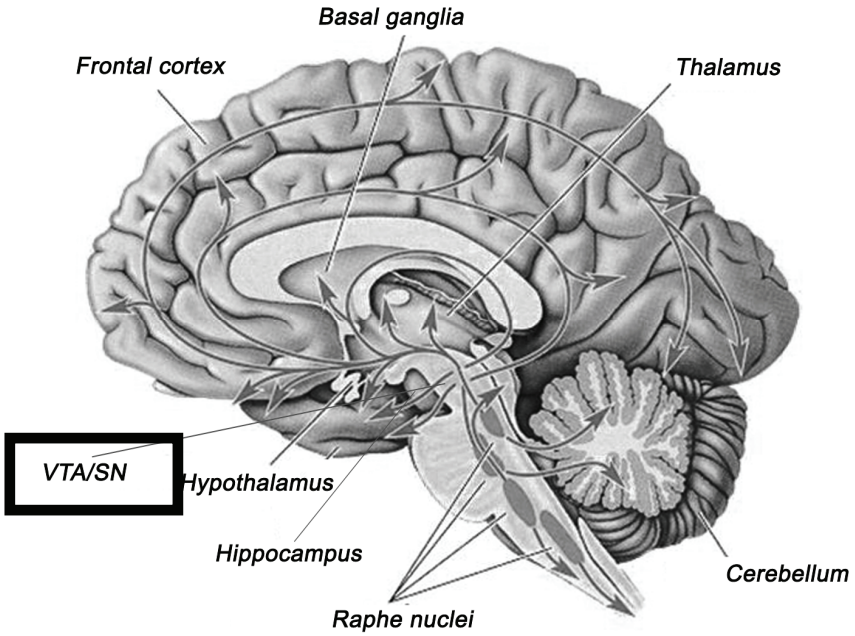

Figure 1.5: Injured brain areas in PD. Adapted from (Deutch and Roth, 1999)

Addittional characteristics of the disease describe an upregulation of the antioxidant enzymes (Marttila et al., 1988a; Saggu et al., 1989; Kalra et al., 1992; Damier et al., 1993) MnSOD (superoxide dismutase mitochondrial isoform) (Saggu et al., 1989), CuZnSOD (Mizuno and Ohta, 1986; Poirier et al., 1994) and several mitochondrial alterations that are further described in table 1.6.

Table 1.6: Common PD related mitochondrial alterations.

PD related mitochondrial alterations	References
Increased MAO-B activity and turnover	(Schneider et al., 1981; Hornykiewicz et al., 1986)
Reduced NADH dehydrogenase activity	(Swerdlow et al., 1996; Parker and Swerdlow, 1998; Betarbet et al., 2002)
Altered intracellular Ca^{2+} homeostasis: MPT induction	(Sheehan et al., 1997)
Increased number of morphologically altered mitochondria	(Trimmer et al., 2000)
Increased levels of anti-apoptotic proteins Bcl-2 and Bcl- XL	(Veech et al., 2000)
Depletion of mitochondrial GSH	(Cohen et al., 1997)
Altered mitochondrial function	(Hsu et al., 2000; Orth et al., 2003; van der Walt et al., 2003)
Deficient activity of mitochondrial complex I	(Schapira et al., 1990a; Schapira et al., 1990b)
S-nitrosylation of mitochondial proteins	(Chung et al., 2004)
Overproduction of NO	(Wu et al., 2002)

Footnotes: MAO-B-monoamine oxidase-B; MPT-mitochondrial permeability transition; GSH-reduced glutathione; NO-nitric oxide.

1.5.3 Myoclonic epilepsy associated with ragged-red muscle fibers (MERF)

MERF is a rare disease of the central nervous system (CNS) and skeletal muscle (Fukuhara et al., 1980; DiMauro et al., 1985; Rosing et al., 1985; Wallace, 1987) that is described as being maternally inherited and the product of oxidative phosphorylation deficiency (Wallace et al., 1988b). A point mutation in the $tRNA^{Lys}$ gene of the mitochondrial genome with an A→G transition is involved (Wallace et al., 1988a; Shoffner et al., 1990; Zeviani et al., 1991) and the common symptoms include myoclonus, seizures, severe cerebellar ataxia, tremor, and ragged-red fibers in muscle (Tritschler and Medori, 1993).

1.5.4 Amyotrophic lateral sclerosis (ALS)

ALS is a disorder characterized by progressive muscle weakness and atrophy as result of the death of motor neurons from spinal cord and brainstem and pyramidal cells of the motor cortex (Cassarino and Bennett, 1999). As AD and PD, the big majority of cases are sporadic and mutations in the CuZnSOD gene with increased $HO^{\bullet}$ production are frequently described as implicated in this disorder (Wong et al., 1995; Yim et al., 1996) although the majority of familial cases do not present this mutation. Additional features of the disease include abnormal mitochondria and mitochondrial degeneration (Bowling and Beal, 1995, Brown, 1997), though increased levels of protein carbonyls, 8-OHdG and nitrotyrosine have also already been associated with the pathology.

1.5.5 Leigh Syndrome

Leigh subacute necrotizing encephalomyelitis is a mitochondrial disorder of infancy and childhood (Leigh, 1951; Cavanagh and Harding, 1994; Munaro et al., 1997) and the typical symptoms include dystonia, tremor, psychomotor retardation and respiratory difficulties (Leigh, 1951).

The common characteristic of patients with the syndrome is a defect in cytochrome c oxidase (DiMauro et al., 1987; Munaro et al., 1997) that results from a mutation in a nuclear gene (Miranda et al., 1989) but deficiencies of pyruvate dehydrogenase complex (Stansbie et al., 1986), respiratory complex I (Fujii et al., 1990; Wijburg et al., 1991; Benit et al., 2001; Benit et al., 2003; Benit et al., 2004), mtDNA point mutations (Tatuch et al., 1992; de Vries et al., 1993; Santorelli et al., 1993) and a mutation in an ATPase gene are also closely associated with the disease. In these patients, specific brain regions present well marked necrotic lesions, ,namely the brainstem, the diencephalon and the cerebellum (DiMauro and De Vivo, 1996).

1.5.6 Leber's hereditary optic neuropathy (LHON)

LHON is a maternally inherited mitochondrial genetic disorder that conduces to a severe mitochondrial respiratory dysfunction (Vilkki et al., 1989; Howell et al., 1991a; Wallace, 1999). The disease is characterized by two point mutations on mitochondrial subunits of mitochondrial complex I: a G→A transition at nucleotide 11778 that converts an arginine to an histidine in the ND4 subunit (Wallace et al., 1988b; Singh et al., 1989; Vilkki et al., 1990) and a T→C transition at nucleotide 4160 that converts a proline to a leucine in the NDI subunit (Howell et al., 1991b).

The onset of the disease is usually before the age of 30 and the characteristic symptoms are severe bilateral optic atrophy and retinal microangiopathy that leads, generally, to loss of vision (Seedorff, 1970; Nikoskelainen et al., 1987). The main target populations are young and adolescent males (Vilkki et al., 1989).

1.5.7 Chronic Uremia

Chronic Uremia is a condition associated with increased ROS production (Roselaar et al., 1995; Odetti et al., 1996; Witko-Sarsat et al., 1996; Miyata et al., 1998) and impaired free radical scavenger system whose main consequences are mitochondrial structural and functional abnormalities (Canaud et al., 1999; Lim et al., 1999; 2002). Studies performed in skeletal muscle found increased levels of a mtDNA 4977 basepair (bp) deletion that involves multiple structural mitochondrial genes (ATPase 6, ATPase 8, COX III, ND3, ND4L, ND4 and ND5) and 5tRNA genes. Additional characteristics of

the disease include increased levels of 8-OHdG and increased levels of protein carbonyls and lipid peroxides in mitochondria and submitochondrial particles of skeletal muscle (Lim et al., 2000; Lim et al., 2002). This neuropathy is also one of the common complications of end stage kidney disease that have recently been connected to changes on membrane potential due to the dysfunction of Na^+/K^+ pumps (Krishnan and Kiernan, 2007).

1.6 Aging and mitochondria

Aging is a biological process that is common to all living organisms.

The most refered cause of age associated alterations is the formation of endogenous free radicals (Harman, 1973; Smith et al., 2000b; Smith et al., 2000a; Harman, 2003) produced in consequence of molecular oxygen utilization as suggested by the first studies on aging that associated increased caloric intake with increased ROS production in mitochondria (Weindruch et al., 1988; Sohal et al., 1994a). Many other sources of oxidation are presently known as also being involved but mitochondrial-related increased production of ROS still consensually indicated as mandatory for aging to occur.

The most common age-related mitochondrial alterations are in table 1.7.

Table 1.7: Common age-related mitochondrial alterations.

Age-related mitochondrial alterations	References
Mitochondrial abnormalities and decreased mitochondrial content	(Frenzel and Feimann, 1984; Atamna et al., 2002; Ames, 2003)
Increased 8-OHdG levels associated with mtDNA mutations	(Richter, 1988; Mecocci et al., 1993)
Defective ETC	(Hattori et al., 1991; Muller-Hocker et al., 1993; Lee et al., 1998; Lopez et al., 2000)
Increased levels of the 4977 basepair (bp) deletion or "common deletion" and other mtDNA mutations	(Ozawa, 1997; Wei, 1998)
Increase appearance of mtDNA deletion mutations associated with age related neurodegenerative disorders	(Hayakawa et al., 1991; Hayakawa et al., 1992; Mecocci et al., 1993; Trifunovic et al., 2005; Poon et al., 2006)
Decline in mtDNA content	(Harman, 1981; Wallace, 1992; Sohal et al., 1994b; Barazzoni et al., 2000)
Mitochondrial matrix vacuolization, shortened cristae and loss of dense granules	(Wilson and Franks, 1975)
Decline of mitochondrial respiratory chain function	(Cottrell and Turnbull, 2000)
Oxidative damage to mitochondrial proteins	(Sohal and Dubey, 1994)
Decreased state3/state 4 ratio	(Hansford, 1983; Hoch, 1992)
Decrease of cardiolipin content	(Shigenaga et al., 1994)
Increase of brain MAO-B activity	(Fowler et al., 1980)
Decrease in mitochondrial GSH levels	(Sastre et al., 2000)
Decline of energy production because of inefficient transport of electrons through ETC and increased ROS production	(Shigenaga et al., 1994)
Decreased catalytic activity of ADP/ATP translocator, ATPsynthase and carnitine transferase, proteins of the mitochondrial inner membrane	(Hoch, 1988)
Accumulation of oxidized lipids in the mitochondrial membrane with resultant increased membrane permeability and absence of mitochondrial respiratory control	(Shigenaga et al., 1994)
Reduced muscle mass in aging skeletal muscle associated with impaired mitochondrial enzymatic activity and decreased synthetic rate of mitochondrial protein fraction	(Holloszy and Coyle, 1984; Trounce et al., 1989; Dutta, 1996; Rooyackers et al., 1996; Dutta et al., 1997)

Footnotes: 8-OHdG-8-hydroxy-2'-deoxiguanosine; ETC-electron transport chain; mtDNA-mitochondrial DNA; MAO-B-monoamine oxidase B; GSH-Reduced glutathione; ROS-reactive oxygen species.

Additional general age related characteristics include increased protein oxidation (Oliver et al., 1987), decrease of cellular membrane fluidity (Huber et al., 1991), increase of superoxide dismutase (SOD) activity, decreased expression of catalase activity (Marttila et al., 1988b; Ciriolo et al., 1991; Benzi and Moretti, 1995), decrease of DNA repair activity (Rao, 1997; Michikawa et al., 1999; Atamna et al., 2000) and decrease of proteasome activity (Hayashi and Goto, 1998; Ponnappan et al., 1999; Keller et al., 2000).

Both ALC and selegiline are considered as powerful tools in preventing age related mechanisms. The protective effects of both the compounds are listed in the table that follows (table 1.8).

Table 1.8: Protective activities of acetyl-L-carnitine and selegiline in the prevention of age related phenomena's.

Compounds	Protective properties against age related alterations	References
Acetyl-L-carnitine	Restoration of the repiratory deficit of state IV in synaptic mitochondria	(Petruzzella et al., 1992)
	Reversion of the cholesterol/phospholipids ratio	(Paradies et al., 1992)
	Reduction of cardiolipin content of the inner mitochondrial membrane	(Paradies et al., 1997)
	Restoration of the transport of adenine nucleotides, pyruvate, phosphate and acylcarnitines	(Paradies et al., 1992; Paradies et al., 1994, 1995)
	Prevention of the reduced activity of cytochrome c oxidase on aged rat heart mitochondria	(Paradies et al., 1994; Paradies et al., 1997)
Selegiline	Increase of the life span of laboratory animals	(Milgram et al., 1990; Yen and Knoll, 1992; Kitani et al., 1993; Freisleben et al., 1994)
	Increased activity of CuZnSOD with consequent reinforced protection against oxidative stress	(Clow et al., 1991)

Footnotes: CuZnSOD- superoxide dismutase (CuZn isoform).

1.7 Monoamine systems and dysfunctions

MAOs are flavoprotein enzymes located in the outer mitochondrial membrane which main function lies in the regulation of intracellular monoamine stores. Deregulation of brain monoamine levels is a condition that is common to several neurodegenerative disorders, psychostimulant abuse and aging mechanisms. In all these states, the study of MAO activity seems to be important for either understanding or developing tools against the neurological deficits produced.

Here, a particular emphasis will be given to the altered neurological states decurrent from MAO dysfunction.

1.7.1 Brief description of monoamine oxidases

There are two known types of MAO isoforms, MAO-A and MAO-B, which differ on substrate affinity and inhibitor sensitivity (Johnston, 1968). MAO-A is found mainly in catecholaminergic neurons and MAO-B in the serotonergic ones and both are involved in the turnover of several neurotransmitters, such as DA, 5-HT and noradrenaline (NA) (Cooper et al., 1996). Excessive MAO activity with associated increased metabolism of monoamine neurotransmitters results in the increased formation of both H_2O_2 and monoamine derived aldehydes, as primary products of oxidation (Sinet et al., 1980; Hauptmann et al., 1996). Under basal metabolic conditions monoamine derived aldehydes are further oxidized by aldehyde dehydrogenase (ADH) to carboxylic acid (Youdim and Bakhle, 2006) and H_2O_2 is destoxified by catalase. However, in situations of increased metabolic activity some H_2O_2 could diffuse across mitochondrial

membranes and increase the levels of radicalar species that are formed during mitochondrial respiration. In these conditions, prevention of increased oxidative stress could be achieved by specific MAO inhibition.

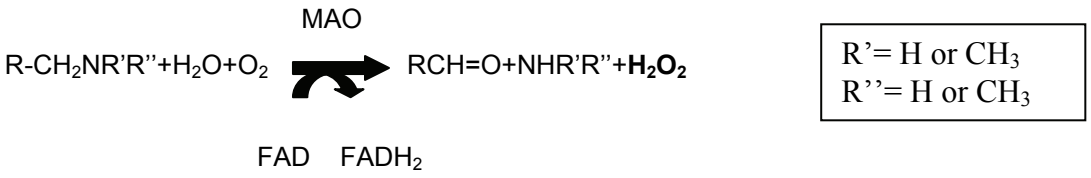

Figure 1.6: General reactions catalysed by MAO (Berry et al., 1994 a).

1.7.2 Selegiline and MAO-B inhibition

Selegiline is an irreversible MAO-B inhibitor (Haefely et al., 1990) and was the first drug known to inhibit MAO-B selectively (Knoll and Magyar, 1972). Selegiline is a propargylamine with a tertiary amino group and an acetylene group that differs from the compounds of the same family by the presence of a chiral carbon. This compound was previously shown to protect against MDMA, induced neurotoxic effects in the brain (Sprague and Nichols, 1995b, a) but its effectiveness in the treatment of neurological disorders, like PD, AD and depression, is the hallmark of its therapeutic usefulness namely in what concerns the improvement of the cognitive effects produced (Cohen, 1987; Portin and Rinne, 1983; Piccinin et al., 1990; Mendlewicz and Youdim, 1983; Quitkin et al., 1984; Mann et al., 1989).

The main neuroprotective actions of selegiline are summarized in the table 1.9.

Table 1.9: Selegiline improvement of neuronal functioning.

Selegiline improvement of neuronal functioning	References
Prevention against MDMA induced neurotoxicity in the brain	(Sprague and Nichols, 1995a, b)
Prevention of H_2O_2 formation	(Gerlach et al., 1996)
Treatment of Parkinson's disease	(Cohen, 1987)
Improvement of cognitive deficits in PD and AD	(Portin and Rinne, 1983; Piccinin et al., 1990)
Up-regulation of SOD and catalase activities	(Carrillo et al., 1994)
Increase in life expectancy	(Knoll, 1988, 1989; Freisleben et al., 1994)
Anti-depressant activity (doses of 30-40mg/day)	(Mendlewicz and Youdim, 1983; Quitkin et al., 1984; Mann et al., 1989)
Prevention of apoptosis	(Tatton and Greenwood, 1991; Wu et al., 1994; Tatton and Chalmers-Redman, 1996; Maruyama et al., 1998; Wadia et al., 1998; Xu et al., 1999; Szende et al., 2001)
Prevention of MPT opening	(Zoratti and Szabo, 1995; Lee et al., 2002)
Prevention against excitotoxic damage	(Mytilineou et al., 1997)
Increase of TH activity in hypothalamus and hypophysis	(De la Cruz et al., 1997)

Footnotes: H_2O_2-hydrogen peroxide; SOD-superoxide dismutase; MPT- Mitochondrial permeability transition, TH-Tyrosine Hydroxilase.

Selegiline improves the cognitive deficits observed in both PD and AD patients where it has the ability to produce mild ameliorations (Piccinin et al., 1990) and its activity in schizophrenia concerns the ability to improve some of the negative symptoms when combined with neuroleptic treatment (Perenyi et al., 1992).

Selegiline activity as an apoptosis preventing agent is mainly based in the regulation of the expression of some genes involved in the process such as Bcl-2 and Bax (Tatton and Chalmers-Redman, 1996; Xu et al., 1999), inhibition of the pro-apoptotic caspase-3 (Szende et al., 2001) and protection against peroxynitrite- and nitric oxide-induced apoptosis (Maruyama et al., 1998). MPT, known as being involved in some of the apoptotic mechanisms, is also inhibited by selegiline (Zoratti and Szabo, 1995; Lee et al., 2002) but through a mechanism different from MAO-B inhibition (De Marchi et al., 2003). Selegiline also protects DA neurons from excitotoxic damage associated with activation of NMDA subtype of glutamate receptors, through a mechanism that is independent from both MAO-B inhibition and competition for NMDA receptor binding (Mytilineou et al., 1997) and that seems to result from prevention of free radical formation and/or increased antioxidant defenses (Mytilineou et al., 1997).

When chronically administered, selegiline looses its selectivity for MAO-B inhibition and starts to inhibit MAO-A (Ekstedt et al., 1979; Zsilla et al., 1986; Vrana et al., 1992). In these conditions, progressive MAO-A inhibition with almost complete MAO-B inhibition will markedly decrease the metabolism of monoamines thus resulting in increased synaptic levels of 5-HT and DA and decreased levels of free radical production (Berry et al., 1994b). This condition is particularly important when the compound is used as an adjuvant to L-DOPA therapy in the treatment of PD and where the elevation of synaptic DA reaches levels that result in the symptomatic relief of patients. Besides these effects, chronic administration of selegiline is also described has being related with an increase of SOD activity (Knoll, 1988; Clow et al., 1991). This might be particularly important in the protection against free radical insults if accompanied by a simultaneous increase in the activities of catalase or GPx (Knoll, 1988; Carrillo et al., 1991; Berry et al., 1994b).

High doses of selegiline (30-40 mg/day) could be neurotoxic instead of neuroprotective and this feature can be explained in two ways: (i) at such high doses, selegiline has non-specific effects particularly by affecting MAO-A fuctioning, and in that way, changes the activity of neurotransmitters other than DA; (ii) the increase in the levels of selegiline-derived amphetamine metabolites may also lead to the negative toxic effects (Tariot et al., 1987).

Additional selegiline benefitial effects, at brain level, includes the improvement of meso-limbo-cortical DA neurons closely associated with cognitive processes (Brandeis et al., 1991); prevention of age-related pigment changes in the substantia nigra (Knoll et al., 1992); blockade of the neurotoxicity of compounds such as 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine used in animal models of Parkinson's disease (Tatton and Greenwood, 1991); protection of substantia nigra against oxidative insult (de la Cruz et al., 1996) and protection of DA neurons against the toxicity induced by glutathione depletion (Chang et al., 1997).

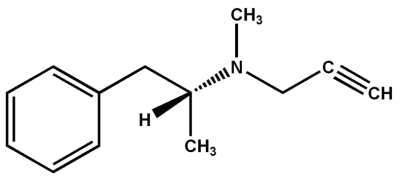

Figure 1.7: Chemical structure of selegiline.

The condition of increased oxidative stress by MAO dependent mechanisms is vulgarly associated with several altered neurological states and is one of the main targets of either prevention or treatment of those disorders. Selegiline, as already referred, could be a powerful tool when the protection against oxidative processes needs to be achieved. However, additional compounds with quite more unspecific antioxidant properties (independently of MAO activity) have also been considered as neuroprotective agents, as is the case of carnitine, which is described in the following section.

1.8 Carnitines

Carnitines exist endogenously in all mammalian species either as unesterified or esterified forms and have been reported as improving antioxidant and antiapoptotic properties. Absorbed from diet or biosynthesized in the liver and kidneys, carnitines are incorporated into the total body carnitine pool that comprises L-carnitine (LC) and short chain esters such as acetyl-L-carnitine (ALC). It's protective role against mitochondrial damage is well described (Virmani et al., 1995; Binienda et al., 2004). Carnitines exert a close interaction with MPT functioning by modulating it activity (Kashiwagi et al., 2001). Mitochondrial dysfunction leads to free Ca^{2+} induced opening of MPT that can therefore lead to apoptosis (Bernardi et al., 1994; Saris and Eriksson, 1995). Carnitines suppress the opening of these pore structures in the mitochondrial membranes (Starkov et al., 1994; Furuno et al., 2001; Kashiwagi et al., 2001), thus protecting against mitochondrial related cell death mechanisms.

Additional features of carnitines in mitochondrial integrity and function include prevention of loss of cardiolipin from mitochondrial membranes and control of both peroxisomal oxidation of fatty acids and production of ketone bodies (Virmani and Binienda, 2004). At the level of mitochondrial membranes, prevention of changes in both palmitolyl-CoA and other long-chain free fatty acid are also important additional properties (Virmani and Binienda, 2004).

From all the carnitine esters, ALC is the one that is more widely distributed in the body, presenting high levels in the brain (Shug et al., 1982) due to its increased ability to cross the blood-brain-barrier (Kido et al., 2001; Virmani and Binienda, 2004). Like the other carnitines, also ALC has important properties at the mitochondrial level (Virmani et al., 1995; Virmani et al., 2001). ALC is particullary abundant in the inner mitochondrial membrane and is primarily responsible for shuttlling long chain fatty acids used in metabolism through ß-oxidation into mitochondria, maintainance of cell membrane stability, increase in the free coenzyme-A (COA) by combining with acetyl and transporting it out of mitochondria, and transport of toxic acyl compounds (Virmani et al., 2003). Extensively used for prevention of aging processes, ALC is also used to attenuate the decrease of cardiolipin (Paradies et al., 1992), mtDNA transcription (Gadaleta et al., 1990), and to prevent the decrease of D1 subclass of striatal DA receptors (Sershen et al., 1991), characteristic of aging and mitochondrial neurodegenerative disorders like AD and PD (Beal, 2003).

The ability of carnitines to enhance the action of some antioxidants with efficacy in protecting against lipid peroxidation, such as GSH, selenium and Vitamin E, and reports on carnitine prevention against methamphetamine induced damage to CNS,

further support its therapeutic properties (Arockia Rani and Panneerselvam, 2001; Virmani et al., 2003).

Carnitines are quite capable to ameliorate mitochondrial activity and have been recently pointed out as being effective in other than metabolic activities. Neuroprotective, neuromodulatory and neurotrofic activities are thought as important characteristics that justify its use in counteracting various neurologic disease states associated with mitochondrial dysfunctions (Beal, 2003; Virmani and Binienda, 2004).

1.9 Involvement of mitochondrial damage in the neurotoxicity of drugs of abuse

1.9.1 Mitochondria as targets of drug toxicity

Drugs abuse, particularly of those that interfere with brain monoaminergic systems, may affect mitochondrial function that is generally correlated with an increased permeability of the inner mitochondrial membrane or/and the oxidation of important mitochondrial matrix and inner membrane components such as the ETC constitutive subunits. All of the drugs here described have serious implications with the monoaminergic systems and, in that way, all of them are able to interfere with the mitochondrial enzymes responsible for monoamine metabolism (MAOs). Drug's interference with mitochondrial metabolic performance possibly begins here.

1.9.1.1 Cocaine

Little is known about the impact of cocaine abuse on mitochondrial function. However, recently, some reports of an association between drug abuse and organelle dysfunction started to emerge. The mechanism is not yet clarified, but the alterations verified are similar to those obtained with methamphetamine and 3,4-methylenedioxymethamphetamine. The pharmacodynamics of cocaine basically begins with binding to monoamine transporters of DA, 5-HT and NA, with the subsequent inhibition of their reuptake into presynaptic nerve endings (Ritz et al., 1990), resulting in sustained high amounts of monoamines in the synaptic cleft. Receptor overstimulation and/or auto-oxidation of monoamine neurotransmitters may then lead to neurotoxicity. There are several reports of increased oxidative stress resulting from cocaine administration, with obvious consequences at the level of energy production focusing biochemical alterations on respiratory function (Gottfried et al., 1986; Boyer and Petersen, 1991) as well as diminished expression of several important subunits of the mitochondrial genome (Couceyro et al., 1997; Dietrich et al., 2004).

Some of the general cocaine-induced biochemical alterations are listed in the table that follows (Table 1.10).

Table 1.10: Common cocaine-associated biochemical alterations.

Cocaine-induced biochemical alterations	References
Downregulation of ND4 gene expression in the nucleus accumbens	(Couceyro et al., 1997)
Downregulation of mitochondrial complexes I and IV transcripts from the cyngulate cortex	(Dietrich et al., 2004)
Increased production of ROS	(Votyakova and Reynolds, 2001; Liu et al., 2002)
Increased formation of H_2O_2 in the frontal cortex	(Dietrich et al., 2005)
Lipid peroxidation in both frontal cortex and striatum	(Dietrich et al., 2005)
Increased activity of SOD and GPx	(Dietrich et al., 2005)
Altered glucose metabolism in prefrontal cortex	(Goldstein et al., 2004)
Increased proteasome enzymatic activity	(Dietrich et al., 2005)

Footnotes: ND4- subunit IV of mitochondrial complex I; ROS.reactive oxygen species; H_2O_2-hydrogen peroxide; SOD-superoxide dismutase; GPx-glutathione peroxidise

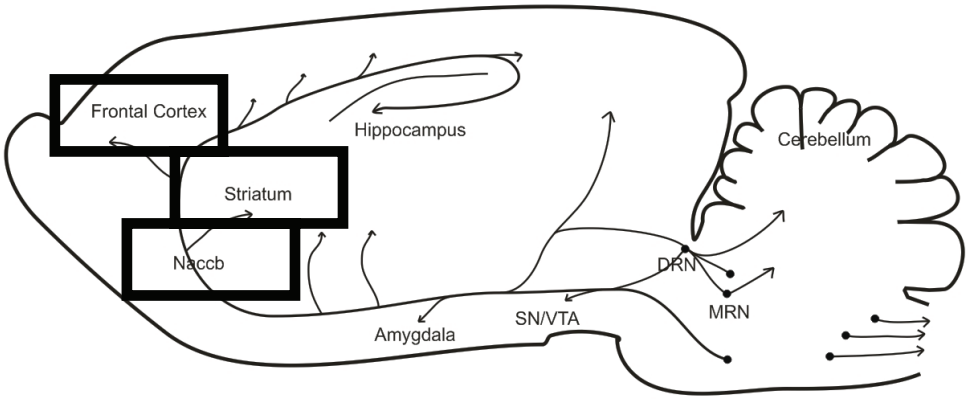

Figure 1.8: Brain targets of cocaine-induced toxicity in the rat.

1.9.1.2 Methamphetamine (METH)

METH (N-methyl-O-phenylisopropylamine) has several neuropsychiatric, neurochemical and neurodegenerative effects associated with its abuse (Wilson et al., 1996; Ernst et al., 2000). METH administration results in the release of monoamine neurotransmitters to the cytoplasm and extracellular space with consequent neuronal receptor overstimulation and dysfunction (Cubells et al., 1994; Ernst et al., 2000; Volkow et al., 2001; Cadet et al., 2003a). General decreases in the brain levels of DA, 5-HT and NA, as well as specific decreases in striatal DA and 5-HT levels and decreased tryptophan hydroxylase (TPH) activity are the neurochemical alterations

more frequently reported (Seiden and Sabol, 1996; Cadet and Brannock, 1998; Cadet et al., 2003b) and brain damage is specifically observed in striatum, cortex and hippocampus (Hip) (Fig 1.9) (Seiden et al., 1993; O'Callaghan and Miller, 1994; Schmued and Bowyer, 1997; Deng et al., 1999).

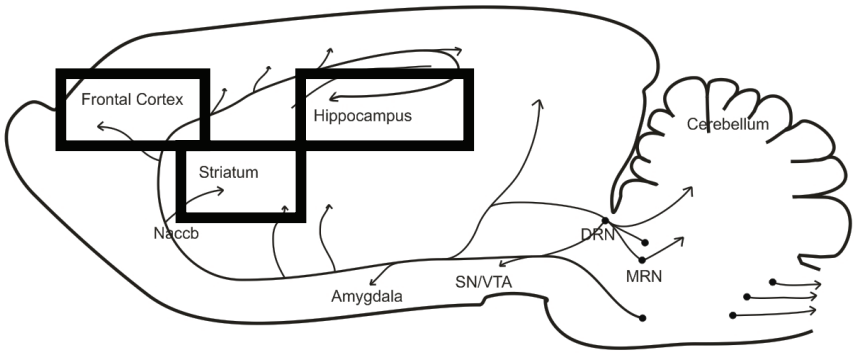

Figure 1.9: Brain targets of METH-induced toxicity in the rat.

Brain injury derived from METH abuse is closely associated with increased ROS and RNS formation and mitochondrial damage (Davidson et al., 2001; Virmani et al., 2002) as suggested by the prevention of the neurotoxic effects with concurrent administration of free radical scavengers (De Vito and Wagner, 1989; Cappon et al., 1996; Hirata et al., 1998).

Acute exposure to METH leads to an increase in the levels of extracellular DA (Sulzer et al., 1995) in the cell wich further leads to the formation of DA quinones (LaVoie and Hastings, 1999), ROS and increased levels of extracellular glutamate (Yamamoto and Zhu, 1998) . The rise in extracellular glutamate leads to an increase in intracellular calcium that could mediate the activation of the NOS pathway with subsequent increased RNS production or/ and to the induction of MPT in mitochondria followed by the activation of caspases. The increased formation of ROS and RNS produces damage to mitochondrial constituents and to the overall cellular structure (Cadet et al., 2003). Decreased production of ATP could further occur and damage of nerve terminals with coupled cell death mechanisms is easily achieved (Stephans et al., 1998; Yamamoto and Zhu, 1998; Imam and Ali, 2000, 2001).

The main biochemical alterations in the brain, induced by METH, are summarized in the table that follows (Table 1.11).

Table 1.11: Common METH-associated biochemical alterations.

METH associated general biochemical alterations	References
Bioactivation of METH by PHS-1 to free radical intermediates with associated increased DNA oxidation	(Jeng et al., 2006)
Increased extracellular concentration of lactate	(Stephans et al., 1998)
Increased formation of ROS	(De Vito and Wagner, 1989; Cadet et al., 1994; Giovanni et al., 1995)
Increase in oxidized and reduced glutathione levels	(Harold et al., 2000)
Lipid peroxidation and protein carbonylation	(Jayanthi et al., 1998; Gluck et al., 2001)
Decrease in cerebral glucose metabolism after high dose regimens	(McBean et al., 1990; Sharkey et al., 1991; Huang et al., 1999)
Increased metabolism of DA to DOPAC by MAO with associated increased ROS production	(Spina and Cohen, 1989; Melo et al., 2005)
Substantial loss of striatal DA transporters (DAT)	(McCann et al., 1998; Volkow et al., 2001)
Decrease in the concentrations of DA uptake sites	(Steranka and Sanders-Bush, 1980; Wagner et al., 1980; Ricaurte et al., 1982)
Decrease of vesicular monoamine transporters (VMAT-2)	(Frey et al., 1997)
Decreased activity of tyrosine hydroxilase	(Hotchkiss and Gibb, 1980a)
Damage of 5-HT axon terminals in rats	(Fukui et al., 1989; Axt and Molliver, 1991)
Reduced activity of TPH	(Hotchkiss and Gibb, 1980b; Fleckenstein et al., 1997)
Significant decreases in synaptossomal 5-HT uptake	(Trulson and Trulson, 1982; Woolverton et al., 1989)
Decrease in 5-HT transporter binding sites	(Seiden and Sabol, 1995)
Formation of inclusion bodies after low doses	(Fornai et al., 2002; Fornai et al., 2004b; Fornai et al., 2004a; Lazzeri et al., 2006)
Hyperthermia	(Bowyer et al., 1994; Miller and O'Callaghan, 1994; Farfel and Seiden, 1995)
Decreases in ATP synthesis and associated secondary excitotoxic mechanisms	(Stephans et al., 1998; Yamamoto and Zhu, 1998; Imam and Ali, 2000, 2001)
Increase in extracellular glutamate concentrations	(O'Dell et al., 1991; Berger et al., 1992; Nash and Yamamoto, 1992) (Nash and Yamamoto, 1992, 1993; Stephans and Yamamoto, 1994; Yamamoto and Zhu, 1998)
NMDA receptor activation	(Nash and Yamamoto, 1992, 1993; Stephans and Yamamoto, 1994; Yamamoto and Zhu, 1998)
Decrease of COXI activity	(Prince et al., 1997; Burrows et al., 2000) (Cleeter et al., 1994; Lizasoain et al., 1996)
Inhibition of the mitochondrial enzymes involved in energy production	(Yagi and Hatefi, 1987; Zhang et al., 1990)
Increased MAO activity	(Spina and Cohen, 1989)
MPT opening and associated Ca^{2+} release	(Davidson et al., 2001; Fornai et al., 2004b)
Alteration of mitochondrial pH with deregulation of ATP synthase functioning and disruption of the mitochondrial membrane integrity	(Davidson et al., 2001)
Activation of mitochondria-mediated caspase-dependent and independent pathways in cell death mechanisms	(Jayanthi et al., 2001; Deng et al., 2002) (Subramaniam et al., 2004)
Increase of pro-apoptotic and decrease of anti-apoptotic members of the Bcl-2 family of proteins	(Stumm et al., 1999)
Inhibition of mitochondrial function	(Brown and Yamamoto, 2003; Brown et al., 2005)

Footnotes: METH-methamphetamine; PHS-1prostaglandine H synthase; ROS-reactive oxygen species; DA-Dopamine; DOPAC-3,4-dihydroxy-phenylacetic acid; MAO-monoamine oxidase; DAT-Dopamine transporters; VMAT-vesicular monoamine transporters; 5-HT-serotonin; TPH-tryptophan hydroxilase; NMDA-N-methyl-D-aspartate; COXI-cytochrome c oxidase, subunit I,

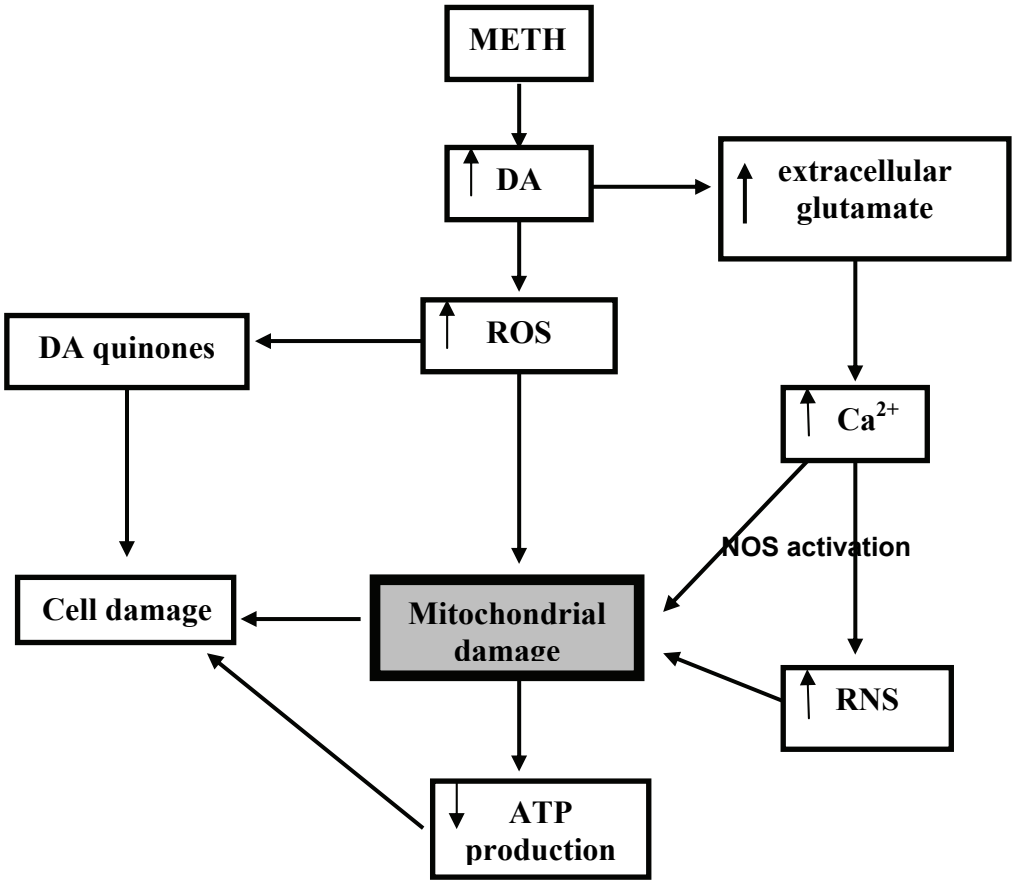

Figure 1.10: Schematic representation of METH-induced toxicity.METH-methamphetamine, DA-dopamine; ROS-reactive oxygen species; RNS-reactive nitrogen species; NOS-nitric oxide synthase.

METH induced neurotoxicity can be reduced by the administration of antioxidant compounds as ascorbate, vitamine E and selenium (De Vito and Wagner, 1989; Kim et al., 1999; Imam and Ali, 2000) and MAO-B inhibitors as selegiline (Bartzokis et al., 1998; Paterson and Tatton, 1998; Tatton and Chalmers-Redman, 1998). L-carnitine (LC) administration significantly reduces the increase in peroxynitrite levels observed in mice striatum (Virmani et al., 2002) and modulates the membrane permeability and MPT opening thus representing a possible way to prevent METH-induced neurotoxicity (Virmani et al., 2003).

1.9.1.3 3,4-methylenedioxymethamphetamine (MDMA)

MDMA is a substituted amphetamine drug whose action at brain level is exerted mainly by elevation of the levels of the monoamine neurotransmitters 5-HT and DA. The alterations in the serotonergic system are the most pronounced and are characterized by an increase in extracellular 5-HT concentrations, resulting from reuptake inhibition

(Steele et al., 1987) and stimulation of release (Berger et al., 1992). The alterations in the dopaminergic system mainly consist in a persistent loss of DA (Ali et al., 1991; Cadet et al., 1995).

The most relevant biochemical consequences of MDMA exposure are listed in the table below (Table 1.12).

Table 1.12: Common MDMA-associated biochemical alterations.

MDMA associated general biochemical alterations	**References**
Hyperthermia	(Schmued, 2003; Freezer et al., 2005)
Inhibition of MAO-A catabolism by acting as a competitive inhibitor	(Leonardi and Azmitia, 1994)
Ocurrence of the "serotonergic syndrome" when taken together with MAO-A inhibitors	(Grahame-Smith, 1971; Parrott, 2002; Ener et al., 2003; Vuori et al., 2003)
Increased concentration of brain tyrosine	(Breier et al., 2006)
Increased glycogen breakdown in vitro	(Poblete and Azmitia, 1995)
Long-term decrease in cerebral glucose metabolism after high dose administration	(McBean et al., 1990; Sharkey et al., 1991; Huang et al., 1999)
Decreases of cytochrome oxidase activity in specific brain areas (caudate, nucleus accumbens and substantia nigra) after acute high dose administration	(Burrows et al., 2000)
Decreased brain 5-HT levels	(Faria et al., 2006)
Increased DA metabolism by MAO-B in depleted 5-HT terminals	(Sprague and Nichols, 1995a)
Lipid peroxidation processes	(Sprague and Nichols, 1995a)

Footnotes: MDMA-3,4methylenedioxymethampetamine; MAO-A-monoamine oxidase A; 5-HT-serotonin; DA-dopamine; MAO-B-monoamine oxidase B.

In this thesis a detailed study concerning the effects of MDMA on brain mitochondria of adolescent male Wistar rats was performed. For a better understanding of its effects, a brief description of the drug and its different mechanisms of neurotoxicity are presented below.

1.10 Brief history of MDMA

MDMA was first synthesized in 1912 as an anticoagulant and patented by Merck in 1914 (Schmued, 2003). It was initially used as an adjuvant of psycotheraphy in the 1970, but its therapeutic utility was never established. In the 1980s, MDMA was given the alternative name of “ecstasy” and appeared as a recreational drug connected to the underground world of dance parties or raves. At the same time, however, evidences started to emerge that classified it as having potentially damaging effects to the brain serotonergic system and in 1985 it was finally classified as a drug with no accepted medical use. 3,4-methylenedioxyamphetamine (MDA), the demethylated metabolite of MDMA was patented in 1960 as tranquilizer and as an appetite inhibitor in 1961 but was never marketed for either of the uses (Climko et al., 1986; Colado et al., 1997; Colado et al., 1999a).

Presently, besides the known detrimental toxic effects of the drug, the number of adolescent and young people who use and consider it as a safe substance is still presenting a large increase fundamentally because of enhanced energy, endurance and sociability acquired after intake (Peroutka et al., 1988). MDMA is rarely used alone and is frequently reported as taken in combination with other drugs, such as marijuana, LSD, viagra and METH. MDMA tablets often differ in composition and the amount of pure MDMA per tablet may vary from 70 to well over 100 mg (Milroy et al., 1996; Sherlock et al., 1999). The sympathomimetic action for which the drug is valued by the users, though is also responsible for both the severe chronic and acute toxic effects. The most common immediate symptoms include increased locomotor activity, which is closely associated with increases in body temperature and related dehydratation and/or exhaustion. Muscle tension, maxillary contraction, altered vision, nausea and sweating are also included in the most commonly observed physiological acute effects (Shulgin, 1986; Peroutka et al., 1988) and additional overdose effects include renal and heart failure, hypertension, and panic attack. At a long-term, the effects more often described are paranoia, anxiety and depression.

1.10.1 General effects on synaptic neurotransmission under MDMA exposure

MDMA primary physiological response is the increased release of several monoamine neurotransmitters, namely 5-HT, NA and DA (Shulgin, 1986; Green et al., 1995; Huether et al., 1997), followed by binding and activation of the respective transporters (Slikker et al., 1989).

5-HT release has a major role in MDMA induced neurotoxicity and related characteristic behavioural and chemical alterations.

Increased DA release (Stone et al., 1986) and decrease of DA trasporter reuptake are some of the consequences observed in the dopaminergic system (Fleckenstein et al., 1999). NA release is responsible for both the effects shared with amphetamine (Kalant, 2001) and for the adverse reactions on the cardiovascular system.

1.10.2 MDMA metabolism

MDMA metabolism is mainly performed in the liver by CYP2D6 (Wu et al., 1997) but several additional enzymes are also involved. The rapid saturation of enzymatic activity with relatively low MDMA concentrations accounts for the increased risk of severe toxic responses with slight increases of drug concentrations and the low rate of MDMA elimination from the body explains the persistence of some symptoms two days after intake (Kalant, 2001).

MDMA metabolism leads to the production of MDA and both MDMA and MDA can be further *O*-demethhylenated to N-methyl-α-methyldopamine (N-Me-α-MeDA) and α-methyldopamine (α-MeDA) (Lim and Foltz, 1988; Kumagai et al., 1991). Both these two metabolites are catechols that could be further oxidized to the correspondent *o*-quinones which may easily suffer redox reactions and lead to the formation of ROS and RNS (Bolton et al., 2000; Remiao et al., 2002; Capela et al., 2006a). Besides, as Michael acceptors these reactive substances could also induce cellular damage of important molecular structures such as proteins and DNA (Capela et al., 2006a). Alternatively, the conjugation with GSH leads to the formation of a glutathionyl adduct (5-(GSH)-α-MeDA) (Hiramatsu et al., 1990; Carvalho et al., 2004) that can also easily suffer redox reactions thus giving an additional contribute for the explanation of the MDMA-induced toxic effects.

MDMA is produced as a racemic mixture of the stereoisomer S(+) -MDMA and the R (-) –MDMA, the first being more potent than the last one (Schechter, 1987). The two stereoisomers differ in the rate of metabolism (Fitzgerald et al., 1989) and this contributes for the differences in the toxic effects produced.

1.10.3 MDMA exposure variables

The neurotoxic effects of MDMA are dependent on some intrinsic and extrinsic variables, the dose and via of administration not being always linearly correlated with the neurotoxic effects produced. Studies with different frequencies of administration described single acute exposures as being harmless than repetitive exposures

because even non neurotoxic doses are able to become neurotoxic when a repetitive protocol is applied (Battaglia et al., 1988b; Battaglia et al., 1988a; O'Shea et al., 1998b). High acute doses are capable to produce a biphasic response in the way that the partial recovery of the initial acute decrease in the 5-HT levels is again followed by a chronic depletion that can persist for two weeks (Schmidt, 1987; Stone et al., 1987).

Regarding the via of administration, the studies performed until the date found that the 5-HT depletions are quite dependent on the animal models used. In the rat, both subcutaneous and oral administration gives rise to similar 5-HT hippocampal depletions (Finnegan et al., 1988) but in non-human primates the results of either oral or subcutaneous administration are dependent on the species used (Ricaurte, 1989).

Differences in drug vulnerability in distinct animal models are also evidenced and nonhuman primates are more susceptible than rats, thus suggesting an even increased susceptibility in humans (Insel et al., 1989; Ricaurte et al., 1992; Ricaurte and McCann, 1992; Ali et al., 1993; Fischer et al., 1995a). The increased vulnerability to the neurotoxic effects of the drug in higher species is hypothesized by many authors as being a direct consequence of the increased time of drug permanence in plasma,. with higher species excreting the drugs slower and thus forming metabolites in greater amounts (Cho et al., 1990; Mas et al., 1999).

The animal's age, as it will be lately discussed, is also an important factor to take into account. The main evidences point to an increased vulnerability to the effects of the drug at around postnatal day (PND) 35 that is thought as being closely related with alterations in the dopaminergic system (Aguirre et al., 1998).

The environment of exposure also needs to be considered and will be discussed with more detail. High ambient temperatures result in increased neurotoxic effects mainly regarding 5-HT depletions (Broening et al., 1995b; Colado et al., 1995).

Additional parameters to take into consideration include animal body temperature, housing and status of hydratation (Gordon and Fogelson, 1994; Dafters, 1995).

1.10.4 MDMA and Hyperthermia

One of the most prominent acute physiological effects of MDMA is the increase of body temperature.

MDMA related alteration of body homeostasis results from complex abnormal interactions between the serotonergic input to the hypothalamo-pituitary-thyroid axis and the sympathetic nervous system (Dafters and Lynch, 1998; Sprague et al., 2003) but many other factors such as neurotransmitters, hormones, neuropeptides, immune system-related proteins (Dinarello, 1999; Roth et al., 2004), the tumor necrosis factor (Connor, 2004) and the uncoupling protein 3 (UCP3) (Mills et al., 2003) are also

reported as being involved. Emergency episodes with temperatures as high as 43ºC are frequently described (Henry et al., 1992; Randall, 1992) and maximum body temperatures are closely correlated with mortality (Gowing et al., 2002). Under these circumstances, both muscle breakdown and kidney failure together with the increased vasoconstriction produced, slow the heat loss from the body (Gordon et al., 1991; Fitzgerald and Reid, 1994). Neurological alterations such as delirium and convulsions, are associated physiological phenomena (Green et al., 2003).

The dose and ambient temperature represent determinant components on deregulation of body thermostability and associated development of toxicity in mature animal models (Malberg and Seiden, 1998). Higher doses and ambient temperatures result in hyperthermia, lower doses and lower ambient temperatures lead to hypothermia and intermediate levels of both the doses and ambient temperatures produce biphasic response patterns of hypothermia followed by hyperthermia (Gordon et al., 1991; Dafters, 1994; Malberg et al., 1996). Gordon et al showed that MDMA (30 mg/kg) produces an hyperthermic response at an ambient temperature of 30ºC that is not observed at an ambient temperature of 20ºC and that is converted to an hypothermic response at ambient temperatures of 10ºC (Gordon et al., 1991). This dose/ temperature correlation, however, is not applicable to humans since the temperature is not always dependent on dose taken (Henry et al., 1992). In humans the conditions that surround the subject during drug exposure such as the intense physical activity, dehydratation and high ambient temperature, all of them typical of rave and dance parties, seem to be involved (Green et al., 2003).

MDMA-induced hyperthermia also plays an important role in the development of long-term toxicity to the 5-HT terminals and these results are confirmed by both the attenuation of the toxic effects (Malberg et al., 1996; Fleckenstein et al., 1997) and free radicals formation under cool ambient temperatures (Globus et al., 1995; Kil et al., 1996) and by the increase in neurotoxicity under hyperthermic conditions. It is important to focus, however, that the neurotoxic damage could occur without hyperthermia (Broening et al., 1995a) since the increases in body temperature, on their own, are not capable of damaging serotonergic structures.

Prolonged hyperthermia and decreased mitochondrial immunoreactivity have also been associated (Burrows et al., 2000), suggesting a possible decrease of energy stores (Madl and Allen, 1995) and joining together the mitochondrial dysfunction in the collection of MDMA-hyperthermic related events.

Prevention of hyperthermia can be achieved by lowering ambient temperature or by coadministration of other drugs such as haloperidol, diazepam and dizocilpine that are also able to protect against both serotonergic and dopaminergic neurotoxicity (Bowyer et al., 1994; Farfel and Seiden, 1995; Miller and O'Callaghan, 1995; Malberg et al., 1996).

1.10.5 MDMA-induced toxicity to the serotonergic system

MDMA- induced damage to serotonergic terminals in both animals and humans is quite extensively reported (McCann et al., 1994; Steele et al., 1994). The most commonly characterized effects on the serotonergic system are summarized on table 1.13.

Table 1.13: MDMA-induced effects on the serotonergic system.

MDMA-induced effects on the serotonergic system	References
Degeneration of serotonergic nerve endings	(McCann et al., 1994; Steele et al., 1994; Green et al., 2003)
Decrease of 5 –HT uptake sites	(Battaglia et al., 1987)
Reduced immunostaining of 5-HT axons in neocortex, striatum and hippocampus	(O'Hearn et al., 1988)
Decrease of TPH activity	(Schmidt, 1987; Stone et al., 1987; O'Hearn et al., 1988; Wilson et al., 1989)
Decrease in the concentration of 5-HT and 5-HIAA	(Battaglia et al., 1987; Stone et al., 1987; Battaglia et al., 1988; McKenna and Peroutka, 1990; Hewitt and Green, 1994; Colado et al., 1997)
Decreased 5-HT_2 receptors density after acute exposure Increased 5-HT_2 receptor density after long-term exposure	(Peroutka and Snyder, 1980a, b; Kellar et al., 1981; Sharif et al., 1989; Stockmeier and Kellar, 1989)
Blockade of SERT	(Shulgin, 1986; Iravani et al., 2000; Liechti et al., 2000)

Footnotes: 5-HT-Serotonin; 5-HIAA-5-hydroxyindole acetic acid; TPH- Tryptophan hydroxilase; $5HT_2$ receptors- serotonin receptors (type 2).

MDMA exposure produces selective serotonergic degeneration of 5-HT nerve endings (Green et al., 2003). 5-HT released stimulates seven classes of 5-HT receptors and fourteen 5-HT receptor subtypes (Barnes and Sharp, 1999). The $5\text{-}HT_{2A}$ receptor subtype, in particular, is quite implied in MDMA induced toxicity as showed by several in vitro studies performed with some receptor antagonists like ketanserin and R-96544 that proved efficacy in protecting against some of the MDMA-induced neurotoxic events (Capela et al., 2006a).

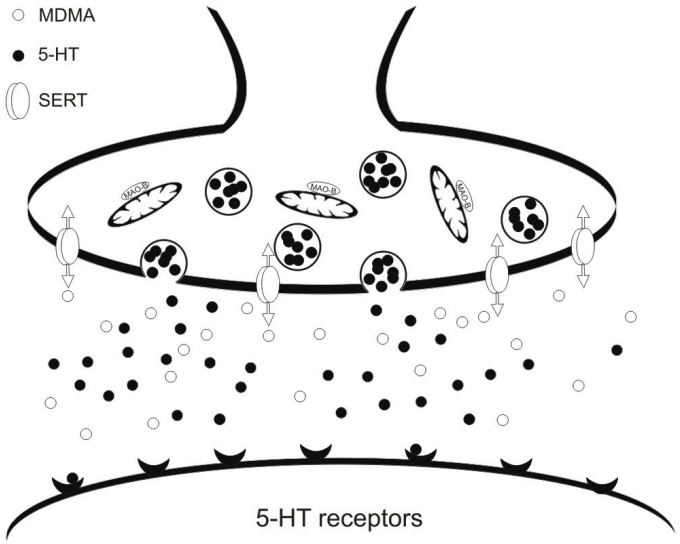

Figure 1.11: Schematic representation of 3,4-methylenedioxymethamphetamine (MDMA) primary action on serotonergic cells. MDMA reaches the synaptic cleft on serotonergic nerve endings and induces the release of serotonin from the synaptic vesicles with subsequent overstimulation of the post-synaptic receptors.

5-HT decreased or increased receptor density depends respectively on acute or long-term studies on drug exposure being 5-HT_{2A} receptor downregulation in acute exposure followed by a long-term up-regulation that is accompanied by respectively high and low levels of synaptic 5-HT (Peroutka and Snyder, 1980a, b; Kellar et al., 1981; Sharif et al., 1989; Stockmeier and Kellar, 1989) .

5-HT receptors are also importantly related with cognition, emotion (Busatto, 1996) and memory impairment and this behavioural alterations are again closely related with both the serotonergic neurotoxicity (Bolla et al., 1998) and alterations in the serotonergic function (McCann et al., 1998).

Besides the initial increase of the 5-HT levels on the synaptic cleft, in a posterior phase, an abrupt augmentation of 5-HT inside the pre-synaptic nerve endings also occurs. At this stage, excess of 5-HT is largely metabolized by MAO's located on the external mitochondrial membranes.

From the existent isoforms of MAO's, MAO-A has a higher afinity for 5-HT as a substract, deaminating it into the neurochemically inactive 5-HIAA (Haefely et al., 1992; Haefely et al., 1993). Nontheless, MDMA-induced chronic 5-HT loss also requires the activity of MAO-B as proved with studies where the 5-HT depletion induced by the drug was suppressed in several brain areas of MAO-B knockout mice (Fornai et al., 2001), and, moreover, although the metabolism of MAO-B is only residual in the presence of

MAO-A, it is fully effective in the absence of the latter, as it happens inside the serotonergic nerve endings (Fowler et al., 1982; Shih et al., 1999a). Oxidative deamination of 5-HT further produces H_2O_2 which might subsequently be converted into the highly reactive hydroxyl radical that is able to damage several macomolecular structures (Fig. 1.12).

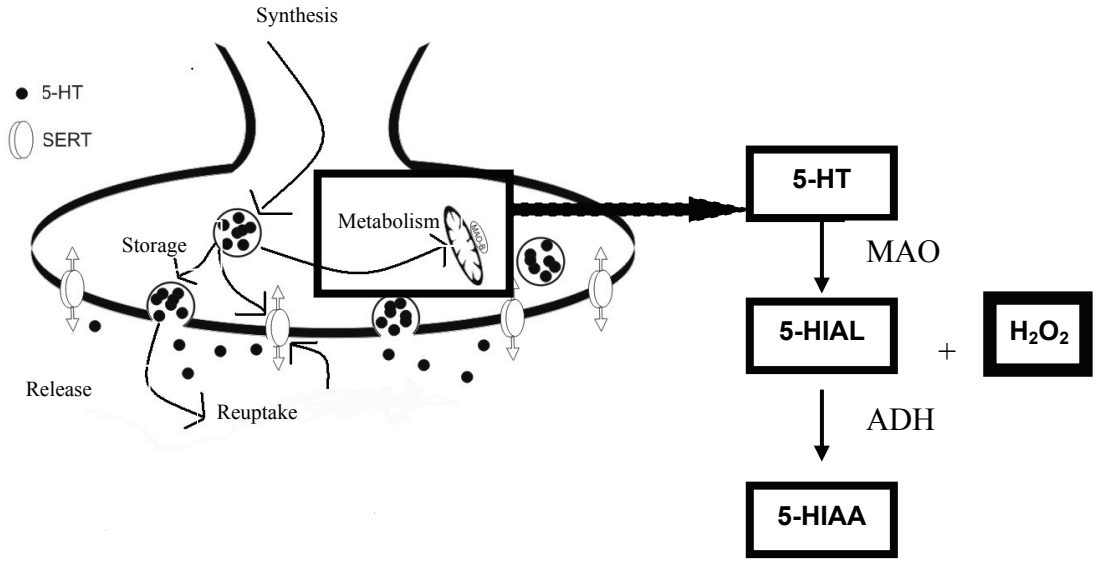

Figure 1.12: MAO mediated 5-HT metabolism inside pre-synaptic nerve endings. Serotonin is metabolized by MAO located in the external mitochondrial membrane. The primary products of oxidation are 5-hydroxyindole acetaldehyde (5-HIAL) and hydrogen peroxide (H_2O_2). 5-HIAL is further destoxifyed by aldehyde dehydrogenase (ADH) to 5-hydroxyindole acetic acid (5-HIAA).

The intracellular oxidative stress produced due to the sustained overproduction of pro-oxidant species is involved in several pathological responses, including drug toxicity and if in basal metabolic conditions, neurons present an intrinsic ability to protect themselves against oxidative stress, when that capacity is overwhelmed, like after MDMA exposure, a toxic response might occur.

MDMA, by itself, is also capable of increasing the generation of ROS and RNS after metabolic breakdown (Hiramatsu et al., 1990; Colado et al., 1997) and hydroxyl formation is particularly evident in both striatum and Hip (Gudelsky et al., 1994; Colado et al., 1997; Colado et al., 1999b; Shankaran et al., 1999) thus being a factor of major concern due to its dangerous indiscriminate and extreme activities. α-MeDA, one of the MDMA metabolites, following oxidation, is able to conjugate with GSH to form the pro-oxidant adduct 5-(glutathione-S-yl)-α-methyldopamine (Hiramatsu et al., 1990; Beitia et al., 1999; Carvalho et al., 2001), the consequent depletion of GSH stores being an additional toxic primary inducing factor (Carvalho et al., 2004). Beside this, the proper

MDMA metabolites are also able to suffer redox cycling into semiquinone radicals that in turn are capable to induce the generation of both ROS and RNS (Bolton et al., 2000; Remiao et al., 2002) and apoptotic cell death mechanisms (Capela et al., 2006b) .

MDMA capacity to decrease SOD and GPx activities (Jayanthi et al., 1999) is also evidenced as part of the oxidative related events related to MDMA-induced cell death mechanisms (Commins et al., 1987; Schmued, 2003).

The enzymatic oxidation of DA by MAO-B previously referred constitutes an additional source of oxidative injury because of the H_2O_2 formed (Cohen, 1987) that in the presence of Fe could give rise to the formation of hydroxyl radicals via the Fenton reaction (Olanow, 1992).However, proper DA autooxidation also needs to be considered as an important factor (Graham, 1978; Fornstedt et al., 1989).

Recently, new evidences of the involvement of RNS in MDMA induced neurotoxicity have started to emerge.

NO, a known cytochrome oxidase inhibitor (Cleeter et al., 1994; Lizasoain et al., 1996) is being pointed as possibly involved in the decrease of the mitochondrial cytochrome oxidase activity after drug exposure (Burrows et al., 2000) and both increased NO release and activation of NOS pathway following METH or MDMA administration are already reported (Abekawa et al., 1996; Zheng and Laverty, 1998b).The mechanism basically consists in an MDMA-induced increase in Ca^{2+} concentration and consequent activation of the Ca^{2+}/calmodulin dependent NOS pathway (Zheng and Laverty, 1998b). The resulting NO formed is able to react with the superoxide anion and thus form the highly reactive free radical $ONOO^-$ that is able to impair mitochondrial activity (Brorson et al., 1999) and induce cellular damage (Dykens, 1994).

Additional consequences of MDMA-induced increase in Ca^{2+} concentrations include alterations in both the phenomena's of MDMA-SERT interactions by means of the translocation of a kinase C dependent Ca^{2+} protein (Kramer et al., 1998) and long-term MDMA-induced DA depletions that are further prevented by the administration of NOS inhibitors (Colado et al., 2001).

MDMA induced oxidative stress could be prevented by both the administration of free radical scavengers or antioxidants (Schmidt and Kehne, 1990; Colado and Green, 1995; Gudelsky, 1996; Capela et al., 2006a) and by sulfhydryl reducing conditions that are able to attenuate the MDMA-induced decrease of TPH activity (Stone et al., 1989a). The long-term depletions of brain 5-HT are significantly diminished by antioxidants administration (Gudelsky, 1996; Shankaran et al., 2001). The use of NOS inhibitors resulted in neuroprotection against METH or MDMA-induced neurotoxic

effects mainly at the level of both DA and 5-HT depletions (Itzhak and Ali, 1996; Ali and Itzhak, 1998; Zheng and Laverty, 1998a; Itzhak et al., 2000; Colado et al., 2001; Darvesh et al., 2005; Capela et al., 2006a).

The pro-oxidant conditions and resulting oxidative stress status induced by MDMA are summarized in the table 1.14.

Table 1.14: Targets of MDMA-induced oxidation.

Targets of MDMA-induced oxidation	Related events	References
Increased formation of $HO^{\cdot}$	MDMA metabolic breakdown	(Hiramatsu et al., 1990; Gudelsky et al., 1994; Colado et al., 1997; Colado et al., 1999; Shankaran et al., 1999)
	Increased metabolism of monoamine neurotransmitters by MAO	(Cohen, 1987; Olanow, 1992)
	Hyperthermic events	(Powers et al., 1992; Flanagan et al., 1998)
GSH depletion	Formation of pro-oxidant adducts with MDMA metabolites	(Hiramatsu et al., 1990; Beitia et al., 1999; Carvalho et al., 2001)
Increased formation of ROS and RNS	MDMA metabolites redox cycling into semiquinone radicals	(Bolton et al., 2000; Remiao et al., 2002)
	Activation of the Ca^{2+}/calmodulin dependent nitric oxide synthase pathway	(Zheng and Laverty, 1998)
	Impairment of mitochondrial activity	(Brorson et al., 1999)
Apoptotic cell death mechanisms	MDMA metabolites redox cycling into semiquinone radicals (further mechanisms described in the table that describes MDMA-induced apoptotic related events)	(Capela et al., 2006b)
Decrease of SOD and GPx activity	Cell death mechanisms Free radical injury	(Commins et al., 1987; Jayanthi et al., 1999; Schmued, 2003)
Lipid peroxides formation	Free radical injury	(Sprague and Nichols, 1995b)
Altered concentration of endogenous antioxidants vitamin E and ascorbic acid	Free radical injury	(Shankaran et al., 2001)
DA autoxidation	Free radical injury	(Graham, 1978; Fornstedt et al., 1989)
Increased intracellular Ca^{2+} concentrations	Activation of Ca^{2+}/calmodulin-dependent NOS pathway	(Zheng and Laverty, 1998)

Footnotes: MDMA-3,4-methylenedioxymethamphetamine; $HO^{\cdot}$-Hydroxil radical; GSH-reduced glutathione; ROS-reactive oxygen species; RNS-reactive nitrogen species,SOD-superoxide dismutase; GPx-glutathione peroxidise; DA-Dopamine; NOS-nitric oxide synthase.

1.10.6 The "Serotonin Syndrome"

The "serotonin syndrome" (SS) occurs typically following the administration of 5-HT releasers and is a consequence of excess serotonergic stimulation of both peripheral and CNS receptors (Sternbach, 1991; Dunkley et al., 2003). Its consequences range from slightly perceptible to lethal and the most severe cases occur when MAO inhibitors (MAOis) specifically those ones that have high affinity for MAO-A (e.g. clorgiline) are used concominantely with drugs such as MDMA that are capable to induce an huge increase of 5-HT release (Demirkiran et al., 1996; Parrott, 2002; Vuori et al., 2003). The main mechanism subjacent to the concomitant administration is thought to involve the increase of the levels of 5-HT, the potentiation of 5-HT mediated behaviour and the increase in body temperature (Freezer et al., 2005).

5-HT_{1A} receptors (Darmani and Ahmad, 1999) are quite implied in the characteristic behaviours of the SS, although the stimulation of 5-HT_{2A} receptors also contribute substantially to the condition (Nisijima et al., 2001; Isbister and Whyte, 2002; Freezer et al., 2005).

The physiological and behavioural consequences of the SS vary with parameters such as the repeated exposure, age and dose, thus accounting for differences between the 5-HT receptors sensitivity among individuals. Nonetheless, the more typical symptoms observed in laboratory animals include hyperactivity, mental confusion, hyperreflexia, tachycardia, shivering, tremor (Huether et al., 1997; Gillman, 1999) forepaw treading, straub tail, head weaving, hyperactivity, "wet dog" shakes (Miller et al., 1997; Darmani and Ahmad, 1999), increased onset of penile erection, pilorection, proptosis of the eyes (Grahame-Smith, 1971), hyperthermia (Boyer and Shannon, 2005) and flat body posture.

The treatment of severe manifestations generally requires physical cooling, paralysis and the use of both 5-HT_2/5-HT_{1A} blocking drugs (Gillman, 1999).

1.10.7 The Serotonin Dopamine continuum in MDMA exposure

Many relevant studies concerning MDMA-induced neurotoxicity point out a close interaction between both the serotonergic and dopaminergic systems to explain the majority of the relevant neurochemical and behavioural effects produced.

Concerning the behavioural alterations, there is a consensus in all the scientific approaches performed until the date: 5-HT neurons innervate and send signals to the DA neurons in brain regions of the reward system that are responsible for the majority of the characteristic behaviours following drug exposure (Bankson and Cunningham, 2001).

At the neurochemical level, however, some divergences exist and there are two fundamental theories that explain the interconnection between both the monoaminergic systems.

The great percentage of authors explains the correlation between both the monoaminergic systems based on the functionality of the 5-HT subtype of receptors 5-HT_{2A}, and 5-HT_{2C}. These receptors are located on gamma-aminobutyric acid (GABA) interneurons and play important roles in the DA function (Bankson and Cunningham, 2001). In conditions of overstimulation, like after MDMA exposure, a marked effect in the decrese of GABAergic transmission and an increase in DA release and synthesis are observed (Sprague and Nichols, 1995a; O'Shea et al., 1998b; Sprague et al., 1998; Colado et al., 1999b; Esteban et al., 2001). Further evidences on this mechanism are again supported by experiments with antagonists of the 5-HT_{2A} receptors that proved to attenuate both the increase in DA synthesis (Nash et al., 1990; Schmidt et al., 1992) and serotonergic neurotoxicity (Schmidt and Lovenberg, 1985).
Alternative explanations point a quite more close involvement of DA and MAO-B, located in the serotonergic terminals (Sprague and Nichols, 1995a). According to these authors, DA is uptaken by the depleted serotonergic terminal through the 5-HT uptake transporter (SERT) that presents a relatively high affinity for DA (Schmidt and Lovenberg, 1985; Waldmeier, 1985; Faraj et al., 1994; Sprague and Nichols, 1995a). The excessive metabolism of DA by MAO-B in the serotonergic terminal result in elevated levels of H_2O_2 that are further responsible for increased oxidative processes (Sprague and Nichols, 1995a). Support for this theory is based on experiments with MAO-B inhibitors, such as selegiline, that proved to be active in protecting against the formation of ROS after MDMA exposure (Sprague and Nichols, 1995a; Colado et al., 1997; Shankaran et al., 2001).

1.10.8 MDMA and mitochondria:

Mitochondria are both a source and a target of ROS-induced toxicity and substituted amphetamines such as METH and MDMA are able to alter the mitochondrial adequate function (Brown and Yamamoto, 2003; Brown et al., 2005) thus increasing the rate of formation of dangerous reactive species.
The events responsible for the altered mitochondrial performance are mainly related to the initial effects of the drug, namely the increased synthesis and release of both the neurotransmitters 5-HT and DA. Neurotransmitters auto-oxidation or MAO mediated metabolism are the secondary events to be taken into consideration fundamentally due to increased H_2O_2 formation that could further lead to mitochondrial macromolecular

constituents oxidation with the resultant diminished organelle performance accounting for a dangerous diminution in brain energy stores. This hypothesis corresponds to the basis of the experimental work performed in the ambit of the present dissertation.
Some of the best described effects of increased ROS formation inside mitochondria after MDMA exposure, or related events are summarized in the following table (table1.15).

Table 1.15: MDMA-induced mitochondrial damage.

MDMA-induced mitochondrial damage	References
Enhancement of MDMA-induced damage by ETC inhibitors	(Nixdorf et al., 2001)
Diminishment of MDMA induced toxicity by substrates of energy metabolism	(Stephans et al., 1998; Wan et al., 1999)
MDMA induced decrease in cytochrome c oxidase activity in DA rich regions (SN, NA and striatum) 2 hours after exposure	(Burrows et al., 2000)
MDMA inhibition of mitochondrial complexes I and II in striatal mitochondria 12 hours after exposure	(Quinton and Yamamoto, 2006)
MDMA induced decrease of ATP concentration in rat striatum and hippocampus	(Darvesh et al., 2005)
Simultaneoous decrease of brain DA and disturbance of mitochondrial respiratory states 3 and 4	(Berman and Hastings, 1999)
Tryptamine 4,5-dione (metabolite of 5-HT) mediated inhibition of cytochrome c oxidase and NADH-coenzyme Q1 reductase	(Jiang et al., 1999)
Depletion of mitochondrial brain energy stores with associated severe neurotoxic damage	(Darvesh et al., 2005)

Footnotes: ETC-electron transport chain; MDMA-3,4-methylenedioxymethamphetamine; SN-substancia nigra; NA-nucleus accumbens; DA-dopamine.

Taking into account the presently described MDMA-induced mitochondrial alterations, this organelle has to be indubitably considered for the study of the effects of the drug and viewed as a valuable target for either prevention or treatment of the toxic effects, thus justifying the relevance given to it in the present work.

1.10.9 MDMA and apoptosis

MDMA exposure and related mitochondrial dysfunction is being associated by several authors with apoptosis observed following exposure (Montiel-Duarte et al., 2002; Capela et al., 2007).
The overall reports on MDMA-induced apoptotic cell death are summarized in the table 1.16.

Table 1.16: MDMA-induced apoptotic-related events.

MDMA-induced apoptotic related events	References
Nuclear condensation and fragmentation	(Loeffler and Kroemer, 2000; Montiel-Duarte et al., 2002)
Disregulation of Bcl-$_{Xl}$ protein, cytochrome c release and caspase 3 activation	(Loeffler and Kroemer, 2000; Montiel-Duarte et al., 2002)
Potentiation of apoptotic neuronal death with MDMA-induced hyperthermic conditions	(Capela et al., 2006a; Capela et al., 2006b)
5-HT$_{2A}$ receptor activation in cortical neurons	(Capela et al., 2007)
Axonal and dendritic degeneration	(Capela et al., 2006a)

Foo: 5-HT$_{2A}$ receptors-serotonin receptors of the type 2A.

1.10.10 MDMA administration and development

Laboratory studies on ecstasy abuse can be consider deficient in two main aspects: (i) there is a lackness of studies concerning evaluations in adolescence, the period of development where comsumption of drugs of abuse commonly begins (ii) the dosage regimen normally used consists of multiple doses in concentrated time periods that are not correspondent to the recreational exposure.

The period of adolescence is characterized by a substantial reorganization of the nervous system with consequent increased susceptibility and vulnerability to drug exposure. The few animal studies performed so far, are summarized in table 1.17.

The measurements made on infant animals are much more frequent but entail some controversial results because they seem to differ depending on the treatment made on periods corresponding to P1-P9, P9-P10 or P11-P20. The discrepancies observed at this early development stage are thought to be related with both the different ability of animals to eliminate MDMA (Williams et al., 2004) from their physiological systems and the development of various receptor systems characteristic of these ages (Williams et al., 2004). At this point, it seems likely that not the 5-HT levels, but the destabilization of the whole 5-HT system are a good reference to understand the different results obtained in early infant ages. Some authors suggest that the combination of both the short half-life in the body and the immature neuronal system could account to the increased resistance of younger animals to the drug (Williams et al., 2004) at both the behavioural and neurochemical levels.

Table 1.17 contains the summarized effects of the drug at the different infancy periods.

The adult period is the best characterized concerning the overall literature available on MDMA induced neurotoxic and behavioural effects.

The main physiological consequence of MDMA exposure in adults is the long-term degeneration of 5-HT nerve terminals (Ricaurte et al., 2000) and associated neurotoxic long-lasting depletion of 5-HT levels.
The behavioural alterations are mainly characterized by both memory and cognitive disturbances and are suggested as being the result of both the aberrant pattern of axon reinnervation following MDMA neurotoxic insults (Fischer et al., 1995b; Meyer and Ali, 2002) and the consequent alterations in 5-HT receptor functioning (McGregor et al., 2003; Bull et al., 2004).
Detailed descriptions of both the physiological and behavioural alterations in adulthood are summarized in table 1.17.

Studies on early gestational MDMA exposure have also been performed. At this stage of development some other significant alterations such as the projection of serotonergic afferents that innervate forebrain targets in cerebral cortex, limbic system, and diencephalon (Lidov and Molliver, 1982) and the transient expression of the serotonin transporter on thalamocortical afferents (Zhou et al., 2000) are elements capable to justify, at some extent, the increased vulnerability to the drug.
A description of the both major effects and the respective consequences in later ages are summarized in table 1.17.

Table 1.17: MDMA-induced effects in different developmental stages.

Developmental periods	Biochemical alterations	References	Behavioral alterations	References
Gestational period (after birth observations)	Increased risk of congenital abnormalities in humans (cardiac malformation and clubfoot) Lasting effects in DA and 5-HT systems in adulthood Apoptotic cell death and reorganization of forebrain serotonergic innervation Elevated brain glucose utilization Decreased body weight	(McElhatton et al., 1999) (Koprich et al., 2003) (Meyer et al., 2004) (Kelly et al., 2002) (Broening et al., 2001)	Increased locomotor activity, lack of habituation to a novel cage environment and altered sequential learning and memory tasks	(Broening et al., 2001)
Infant period(P1-P9)	Long-lasting 5-HT deficits Independence of the effects on temperature	(Meyer and Ali, 2002)(Meyer and Ali, 2002)	Absence of deficits in spatial and sequential learning tasks	(Broening et al., 2001)
Infant period (P9-P10)	Absence of 5-HT lasting changes Independence of the effects on temperature	(Broening et al., 1995) (Broening et al., 1995)	Absence of deficits in spatial and sequential learning tasks	(Broening et al., 2001)
Infant period (P11-P20)	5-HT hippocampal deficits Increased 5-HT_{1A} hippocampal receptor activity	(Williams et al., 2005; Schaefer et al., 2006) (Crawford et al., 2006)	Deficits in spatial and sequential learning tasks	(Winslow and Insel, 1990; Broening et al., 2001; Williams et al., 2003)
Adolescent period	Anorexia Lower levels of SERT binding in hippocampus	(Parrott, 2002) (Piper and Meyer, 2004)	Impaired working memory Decrease of anxiety like behaviours in relation to adults	(Parrott, 2000; Bhattachary and Powell, 2001; Piper and Meyer, 2004) (Piper and Meyer, 2004)
Adul period	Long-term degeneration of 5-HT nerve endings Hyperthermia Deficient pattern of axonal reinnervation Altered 5-HT receptor functioning Anorexia	Ricaurte et al. 2000 Malberg and Seiden 1998 Fisher et al. 1995; Meyer and Ali 2002 McGregor et al. 2003; Bull et al. 2004 Frith et al 1987; Parrot et al. 2002	Impaired working memory and chronic memory disorders Alteration of cognitive abilities Anxiety like behaviours	(Parrott, 2000; Bhattachary and Powell, 2001) (Williams et al., 2004) (Gurtman et al., 2002)

Footnotes: 5-HT-serotonin; DA-dopamine; SERT-serotonin transporter.

1.10.11 The reward system:

All the drugs of abuse interfere with the brain limbic system. The release of DA in the Nucleus accumbens is responsible for pleasurable feelings by means of DA release and is commonly referred as an important component of the “reward system”.

The brain reward system exists to reinforce (reward) behaviours necessary for individual’s survival. Such behaviours include simple actions such as drinking and eating and drug effect on the system fools the brain into believing that the drug is also a natural pleasure, and, in that way, essential for survival. The reward system is a collection of brain structures from which the major chemical pathway is the mesocorticolimbic pathway. The basic anatomy of the brain reward pathway is centered on the limbic system (Koob and Nestler, 1997), from which the most important areas include the hypothalamus, amygdala, Hip, septal nuclei, anterior cyngulate gyrus,NAccb, ventral caudate nucleus and the putamen. From these areas, the NAccb is one of the main target areas of drugs of abuse thus being together with amygdala, VTA and the ventromedial and lateral nuclei of hypothalamus, one of the most important structures involved in reward mechanisms (Koob and Nestler, 1997; Dager et al., 1999; Little et al., 1999). Some other important functions of the brain, like the autonomic nervous system and the neuroendocrine system have also important modulative effects upon rewarding, by means of interactions with limbic structures, and the median forebrain bundle, made of dopaminergic neurons, represents the structure responsible for the connections established between all the brain areas involved (Koob and Nestler, 1997).

MDMA, like the majority of the other drugs of abuse acts trough many different mechanisms and locations of the brain reward system. As already referred, although the major neurotransmitter released after consume is 5-HT, MDMA is also capable to induce the release of both DA and NA. In this way, the primary neurotransmitter of the reward pathway, DA, by means of either blockade of reuptake or modulated release through intertwining with both 5-HT and NA, has its levels increased in the reward pathway after MDMA exposure thus explaining the pleasant feelings often described by abusers.

From the presently knowledge on MDMA induced neuronal degeneration it is presently assumed that it affects a big part of the brain areas of the reward system. Studies on specific brain regions, concerning degeneration processes after MDMA exposure describe the parietal cortex, insular/perirhinal cortex, ventromedial/ventrolateral thalamus and the tenia tecta brain areas (Commins et al., 1987; Schmued, 2003) as

the most harvested ones. The visual cortex is also quite influenced by MDMA exposure since both this brain region and the pyriform cortex suffer 5-HT depletions that could persist for up to 7 years (Hatzidimitriou et al., 1999). In contrary, areas near the rostral RN gave evidences of completely recovery (Hatzidimitriou et al., 1999).

In the present work we studied some of the brain areas closely connected to the reward mechanisms. In particular the VTA/SN, Hip, CPU, FCx, RN and amygdala were analysed.

Chapter I

2. The Animal Model

2. The animal model

The selection of an appropriate animal model is indubitably the first step of major concern when designing an experimental protocol on drugs of abuse in order to obtain consistent and conclusive results (Vorhees, 1995; Vorhees and Pu, 1995). Extensive research on MDMA-induced effects in different stages of development has already been discussed (see table 1.17). As mentioned before, published data regarding the effects of exposure to MDMA (or other drugs) through the period of adolescence, is still scarce. Drug consumption in late developmental stages may lead to different outcomes than in the adulthood, including persistent cumulative neurotoxicity. Adolescence is a stage of active maturation of the nervous system, where levels of MDMA consumption are well documented and highly increased (Pope et al., 2001; Lieb et al., 2002; Jacobsen et al., 2004). Therefore, the study of immediate and long-term effects and consequences of exposure to MDMA in the adolescent period constitutes a good and relevant model.

In the present study, the effects of exposure to a neurotoxic dose of MDMA in an adolescent rat model (PND 45) and its long-term consequences in the adulthood (two weeks latter) were examined. The dose regimen used was already described as being able to induce neurotoxic insults in the rat (Commins et al., 1987; Broening et al., 1995; Gowing et al., 2002; Rusyniak et al., 2005) and simulates the human binge pattern of administration of MDMA (Parrott, 2005). The period of evaluation was chosen based on previous reports where serotonergic damage was described as still being clearly observable two weeks after the neurotoxic insult (Battaglia et al., 1987; Commins et al., 1987; Stone et al., 1987; O'Hearn et al., 1988).

Studies on interspecies scaling describe the neurotoxic dose used in the present study as having a direct correspondence with the recreational doses used in humans (Ricaurte et al., 2000). Furthermore, this dose was shown to be comparable to 2,5mg/Kg dose, which is vulgarly ingested by humans (Greer and Strassman, 1985), reinforcing the choice of this regimen. As regular users typically take 2 or 3 tablets in one night, repeated exposure (4 injections of 10mg/Kg two hours apart) was used to mimic this sequential dosing regimen (Parrott, 2002, 2005) .

Males, both in rats and humans (Cadet et al., 1994; Miller and O'Callaghan, 1995; Reneman et al., 2001; Koenig et al., 2005) were suggested to be differently susceptible to the effects of drug abuse. Because of a possible gender dimorphism in MDMA-induced effects, in the present work only males were assessed. Furthermore, female oestrus cycles can interfere with the effects of drug exposure, adding confounding variables to the study.

Chapter I

3. General and specific objectives of the dissertation

3. General and specific objectives of the dissertation

The general objective of this dissertation was to understand the role of brain mitochondria in MDMA-induced damage to adolescent Wistar rats.

The following specific objectives were pursued:

● To evaluate long-term MDMA neurotoxic effects at the mitochondrial level, namely in what concerns to lipid peroxidation, protein carbonylation, specific mtDNA deletions and corresponding decrease in the expression of NDII and COXI mitochondrial subunits.

● To investigate the contribution of MAO-B to the overall neurotoxic process mediated by MDMA at the mitochondrial level

● To investigate the contribution of MAO-A to the overall neurotoxic process mediated by MDMA at the mitochondrial level

● To investigate the putative protective ability of ALC at the mitochondrial level

● To analyse the alterations in body temperature that could contribute for the effects observed for MDMA and/or the other tested compounds.

● To analyse the alterations in body weight gain throughout the 13 days following injections.

Chapter II

Manuscripts

1. Manuscript I

Monoamine oxidase-B mediates ecstasy-induced neurotoxic effects to adolescent rat brain mitochondria

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Neurobiology of Disease

Monoamine Oxidase-B Mediates Ecstasy-Induced Neurotoxic Effects to Adolescent Rat Brain Mitochondria

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3,4-Methylenedioxymethamphetamine (MDMA)-induced neurotoxicity and the protective role of monoamine oxidase-B (MAO-B) inhibition were evaluated at the mitochondrial level in various regions of the adolescent rat brain. Four groups of adolescent male Wistar rats were used: (1) saline control, (2) exposed to MDMA (4 × 10 mg/kg, i.p.; two hourly), (3) treated with selegiline (2 mg/kg, i.p.) 30 min before the same dosing of MDMA, and (4) treated with selegiline (2 mg/kg, i.p.). Body temperatures were monitored throughout the whole experiment. Animals were killed 2 weeks later, and mitochondria were isolated from several brain regions. Our results showed that "binge" MDMA administration causes, along with sustained hyperthermia, long-term alterations in brain mitochondria as evidenced by increased levels of lipid peroxides and protein carbonyls. Additionally, analysis of mitochondrial DNA (mtDNA) revealed that NDI (nicotinamide adenine dinucleotide phosphate dehydrogenase subunit I) and NDII (nicotinamide adenine dinucleotide phosphate dehydrogenase subunit II) subunits of mitochondrial complex I and cytochrome *c* oxidase subunit I of complex IV suffered deletions in MDMA-exposed animals. Inhibition of MAO-B by selegiline did not reduce hyperthermia but reversed MDMA-induced effects in the oxidative stress markers, mtDNA, and related protein expression. These results indicate that monoamine oxidation by MAO-B with subsequent mitochondrial damage may be an important contributing factor for MDMA-induced neurotoxicity.

Key words: **3,4-methylenedioxymethamphetamine; neurotoxicity; adolescent rat model; brain mitochondria; monoamine oxidase B; oxidative stress**

Introduction

Studies in rodents and nonhuman primates indicate that exposure to 3,4-methylenedioxymethamphetamine (MDMA) may elicit neurotoxicity to serotonin (5-HT)-containing axon terminals (Stone et al., 1986; Battaglia et al., 1987; Commins et al., 1987; Hatzidimitriou et al., 1999). MDMA binds to the 5-HT reuptake transporter and, inside axon terminals, causes an acute and powerful release of neurotransmitters (mainly 5-HT, but also noradrenaline and dopamine) from storage vesicles (Green et al., 1995; Sulzer et al., 2005). MDMA also contributes to a steady concentration of these neurotransmitters in the synaptic cleft by partially inhibiting their oxidation through monoamine oxidase (MAO) while inside nerve endings and by blocking their reuptake into nerve terminals (Leonardi and Azmitia, 1994; Green et al., 2003). During the phase of abrupt increase of the extravesicular levels of monoamine neurotransmitters inside nerve endings, a large amount is metabolized by MAO. Two isoforms of MAO exist: MAO-A and MAO-B. In the brain, MAO-A is expressed predominantly in catecholaminergic neurons, whereas MAO-B is expressed in serotoninergic neurons, astrocytes, and glia (Shih et al., 1999). 5-HT has been shown to be metabolized *in vitro* by MAO-A (*Km*, 178 ± 2 μM) and MAO-B (*Km*, 1170 ± 432 μM) (Shih et al., 1999). Although metabolism by MAO-B is only residual in the presence of MAO-A, it is fully effective in the absence of the latter, as it happens inside serotonergic nerves (Fowler and Tipton, 1982; Shih et al., 1999). Oxidative deamination of monoamine neurotransmitters by MAO produces hydrogen peroxide (H_2O_2), which subsequently may be converted into the highly reactive hydroxyl radical (HO), and consequent oxidative stress-related damage may occur. Corroborating this hypothesis, previous studies have shown that MDMA-induced chronic 5-HT loss requires the activity of MAO-B and involves oxidative stress (Schmidt, 1987; Sprague and Nichols, 1995; Sprague et al., 1998). More recently, it was demonstrated that MDMA-induced 5-HT depletion observed in wild-type mice did not occur in MAO-B-deficient mice (Fornai et al., 2001) and that an antisense oligonucleotide targeted at MAO-B attenuates rat striatal serotonergic neurotoxicity induced by MDMA (Falk et al., 2002). Moreover, it

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was also shown that dopamine uptake into serotonergic nerve endings, followed by MAO-B metabolism and subsequent oxidative stress also contributes to the neurotoxic effects of MDMA (Hrometz et al., 2004; Jones et al., 2004). Thus, it may be assumed that MAO-B-mediated metabolism of monoamine neurotransmitters following their vesicular release by MDMA is an important driving factor for MDMA-induced neurotoxicity. However, the mechanisms mediating the deleterious effects of MAO-B derived H_2O_2 at nerve endings are hitherto not known.

Considering that MAO enzymes are located at the outer membrane of mitochondria, our hypothesis is that H_2O_2 diffuses through mitochondrial membranes leading to oxidative damage on mitochondrial macromolecules, namely lipids, proteins, and DNA. Thus, the aim of the study was to expose an adolescent rat model to a neurotoxic "binge" administration of MDMA and to evaluate the neurotoxicity at the brain mitochondrial level. An additional important aim of this work was to verify whether inhibition of MAO-B by selegiline could prevent the toxic effects of MDMA.

Materials and Methods

Animal model. Adolescent male Wistar rats were used. Animals were kept under controlled environmental conditions (temperature, 20 ± 2°C; relative humidity, 45–55%; 12 h light/dark cycle) and housed with food and water supplied *ad libitum*. All procedures were approved by the Portuguese Agency for Animal Welfare (General Board of Veterinary Medicine in compliance with the Institutional Guidelines and the European Convention).

On postnatal day 40 (P40), animals were divided into four experimental groups (MDMA group, selegiline plus MDMA group, selegiline control group, and an isovolumetric saline control group) and caged in pairs. Two days later, a subcutaneous probe (Implantable Programmable Temperature Transponder, IPTT-200; BMD, Einsteinweg, The Netherlands) (Kort et al., 1998) was inserted in the lumbar region to allow the measurement of body temperature throughout the experiment. On P45, the animals assigned to the MDMA group received a freshly prepared solution of 10 mg MDMA/kg body weight, intraperitoneally, every 2 h in a total of four injections. The selegiline plus MDMA group was submitted to the same MDMA dosing scheme, the first administration of MDMA preceded 30 min by the administration of seleginine (2 mg/kg, i.p.). Control animals received equal doses of saline vehicle (0.9% w/v) in the same protocol of administration, and the selegiline group was administrated a single dose of selegiline (2 mg/kg, i.p.). Body temperature was measured just before the first MDMA or isovolumetric saline injection, and then, every 15 min, for a period of 9 h. Body temperature was recorded daily throughout the course of the experiment, always between 10:00 and 12:00 A.M.

MDMA (HCl salt) was extracted and purified from high-purity MDMA tablets that were provided by the Portuguese Criminal Police Department. The obtained salt was pure and fully characterized by NMR and mass spectrometry methodologies. Selegiline was supplied by Sigma (Steinheim, Germany).

Mitochondria isolation for determination of lipid peroxides and protein carbonyls. Two weeks after exposure, animals were killed by decapitation, and brains were removed rapidly on ice. Mitochondria were isolated from whole brain by the method of Rosenthal et al. (1987), with slight modifications. Briefly, the whole encephalon was washed, minced, and homogenized at 4°C in isolation medium (250 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mg/ml BSA, pH 7.4) containing 5 mg of bacterial protease (P-5380; Sigma). Single brain homogenates were brought to 30 ml and centrifuged at 1260 × *g* (SORVAL RC 5B Plus; Kendro Laboratory Products, Newtown, CT) for 5 min. The supernatant was centrifuged at 20,000 × *g* for 10 min. The pellet including the fluffy synaptosomal layer was resuspended in 10 ml of isolation medium containing 0.02% digitonin (to release mitochondria from the synaptosomal fraction) and centrifuged at 20,000 × *g* for 10 min. The brown mitochondrial pellet (without the synaptosomal layer) was resuspended again in 10 ml of washing medium (225 mM sucrose, 5 mM HEPES, pH 7.4) and recentrifuged at 20,000 × *g* for 10 min. The main portion of the mitochondrial pellet was resuspended in 300 μl of washing medium. Mitochondrial proteins were determined by the biuret method, calibrated with BSA (Gornall et al., 1949).

Quantification of lipid peroxidation. Lipid peroxidation was determined by measuring malondialdehyde (MDA) equivalents, using the thiobarbituric acid (TBA) assay, according to a modified procedure (Rohn et al., 1993). Mitochondrial protein (3 mg) was incubated for 30 min at 25°C in 3 ml of medium (175 mM KCL, 10 mM Tris, pH 7.4, and 3 μM rotenone). Samples of 0.3 ml were then incubated with 2.7 ml of TBA reactive substances reagent (9% TBA, 0.6 N HCl, and 0.0056% butylated hydroxyl toluene). The mixture was warmed to 80–90°C for 15 min and cooled by immersion in ice during 10 min before centrifuge at 1500 × *g* for 5 min. Lipid peroxidation was estimated by spectrophotometric determination, at 535 nm, of the MDA equivalents produced. The amount of MDA equivalents formed was calculated using a molar extinction coefficient of 1.56×10^5 $mol^{-1}cm^{-1}$ and expressed as nmol MDA equivalents/mg protein (Buege and Aust, 1978).

Quantification of protein carbonyls. Protein carbonyls were quantified through the spectrophotometric method for carbonyl assay, using 2,4-dinitrophenylhydrazine (DNPH) (adapted from Reznick and Packer, 1994). Two samples of 1 ml of each 1 mg/ml mitochondrial extract were placed in glass tubes. To one tube, 4 ml of 10 mM DNPH in 2.5 M HCl solution was added, and to the other tube of the same sample, only 4 ml of 2.5 M HCl (blank tube) was added. Tubes were left for 1 h at room temperature in the dark and vortexed every 15 min. At this point, 5 ml of 20% trichloroacetic acid (TCA) (w/v) solution was added to both DNPH and HCl samples to a final concentration of 10% (w/v) TCA. The tubes were left in ice for 10 min and then centrifuged for 5 min. The resultant supernatant was discarded. Next, another wash was performed with 4 ml of 10% TCA, and the protein pellets were broken mechanically. The protein pellets were washed three times with ethanol-ethyl acetate (1:1) (v/v). The final pellet was dissolved in 6 M guanidine hydrochloride solution and left for 10 min at 37°C in agitation in a water bath. All samples were centrifuged to remove any insoluble material remaining in suspension. The concentration of DNPH was determined at 360 nm, and the molar absorption coefficient of 22×10^3 $mol^{-1}cm^{-1}$ was used to quantify the levels of protein carbonyls. Protein concentration in the samples was calculated by determining the absorbance at 280 nm. Protein carbonyl content was expressed as nmol protein carbonyl formed/mg mitochondrial protein (Reznick and Packer, 1994).

DNA isolation for PCR. Two weeks after exposure, animals were killed by decapitation, brains were rapidly removed, and dissection of the different brain regions (prefrontal cortex, striatum, hippocampus, amygdala, ventral mesencephalon [comprising substantia nigra and ventral tegmental area (VTA/SN)] and raphe nuclei) was performed on ice. DNA from the different brain regions was extracted with GenomicPrep Cells and Tissue DNA Isolation kit (GE Healthcare, Buckinghamshire, UK) according to the instructions of the manufacturer. No pools of animals were necessary. Extracted DNA (5 μl) was applied on a 1% agarose gel to quantify the amount of DNA used on the subsequent PCR protocols.

Photographs were taken under UV transillumination (Typhoon 8600; Molecular Dynamics, Buckinghamshire, UK), and the semiquantitative analysis of extracted DNA was made with the software Image Quant 5.1.

PCR. Previously isolated brain areas were analyzed for a deletion between direct repeats corresponding to base pairs 1095–4095 of rat mitochondrial DNA (mtDNA). Deletion primers were designed based on the sequence of the rat mtDNA (GenBank accession number NC-001665) to detect a deletion corresponding to nicotinamide adenine dinucleotide phosphate dehydrogenase subunit I (NDI) and nicotinamide adenine dinucleotide phosphate dehydrogenase subunit II (NDII) subunits of mitochondrial complex I (NADH dehydrogenase) and COXI subunit of mitochondrial complex IV (cytochrome *c* oxidase) (Suliman et al., 2003).

The following mtDNA deletion primers were used: 5′-AGTCGTAACAAGGTAAGCAT-3′ (base pairs 982–1001) mtf1 primer and 5′-ATTTCTACTCTTTTAGCAT-3′ (base pairs 5632–5651) mtr2 primer (Suliman et al., 2003). The reaction mixture consisted of primers in a concentration of 400 μM (1 μl of stock 20 pmol; MWG-Biotech,

Ebersberg, Germany), 40 ng of template DNA, 1.5 mM $MgCl_2$ (50 mM stock; Bio-Rad, Hercules, CA), 1 μl of 10 mM PCR nucleotide mix (Eppendorf, Hamburg, Germany), 0.25 μl of *Taq* Polymerase (5 U/μl) (Bio-Rad), and 5 μl of enzyme buffer 10× (Bio-Rad). The final volume of the PCR was 50 μl, and the program used was 94°C for 2 min, 50°C for 30 s, 72°C for 2 min (35 cycles), and 72°C for 7 min (1 cycle) (MyCyclerthermocycler; Bio-Rad).

Negative controls were included containing all the above mentioned PCR components except template DNA. Ten microliter aliquots of the PCR products were electrophoresed through a 1–1.5% agarose gel in Tris-acetate containing ethidium bromide at 45 V/cm. Photographs were taken under UV transillumination (Typhoon; Molecular Dynamics), and the semiquantitative analysis of amplified DNA was made with the software Image Quant 5.1.

Western blot analysis of isolated whole-brain mitochondria. Two weeks after exposure, animals were killed by decapitation, and brains were removed rapidly on ice as described above. Isolated whole-brain mitochondria were resuspended in extraction buffer (20 mM Tris-HCl, pH 7.6, 250 mM sucrose, 40 mM KCl, 2 mM EGTA). The homogenate was centrifuged at 600 × *g* for 10 min at 4°C, and the supernatant was taken for mitochondrial Western blot analysis, 15 μg of protein was loaded per lane and separated on 10% SDS-polyacrylamide gels. The gels were transferred to a polyvinylidene fluoride membrane for protein blotting (0.2 μm; Bio-Rad) membranes by electroblotting 1 h at 150 mA. The filters were blocked in 5% nonfat dry milk and 0.1% Tween 20 overnight at 4°C. Blots were then incubated with mouse monoclonal antibody against COXI (2 μg/ml) (Invitrogen, Eugene, OR) or NDII (0.5 μg/ml) (Invitrogen) diluted in 0.1% TBS Tween 20 (20 mM Tris, 137 mM NaCl, pH 7.6) for 1 h at room temperature. Membranes were washed three times for 10 min in the same buffer and incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Imun-Star, 1:20,000; Bio-Rad). Immunoreactive proteins were revealed using enhanced chemiluminescence method (Immun-Star HRP Chemiluminescent kit; Bio-Rad). Blots were analyzed with Quantity One Software version 4.5 (Bio-Rad).

Statistical analyses. Data concerning evolution of body temperature were analyzed using a two-way ANOVA (treatment vs time). Significant differences were further tested using the *post hoc* Tukey's HSD for unequal *n*.

Data concerning MDA equivalents, protein carbonyls, mtDNA deletions, and Western blot quantifications were analyzed using a one-way ANOVA (treatment). Significant main effects and interactions were further explored using the *post hoc* Scheffé's test. The statistical level of significance was considered at $p < 0.05$.

Results

Effects of MDMA administration on body temperature

Hyperthermia is one of the most dangerous clinical symptoms of MDMA intoxication. Accordingly, in the present study, MDMA administration resulted in thermoregulation alterations. Analysis of data obtained for body temperature profile throughout the day of exposure shows that rats treated with MDMA presented significantly higher average body temperatures 30 min after the first injection and until the end of the measuring period, when compared with saline controls ($p < 0.001$ for most values measured) (Fig. 1). Animals exposed to MDMA plus selegiline also presented a higher average body temperature when compared with the control group ($p < 0.001$) or the group exposed only to selegiline ($p < 0.001$) (Fig. 1). However, no significant differences were observed between body temperatures of MDMA and MDMA plus selegiline exposed rats. Selegiline administered animals did not show significant differences in body temperature when compared with the control group (Fig. 1). Although after the third injection, the effects of increased core temperature were less evident in MDMA group, rats treated with MDMA preceded by selegiline continued to exhibit, to a certain extent, temperatures similar to the ones observed after the first and the second injections (Fig. 1).

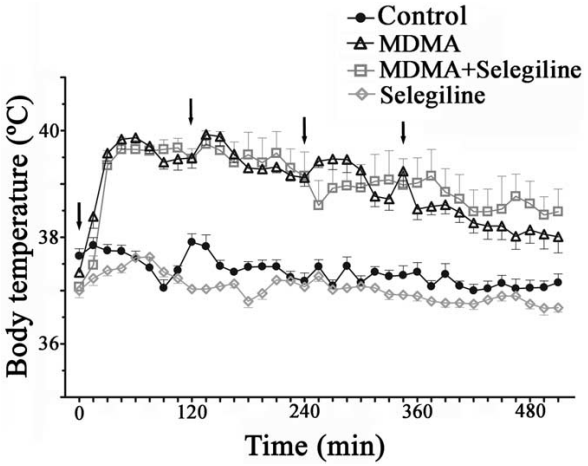

Figure 1. MDMA induced hyperthermia in adolescent male Wistar rats. The image represents body temperature evolution (measured by scanning a subcutaneously inserted probe every 15 min for 9 h) throughout the period of exposure to the following: MDMA (4 × 10 mg/kg), selegiline (2 mg/kg) plus MDMA (4 × 10 mg/kg), control saline (isovolumetric saline), and selegiline (2 mg/kg). Selegiline was administered 30 min before exposure to MDMA. Results are reported as mean ± SEM for $n = 10$ in control and MDMA exposed rats and $n = 6$ in the other groups. Evolution curves for MDMA and MDMA plus selegiline exposed rats started to display significantly higher values than the curves of control and selegiline exposed rats 30 min after the first dose ($p < 0.001$; two-way ANOVA followed by a *post hoc* Tukey's HSD for unequal *n*). Arrows indicate injection timings.

MDMA-treated animals demonstrated a chronic alteration in thermoregulation throughout the 13 d after the day of exposure, showing small but significantly increased values ($p < 0.05$) that were prevented by selegiline (data not shown).

Effects of MDMA administration on lipid peroxidation

The extension of lipid peroxidation was measured in the mitochondrial fraction of whole brain homogenates of adolescent rats 2 weeks after exposure to MDMA, MDMA plus selegiline, selegiline, and isovolumetric saline. MDMA-treated rats had significantly higher levels of malondialdehyde equivalents ($p < 0.001$), when compared with saline controls, selegiline, or MDMA plus selegiline. The levels of lipid peroxidation on animals injected with MDMA plus selegiline were not significantly different from the control groups (selegiline and saline) (Fig. 2).

Effects of MDMA administration on the formation of protein carbonyls

Two weeks after exposure, the levels of protein carbonyls were evaluated in the mitochondrial fraction of whole brain homogenates of adolescent male rats injected with MDMA, MDMA plus selegiline, selegiline, and isovolumetric saline. The administration of a neurotoxic dose of MDMA produced a significant increase on protein carbonyls of whole-brain mitochondria ($p < 0.001$) comparatively to all of the other groups. Animals treated with selegiline before MDMA administration presented carbonyl levels that were similar to those measured in control groups (Fig. 3).

Effects of MDMA administration on mtDNA

PCR

DNA from different brain areas (frontal cortex, striatum, amygdala, VTA/SN, hippocampus, and raphe nuclei) were submitted to an amplification procedure with *Taq*DNA polymerase to verify

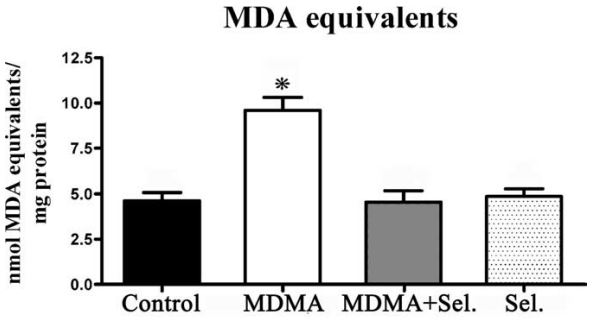

Figure 2. MDMA induced lipid peroxidation in whole-brain mitochondria and the protective effect of selegiline. Lipid peroxidation was determined by measuring MDA equivalents, using the TBA assay. Animals were killed 14 d after exposure to: MDMA (4 × 10 mg/kg), selegiline (Sel.) (2 mg/kg) plus MDMA (4 × 10 mg/kg), saline (isovolumetric saline), or selegiline (2 mg/kg). Selegiline was administered 30 min before exposure to MDMA. Columns represent mean + SEM, expressed in nanomoles MDA equivalents per milligram of protein for each experimental group ($n = 10$ for control and MDMA; $n = 6$ for MDMA plus selegiline and selegiline). Animals exposed to MDMA presented significantly higher values of MDA equivalents than all other groups (*$p < 0.001$, one-way ANOVA followed by a *post hoc* Scheffé's test).

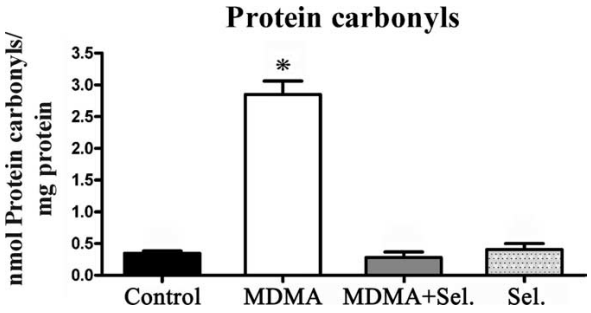

Figure 3. MDMA induced carbonyl formation in whole-brain mitochondria and the protective effect of selegiline. Protein carbonyls were quantified by reaction with DNPH using a spectrophotometric assay for carbonyls. Animals were killed 14 d after exposure to: MDMA (4 × 10 mg/kg), selegiline (Sel.) (2 mg/kg) plus MDMA (4 × 10 mg/kg), saline (isovolumetric saline), or selegiline (2 mg/kg). Selegiline was administered 30 min before exposure to MDMA. Columns represent mean + SEM, expressed in nanomoles of protein carbonyls per milligram of total protein for each experimental group ($n = 10$ for control and MDMA; $n = 6$ for MDMA plus selegiline and selegiline). Animals exposed to MDMA presented significantly higher levels of carbonyl formation than all other groups (*$p < 0.001$, one-way ANOVA followed by a *post hoc* Scheffé's test).

the presence of a deletion corresponding to the genes NDI and NDII of the mitochondrial complex I and COXI of complex IV. With the set of primers used, a band corresponding to NDI, NDII, and COXI deletion was expected. This deletion was substantially more evident on animals injected with MDMA ($p < 0.001$ for all tested areas except the prefrontal cortex) comparatively to all the other groups. In animals treated with selegiline, there was a significant attenuation on the level of deletion ($p < 0.001$) in most brain areas, although these levels were still higher than those observed for the control and the selegiline groups (Fig. 4).

Figure 4*A* is representative of the PCR for the mentioned deletion (the image shown corresponds to a raphe nuclei sample). In the prefrontal cortex, animals treated with MDMA presented lower levels of the deletion than the other tested areas, although the difference between MDMA exposed animals and the other groups was still highly significant ($p < 0.01$) (Fig. 4*B*). In the VTA/SN, striatum, raphe nuclei, amygdala, and hippocampus, the group exposed to MDMA was significantly more affected than the other, presenting increased levels of deletion ($p < 0.001$) (Fig. 4*B*). A significant protection was obtained in animals previously dosed with selegiline, where levels of deletion, although higher than in control and selegiline groups, were significantly lower than for the MDMA group in all tested regions except the prefrontal cortex (Fig. 4*B*).

Western blot analysis for the expression of mitochondrial subunit NDII and COXI

Expression of NDII was considerably diminished ($p < 0.001$) on MDMA group compared with control groups (isovolumetric saline and selegiline) and with MDMA plus selegiline groups. Animals treated with MDMA plus selegiline presented values of expression that were not significantly different from controls (Fig. 5).

COXI was also less expressed on MDMA administered animals relatively to controls (isovolumetric saline and selegiline) (Fig. 6). Selegiline protected, to some extent, the deficient expression of these two subunits caused by exposure to MDMA.

Discussion

The neurotoxic effects that may result from MDMA consumption have been a matter of continuous research in the last decades. Several factors may contribute to MDMA-induced neurotoxicity, namely MDMA metabolism, sustained receptor stimulation, hyperthermia, enzymatic and nonenzymatic oxidation of neurotransmitters, inhibition of neurotransmitters synthesis, inflammation, and oxidative stress. The results obtained in the present study provide a new insight on the deleterious effects of MDMA to the CNS. To our knowledge, this is the first study to report a MAO-B-dependent, MDMA-induced damage on mitochondrial macromolecules, especially to mtDNA and subsequent impairment in the correspondent protein expression.

The exposure of the adolescence rat model to a neurotoxic "binge" administration of MDMA induced an oxidative stress status in brain mitochondria, as reflected by a robust increase in mitochondrial lipid peroxidation and protein carbonylation, 14 d after administration. Additionally, more studies in the prefrontal cortex, striatum, amygdala, ventral mesencephalon, hippocampus, cerebellum, and raphe nuclei revealed that mtDNA from these regions codifying the NDI and NDII subunits of the mitochondrial complex I (NADH dehydrogenase) and COXI of complex IV (cytochrome *c* oxidase) were partially deleted in MDMA-exposed animals, leading to the subsequent decrease of protein expression for these enzymatic systems. The contribution of MAO-B to MDMA-induced mitochondrial damage in the CNS was also clearly evidenced, because the inhibition of this enzyme by selegiline protected brain mitochondria against lipid peroxidation and protein carbonylation, as well as partially prevented mtDNA deletion and consequent decrease of protein expression. Selegiline was administered in a dose that is known to inhibit MAO-B. Although some authors argue that the often observed protective role of selegiline may also be attributable to its hydroxyl radical scavenging activity (Muralikrishnan et al., 2003; Sharma et al., 2003), others had not found such effect (Ferger et al., 1998).

MAO is localized in the outer membrane of mitochondria. Thus, the formation of H_2O_2 resulting from the metabolism of MDMA-released monoamine neurotransmitters by MAO will mostly affect mitochondria itself. When H_2O_2 removal pathways are lacking or inactivated, H_2O_2 accumulates and is converted

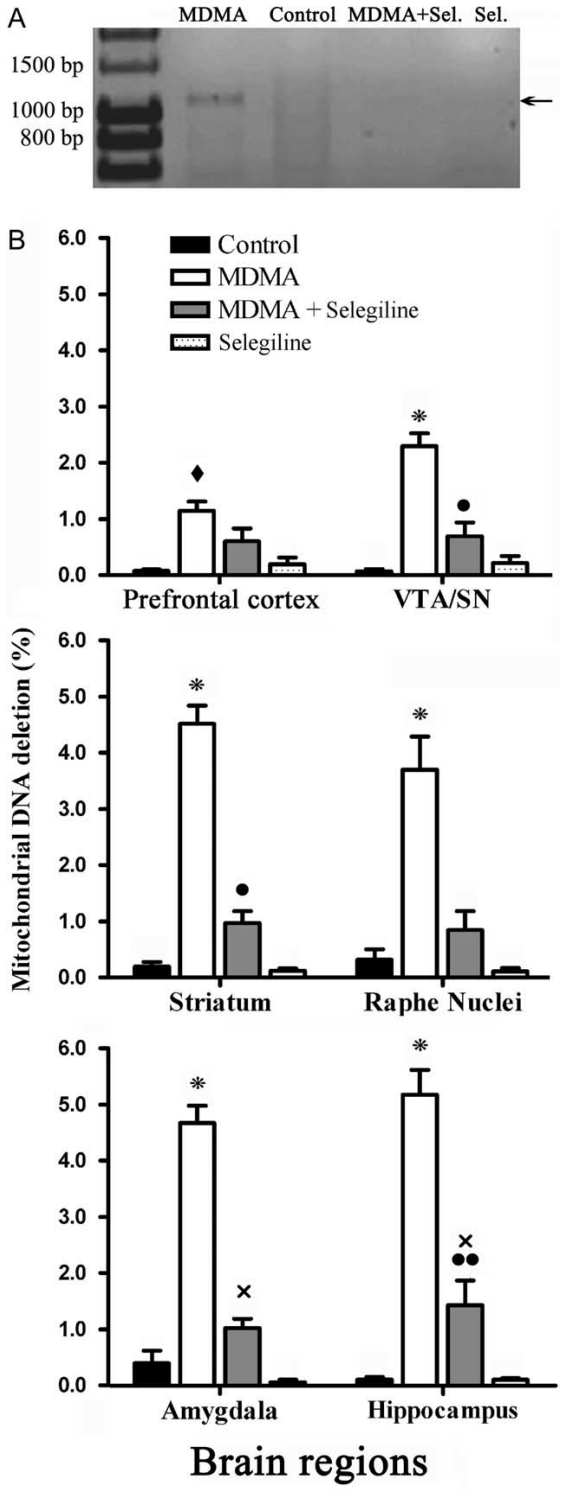

Figure 4. Protective effects of selegiline over the deletion of the mitochondrial gene sequences for NDI, NDII, and COXI in male Wistar rats exposed to a neurotoxic dose of MDMA. ***A***, A representative densitometry analysis is shown for the raphe nuclei; lanes, from left to right, correspond to standard Hyper ladder I, MDMA, control, MDMA plus selegiline (Sel.), and selegiline. The PCR was performed with the mtf1 and mtr2 primers, amplifying a fragment correspondent to the deletion of the sequences for NDI, NDII, and COXI. Animals were killed 14 d after exposure to MDMA (4 × 10 mg/kg), selegiline (2 mg/kg) plus MDMA (4 × 10 mg/kg), saline (isovolumetric saline), or selegiline (2 mg/kg). Selegiline was administered 30 min before exposure to MDMA. ***B***, Graphic representation of the levels of deletion of the mitochondrial gene sequences for NDI, NDII, and COXI for the four experimental groups, in different brain regions: prefrontal cortex, VTA/SN, striatum, raphe nuclei, amygdala, and hippocampus. Columns represent mean + SEM, expressed as percentage of DNA deletion, for each experimental group ($n = 6$). Significant differences between treatments are represented as: $^{*}p < 0.001$, used for MDMA, significantly higher than all other groups; $^{\blacklozenge}p < 0.01$, used for MDMA, significantly higher than control and selegiline groups; $^{\bullet}p < 0.05$ and $^{\bullet\bullet}p < 0.01$, used for MDMA plus selegiline, significantly higher than control; $^{\times}p < 0.01$, used for MDMA plus selegiline, significantly higher than selegiline alone (one-way ANOVA followed by a *post hoc* Scheffé's test).

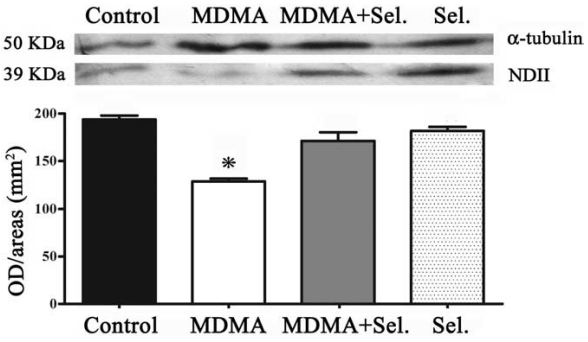

Figure 5. MDMA induced decreased expression of the subunit NDII in whole-brain mitochondria and the protective effect of selegiline. NDII expression was determined by Western blot analysis performed with isolated whole-brain mitochondria of male Wistar rats, using a mouse monoclonal antibody anti-NDII subunit. Animals were killed 14 d after exposure to: MDMA (4 × 10 mg/kg), selegiline (Sel.) (2 mg/kg) plus MDMA (4 × 10 mg/kg), saline (isovolumetric saline), or selegiline (2 mg/kg). Selegiline was administered 30 min before exposure to MDMA. Columns represent densitometric analysis of Western blots (mean + SEM; $n = 6$) expressed in outer diameter/area (mm^2). Densitometric analysis of α-tubulin served as loading control. Expression of NDII in the MDMA group was significantly decreased when compared with the other experimental groups ($^{*}p < 0.001$, one-way ANOVA followed by a *post hoc* Scheffé's test).

into the highly reactive HO, inducing intense oxidative stress in mitochondria (Mathai and Sitaramam, 1994; Agostinelli et al., 2004). Rat mtDNA, like human mtDNA, is vulnerable to reactive oxygen species (ROS), because it lacks the protection of histones and DNA-binding proteins (Shigenaga et al., 1994). Recently, a new oxidant-sensitive deletion in hepatic mtDNA of 3.81 kb was identified in the region between 1095 and 4905 bp involving two direct repeats in rat mtDNA (Suliman et al., 2002; Suliman et al., 2003). In the present study, the same type of mtDNA depletion was observed, for the first time, in MDMA-treated rats. The deleted portion of the mitochondrial genome includes codons for NADH dehydrogenase (NDI, NDII), cytochrome *c* oxidase (COX1), and tRNA 16S and rRNA for six amino acids (Suliman et al., 2002, 2003). Mitochondria carrying this deletion in sufficient copy number would have both impaired entry of reducing equivalents into the respiratory chain at complex I and functional alterations in molecular O_2 reduction at complex IV, which is also indicated in our study through the decreased expression of NDII and COXI. As the percentage of mtDNA deletions increases, mitochondrial energy output declines, ROS production increases further, and mitochondrial decline and mtDNA damage accumulate within the cells, leading to activation of apoptotic and necrotic cell death.

In addition to the immediate metabolism of vesicular released

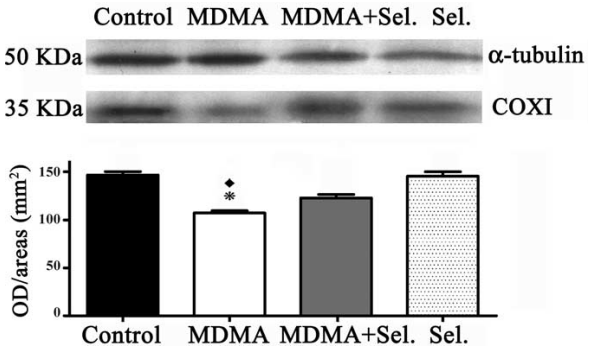

Figure 6. MDMA induced decreased expression of the subunit COXI in whole-brain mitochondria and the protective effect of selegiline. COXI expression was determined by Western blot analysis performed with isolated whole-brain mitochondria of male Wistar rats, using a mouse monoclonal antibody anti-COXI subunit. Animals were killed 14 d after exposure to: MDMA (4 × 10 mg/kg), selegiline (Sel.) (2 mg/kg) plus MDMA (4 × 10 mg/kg), saline (isovolumetric saline), or selegiline (2 mg/kg). Selegiline was administered 30 min before exposure to MDMA. Columns represent densitometric analysis of Western blots (mean + SEM; $n = 6$) expressed in outer diameter/area (mm^2). Densitometric analysis of α-tubulin served as loading control. Expression of COXI in the MDMA group was more significantly decreased (*$p < 0.001$) when compared with control and selegiline groups than when compared with the MDMA plus selegiline group (◆ $p < 0.01$, one-way ANOVA followed by a *post hoc* Scheffé's test).

5-HT, reuptake of dopamine into the 5-HT terminal may also contribute to MDMA-induced neurotoxicity. As discussed by Hrometz et al. (2004), *in vivo*, MDMA administration leads to a rapid release of 5-HT and inhibition of tryptophan hydroxylase, which results in the depletion of neuronal 5-HT. The initially released 5-HT activates 5-HT_{2A} postsynaptic receptors that lead to the upregulation of dopamine biosynthesis and release. Under these circumstances, dopamine may be transported into serotonergic nerve terminals by 5-HT transporter (SERT), which means that further MAO-B metabolism of dopamine may also contribute to the oxidative stress-related neurotoxic effect of MDMA (Sprague and Nichols, 1995). Although this hypothesis was not tested in the present study, it was demonstrated previously that a correlation exists between the extent of dopamine release and the extent of 5-HT depletion induced by MDMA, these effects being prevented by treatment with dopamine uptake inhibitors or inhibition of dopamine synthesis (Gudelsky and Yamamoto, 2003).

The differential effects in various brain regions have been a characteristic feature of MDMA neurotoxicity. MDMA is known to induce 5-HT depletion in selected brain regions in various animal species (Schmidt, 1987; Finnegan et al., 1988; O'Hearn et al., 1988; Ricaurte et al., 1988). In a previous study, O'Hearn et al. (1988) performed immunocytochemical analysis of regional brain sections 2 weeks after high-dose MDMA administration to rats and observed that the terminal portions of 5-HT axons were selectively vulnerable to the neurotoxic effects of MDMA, whereas preterminal axons, fibers of passage, and raphe cell bodies were not damaged. Regional differences in neurotoxicity were manifested by a greater loss of 5-HT axons in several forebrain areas, particularly neocortex, striatum, and thalamus, whereas more fibers were spared in hippocampus, septum, and amygdala. Battaglia et al. (1991) also demonstrated that, in rat, brain regions containing 5-HT pathways or perikarya were little affected by MDMA, the predominant effects being mediated on axons and terminals. Later, Aguirre et al. (1995) corroborated the previous data by showing the lack of 5-HT depletion in the dorsal raphe region of the brain stem, which includes part of the serotonergic cell bodies. The regional data obtained in our study comprises the striatum and the hippocampus as prone to MDMA-induced mtDNA deletion, but this time a deleterious effect with similar potency was observed for raphe nuclei and the hippocampus, whereas the frontal cortex and the VTA/SN were less affected. Our data imply that the classical view of MDMA neurotoxicity may not be applied to mitochondrial effects because other areas were also strongly affected. Recently, it was demonstrated that, in the dorsal raphe nucleus, serotonin-positive neuronal cell bodies were positive for MAO-B, which was present in all mitochondria. In contrast, within the neuronal cell bodies and dendrites that were positive for MAO-B, whereas most mitochondria contained this enzyme, a substantial quantity of mitochondria lacked it (Arai et al., 2002). Thus, the localization of MAO-B in the mitochondrial outer membrane of the neuronal cell bodies may help to understand the mitochondrial damage occurring at these sites.

Another potentially deleterious effect mediated by MDMA, hyperthermia, was observed in the present study but, under the present experimental conditions, did not substantially contribute to the changes in the measured toxicological biomarkers. The exposure of the adolescence rat model to a neurotoxic binge administration of MDMA provoked acute and long-term hyperthermia. Acute hyperthermia corresponds to a characteristic MDMA effect on laboratory animals maintained at ambient temperatures of approximately ≥20°C and is aggravated if ambient temperature increases (Malberg and Seiden, 1998; Carvalho et al., 2002; Green et al., 2005). Note that hyperthermia has been shown to potentiate the neurotoxic effects of MDMA both *in vivo* (Malberg and Seiden, 1998) and *in vitro* (Capela et al., 2006a,b). However, considering that the strong neuroprotective effect of MAO-B inhibition was independent of body temperature, the obtained data also contribute to a better clarification of MDMA effects, showing that the mechanism of MDMA neurotoxicity extends beyond hyperthermia.

In this study, we used a rat adolescence model. The impact of MDMA on adolescent abusers may be particularly harmful because of the vulnerability of their cerebral and hormonal systems, which are still undergoing crucial maturational changes (Montoya et al., 2002). For example, the density of the 5-HT transporter (SERT) rises during adolescence (Moll et al., 2000). A few experimental studies using adolescent rat models have shown that MDMA induces changes in several behavioral and physiological markers (Fone et al., 2002; Koenig et al., 2005; Piper et al., 2005) and revealed differences in the response when adolescence models are compared with adult models, namely in terms of the thermoregulation (Piper et al., 2005).

In conclusion, the results obtained in the present study demonstrate, for the first time, that the exposure of an adolescence rat model to a neurotoxic binge administration of MDMA induces not only an oxidative stress status in brain mitochondria but also deletion of mtDNA and subsequent impairment in the genes required for protein synthesis, which are essential complexes of the mitochondrial respiratory system required for energy production. The tissue-specific manifestations induced by MDMA may result from varying energetic roles and needs of the different tissues. The contribution of MAO-B to MDMA-induced mitochondrial damage in CNS was clearly evidenced, given that the inhibition of this enzyme by selegiline completely protected brain mitochondria against

lipid peroxidation and protein carbonylation and significantly prevented mtDNA deletion and correspondent protein expression.

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2. Manuscript II

Ecstasy-induced oxidative stress to adolescent rat brain mitochondria *in vivo*: influence of monoamine oxidase type A

Manuscript submitted

Ecstasy-induced Oxidative Stress to Adolescent Rat Brain Mitochondria *in vivo*: Influence of Monoamine Oxidase Type A

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Abstract

The administration of a neurotoxic dose of 3,4-methylenedioxymethamphetamine (MDMA; “ecstasy”) to rats results in mitochondrial oxidative damage in the CNS, namely lipid and protein oxidation, and mtDNA deletions with subsequent impairment in the correspondent protein expression. Although these toxic effects were shown to be prevented by monoamine oxidase B (MAO-B) inhibition, the role of MAO-A in MDMA-mediated mitochondrial damage remains to be evaluated. Thus, the aim of the present study was to clarify the potential interference mediated through the specific inhibition of MAO-A by clorgyline, on the deleterious effects produced by a binge administration of a neurotoxic dose of MDMA (10 mg MDMA/Kg of body weight, i.p., every 2 h in a total of 4 injections) to an adolescent rat model. The parameters evaluated were mitochondrial lipid peroxidation, protein carbonylation and expression of the respiratory chain protein subunits II of NADH dehydrogenase (NDII) and I of cytochrome c oxidase (COXI). Considering that hyperthermia has been shown to contribute to the neurotoxic effects of MDMA, another objective of the present study was to evaluate the body temperature changes mediated by MDMA with and without MAO-A selective inhibition by clorgyline. The obtained results demonstrated that the administration of a neurotoxic binge dose of MDMA to an adolescent rat model previously treated with the specific MAO-A inhibitor, clorgyline, resulted in synergistic effects on 5-HT mediated behaviour, body temperature and provokes high mortality. Inhibition of MAO-A by clorgyline administration had no protective effect on MDMA-induced alterations on brain mitochondria (increased lipid peroxidation, protein carbonylation and decrease in the expression of the respiratory chain subunits NDII and COXI), while it aggravated MDMA-induced decrease in the expression of COXI. These results reinforce the notion that the concomitant use of MAO-A inhibitors and MDMA is counter indicated because of the resulting severe synergic toxicity.

Key words: 3,4-Methylenedioxymethamphetamine; neurotoxicity; brain mitochondria; monoamine oxidase A; oxidative stress; hyperthermia.

Introduction

The widespread consumption of 3,4-methylenedioxymethamphetamine (MDMA; "ecstasy"), especially among adolescents and young adults, is presently a matter of high concern, due to the acute and chronic toxic effects mediated by this drug. Among the toxic effects mediated by MDMA, its potential to induce damage to the serotonergic nerve terminals is considered a soaring risk, due to its irreversible nature, which may result in permanent neurological deficits, like sleep disorders, depressed mood, persistent elevation of anxiety, impulsiveness and hostility, and selective impairment of episodic memory, working memory and attention (Morgan, 2000; Morton, 2005).

Several factors may contribute to MDMA-induced neurotoxicity, namely MDMA metabolism, sustained receptor stimulation, hyperthermia, enzymatic and non-enzymatic oxidation of neurotransmitters, inhibition of neurotransmitters synthesis, inflammation, and oxidative stress. In addition, we have recently given a new insight on the neurotoxicity of MDMA, by showing that the administration of a neurotoxic dose to rats results in monoamine oxidase B (MAO-B)-dependent mitochondrial oxidative damage in the CNS, namely lipid and protein oxidation, and mtDNA deletion with subsequent impairment in the correspondent protein expression (Alves et al., 2007). The corresponding mechanism involves MDMA mediated release of neurotransmitters [mainly serotonin (5-HT), but also noradrenaline and dopamine] from storage vesicles and their oxidative deamination by MAO-B, with the consequent production of hydrogen peroxide (H_2O_2). H_2O_2 may then be converted into the highly reactive hydroxyl radical ($HO^{\cdot}$), leading to $HO^{\cdot}$ mediated oxidative stress damage (Alves et al., 2007). Since monoamine oxidase enzymes are located in the outer membrane of mitochondria (Zhuang et al., 1988; Zhuang et al., 1992), H_2O_2 diffuses through mitochondrial membranes leading to oxidative damage of mitochondrial macromolecules, namely lipids, proteins and DNA (Alves et al., 2007). This study corroborated previous findings showing that MDMA-induced chronic 5-HT loss requires the activity of MAO-B and involves oxidative stress (Schmidt, 1987; Sprague and Nichols, 1995b; Sprague et al., 1998). Noteworthy, the strong neuroprotective effect of MAO-B inhibition was independent of body temperature (Alves et al., 2007).

At the central nervous system level there are two isoforms of MAO: MAO-A and MAO-B. MAO-A is expressed predominantly in catecholaminergic neurons, whereas MAO-B is expressed in serotoninergic neurons, astrocytes and glia (Shih JC, 1999), which implies that the metabolism of monoamine neurotransmitters inside serotonergic nerve terminals is mediated by MAO-B, and explains the protective effect of MAO-B inhibitors against MDMA mediated neurotoxicity. Nevertheless, it has also been demonstrated that the selective inhibition of MAO-A also results in a significant increase of extraneuronal 5-HT in some areas of the rat brain after administration of MDMA (Hewton et al., 2007; Stanley et al., 2007). Considering that the post-synaptic effect mediated by acute MAO-A inhibition is physiologically significant under MDMA use, it may be important to evaluate the putative protective effects of MAO-A selective inhibitors against the MDMA-induced neurotoxicity at the mitochondrial level.

Thus, the aim of the present study was to evaluate the interference of the specific inhibition of MAO-A, by clorgyline, on the deleterious effects produced by a binge administration of a neurotoxic dose of MDMA (10 mg MDMA/Kg of body weight, i.p., every 2 h in a total of 4 injections) in brain mitochondria of adolescent male Wistar rats. The parameters evaluated were lipid peroxidation, protein carbonylation and expression of the respiratory chain protein subunits II of NADH dehydrogenase (NDII) and I of cytochrome c oxidase (COXI). Considering that hyperthermia has been shown to contribute to the neurotoxic effects of MDMA both in vivo (Malberg and Seiden, 1998) and in vitro (Capela et al., 2006a; Capela et al., 2006b), another objective of the present study was to evaluate the body temperature changes mediated by MDMA with and without MAO-A selective inhibition by clorgyline.

Materials and methods:

Animal Model

Adolescent male Wistar rats were used. Institutional guidelines were followed for animal care. Animals were kept under controlled environmental conditions (temperature, 20±2ºC; relative humidity, 45-55%; 12 h light/dark cycle) and housed with food and water supplied “ad libitum”.
On postnatal day 40 (PND 40), two male Wistar rats were caged together. Two days later, a subcutaneous probe (Implantable Programmable Temperature Transponder, IPTT^{TM}-200,

BMDS) (Kort et al., 1998) was inserted in the lumbar region, to allow the measurement of the body temperature throughout the experiment.

Animals were divided into four experimental groups: MDMA group, clorgyline plus MDMA group, clorgyline control group and an isovolumetric saline control group. On PND 45, the animals assigned to the MDMA-group received a freshly prepared solution of 10 mg MDMA/Kg of body weight, intraperitoneally (i.p.) every 2 h in a total of 4 injections. The dose schedule was chosen because a common pattern of recreational MDMA use is taking several doses in one session ('binge'), which can be experimentally simulated in animals using repeated drug administration in a single day (Sanchez et al., 2004). Clorgyline plus MDMA group received the same dose preceded 30 minutes by an injection of 1 mg/Kg of clorgyline. Control animals received equal doses of saline vehicle (0.9% w/v), in the same protocol of administration and the clorgyline group was administrated a single dose of clorgyline (1 mg/Kg). Body temperature was measured before the first MDMA or isovolumetric saline injection, and then, every 15 min for a period of 9 h. Humane end-points were clearly defined to avoid unnecessary pain and distress of experimental animals, signs that would predict imminent death were used to decide the use of timely euthanasia (Toth, 2000).

Mitochondria isolation

Fourteen days after exposure (PND 59), animals were killed by decapitation. The encephalon was rapidly removed and processed for brain mitochondria isolation. Mitochondria were isolated from whole rat brain by the method of (Rosenthal et al., 1987) with slight modifications. Rapidly, the whole encephalon was washed, minced and homogenised at 4ºC in isolation medium (250 mM sucrose, 5 mM Hepes, 1 mM EGTA, 1 mg/mL bovine serum albumine (BSA), pH=7.4) containing 5 mg of bacterial protease. Single brain homogenates were brought to 30 mL and centrifuged at 1260 g (*SORVAL RC 5B Plus; Kendro Laboratory Products, Newtown, CT, USA*) for 5 min. The pellet was discarded and the supernatant was centrifuged at 20 000 g for 10 min. The pellet including the fluffy synaptossomal layer was ressuspended in 10 mL of isolation medium containing 0.02% digitonin (to release mitochondria from the synaptossomal fraction) and centrifuged at 20 000 g for 10 min. The brown mitochondrial pellet (without the synaptossomal layer) was ressuspended again in 10 ml of washing medium (250 mM sucrose,

5 mM Hepes, pH=7.4) and recentrifuged at 20 000 g for 10 min. The main portion of the mitochondrial pellet was ressuspended in 300µl of washing medium. Mitochondrial protein was determined by the biuret method calibrated with BSA (Gornall et al., 1949b).

Quantification of lipid peroxides

Lipid peroxidation was determined by measuring malondialdehyde (MDA) equivalents, using the thiobarbituric acid (TBA) assay, according to a modified procedure described by (Rohn et al., 1993b). Mitochondrial protein (3 mg) was incubated 30 minutes at 25ºC in 3 mL of medium (175 mM KCL, 10 mM Tris, (pH=7.4) and 3 µM rotenone). Samples of 0.3 mL were then incubated with 2.7 mL of TBARS reagent (9 % TBA, 0.6 N hydrochloric acid (HCl) and 0.0056 %butylated hydroxyl toluene (BHT)). The mixture was warmed to 80-90ºC, for 15 min and cooled by immersion in ice during 10 minutes before centrifuge at 1500 g for 5 min. Lipid peroxidation was estimated by spectrophotometric determination at 535 nm of the MDA equivalents produced. The amount of MDA equivalents formed was calculated using a molar extinction coefficient of 1.56×10^5 $mol^{-1}cm^{-1}$ and expressed as nmol MDA equivalents/mg protein (Buege and Aust, 1978).

Quantification of protein carbonyls

Protein carbonyls were quantified by reaction with 2,4-dinitrophenylhydrazine (DNPH) with the spectrophotometric method for carbonyl assay adapted from (Reznick and Packer, 1994). Two samples of 1 mL of each mitochondrial extract 1 mg/mL were placed in glass tubes. To one tube 4 mL of 10 mM DNPH in 2.5 M HCl solution was added, and to the other tube of the same sample, only 4 mL of 2.5 M HCl (blank tube). Tubes were left for 1 hour at room temperature in the dark and vortexed every 15 min. At this point, 5 mL of 20 % trichloroacetic acid (TCA) (w/v) solution was added to both DNPH and HCl samples to a final concentration of 10% (w/v) TCA. The tubes were left in ice for 10 minutes and then centrifuged at 2300 *g* for 5 minutes. The resultant supernatant was discarded. Next, another wash was performed with 4 ml of 10% TCA and the protein pellets were broken mechanically. The protein pellets were washed three times with ethanol-ethyl acetate (1:1) (v/v). The final pellet was dissolved with 6 M guanidine hydrochloride solution and left for 10 minutes at 37ºC in agitation in a water bath. All samples

were then centrifuged to remove any insoluble material remaining in suspension. The concentration of DNPH was determined at 360 nm and a molar absorption coefficient of 22 x 10^3 M^{-1} cm^{-1} was used to quantify the levels of protein carbonyls. Protein concentration in the samples was calculated by determining the absorbance at 280 nm. Protein carbonyl content was expressed as nmol protein carbonyl formed/mg mitochondrial protein (Reznick and Packer, 1994).

Western Blot analysis of isolated whole brain mitochondria

Isolated whole brain mitochondria were ressuspended in extraction buffer (20 mM Tris-HCl, pH 7.6, 250 mM sucrose, 40 mM KCl, 2 mM ethylenedioxybis (ethyleneamino) tetraacetic acid (EGTA)). The homogenate was centrifuged at 600 g for 10 min at 4º C and the supernatant was taken for mitochondrial Western Blot analysis, 15 µg of protein were loaded per lane and separated on 10 % Sodium Dodecyl Sulfate (SDS)-polyacrylamide gels. The gels were transferred to a polyvinylidene fluoride (PVDF) membrane for protein Blotting (0.2 µm, *Bio-Rad Laboratories*) membranes by electroblotting 1 hr at 150 mA. The filters were blocked in 5 % non fat dry milk and 0.1% Tween 20 overnight at 4º C. Blots were then incubated with mouse monoclonal antibody against complex IV subunit I (A-6403; Molecular Probes; 2 µg/ml) or complex I subunit II (A-21344; Molecular Probes; 0,5 µg/ml) diluted in Tris Buffered Saline Tween 20 (TBST) (0.1% TTBS; 20 mM Tris, 137 mM NaCl, pH 7.6) for 1 hr at room temperature. Membranes were washed three times for 10 min in the same buffer and incubated for 1 hr with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Imun-Star™ Bio-Rad; 1:20000). Immunoreactive proteins were revealed using enhanced chemiluminescence method (Immun-Star™ HRP Chemiluminescent Kit, *BIO-RAD*).

Blots were analyzed with Quantity One® Software (*BIO-RAD, Hercules, CA, USA*) version 4.5.

Statistical analysis

Data concerning evolution of body temperature were analysed using a 2-way ANOVA (treatment *vs* time). Significant differences were further tested using the post-hoc Tukey HSD for unequal n.

Data concerning MDA equivalents, protein carbonyls, and western blots quantifications were analysed using an appropriated non-parametric alternative to the one-way independent-samples ANOVA, the Kruskal-Wallis Test, since these data failed to met the necessary assumptions for the use of an ANOVA. The statistical level of significance was considered at $p<0.05$.

Results

Effects of MDMA plus clorgyline administration on body temperature and survival

On the day of injection (PND 45) rats treated with MDMA (4x10 mg/Kg, n=6) and MDMA plus clorgyline (1 mg/Kg, 30 minutes before the drug, n=3), showed significantly higher body temperatures when compared to saline controls (isovolumetric 0.9%NaCl solution, n=6) and clorgyline (1mg/Kg, n=6) administered animals (Fig. 1.1). Clorgyline administered animals did not show significant differences in body temperature when compared to the control group (Fig. 1.1). After the third injection, body temperature started to decline for MDMA group and attained control values about 7.5 h after the first administration, while for clorgyline plus MDMA treated animals, the hyperthermic effect was maintained fairly high (Fig. 1.1). Importantly, about 57% of the animals injected with clorgyline plus MDMA (four out of seven rats) died or where euthanized (because of humane end points previously defined) before the last administration of MDMA. Death of these animals was preceded by characteristic behaviour of the “serotonin syndrome” (SS), namely head weaving, forepaw treading, tremor, flat body posture, and backward movement; and noradrenergic dysfunction observed by increased secretion of fluids by nose and mouth (increased salivation). Noteworthy, the body temperature of these animals, just before deceasing, was around 41ºC (Fig. 1.2), well above those that survived, from MDMA or clorgyline plus MDMA groups.

Effects of MDMA plus clorgyline administration on lipid peroxidation

Lipid peroxidation was estimated, 14 days after injection, on whole brain mitochondrial homogenates. The levels of MDA equivalents were measured on the four groups under study: MDMA (40mg/Kg, n=6), MDMA plus clorgyline (40mg/Kg + 1mg/Kg, n=2), clorgyline (1mg/Kg, n=8) and isovolumetric saline (0.9% NaCl, n=5). MDMA exposed rats had significantly higher

levels of MDA equivalents, when compared to those administered with isovolumetric saline ($p<0.01$). Rats administered with clorgyline before exposure to MDMA also presented significant higher levels of MDA equivalents than rats solely administered with clorgyline ($p<0.05$). Significantly different values were not detected between MDMA and MDMA plus clorgyline neither between control saline and clorgyline groups (Fig. 2).

Effects of MDMA plus clorgyline administration on the formation of protein carbonyls

Protein carbonyls were measured, 14 days after injection, on whole brain mitochondrial homogenates on the four groups under study: MDMA (40 mg/Kg, n=7); MDMA plus Clorgyline (40 mg/Kg+1 mg/Kg, n=2); clorgyline (1 mg/Kg, n=8) and isovolumetric saline (0.9% NaCl, n=5). Protein carbonylation was significantly higher in MDMA ($p<0.05$) and MDMA plus clorgyline ($p<0.01$) groups when compared to both control groups. The MDMA plus clorgyline group also presented higher protein carbonylation than the clorgyline group ($p<0.05$). Significantly different values were not detected between control saline and clorgyline groups (Fig.3).

Western blot analysis for the expression of mitochondrial subunit NDII

The expression of the subunit II of NADH dehydrogenase (complex I of the mitochondrial respiratory chain) was measured, 14 days after injection, on whole brain mitochondrial homogenates.

Subunit II of NADH dehydrogenase suffered a significant decrease ($p<0.05$) on its expression after exposure to a neurotoxic dose of MDMA (40 mg/kg, n=6). The administration of clorgyline before MDMA (1 mg/Kg, n=2) did not produce any significant alteration on the pattern of expression of this mitochondrial subunit in MDMA treated animals. The results for both controls (isovolumetric saline (0.9% NaCl, n=6) and clorgyline (1 mg/Kg, n=6) were not different at the tested level (Fig. 4).

Western blot analysis for the expression of mitochondrial subunit COXI

The expression of the subunit I of cytochrome oxidase (complex IV of mitochondrial respiratory chain) was measured, 14 days after injection, on whole brain mitochondrial homogenates.

The expression of subunit I of cytochrome oxidase was significantly reduced ($p<0.01$) on MDMA group (40 mg/Kg, n=6) comparatively to control (0.9% NaCl, n=6). The administration of clorgyline before MDMA (1mg/Kg, n=2) produced a significant decrease ($p<0.05$) on the expression of this mitochondrial subunit compared with MDMA treated animals and clorgyline administered animals ($p<0.05$). No differences were observed between isovolumetric saline and clorgyline groups (Fig. 5).

Figures:

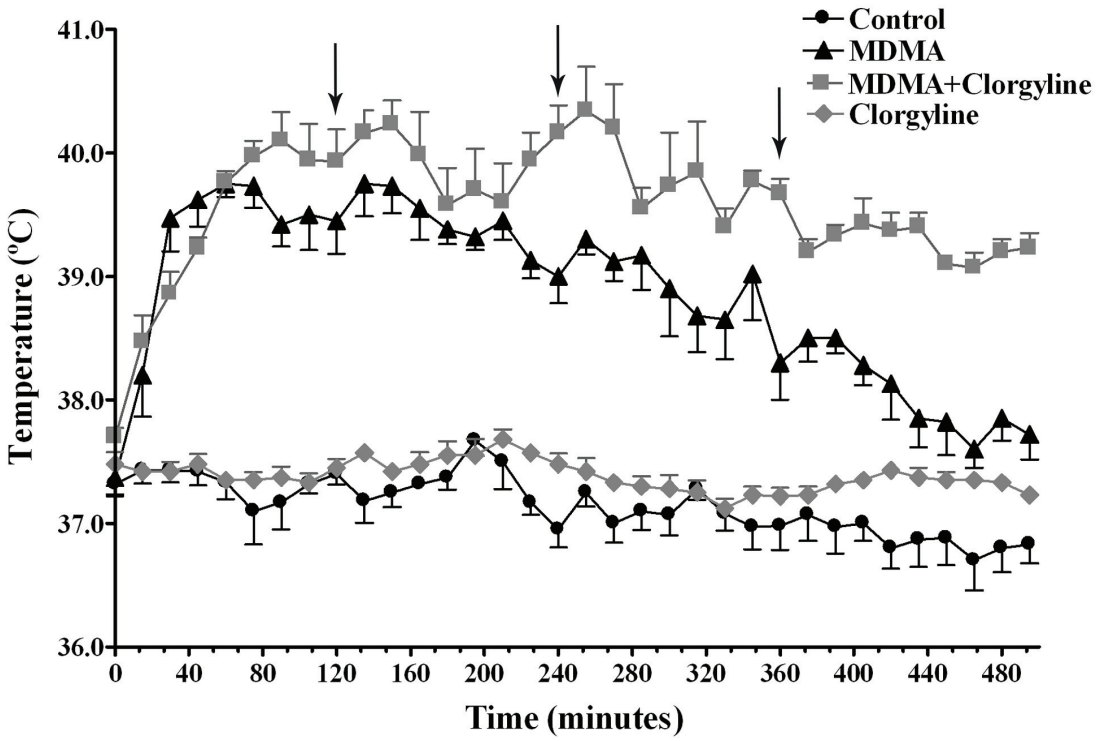

Figure 1.1 - MDMA induced hyperthermia in adolescent male Wistar rats. The image represents body temperature evolution (measured by scanning an inserted subcutaneously probe, every 15 min for 9 hours) throughout the period of exposure to: MDMA (4x10 mg/Kg); clorgyline (1 mg/Kg) + MDMA (4x10 mg/Kg), control saline (isovolumetric saline) and clorgyline (2 mg/Kg). Clorgyline was administered 30 min before exposure to MDMA. Results are reported as mean ± SEM for n=6 in all groups except clorgyline+MDMA, where n=3. Evolution curves for MDMA and MDMA+clorgyline exposed rats started to display significantly higher values than the curves of control and selegiline exposed rats 30 min after the first dose ($p<0.001$ for most time points, two-way ANOVA followed by a post-hoc Tukey HSD for unequal n). Arrows indicate injection timings.

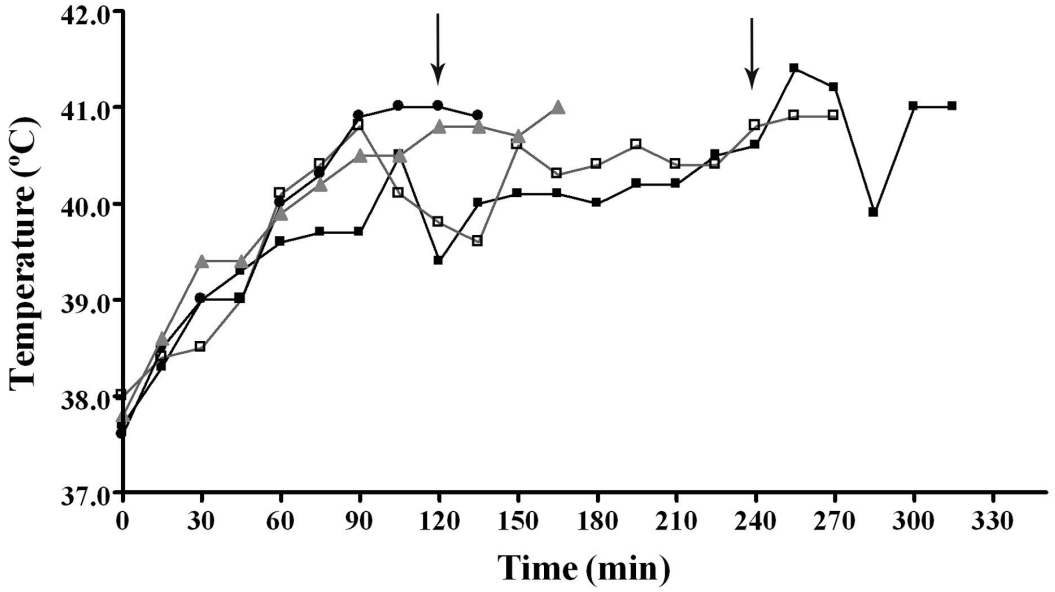

Figure 1.2- Graphic representation of MDMA+clorgyline induced hyperthermia in the animals that died through the injection period (each line represents a different subject). Temperatures were measured every 15 min until deceasing, by scanning a probe subcutaneously inserted 3 days before exposure. Clorgyline (1 mg/Kg) was administered 30 min before exposure to MDMA (10mg/Kg every 2 hours, though none of these animals survived to the time of the last administration, i.e. 360 min). Results are expressed as individual measures of body temperature (ºC) for each of the four animals represented. Arrows indicate injection timings.

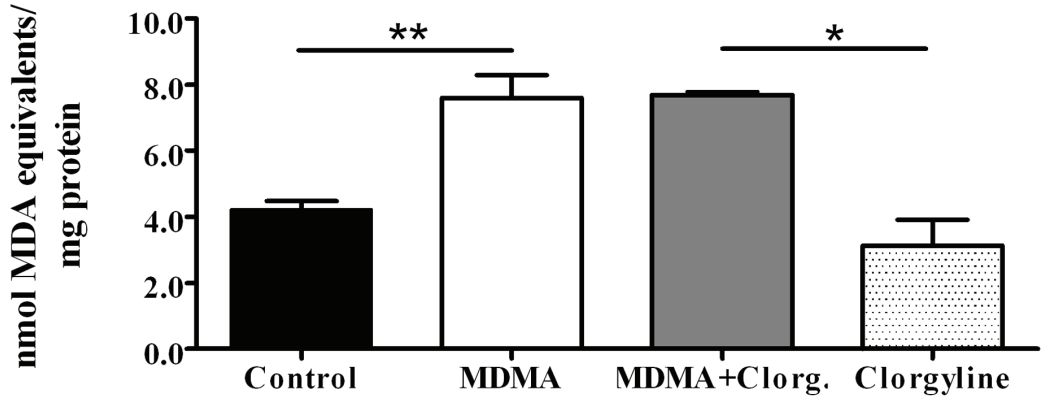

Figure 2 - MDMA induced lipid peroxidation in whole brain mitochondria. Lipid peroxidation was determined by measuring malondialdehyde (MDA) equivalents, using the thiobarbituric acid (TBA) assay. Animals were killed 14 days after exposure to: MDMA (4x10mg/Kg), clorgyline (1 mg/Kg) + MDMA (4x10 mg/Kg), saline (isovolumetric saline) or clorgyline (1 mg/Kg). Clorgyline was administered 30 min before exposure to MDMA. Columns represent mean+SEM,

expressed in nmol MDA equivalents/mg protein, for each experimental group (n=5 for control saline, n=6 for MDMA, n=2 for MDMA+clorgyline and n=8 for clorgyline). Significant differences between groups are signed as *p<0.05 and **p<0.01 as evaluated by the non parametric test Kruskal-Wallis.

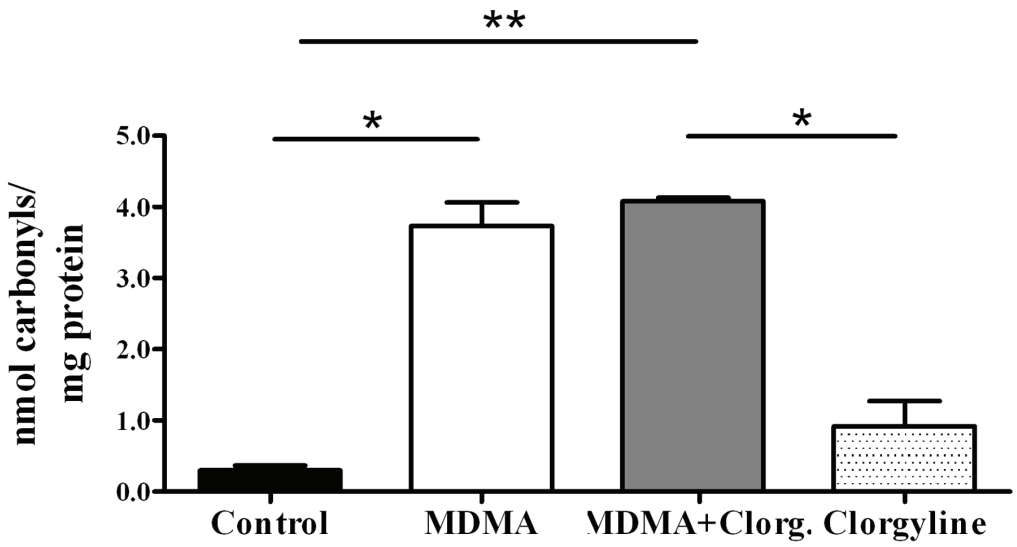

Figure 3: MDMA induced carbonyl formation in whole brain. Protein carbonyls were quantified by reaction with 2,4-dinitrophenylhydrazine (DNPH) using a spectrophotometric assay for carbonyls. Animals were killed 14 days after exposure to: MDMA (4x10mg/Kg), clorgyline (1 mg/Kg) + MDMA (4x10 mg/Kg), saline (isovolumetric saline) or clorgyline (1 mg/Kg). Clorgyline was administered 30 min before exposure to MDMA. Columns represent mean+SEM, expressed in nmol MDA equivalents/mg protein, for each experimental group (n=5 for control saline, n=6 for MDMA, n=2 for MDMA+clorgyline and n=8 for clorgyline). Significant differences between groups are signed as *p<0.05 and **p<0.01 as evaluated by the non parametric test Kruskal-Wallis.

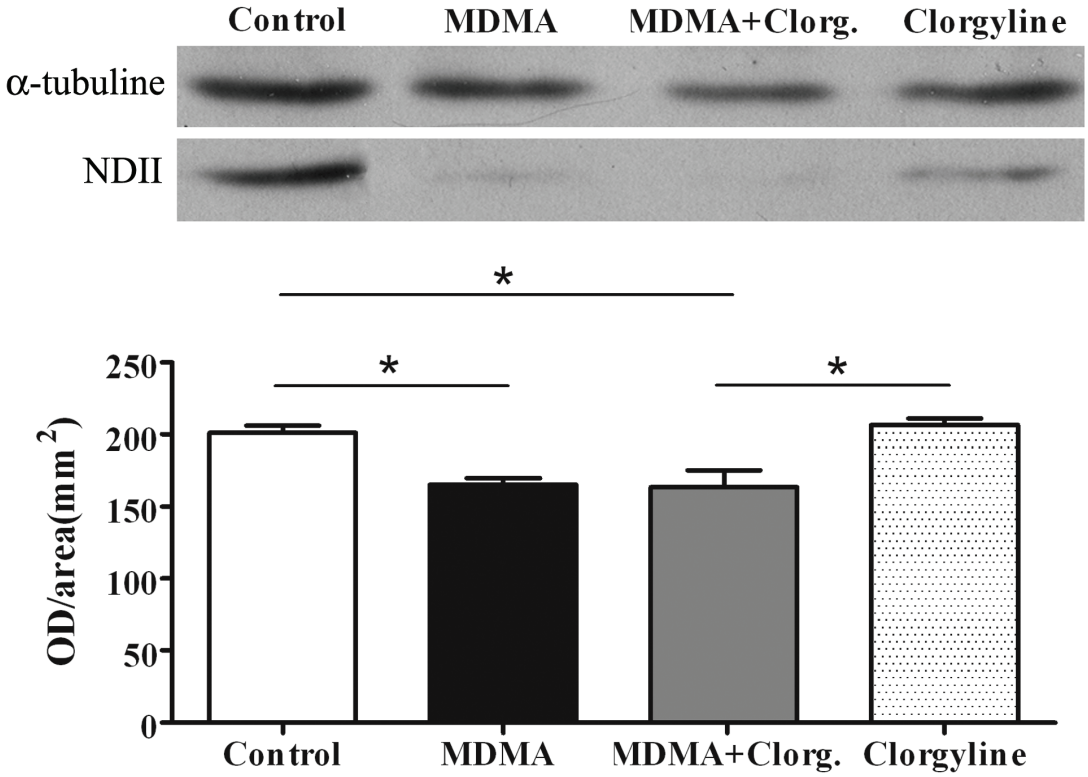

Figure 4. MDMA induced decreased expression of the subunit NDII in whole brain mitochondria. NDII expression was determined by Western blot analysis performed with isolated whole brain mitochondria of male Wistar rats, using a mouse monoclonal antibody anti- NDII subunit. Animals were killed 14 days after exposure to: MDMA (4x10 mg/Kg), clorgyline (1 mg/Kg) + MDMA (4x10 mg/Kg), saline (isovolumetric saline) or clorgyline (1 mg/Kg). Clorgyline was administered 30 min before exposure to MDMA. Columns represent densitometric analysis of Western blots (mean+SEM, n=6 for all the groups with exception of MDMA + clorgyline where n = 2) expressed in OD/area (mm^2). Densitometric analysis of α-tubulin served as loading control. Significant differences in the expression of NDII between groups are signed as *$p<0.05$ and *$p<0.01$ as evaluated by the non parametric test Kruskal-Wallis.

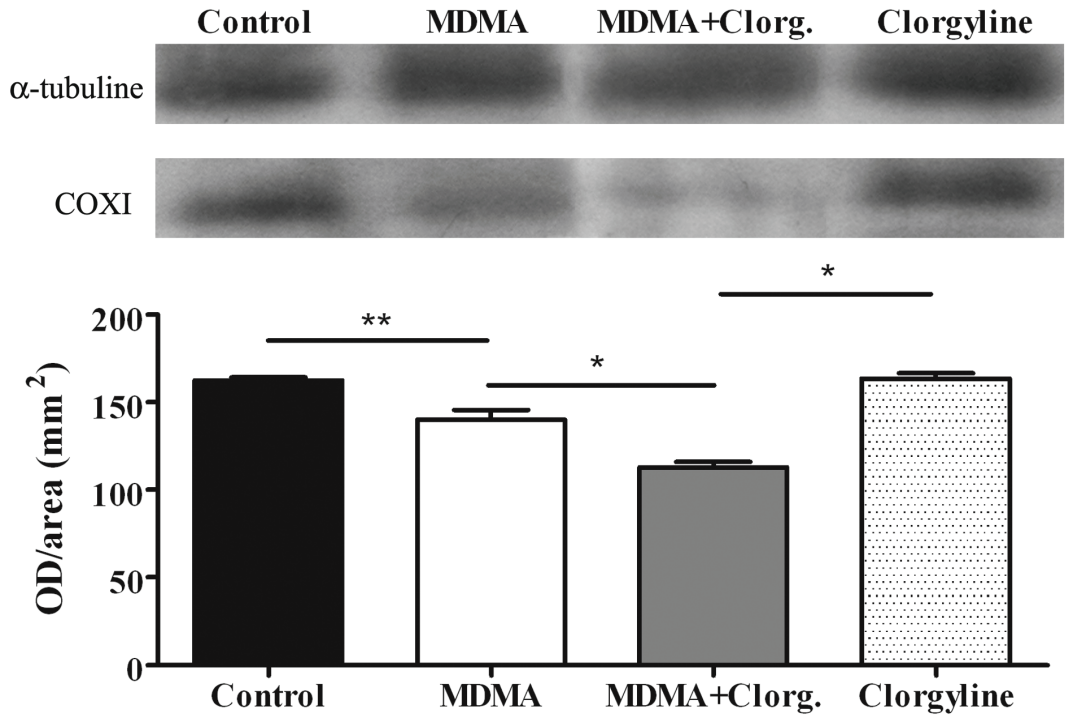

Figure 5. MDMA induced decreased expression of the subunit COXI in whole brain mitochondria. COXI expression was determined by Western blot analysis performed with isolated whole brain mitochondria of male Wistar rats, using a mouse monoclonal antibody anti-COXI subunit. Animals were killed 14 days after exposure to: MDMA (4x10mg/Kg), clorgyline (2mg/Kg) + MDMA (4x10mg/Kg), saline (isovolumetric saline) or clorgyline (2 mg/Kg). Clorgyline was administered 30 min before exposure to MDMA. Columns represent densitometric analysis of Western blots (mean+SEM, n=6 for all the groups with exception of MDMA + clorgyline where n = 2) expressed in OD/area (mm^2). Densitometric analysis of α-tubulin served as loading control. Significant differences in the expression of NDII between groups are signed as *$p<0.05$ and **$p<0.01$ as evaluated by the non parametric test Kruskal-Wallis.

Discussion

The results obtained in the present study demonstrate that the selective inhibition of MAO-A by clorgyline has no beneficial influence on the MDMA-mediated oxidative damage in whole brain mitochondria of rats *in vivo*. In fact, while the administration of a neurotoxic binge dose of MDMA (10 mg MDMA/Kg of body weight, i.p., every 2 h in a total of 4 injections) resulted in significant lipid peroxidation, protein carbonylation and decrease in the expression of the respiratory chain proteins NDII and COXI, the inhibition of MAO-A by clorgyline (1mg/Kg), 30 min before MDMA administration, had no protective effect on the modified parameters. On the contrary, it was observed that MAO-A inhibition resulted in about 57% mortality during the course of MDMA administration. Importantly, death of these animals was preceded by an increased characteristic behaviour of the "serotonin syndrome" (SS) and adrenergic dysfunction.

Also of note, the hyperthermic effect on the clorgyline+MDMA treated animals was maintained fairly stable during the whole time of MDMA administration, while that of the animals treated with only MDMA started to decline after the third administration. Furthermore, the body temperature of these animals, just before deceasing, was around 41ºC, well above those that survived, from MDMA or clorgyline plus MDMA groups. One of the first physiological phenomena observed after MDMA exposure is the increase in body temperature (Carvalho et al., 2002; Alves et al., 2007). Our results showed that the sustained hyperthermic effect in animals administered with clorgyline and MDMA was associated with high lethality, thus confirming previous studies where MAO-A inhibition together with MDMA exposure resulted in higher temperatures and increased toxicity of the drug associated with a higher rate of mortality (Huether et al., 1997).

Our group has recently demonstrated that the exposure of an adolescent rat model to a neurotoxic "binge" administration of MDMA, induces not only an oxidative stress status in mitochondria, but also deletion of mtDNA and consequent impairment in the genes required for protein synthesis, which are essential complexes of the mitochondrial respiratory system required for energy production (Alves et al., 2007). Although deletion of mtDNA was not measured in the present study, due to the high mortality observed for the MDMA+clorgyline

group, the decrease in the expression of the respiratory chain proteins NDII and COXI in the MDMA and MDMA+clorgyline groups much probably results from the deletion of the portion of the mitochondrial genome that includes codons for these proteins (Suliman et al., 2002; Suliman et al., 2003). Of note, the pre-treatment with clorgyline potentiated the MDMA-induced decrease on the expression of COXI, compared with MDMA treated animals. Thus, although MAO-A inhibition had no effect on MDMA-induced oxidative stress in mitochondria, it still potentiated MDMA-induced mitochondrial damage.

Notwithstanding the protection afforded by MAO-B inhibition, reported in previous studies, against MDMA-induced neurotoxicity, it has long being known that the use of MAO inhibitors may result in a steady increase of intrasynaptic serotonin levels mediated by MDMA (Parrott, 2002; Silins et al., 2007) aggravating this way the “serotonin syndrome” (SS) often observed in ecstasy users, which ultimately may be fatal (Silins et al., 2007). In rodents, the signs of SS include head weaving, forepaw treading, hind limb abduction, dorsiflexion of the tail (straub tail), tremor, flat body posture, and backward movement (Izumi et al., 2007), which was corroborated in the present study. Noteworthy, in previous studies, including our own, the observed moderate MDMA-induced SS effect was not affected by MAO-B inhibition. Thus, it is possible that the beneficial effects of specific MAO-B inhibitors overwhelm the deleterious effects of the SS or that the inhibition of the MAO-A isoform is the main responsible for the high risk reported for the concomitant use of ecstasy with MAO inhibitors such as phenelezine and tranylcypromine, nialamide, isoniazid, moclobemide and clorgyline (Vuori et al., 2003; Silins et al., 2007). Corroborating this hypothesis, in the present study, it was observed that MAO-A inhibition led to the aggravation of MDMA-induced behavioural effects, characteristic of both serotonergic (head weaving, forepaw treading, tremor, flat body posture, and backward movement) and over stimulated adrenergic signs, such as of piloerection and increased salivation (Spanos and Yamamoto, 1989a).

It has first been suggested, by Sprague and Nichols, that the neurotoxicity induced by MDMA may be explained with the following sequence of events: depletion of 5-HT from serotonergic neurons; increase in dopamine synthesis and release, in part attributable to the stimulation of 5-HT_{2A} receptors; increase in extracellular dopamine; transport of dopamine into 5-HT nerve terminals by the 5-HT transporter; deamination of dopamine inside 5-HT nerve

terminals by MAO-B, with the consequent formation of H_2O_2 and related oxidative stress, leading to the selective 5-HT neuronal degeneration (Sprague and Nichols, 1995b). Taking into consideration that MAO-A is expressed predominantly in catecholaminergic neurons (Shih et al., 1999b), it may be considered that its inhibition will increase the availability of dopamine for the uptake by serotonergic neurons and subsequent MAO-B metabolism. This hypothesis may explain the lack of protective effects mediated by MAO-A inhibition against MDMA-induced neurotoxicity.

In conclusion, the administration of a neurotoxic binge dose of MDMA to an adolescence rat model previously treated with the specific MAO-A inhibitor clorgyline is able to potentiate its effects on serotonergic and adrenergic mediated behaviour, body temperature and provokes high mortality. Also, inhibition of MAO-A had no protective effect on the MDMA-mediated oxidative damage in whole brain mitochondria of these rats, while it aggravated MDMA-induced decrease in the expression of COXI. These results reinforce the notion that the concomitant use of MAO-A inhibitors and MDMA is counter indicated because of the resulting severe synergic toxicity.

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Manuscript III

Acetyl-L-carnitine provides effective *in vivo* neuroprotection over MDMA-induced neurotoxicity in adolescent rat brain mitochondria

Manuscript in preparation

Acetyl-L-carnitine provides effective *in vivo* neuroprotection over MDMA-induced neurotoxicity in adolescent rat brain mitochondria

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Abstract

3,4-Methylenedioximethamphetamine (MDMA) is a worldwide abused stimulant drug with neurotoxic effects. One of the neurotoxic mechanisms involves a massive release of serotonin from storage vesicles, with associated increase in its metabolism by mitochondrial MAO-B, leading to formation of hydrogen peroxide and consequent oxidative stress in this organelle. Carnitine has been shown to enhance cellular antioxidant and antiapoptotic properties. L-carnitine and its ester, acetyl-L-carnitine (ALC), facilitate the transport of long chain free fatty acids (FFAs) across the mitochondrial membrane for ß-oxidation. Here, we evaluated the potential neuroprotection of ALC against the brain neurotoxic effects produced by MDMA exposure at the mitochondrial level. Adolescent males Wistar rats were randomly assigned to four groups, MDMA (4x10mg/Kg MDMA, i.p.); control (4x saline, i.p.); ALC/MDMA (100mg/Kg of ALC. 30 min prior to MDMA, i.p.) and ALC (100mg/Kg, i.p.). Animal body temperatures were continuously monitored on the day of exposure. Rats were sacrificed 14 days post-treatment. Their brains were processed for evaluation of the oxidized lipid and protein content in the mitochondrial fraction. Brains were also dissected into specific regions for analysis of mitochondrial DNA (mtDNA) by polymerase chain reaction (PCR). Additionally, the pattern of expression of the mitochondrial subunits II (NDII) and I (COXI) of respiratory mitochondrial complexes I and IV, respectively, was assessed. MDMA exposure resulted in a significant increase of mitochondrial lipid peroxidation, protein carbonylation, diminished expression of NDII and COXI and deletion of part of the mitochondrial genomic sequence. The PCR revealed a significant reduction of the percentage of deletion of the mitochondrial genomic sequence in ALC+MDMA rats for most tested regions (amygdale; $p<0.05$), prefrontal cortex ($p<0.05$), raphe nuclei ($p<0.05$), corpora striata ($p<0.001$), hippocampus ($p<0.001$) and ventral mesencephalon ($p<0.001$). The expression of NDII and COXI subunits was significantly protected in ALC+MDMA group as well ($p<0.01$). Data indicate protective effects of ALC in the mitochondrial neurotoxicity induced by MDMA exposure.

Introduction

The amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA or Ecstasy), is presently one of the most abused recreational drugs among adolescents and young adults. Despite the increasing number of publications reporting the toxic effects of MDMA, long term consequences of this drug are still mostly unknown among consumers. Acute doses of MDMA markedly affect the levels of serotonin (5-HT) and associated behaviours, leading to hyperactivity and increased social interaction, but decreasing alertness and blurring perception (Morgan, 2000; Baylen and Rosenberg, 2006).

Long-term exposure to MDMA has been implicated in the etiology of several neuropsychological disorders such as depression, increased and phobic anxiety, obsessive traits, paranoid thoughts, sleep disorders, memory deficits, impulsiveness and addiction (for review see (Britt and McCance-Katz, 2005)). After the initial marked release of 5-HT and associated increase of dopamine (DA) transmission, the multifactorial mechanism of MDMA-induced neurotoxicity leads to persistent depletion of tryptophan hydroxylase, 5-HT and its major metabolite 5-hydroxyindolacetic acid (Escobedo et al., 2005; O'Shea et al., 2006). Consequently, the expression of serotonin transporter (SERT) is reduced (Jayanthi and Ramamoorthy, 2005; Xie et al., 2006). Although, several factors contribute to this outcome, oxidative stress plays here a major role. The MDMA-evoked monoamine surge is leading to formation of toxic quinones, peroxides and other derivatives that are implicated in the sustained increase of free radicals and reactive oxygen species (ROS) (Jayanthi et al., 1999; Zhou et al., 2003; Hrometz et al., 2004).

Recently, we have shown that exposure of adolescent rats to a neurotoxic dose of MDMA results in mitochondrial oxidative damage to several brain regions. Increased lipid and protein peroxidation, mtDNA deletion and subsequent impaired expression of NDII and COXI subunits of mitochondrial complexes I and IV (NADH dehydrogenase and cytochrome c oxidase) is concomitantly observed (Alves et al., 2007). Moreover, we demonstrated that selegiline, a selective inhibitior of monoamine oxidase B (MAO-B), was able to robustly prevent all these events, evidencing the role of MAO-B in the observed oxidative stress (Alves et al., 2007). MDMA induced release of monoamine neurotransmitters (mainly 5-HT) from storage

vesicles was shown to increase MAO-B catalyzed oxidative deamination that is followed by the production of hydrogen peroxide and highly reactive hydroxyl radicals ($HO^{\cdot}$) that mediate stress damage (Alves et al., 2007). MAO´s location in the outer membrane of the mitochondria (Zhuang et al., 1988; Zhuang et al., 1992) facilitates oxidative damage of mitochondrial macromolecules through diffusion of peroxides into the mitochondrial matrix.

Additionally, it has been shown that exposure to MDMA reduces concentrations of neuronal antioxidant elements, such as ascorbic acid (Shankaran et al., 2001) (Shankaran et al., 2001), glutathione (Capela et al, 2007a) and vitamin E (Johnson et al., 2002), and increases the concentration of ROS, evidencing the neuroprotective role of antioxidants against MDMA neurotoxicity. Likewise, increasing the levels of ascorbic acid was shown to prevent the neurochemical and behavioral responses to MDMA administration (Shankaran et al., 2001) and N-acetylcysteine, an antioxidant and glutathione precursor, also reduced MDMA-induced neurotoxicity (Capela et al, 2006; Capela et al, 2007a; Capela et al, 2007b).

Neuroprotective effects of carnitines in various conditions of metabolic stress have been reported (Binienda and Virmani, 2003; Virmani and Binienda, 2004). Absorbed from diet or biosynthesized in the liver and kidneys, carnitine is incorporated into the total body carnitine pool that comprises L-carnitine (LC) and short chain esters such as acetyl-L-carnitine (ALC). Both LC and ALC, as a “shuttle”, facilitate transport of long chain free fatty acids (FFAs) across the mitochondrial membrane for β-oxidation. While, LC deficiency may impair the FFAs oxidation and the utilization of carbohydrates (Schulz, 1994), LC has been shown to prevent oxidative stress related damage induced by metamphetamine (Virmani et al., 2003). It has been suggested that LC administration enhanced glutathione activity preventing lipid peroxidation in aging rats (Arockia Rani and Panneerselvam, 2001). Studies have shown that administered therapeutically ALC is easier than LC transported across the blood-brain barrier suggesting ALC application in acute and chronic neurological disorders (Kido et al., 2001).

Based on the characteristics of ALC, the aim of present study was to evaluate the ALC neuroprotective potential against the MDMA-induced oxidative stress in the brain mitochondria. A model of MDMA “binge” administration in adolescent rats that previously demonstrated to induce significant neurotoxicity was used (Alves et al., 2007).

Material and Methods

Animal Model

Wistar rats born from nulliparous females purchased from Charles River Laboratories España S.A. (Barcelona, Spain) were used. Animals were maintained under a 12-h light/ dark cycle, in a temperature- and humidity-controlled room and given *ad libitum* access to food and water. Institutional guidelines regarding animal experimentation were followed. Rats were handled daily and regularly weighed. Cylindrical plastic tubes and soft paper for nest construction were made available to reduce stress. All procedures used were approved by the Portuguese Agency for Animal Welfare (general board of Veterinary Medicine in compliance with the Institutional Guidelines and the European Convention). On postnatal day 40 (PND 40), animals were assigned to the different experimental groups and caged in pairs. Two days later, a subcutaneous probe (Implantable Programmable Temperature Transponder, IPTT^{TM}-200, BMDS) (Kort et al., 1998) was inserted in the dorsal region, to allow the measurement of body temperature throughout the experiment. On PND 45, the animals assigned to the MDMA group received a freshly prepared solution of MDMA at 10 mg/Kg of body weight, intraperitoneally (i.p.) every 2 h in a total of 4 injections. Rats in the ALC/MDMA group received the same MDMA dose preceded in 30 min by an i.p. injection of 100mg/Kg ALC. Control animals received equal i.p. doses of saline vehicle (0.9% W/V) in the same protocol and the ALC group of rats were administrated i.p. a single dose of ALC (100 mg/Kg).

MDMA (HCl salt) was extracted and purified from high purity MDMA tablets that were provided by the Portuguese Criminal Police Department. The obtained salt was pure and fully characterized by NMR and mass spectrometry methodologies. ALC was kindly provided by Sigma-tau Health Science S.p.A., Pomezia, Italy.

Mitochondria isolation for determination of lipid peroxides and protein carbonyls

Two weeks after exposure, animals were sacrificed by decapitation and brains were rapidly removed on ice. Mitochondria were isolated from whole brain by the method of

Rosenthal et al (1987), with slight modifications. Briefly, the whole encephalon was washed, minced and homogenized at 4ºC in isolation medium (250 mM sucrose, 5 mM Hepes, 1 mM

ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), 1mg/mL bovine serum albumin (BSA), pH 7.4) containing 5 mg of bacterial protease (P-5380, SIGMA, Steinheim, Germany). Single brain homogenates were brought to 30 mL and centrifuged at 1260 g (*SORVAL* RC 5B Plus*,* Kendra Laboratory Products, USA) for 5 min. The supernatant was centrifuged at 20 000g for 10 min. The pellet including the fluffy synaptosomal layer was ressuspended in 10 mL of isolation medium containing 0.02% digitonin (to release mitochondria from the synaptosomal fraction) and centrifuged at 20 000 g for 10 min. The brown mitochondrial pellet (without the synaptosomal layer) was ressuspended in 10 mL of washing medium (225 mM sucrose, 5 mM HEPES, pH 7.4) and recentrifuged at 20 000 g for 10 min. The main portion of the mitochondrial pellet was ressuspended in 300 µl of washing medium. Mitochondrial protein were determined by the biuret method, calibrated with BSA (Gornall et al., 1949a).

Quantification of lipid peroxidation

Lipid peroxidation was determined by measuring malondialdehyde (MDA) equivalents, using the thiobarbituric acid (TBA) assay, according to a modified procedure (Rohn et al., 1993a). Mitochondrial protein (3 mg) was incubated for 30 min at 25ºC in 3 mL of medium (KCL 175 mM, Tris 10 mM, pH 7.4 and rotenone 3 µM). Aliquot of 0.3 mL were then incubated with 2.7 mL of thiobarbituric acid reactive substances (TBARS) reagent (TBA 9 %, hydrochloric acid (HCl) 0.6 N and butylated hydroxyl toluene (BHT), 0.0056 %). The mixtures were warmed to 80-90ºC, for 15 min, and cooled by immersion in ice during 10 min before centrifugation at 1500 g for 5 min. Lipid peroxidation, reflected by the production of MDA equivalents, was estimated by spectrophotometryc determination, at 535 nm, of the MDA equivalents produced. The amount of MDA equivalents formed was calculated using a molar extinction coefficient of 1.56×10^5 $mol^{-1}cm^{-1}$ and expressed as nmol MDA equivalents / mg protein (Buege and Aust, 1978).

Quantification of protein carbonyls

Protein carbonyls were quantified through the spectrophotometric method for carbonyl assay, using 2,4-dinitrophenylhydrazine (DNPH) (adapted from (Reznick and Packer, 1994). Two samples of 1 mL of each mitochondrial extract 1 mg/mL were placed in glass tubes. To one

tube 4 mL of 10 mM DNPH in 2.5 M HCl solution was added, and to the other tube of the same sample, only 4 mL of 2.5 M HCl (blank tube). Tubes were left for 1 hour at room temperature in the dark and vortexed every 15 min. At this point, 5 mL of 20 % trichloroacetic acid (TCA) (w/v) solution was added to both DNPH and HCl samples to a final concentration of 10% (w/v) TCA. The tubes were left in ice for 10 min and then centrifuged for 5 min. The resultant supernatant was discarded. Next, another wash was performed with 4 mL of 10% TCA and the protein pellets were broken mechanically. The protein pellets were washed three times with ethanol-ethyl acetate (1:1) (v/v). The final pellet was dissolved in 6 M guanidine hydrochloride solution and left for 10 min at 37ºC under agitation in a water bath. All samples were centrifuged to remove any insoluble material remaining in suspension. The concentration of DNPH was determined at 360 nm, and the molar absorption coefficient of 22 x 10^3 M^{-1} cm^{-1} was used to quantify the levels of protein carbonyls. Protein concentration in the samples was calculated by determining the absorbance at 280 nm. Protein carbonyl content was expressed as nmol protein carbonyl formed/mg mitochondrial protein (Reznick and Packer, 1994).

DNA isolation for polimerase chain reaction

Two weeks after exposure, animals were sacrificed by decapitation, brains were rapidly removed and dissection of the different brain regions (prefrontal cortex, striatum, hippocampus, amygdala, ventral mesencephalon (comprising substancia nigra and ventral tegmental area, VTA/SN) and raphe nuclei) was performed on ice. DNA from the different brain regions was extracted with GenomicPrep Cells and Tissue DNA Isolation Kit (Amersham Biosciences, Buckinghamshire, UK) according to instructions of manufacturer. No pools of animals were necessary. Extracted DNA (5 µL) was applied on a 1 % agarose gel to quantify the amount of DNA used on the subsequent PCR protocols.

Photographs were taken under UV transillumination (Typhoon 8600, Molecular Dynamics, Amersham Pharmacia Biotech, Buckinghamshire, UK) and the semiquantitative analysis of extracted DNA was made with the software Image Quant 5.1

Polymerase chain reaction

Previously isolated brain areas were analyzed for a deletion between direct repeats corresponding to base pairs (bp) 1095-4095 of rat mtDNA. Deletion primers were designed based on the sequence of the rat mtDNA (Acession No. NC-001665, Genbank) to detect a deletion corresponding to NDI and NDII subunits of mitochondrial complex I (NADH dehydrogenase) and COXI subunit of mitochondrial complex IV (cytochrome c oxidase) (Suliman et al., 2003).

The following mtDNA deletion primers were used: 5'-AGTCGTAACAAGGTAAGCAT-3' (bp 982-1001) mtf1 primer and 5'-ATTTCTACTCTTTTAGCAT-3' (bp 5632-5651) mtr2 primer (Suliman et al., 2003). The reaction mixture consisted of primers in a concentration of 400 µM (1 µl of stock 20 pmol) (MWG-Biotech AG, Germany), 40 ng of template DNA, $MgCl_2$ 1.5 mM (50 mM stock) (Bio-Rad, Hercules, CA), 1 µl of 10 mM PCR nucleotide mix (Eppendorf, Hamburg, Germany), 0.25 µl of Taq Polymerase (5 U/µl) (Bio-Rad, Hercules, CA) and 5 µl of enzyme buffer 10X (Bio-Rad, Hercules, CA). The final volume of the PCR reaction was 50 µl and the program used was 94º C for 2 min, 50º C for 30 s, 72ºC for 2 min (35 cycles) and 72º C for 7 min (1 cycle) (MyCycler^{TM}thermocycler, Bio-Rad, Hercules, CA).

Negative controls were included containing all the above mentioned PCR components except template DNA. Ten-microliter aliquots of the PCR products were electrophoresed through a 1-1.5 % agarose gel in Tris-acetate (TAE) containing ethidium bromide at 45 V/cm. Photographs were taken under UV transillumination (Typhoon, Molecular Dynamics, Amersham Pharmacia Biotech, Buckinghamshire, UK) and the semiquantitative analysis of amplified DNA was made with the software Image Quant 5.1.

Western Blot analysis of isolated whole brain mitochondria

Two weeks after exposure, animals were sacrificed by decapitation and brains were rapidly removed on ice as described above**.** Isolated whole brain mitochondria were ressuspended in extraction buffer (20 mM Tris- hydrochloric acid (Tris-HCl), pH 7.6, 250 mM sucrose, 40 mM potassium chloride (KCl), 2 mM EGTA. The homogenate was centrifuged at 600 g for 10 min at 4º C and the supernatant was taken for mitochondrial Western Blot analysis, 15 µg of protein were loaded per lane and separated on 10 % Sodium Dodecyl Sulfate (SDS)-polyacrylamide

gels. The gels were transferred to a polyvinylidene fluoride (PVDF) membrane for protein blotting (0.2 µm, Bio-Rad, Hercules, CA) membranes by electroblotting 1 h at 150 mA. The filters were blocked in 5 % non fat dry milk and 0.1% Tween 20 overnight at 4º C. Blots were then incubated with mouse monoclonal antibody against complex IV (cytochrome c oxidase) subunit I (COXI) (Molecular Probes, Oregon, 2 µg/ml) or complex I (NADH dehydrogenase) subunit 2 (NDII) (Molecular Probes, Oregon, 0.5 µg/ml) diluted in Tris Buffered Saline Tween 20 (TBST) 0.1% (TBS; 20 mM Tris, 137 mM NaCl, pH 7.6) for 1 h at room temperature. Membranes were washed three times for 10 min in the same buffer and incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Imun-Star^{TM,} 1:20000, Bio-Rad, Hercules, CA). Immunoreactive proteins were revealed using enhanced chemiluminescence method (Immun-Star^{TM} HRP Chemiluminescent Kit, Bio-Rad, Hercules, CA).Blots were analyzed with Quantity One Software (*Bio-rad,* Hercules, CA) version 4.5.

Statistical analyses

Weight evolution was analysed using SPSS for Windows (SPSS Statistical Software Programs version 15.0). A one-way analysis of variance (ANOVA, treatment) was used, since different animal were used for each analysed PND. Significant differences were further tested using the post-hoc Tukey HSD for unequal n. Data concerning evolution of body temperature were analyzed using a 2-way ANOVA (treatment x time) with time as a repeated measure, significant differences were further tested using the post-hoc Tukey HSD for unequal n.

Data concerning MDA equivalents, protein carbonyls, mtDNA deletions and western blots quantifications were analysed using a one-way ANOVA (treatment). Significant main effects and interactions were further explored using the post-hoc Tukey HSD test. Differences were considered to be statistically significant at $p<0.05$ level. All analyses were performed using SPSS 12.0.0 (SPSS, Inc., Chicago, Illinois).

Results

MDMA-induced decreased weight gain

Daily changes in animal body weight gain were monitored throughout the experiment until sacrifice. Compared with saline control rats, there was a decresed weight gain in the MDMA group on PND 49 ($p<0.05$), 53 ($p<0.01$) and 59 ($p<0.001$) (Fig.1). The ALC/MDMA group also displayed significantly reduced weight gain values on PND 49 ($p<0.05$), 53 ($p<0.001$, 57 ($p<0,05$)) and 59 ($p<0.01$) when compared with the control group. Interestingly, the decreased body weight again in the ALC/MDMA animals was more accentuated than in animals exposed only to MDMA. However, the weight gain in the ALC group was different from the control group, and was significantly higher than the ALC/MDMA group (PND53, $p<0.01$ and PND57, $p<0.05$) (Fig. 1).

MDMA-induced hyperthermia

MDMA administration resulted in hyperthermia. Analysis of body temperature data, throughout the day of exposure indicated that ALC was unable to modify the hyperthermic MDMA effect when administered 30 min prior to MDMA (Fig. 2). Rats exposed to MDMA,, as well as rats administered ALC prior to MDMA, had significantly higher body temperature ($p<0.001$) soon after the first injection of MDMA and until the end of the measuring period, as compared to both the saline and the ALC group for most time points (Fig. 2).

ALC administration does not attenuate the MDMA-induced increase in lipid peroxidation

Lipid peroxidation was assessed by means of MDA equivalents formation in isolated whole brain mitochondria homogenates of adolescent male Wistar rats 14 days post exposure. Animals treaded with MDMA presented significantly higher levels of malondialdehyde equivalents ($p<0.01$) as compared to the saline control. No differences between MDMA and ALC/MDMA groups were observed at the tested level (Fig. 3). However, the levels of MDA equivalents formed under exposure to ALC/MDMA or ALC were also increased ($p<0.01$ and $p<0.05$) showing a clear change in the processing of lipids in the presence of ALC.

ALC administration prevented the MDMA-induced increased formation of protein carbonyls

The levels of protein carbonyls were evaluated in the mitochondrial fraction of whole brain homogenates of adolescent male rats 14 days after exposure. The administration of a neurotoxic dose of MDMA produced a significant increase in protein carbonyls of whole brain mitochondria when compared with the control group ($p<0.001$). In animals treated with ALC prior to MDMA, carbonyl levels were significantly lower ($p<0.05$) than those of the MDMA group and simultaneously very similar to the levels of carbonyls formed in the ALC group (Fig. 4). Therefore, the administration of ALC was clearly efficient in protecting the cells from the action of MDMA, leading to a significant reduction of protein carbonyls in the mitochondria.

ALC administration prevented the MDMA-induced deletion of mtDNA

Mitochondrial DNA from the prefrontal cortex, the striatum, the amygdala, the ventral mesencephalon (dissected as the ventral tegmental area and substantia nigra, VTA/SN), the hippocampus and the raphe nuclei, were analyzed by PCR using the Taq DNA polymerase to verify the presence of a deletion corresponding to the genes NDI and NDII of the mitochondrial complex I and COXI of complex IV. With the set of primers used, a band corresponding to NDI, NDII and COXI deletion was expected (see example of striatum PCR results, above Fig. 5). The deletion was substantially more evident in animals exposed to MDMA ($p<0.001$ in all analyzed brain regions) as compared with the other experimental groups. A previous administration of ALC produced a significant attenuation on the level of mtDNA deletion ($p<0.001$), in all tested brain regions, as evidenced in Fig. 5 in ALC/MDMA rats.

ALC administration prevented the MDMA-induced decrease in the expression of the mitochondrial subunit NDII and COXI

Expression of the mitochondrial respiratory chain components COXI and NDII was evaluated by Western blot, two weeks after exposure to MDMA and ALC. Expression of COXI was considerably decreased ($p<0.001$) in the MDMA group compared with the other experimental groups. Animals treated with ALC prior to MDMA presented values of expression that were not significantly different from controls (Fig. 6). Likewise, NDII expression was also significantly

decreased in MDMA rats compared with control and ALC/MDMA ($p<0.01$) and ALC ($p<0.001$) (Fig. 7). These results evidence that pre-administration of ALC successfully prevented the MDMA-induced decreased expression of COXI and NDII.

Figures:

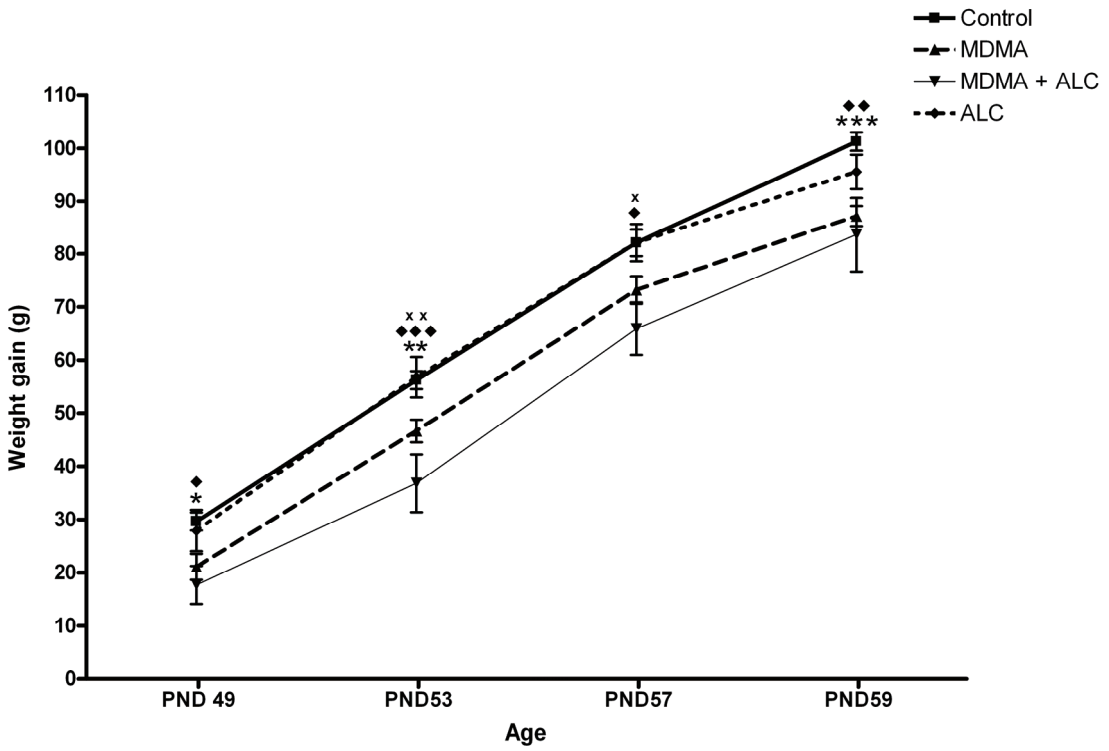

Figure 1: .Weight gain between the day of exposure (PND45) and the day of sacrifice (PND59) measured on the four groups of animals under analysis: MDMA (10 mg/Kg x 4 injections), acetyl-L-carnitine (100 mg/Kg) + MDMA (10 mg/Kg x 4 injections), control (isovolumetric saline x 4 injections) and acetyl-L-carnitine (100 mg/Kg). MDMA and isovolumetric saline groups were given four injections with two hours intervals. Acetyl-L-carnitine plus MDMA group was injected with the same protocol of administration used on MDMA group of animals, but preceded 30 minutes by acetyl-L-carnitine (100 mg/Kg) administration.

Each value is expressed as the mean ± SEM of body weight for each group (MDMA, n=49; MDMA+acetyl-L-carnitine, n=14; acetyl-L-carnitine, n=18; Control, n=45). Statistical analysis was performed using a one-way ANOVA and the main effects and interactions were further analysed using the post-hoc Tukey HSD for unequal n test (*$p<0.05$, ** $p<0.01$*** $p<0.001$, for

control saline compared with MDMA; $^{\blacklozenge}$ p<0.05, $^{\blacklozenge\blacklozenge}$p<0.01, $^{\blacklozenge\blacklozenge\blacklozenge}$p<0.001 for control saline compared with ALC/MDMA, ^{x}p<0.05, ^{xx}p<0.01 for ALC/MDMA compared with ALC).

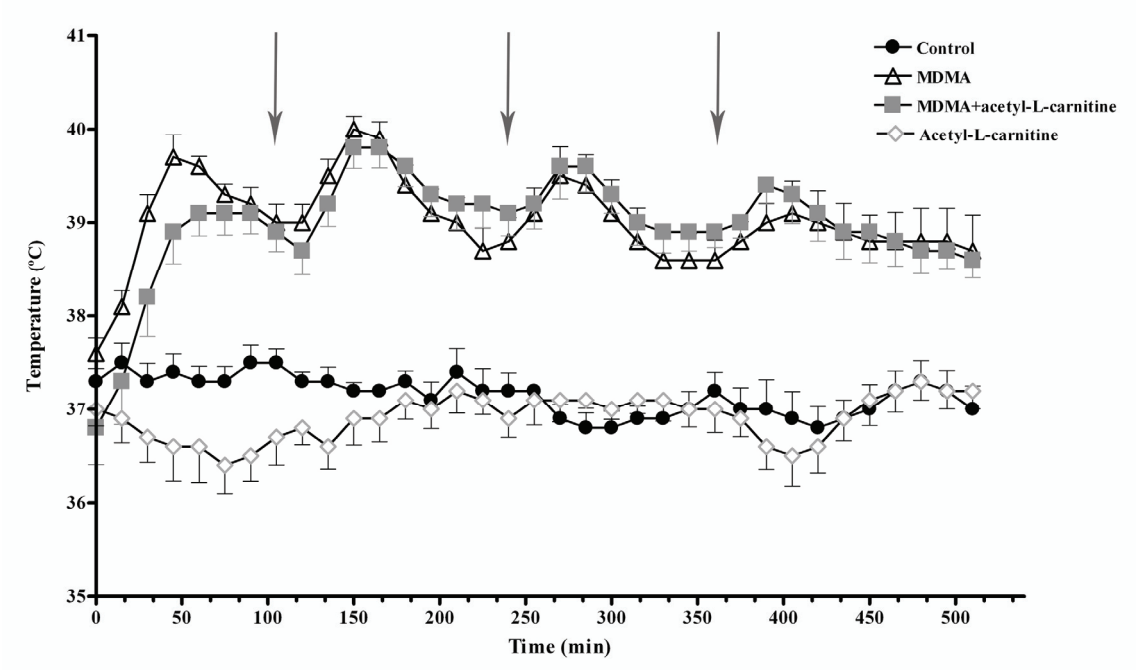

Figure 2: MDMA induced hyperthermia in adolescent male Wistar rats. Body temperature evolution throughout the period of exposure. Temperatures were measured by scanning a subcutaneously probe dorsally inserted, every 15 min for 9 hours. Each tested animal was injected within one of the following experimental group: MDMA (10mg/Kg x 4 injections); acetyl-L-carnitine (100 mg/Kg) + MDMA (10mg/Kg x 4 injections), control (isovolumetric saline x 4 injections) and acetyl-L-carnitine (100 mg/Kg). Acetyl-L-carnitine was administerd once, 30 min before exposure to MDMA. Results are reported as mean ± SEM for n=7 rats per experimental group. Evolution curve for MDMA and acetyl-L-carnitine + MDMA started to display significantly higher values than the groups of control and acetyl-L-carnitine exposed rats 30 min after the first dose (p<0.001; two-way ANOVA). Arrows indicate injection timings.

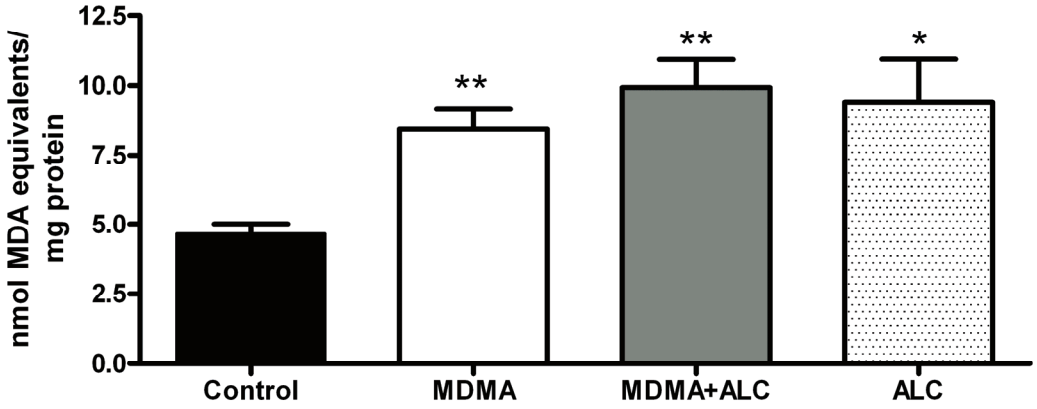

Figure 3: Effects of acetyl-L-carnitine in whole brain mitochondria lipid peroxidation induced by exposure to MDMA. Lipid peroxidation was determined by measuring malondialdehyde (MDA) equivalents, using the thiobarbituric acid (TBA) assay. Animals were sacrificed 14 days after exposure to MDMA (10mg/Kg x 4), acetyl-L-carnitine (100 mg/Kg) + MDMA (10mg/Kg x 4 injections), saline (isovolumetric saline x 4 injections) or acetyl-L-carnitine (100 mg/Kg). Acetyl-L-carnitine was administerd once, 30 min before exposure to MDMA. Columns represent mean ± SEM, expressed in nmol MDA equivalents per mg protein for each experimental group (n=6-19). Animals exposed to MDMA and acetyl-L-carnitine + MDMA presented significantly different values of MDA equivalents than control saline ($^{**}p<0.01$; $^{*}p<0.05$, one way ANOVA followed by a *post hoc* post-hoc Tukey HSD for unequal n).

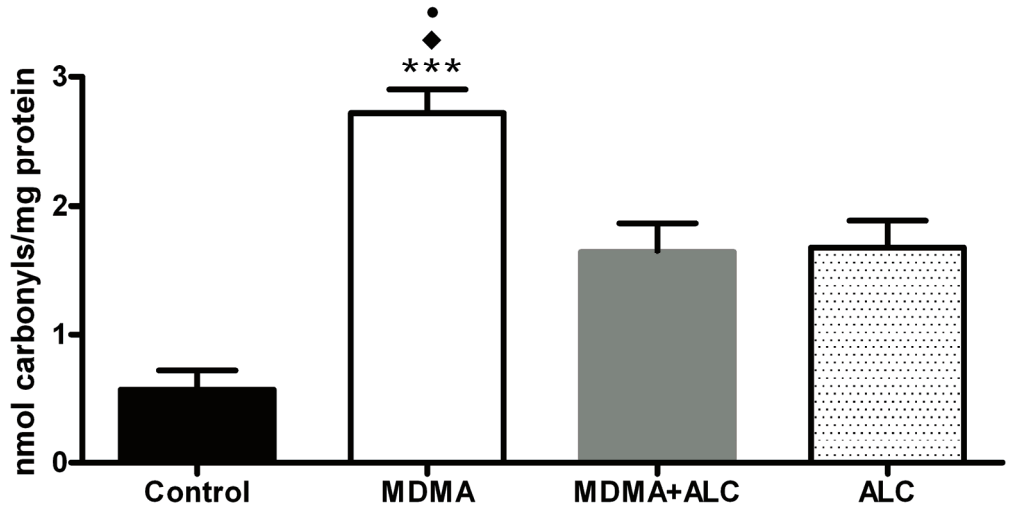

Figure 4: Effects of acetyl-L-carnitine in whole brain mitochondria protein carbonylation of male Wistar rats treated with MDMA. Protein carbonyls were quantified by reaction with 2,4-

dinitrophenylhydrazine (DNPH) using the spectrophotometric method for carbonyl assay. Animals were sacrificed 14 days after exposure to MDMA (10mg/Kg x 4 injections), acetyl-L-carnitine (100 mg/Kg) + MDMA (10mg/Kg x 4 injections), saline (isovolumetric saline x 4 injections) or acetyl-L-carnitine (100 mg/Kg). Acetyl-L-carnitine was administered once, 30 min before exposure to MDMA. Columns represent mean ± SEM, expressed in nmol MDA equivalents per mg of protein for each experimental group (n=6-23). Significantlydifferent from control at $^{***}p<0.001$; significantly different from acetyl-L-carnitine + MDMA at $^{\blacklozenge}$ p< 0.05; significantly different from acetyl-L-carnitine at $^{\bullet}$ p< 0.05 (one way ANOVA followed by a post-hoc Tukey HSD for unequal n).

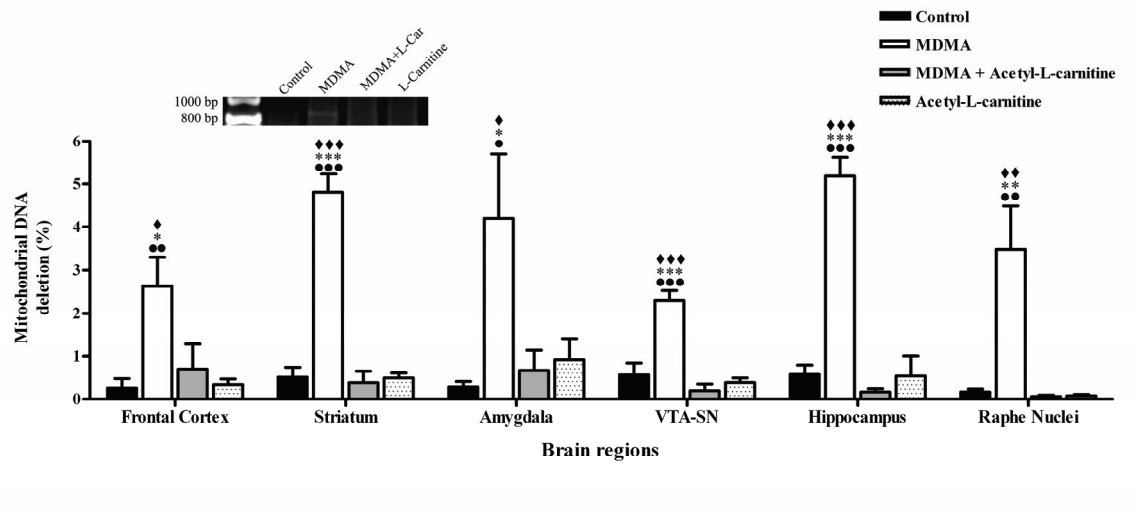

Figure 5: Graphic representation of the protective effects of acetyl-L-carnitine on the deletion of the mitochondrial gene sequences for NDII and COXI in male Wistar rats exposed to a neurotoxic dose of MDMA. Damage was determined by densitometry analysis of Polymerase Chain Reaction (PCR) in different brain regions: prefrontal cortex, ventral mesencephalon (VTA/SN), striatum, raphe nuclei, amygdala and hippocampus. The primers used were mtf1 and mtr2.Animals were sacrificed 14 days after exposure to MDMA (10 mg/Kg x 4), acetyl-L-carnitine (100 mg/Kg) + MDMA (10 mg/Kg x 4), saline (isovolumetric saline x 4) or acetyl-L-carnitine (100 mg/Kg). Acetyl-L-carnitine was administered once, 30 min before exposure to MDMA. Columns represent mean+SEM, expressed as percentage of DNA delection for each experimental group (n=6). A representative image for the PCR analysis is shown for : striatum,

lanes, from left to right, correspond to standard Hyper lader I; control saline; MDMA, acetyl-L-carnitine + MDMA and acetyl-L-carnitine. Significant differences between treatments are represented as: •••p<0.001, ••p<0.01, •p<0.05 as compared with control group; ***p<0.001, **p<0.01, *p<0.05 as compared with MDMA+ acetyl-L-carnitine ; ♦♦♦p<0.001, ♦♦p<0.01, ♦p<0.05 as compared with acetyl-L-carnitine (one-way ANOVA).

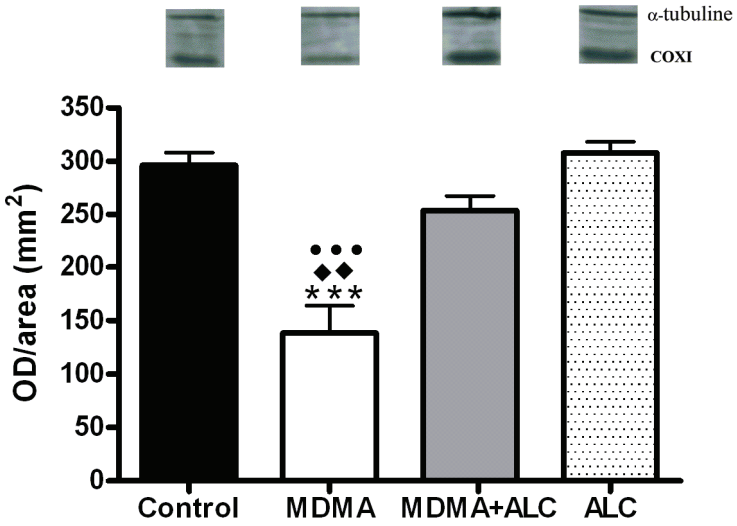

Figure 6: Acetyl-L-carnitine effects on MDMA induced decrease of the mitochondrial subunit COXI expression, as determined by western blot analysis performed in whole brain mitochondria of male Wistar rats. An antibody against COXI subunit was used. Animals were sacrificed 14 days after exposure to MDMA (10mg/Kg x 4), acetyl-L-carnitine (100 mg/Kg) + MDMA (10 mg/Kg x 4), saline (isovolumetric saline x 4) or acetyl-L-carnitine (100 mg/Kg). Acetyl-L-carnitine was administered once, 30 min before exposure to MDMA. Columns represent densitometric analysis of Western blots (mean ± SEM, n=6-8) expressed in OD/area (mm^2). Detection of tubulin served as a loading control. Significant differences between treatments are represented as: ***p<0.001 as compared with control saline; ♦♦p<0.01 as compared with acetyl-L-carnitine + MDMA; •••p<0.001 as compared with acetyl-L-carnitine (one way ANOVA followed by post-hoc Tukey HSD for unequal n).

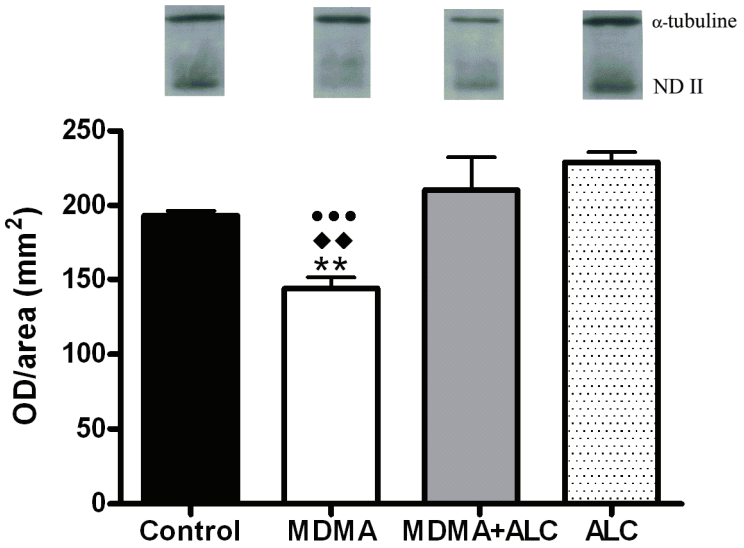

Figure 7: Acetyl-L-carnitine effects on MDMA induced decrease of the mitochondrial subunit NDII expression, as determined by western blot analysis performed in whole brain mitochondria of male Wistar rats. An antibody against NDII subunit was used. Animals were sacrificed 14 days after exposure to MDMA (10 mg/Kg x 4 injections), acetyl-L-carnitine (100 mg/Kg) + MDMA (10 mg/Kg x 4 injections), saline (isovolumetric saline x 4 injections) or acetyl-L-carnitine (100 mg/Kg). Acetyl-L-carnitine was administered once, 30 min before exposure to MDMA. Columns represent densitometric analysis of Western blots (mean ± SEM, n=6-12) expressed in OD/area (mm^2). Detection of tubulin served as a loading control. Significant differences between treatments are represented as: $^{**}p<0.01$ as compared with control saline; ♦♦$p<0.001$ as compared with acetyl-L-carnitine + MDMA; ●●●$p<0.001$ as compared with acetyl-L-carnitine (one way ANOVA followed by a post-hoc Tukey HSD for unequal n).

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Discussion

The findings described in the present study confirm our previous report of MDMA-induced increased oxidative stress at the level of the brain mitochondria (Alves et al., 2007). The protection afford by ALC against MDMA exposure, also reinforce the notion that the mechanisms involved in the neurotoxicity of MDMA abuse are strongly related with oxidative stress events that primarily act on the mitochondria. To our knowledge, this is the first publication that reports the neuroprotective properties of ALC against the neurotoxic effects of MDMA exposure.

Exposure to MDMA significantly attenuated body weight gain throughout the 2 weeks that followed exposure. This result is consistent with previous studies where an anorectic effect emerged after the fourth day of dosing and was still apparent almost 2 weeks after the last MDMA administration (Piper et al., 2005). Acutely, MDMA exposure is characterized by increased urinary and faecal excretion, as well as water loss through sudoresis, inducing a decrease in body weight (Bilsky et al., 1991; Green et al., 2003). Decreased food intake may also be involved (Frith et al., 1987). Increased release of 5-HT is also known to be related with induction of anorectic behavior and, particularly, the activation of the 5-HT_{1B} and 5-HT_{2C} receptors is involved in the regulation of feeding (Bendotti and Samanin, 1987; Kennett et al., 1997; Lucas et al., 1998; Conductier et al., 2005). MDMA is a potent 5-HT releaser (McKenna and Peroutka, 1990; Colado et al., 2004) strongly activating these receptors (Lyon et al., 1986; Battaglia and De Souza, 1989) and leading to altered feeding behaviour. MDMA exposure was also shown to disrupt the appetite for food in adult mice following exposure to a 10 mg/Kg dose (Conductier et al., 2005).

Co-administration of ALC seems to intensify (although not significantly) the decrease in body gain observed after MDMA exposure, which may indicate that other factors are possibly implicated in this process. The half–life of ALC is close to 12 hours and therefore, albeit its role in fatty acid utilization (Penn et al., 1997; Mollica et al., 2001; Iossa et al., 2002), a single ALC administration would not account for a decrease in body weight over a two-week period. However, intraperitoneal injection of ALC itself, may cause a stress reaction accounting for overall body weight loss. It was observed that L-carnitine i.p. administration was associated with a localized inflammatory macrophages activation in the peritoneal cavity, (Dionyssopoulou

et al., 2005), However, our results also show that ALC alone did not affect the normal weight gain, liminting its possible negative effects.

Hyperthermia is a common feature of exposure to MDMA, involving a complex interaction between the hypothalamic-pituitary-thyroid axis and the sympathetic nervous system (Sprague et al., 2003). Despite former reports where no hyperthermic response was observed in adolescent models of MDMA-exposure (Piper and Meyer, 2004), we have previously shown that, under the present protocol, adolescent rats display a robust increase in body temperature as monitored trough subcutaneously inserted probes (Alves et al., 2007). Thermogenesis is a multifaceted process that involves the action of the central nervous system, the peripheral nervous system, as well as cell signalling (for review see (Mills et al., 2004)). The activation of mitochondrial uncoupling protein 3 (UCP3) was pointed as a key factor in the abnormal thermoregulation induced by MDMA (Mills et al., 2003). Increased noradrenaline levels were also shown to be involved in the development of hyperthermia, both by direct activation of UCP3 trough the α1 and β3 adrenergic receptors, and by vasoconstrictive prevention of heat dissipation (Sprague et al., 2007). Likewise, increased levels of plasma FFAs were implicated in the activation of UCP3 in skeletal muscle mitochondria (Sprague et al., 2007). In that sense, administration of ALC before MDMA was not expected to affect the MDMA-induced hyperthermic response. Carnitine was previously shown to increase levels of FFAs in the frontal cortex of rats treated with this same dose of ALC, and may, therefore, contribute to an increased activation of UCP3 (Binienda et al., 1999). Increased levels of FFAs may result from an ALC-induced improved ratio of free-to-esterified CoA, with a consequent increase in oxidative phosphorylation. In agreement with this hypothesis, we have observed a significant augment in the formation of lipid peroxides in groups that were administered with ALC.

The neuroprotective effects of ALC may be exerted through attenuation of mitochondrial membrane permeability transition pore (MPT) opening. Mitochondria control apoptosis via release of cytochrome c into the cytosol through the MPT. Consequently, the administration of AlC may reduce the activation of the caspase cascades, leading to restrained apoptosis (Wieckowski et al., 2000).The overall benefit of ALC administration against MDMA neurotoxicity is probably related with an increased protection of the mitochondria membrane integrity (Kashiwagi et al., 2001). The β-oxidation of FFA involves the formation of long-chain fatty acid

esters of acetyl-coenzyme A (CoA) and their transport into the mitochondria. Previous studies have hypothesized that the protective actions of LC could be conveyed by restoring mitochondrial production of energy via changes in cell membrane viscosity (Binienda et al., 1999). We have recently reported that a neurotoxic dose of MDMA in adolescent rats results in a strong mitochondrial oxidative damage increasing mitochondrial peroxidation, protein carbonylation, mtDNA deletion and impaired expression of NDII and COXI (Alves et al., 2007) with a consequent impairment of energy production (reduced levels of ATP, data not shown). Here, we demonstrate that the previous administration of ALC was able to significantly prevent the deletion of the mtDNA portion that encodes the expression of COXI and NDII proteins. In accordance, there is a recovery in the expression of the COXI and NDII proteins. Preventing the diminished expression of COXI and NDII enhances the functionality of the respiratory chain and favours the production of energy, enlightening the protective role of ALC in mitochondria.

Despite the already discussed increase in lipid peroxidation, in the present work we show a significant reduction on the formation of protein carbonyls in the mitochondria, demonstrating the protective role of ALC at this level. Proteins are major targets for ROS, leading to the formation of oxidized forms of proteins that are easily recognized by proteases, increasing degradation levels and resulting in loss of enzymatic activity (Grune et al., 2001). Increased levels of protein carbonyls are a characteristic feature of aging, which may be explained by increased mitochondrial oxidant production and a progressive decline in proteasome activity (Davies et al., 2001; Grune et al., 2001). Confirming the role of ALC in preventing the increase in protein carbonyls, there are several studies that report the use of ALC to delay age-related processes (Abdul et al., 2006; Calabrese et al., 2006; Sethumadhavan and Chinnakannu, 2006; Savitha et al., 2007; Tamilselvan et al., 2007).

In consequence of its properties ALC has become a compound of great interest in various neurological problems, especially chronic neurodegenerative disorders. The substantia nigra (SN) is a brain region with an increased vulnerability to oxidative damage, because of its high content of oxidizable contents, high metabolic rate and relatively low antioxidant complement. Therefore, treatment with carnitine may contribute to a delay in the development of Parkinson's disease (Kidd, 2000). Likewise, ALC may be useful as a possible therapeutic agent for patients with Alzheimer's disease. The presence of ALC in primary cortical neuronal cultures,

significantly attenuated amyloid-beta peptide-induced cytotoxicity, decreasing protein oxidation, lipid peroxidation and apoptosis in a dose-dependent manner (Abdul et al., 2006). Moreover, ALC was also shown to elevate cellular GSH and heat shock protein levels (Abdul et al., 2006) which reinforces its neuroprotective potential in neurodegeneration associated with mitochondrial oxidative damage.

The present work successfully demonstrates that a pre-administration of ALC confers effective neuroprotection against the MDMA-induced neurotoxicity in the rat brain. We report a significant prevention of mitochondrial oxidative damage, with reduced carbonyl formation, decreased mtDNA deletion and improved expression of respiratory chain components. These results contribute to reinforce the beneficial potential of ALC against neurodegenerative disorders.

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Chapter III

Unpublished results

1.1. Introduction

1.2. Methods

1.3. Results

1.1. Introduction

In previous works (Alves et al., 2007) we demonstrated that the administration of a neurotoxic dose of MDMA to rats results in mitochondrial oxidative damage in the CNS, namely lipid and protein oxidation, and mtDNA deletion with subsequent impairment in the correspondent protein expression, of the mitochondrial enzymes NADH dehydrogenase and cytochrome c oxidase. Both enzymes are constitutive elements of the enzymatic battery that compose the ETC where coupling of OXPHOS and respiration leads to the production of ATP, the main energy source of brain cells. Damage at these levels could result in an inadequate supply of energy to neurons that may lead, in last instances, to loss of function and activation of the necrotic and apoptotic cell death pathways.

Triphenyl tetrazolium salts, like TTC, have been shown to be an excellent tool to assess the functional activity of mitochondria. Indeed, TTC staining permits to assess the mitochondrial viability besides being an easy and cheap technique. The colourless TTC is enzymatically reduced to a lipid soluble red formazan by-product by mitochondrial dehydrogenases such as succinate dehydrogenase (Borges et al., 2004). The extent and intensity of the red colour produced is directly proportional to the functional activity of mitochondria (Lippold, 1982; Goldlust et al., 1996). If TTC is reduced, a reasonable conclusion is that the enzyme is intact thus being able to supply reducing equivalents and conducing to the formation of a red formazan by-product of this compound. When brain damage surmounts to such a condition, the mitochondria ability to enzymatically convert TTC to formazan is decreased, and white staining develops. In view of this mechanism, tetrazolium salts have been extensively applied to quantitatively delineate the infarcted area in animal stroke experiments (Memezawa et al., 1992; Kuge et al., 1995; Takano et al., 1996). Of note, several conditions such as the accumulation of toxic metabolites, continuous activation of the synapses by increased release of neurotransmitters, repair of proteins and enzymes previously damaged by oxidative stress conditions and restoration of membrane potential due to previous opening of MPT, are all capable of disrupting the mitochondrial metabolism. The majority of these dysfunctions have already been described for MDMA-induced neurotoxicity thus justifying the use of the technique in our experiments. Moreover, the generalized use of TTC in measuring the efficacy of putative neuroprotection (Bose et al., 1988; Heinel et al., 1994) lead us to consider its application in our experiments in order to assess the neuroprotective results obtained relatively to both selegiline and ALC against MDMA-induced neurotoxic effects.

ATP, as the final product of the ETC, represents a good indicator of the overall damage produced, being a tool that allows to estimate the damage inflicted to the organelle

itself and the extension of damage to the overall cellular function. Like TTC staining, ATP measurement, is an unspecific technique that complements our previous reported data. It also gives new insights on the effect of the drug on the energetic metabolism of the affected tissues.

Considering the above mentioned rationale, the aim of the present study was to evaluate the metabolic activity of brain tissue of adolescent male Wistar rats, namely with respect to succinate dehydrogenase activity and ATP levels, after exposure to a neurotoxic dose of MDMA, as well as the protective effects of both selegiline and ALC upon the damage produced.

1.2. Methods

Animal model

The animal model used consisted of adolescent male Wistar rats kept under controlled environmental conditions (temperature, 20±2ºC; relative humidity, 45-55%; 12 h light/dark cycle) and housed with food and water supplied “ad libitum”. All procedures used were approved by the Portuguese Agency for Animal Welfare (General Board of Veterinary Medicine in compliance with the Institutional Guidelines and the European Convention).

On PND 40, animals were caged in pairs and divided in six experimental groups: MDMA, control saline, selegiline plus MDMA, selegiline, ALC plus MDMA and ALC.

On PND 45, the animals assigned to the MDMA group received a freshly prepared solution of 10 mg MDMA/Kg of body weight, intraperitoneally (i.p.), every 2 h in a total of 4 injections. Selegiline plus MDMA group received the same dose preceded 30 min by an i.p. injection of 2 mg/Kg of selegiline. Control animals received equal doses of saline vehicle (0.9% W/V), in the same protocol of administration. Selegiline group was administrated a single dose of selegiline (2 mg/Kg). ALC plus MDMA group received MDMA in the same protocol of administration of MDMA group preceded 30 min by an i.p. injection of 100mg/Kg of ALC. ALC group was administered with a single dose of ALC (100mg/Kg). MDMA (HCl salt) was extracted and purified from MDMA tablets that were provided by the Portuguese Criminal Police Department. The obtained salt was pure and fully characterized by NMR and mass spectrometry methodologies. Selegiline was supplied by Sigma (Steinheim, Germany) and acetyl-L-carnitine was supplied by Sigma tau (Italy).

ATP quantification

On PND 59 animals were sacrificed by decapitation and brains were immediately transferred and dissected on ice. The following brain areas were removed: FCx, CPU, SN, RN, Hip and cereb.

Brain areas were added to eppendorfs previously filled with 1ml of percloric acid at 5% and stored at -80ºC until further analysis.

Cellular ATP levels were determined by a bioluminescence assay using the firefly ATP-dependent luciferin-luciferase system. The intensity of the emitted light is linearly related to ATP concentration and it was measured using a 96-well Microplate Luminometer (BioTek Instruments). Briefly, samples were homogeneized in 5% perchloric acid and supernatants were then neutralized with an equimolar solution of $KHCO_3$. After centrifugation, samples were transfered to 96-well opaque microplates and an equal volume of the luciferin-luciferase assay solution (pH 7.6) was then added (final concentrations: luciferin 0.15 mM, luciferase 30000 light units, glycine 50 mM, $MgSO_4$ 10 mM, Tris 1 mM, EDTA 0.55 mM, BSA 1%). An ATP calibration curve was performed and ATP contents were normalized to the total protein content of samples. Results were expressed in nmol ATP per mg of protein analysed.

2,3,5-Triphenyltetrazolium Chloride-Staining of rat brain slices

On PND 59 animals were killed by decapitation and the whole brain was removed and placed at -20ºC for 10 minutes. Four slices of approximately 2mm were dissected (interaural 8.20mm/bregma -0.80mm; interaural 3.80mm/bregma -5.20mm; interaural 1.36mm/bregma -7,64; interaural-2.60/bregma -11.60) and immediately transferred to a 0.5% TTC solution warmed at 37ºC. Submersed slices were kept for 20 min in a water bath at 37ºC with agitation. After staining, slices were washed 3 times with PBS 1X for 1 minute and incubated with agitation at room temperature with 0.1 mol/L- phosphate buffered 4% formaldehyde (PBF) and protected from light.

Photographs were taken with a Leica microscope and analysed with an image analysis software program (Image J version 1.37). Each brain slice was analysed for effects in different brain regions: CPU, NAccb, SN, VTA, Hip, RN and cereb.. The results were expressed as the mean values for each brain region in percentages of white area relatively to control saline.

Data analysis

Data concerning evolution of body temperature were analysed using a two-way ANOVA (treatment *vs* time). Significant differences were further tested using the post-hoc Tukey HSD for unequal n.

Data concerning weight gain was analysed using SPSS for Windows (SPSS Statistical Software Programs version 15.0) using a one-way ANOVA (treatment) Significant differences were further tested using the post-hoc Tukey HSD for unequal n.

Data concerning ATP measurements and TTC staining were analysed using SPSS for Windows (SPSS Statistical Software Programs version 15.0). A one-way ANOVA was used and the main effects and interactions were further analysed using the post-hoc Tukey HSD test. Level of significance was considered at $p<0.05$.

1.3 Results

Body weight gain after exposure to MDMA, effect of selegiline

Evolution of animal body weight was monitored on the day of exposure and through the following days until sacrifice.

Body weight monitoring between the day of injection and the day of sacrifice evidenced that MDMA treatment (4 x 10mg/Kg) induced a significant decrease in body weight gain when compared with controls. MDMA-induced diminishment of weight gain is aggravated through the two week period ($p<0.05$ on PND49, $p<0.01$ on PND53 and $p<0.001$ on PND59) (Fig.1). Control groups were not statistically different between them. Significant differences were also not found between MDMA plus selegiline and MDMA.

The results obtained are summarized in the graph that follows (Fig. 1).

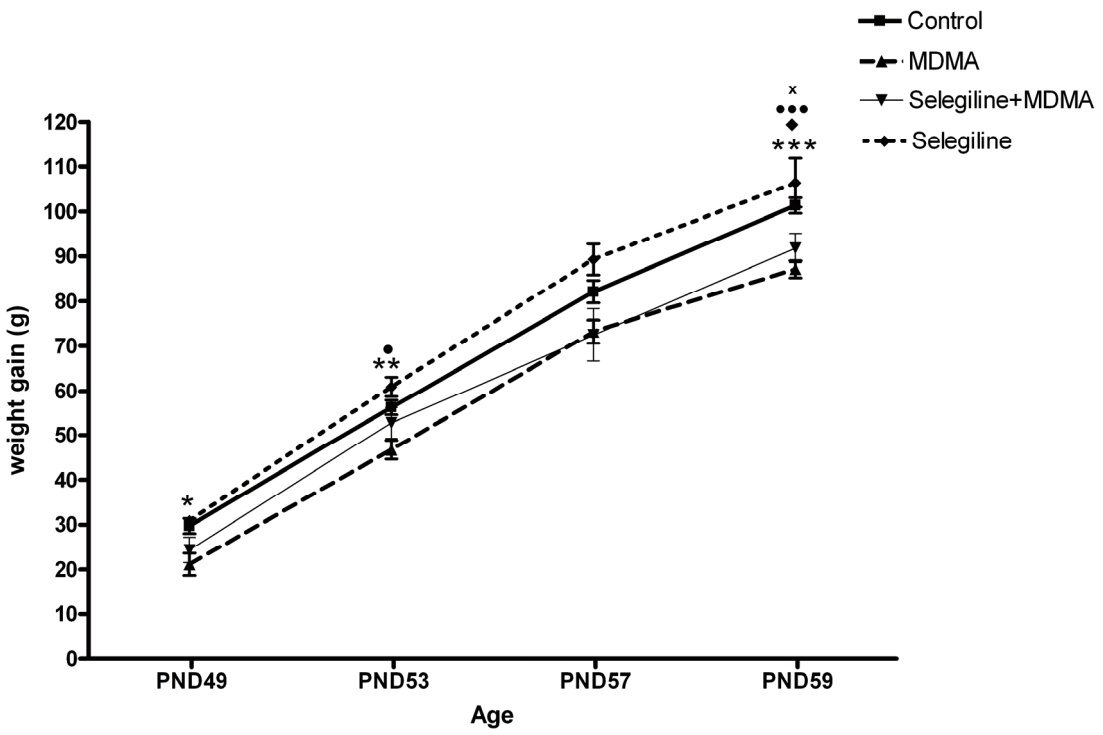

Figure 1: Weight gain between the day of exposure (PND45) and the day of sacrifice (PND59) measured on the four groups of animals under analysis: MDMA (4x10 mg/Kg), selegiline (2 mg/Kg) + MDMA (4x10 mg/Kg), control (isovolumetric saline) and selegiline (2 mg/Kg). The selegiline plus MDMA group was injected with the same protocol of administration used on MDMA group of animals, but preceded 30 minutes by selegiline (2 mg/Kg) administration. Each value is expressed as the mean ± SEM of body weight for each group (MDMA, n=49; MDMA+selegiline, n=19; selegiline, n=10; control, n=45). Statistical analysis was performed using a 1-way ANOVA and the main effects and interactions were further analysed using the post-hoc Tukey HSD test (*p<0.05, **p<0.01, *** p<0.001 for MDMA when compared with control; $^{\bullet}$p<0.05 for selegiline when compared with MDMAl; $^{\blacklozenge}$p<0.05 for selegiline+MDMA when compared with control; $^{\bullet\bullet\bullet}$p<0.001 for selegiline when compared with MDMA; ^{x}p<0.05 for selegiline+MDMA when compared with selegiline alone).

Measurements of body temperature

Daily measurements of body temperature, evidenced that MDMA (4 x 10mg/Kg) treated rats presented signs of improper thermoregulation throughout the 13 days that followed exposure, showing significantly increased values relatively to selegiline and control saline groups (p<0.05). This was not observed in animals treated with MDMA plus selegiline. (Fig. 2).

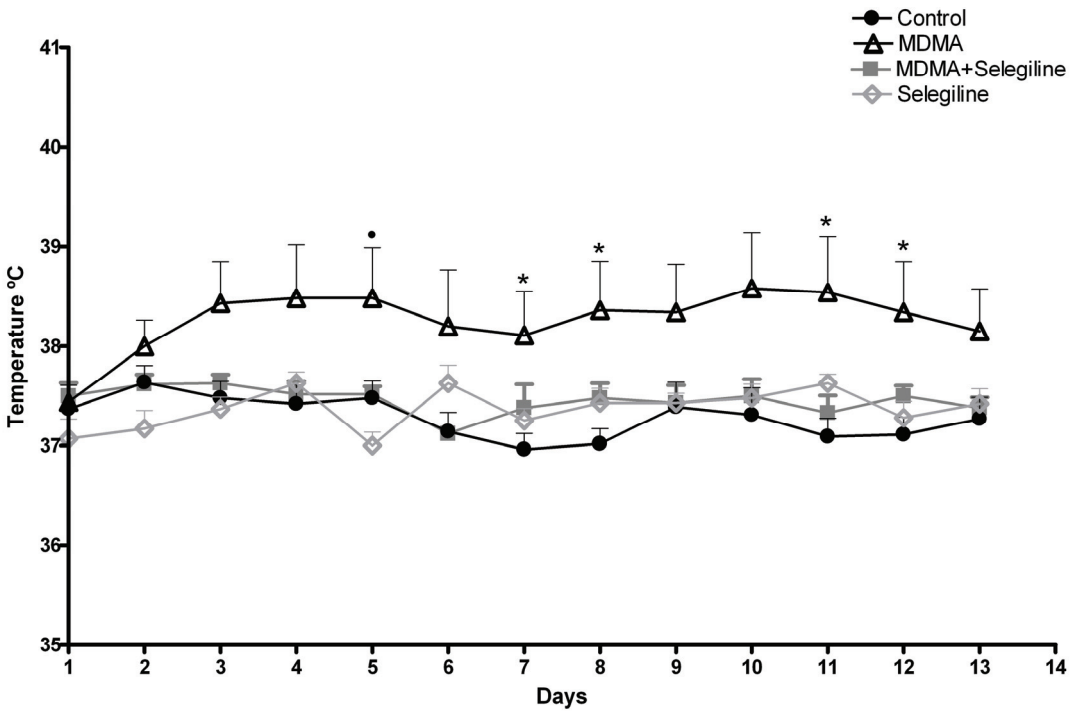

Figure 2: Body temperature measured subcutaneously throughout the 13 days following exposure within the different experimental groups (PND46 to PND59). Representative graph of temperatures measured on the four groups of animals under analysis: MDMA (10 mg/Kg x4); selegiline (2 mg/Kg) + MDMA (4x10 mg/Kg), control (isovolumetric saline) and selegiline (2 mg/Kg). Selegiline preceded MDMA exposure in 30 minutes. Body temperature was measured by scanning an inserted subcutaneously probe, every day, at the same hour and until the day of sacrifice. Each value is expressed as the mean ± SEM of temperatures measured in centigrade degrees on each group (MDMA, n=8; MDMA+selegiline, n=6; selegiline, n=6; Control, n=9). * $p<0.05$, as compared with controls; • $p<0.05$, as compared with selegiline. Statistical analysis was performed using a two-way ANOVA followed by a post-hoc Tukey HSD for unequal n.

ATP measurements in brain areas

Analysis of ATP contain revealed no significant differences between the four groups in almost all brain areas under analysis. Nonetheless, the values of ATP were tendentially lower in animals administered with MDMA comparatively to control saline in all the regions analysed despite in the majority of them no recovery was found between MDMA and MDMA+selegiline group. The few significant differences observed respect to Hip where animals treated with MDMA showed significantly lower values in comparison with control saline ($p<0,01$) and in MDMA+selegiline group in both SN and Hip where the values of ATP/mg protein were significantly lower respectively to either control saline ($p<0,01$; $p<0,001$) or selegiline ($p<0,05$) groups. The graph bellow summarizes the values obtained.

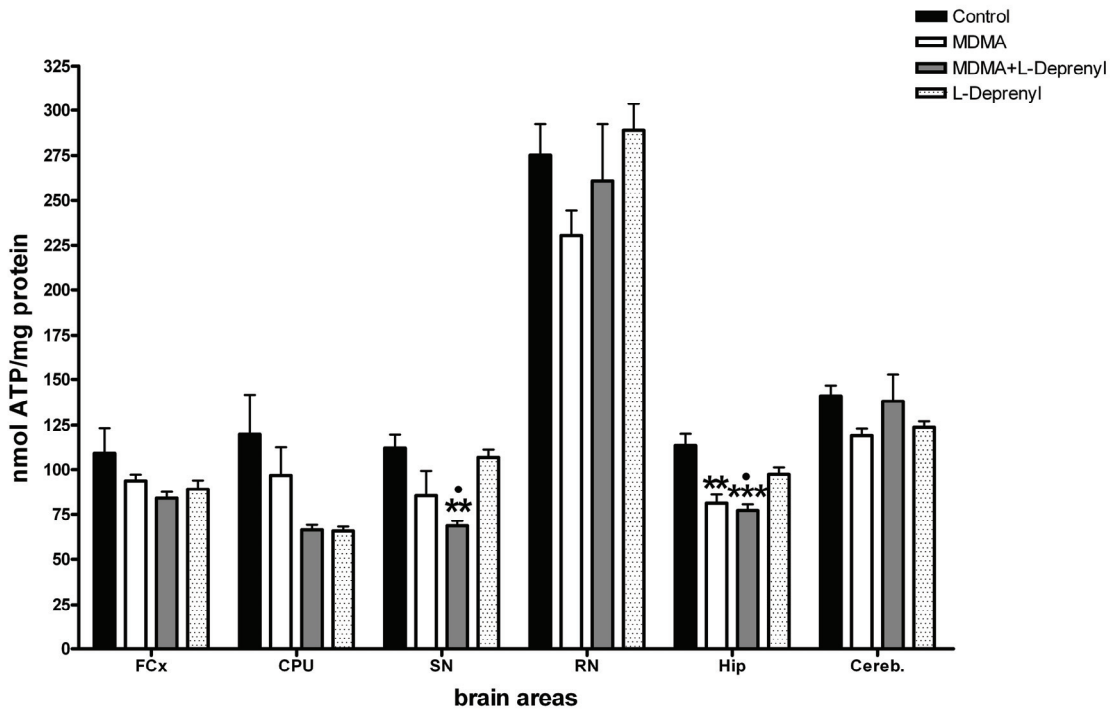

Figure 3: Amount of ATP per miligram of protein in brain areas of adolescent male Wistar rats 14 days after drug exposure. FCx-frontal cortex, CPU- caudate putamens, SN- substancia nigra, RN-raphe nuclei, Hip-hippocampus, Cereb.-cerebellum. Four experimental groups were analysed: MDMA (4x10mg/Kg); selegiline (2mg/Kg) + MDMA (4x10mg/Kg), control saline (isovolumetric saline) and selegiline (2mg/Kg). Selegiline was administered 30 min before exposure to MDMA. Results are expressed as mean ± SE for n = 5 for each group in all brain areas analysed (**p<0,01 and ***p<0,001 (MDMA and MDMA+selegiline compared with control); •p<0,05 (MDMA+selegiline compared with selegiline), one-way ANOVA followed by a post-hoc Tukey HSD).

RN distinguishes from all the brain areas due to the ATP values obtained that even on MDMA group of animals was more than the double of the values obtained for controls in the remaining areas analysed.

Cereb and RN showed a different pattern of behaviour concerning comparisons between MDMA and MDMA+selegiline groups. Although not significantly, these were the only areas where a recovery of ATP levels was tendentially achieved with MAO-B inhibition.

TTC staining in experiments with selegiline:

Brain slices of animals treated with MDMA, selegiline + MDMA, selegiline and controls saline were analysed by TTC staining.

In all the brain areas analysed the values obtained were tendentially in accordance with the expected results, concerning an increased percentage of white area,.However, the

differences were not significant at the tested level. The exceptions were SN and RN where an inversion of these values was observed.

The values of white area measured for MDMA+selegiline treated animals were tendentially lower, although also not statistically different, from those observed for MDMA, in all brain areas analysed with the exception of RN and Cereb. The few statistical differences obtained were observed for VTA [(p<0,05 (MDMA compared with selegiline)] and SN [(p<0,05 (MDMA+selegiline compared with control saline)]. The graph below summarizes the results obtained.

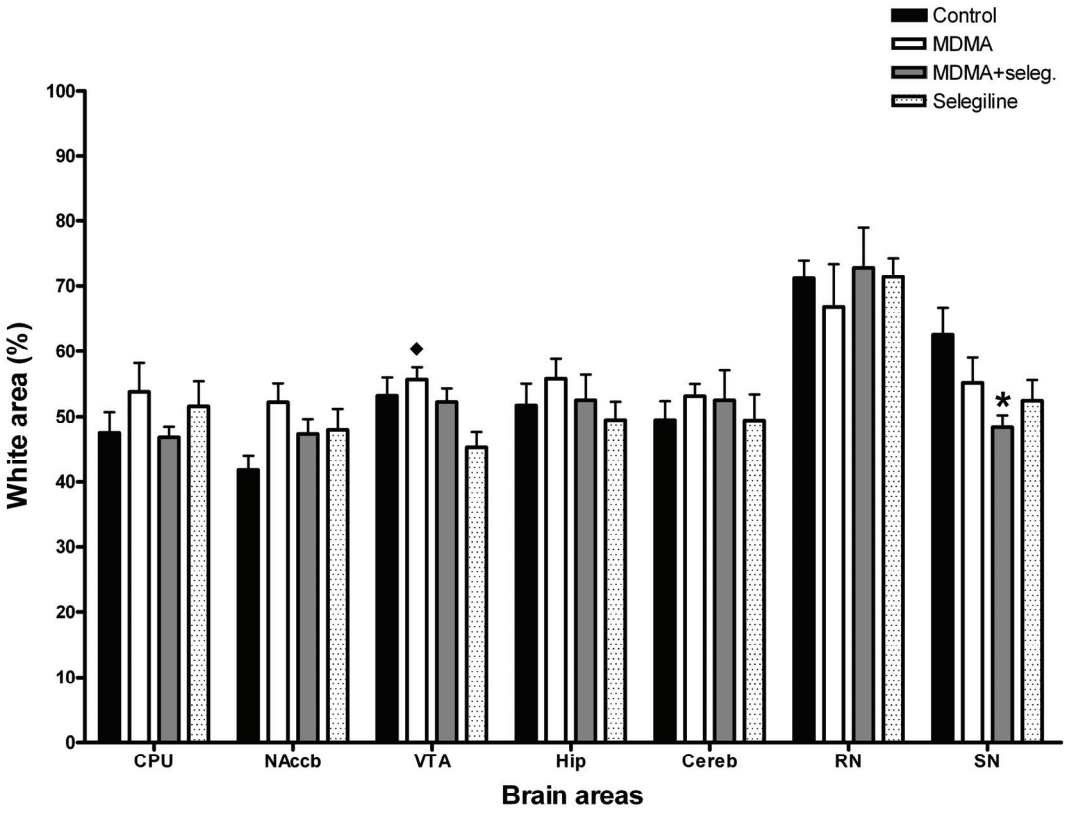

Figure 4: Percentage of white area analysed in brain slices of adolescent male Wistar rats 14 days after drug exposure. CPU-caudate putamens, NAccb-nucleus accumbens, SN-substancia nigra, VTA-ventral tegmental area, Hip-hippocampus, RN-raphe nuclei, Cereb-cerebellum. The results represent the mean ± SE (n = 6 for control, n = 8 for MDMA, n = 5 for MDMA+selegiline, n = 9 for selegiline in CPU and NAccb; n = 6 for control, n = 8 for MDMA, n = 7 for MDMA+selegiline, n = 7 for selegiline in SN, VTA and Hip; n = 5 for control, n = 6 for MDMA, n = 8 for MDMA+selegiline, n = 9 for selegiline in RN and n = 6 for control, n = 9 for MDMA, n = 5 for MDMA+selegiline, n = 7 for selegiline in cereb (♦p< 0,05 (MDMA compared with selegiline; *p< 0,05 (MDMA+selegiline compared with control)).

TTC staining in experiments with ALC:

Brain slices of animals treated with MDMA, ALC+MDMA, ALC and controls saline were analysed by TTC staining.

The effect of ALC was traduced in two main different results depending on brain regions but just a few significant differences were encountered. On CPU, NAccb and cereb, ALC treatment previously to MDMA, seemed to protect against MDMA-induced increment of white area, nonetheless, in the remaining brain areas analysed, the trend was exactly opposite. In SN, VTA, Hip and RN, the values obtained in animals treated with ALC were all well above those ones of any of the other groups and in SN, VTA and Hip, the values obtained for animals treated with ALC plus MDMA were incremented relatively to the group treated with MDMA thus accounting for an absence of protective action at this level. In VTA, the values were particularly curious and alarming since the white areas observed among ALC treated animals were statistically increased relatively to controls and MDMA groups ($p<0.05$) thus accounting for a quite negative effect of ALC administration on the activity of some of the mitochondrial enzymes with possible deleterious effects on mitochondrial function.

The graph that follows summarizes the results obtained.

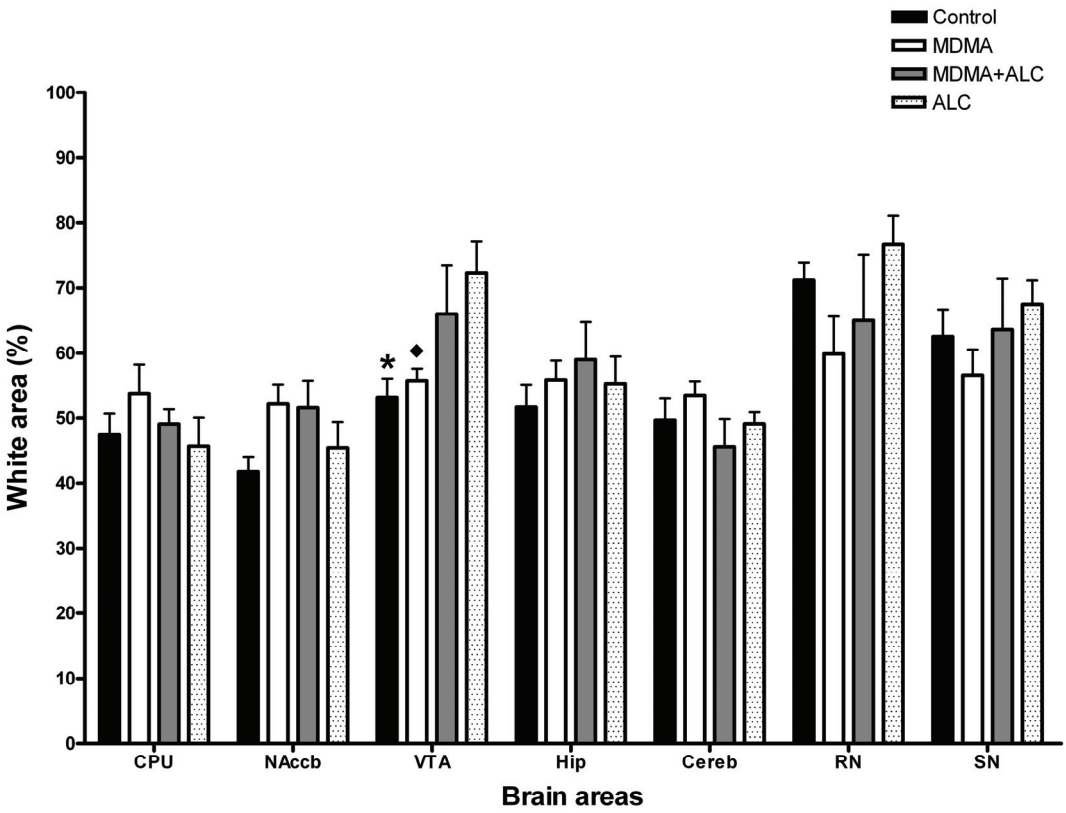

Figure 5: Percentage of white area analysed in brain slices of adolescent male Wistar rats 14 days after drug exposure. CPU-caudate putamens, NAccb-nucleus accumbens, SN-substancia nigra, VTA-ventral tegmental area, Hip-hippocampus, RN-raphe nuclei, Cereb.-cerebellum. The results represent the mean ± SE (n = 6 for control, n = 8 for MDMA, n = 5 for MDMA+ALC, n = 5 for ALC in CPU, NAccb, SN, VTA and Hip; n = 5 for control, n = 7 for MDMA, n = 5 for MDMA+ALC, n = 5 for ALC in RN; n = 6 for control, n = 9 for MDMA, n = 5 for MDMA+ALC, n = 5 for ALC in cereb. (♦p< 0,05 ALC compared with MDMA; *p< 0,05 ALC compared with control).

Chapter IV

1. Integrated overview of the performed studies

2. Conclusions

1. Intergrated overview of the performed studies

One of the most alarming facts about MDMA abuse is the increased number of adolescents and young adults that still use the drug despite the knowledge on its detrimental consequences. This reality renders importance to the study of drug effects on this developmental stage, notwithstanding few data is available until the date. In the present thesis, by using an adolescent animal model in all the experiments performed, we intend to give new insights on the MDMA-induced neurotoxic effects during adolescence. A rat model was used for two additional main reasons: (i) rodent brain systems develop at quite fast rates thus allowing to observe long-term changes through short periods of time; (ii) the rat model seems to be quite more resistant to drug effects than other animal models thus allowing high dose exposures with less risks of mortality.

One of the prominent acute effects of MDMA, either in adolescence or in any other developmental period, regards the deregulation of body temperature. This characteristic depends on many factors from which the administrated dose and the surrounding ambient temperatures (Dafters and Lynch, 1998; Marston et al., 1999) are important variables directly related with the effects produced. In the present work, after binge administration of a high dose of the drug, animal body temperatures were measured both on the day of injection and in the following 13 days. The results obtained confirmed previous reports concerning the development of hyperthermic responses immediately after drug exposure and long-term abnormal thermoregulation thereafter (Dafters and Lynch, 1998; Marston et al., 1999) thus reinforcing the role of hyperthermia for the drug induced neurotoxicity and devaluating previous data that described the absence of this characteristic feature during adolescence (Piper and Meyer, 2004).

There have been a number of studies demonstrating that MDMA increases the synaptic concentrations of monoamine neurotransmitters via their release from vesicle stores and the inhibition of their reuptake. Indeed, MDMA binds to SERT and, inside axon terminals, causes an acute and powerful release of neurotransmitters (mainly 5-HT, but also NA and DA) from storage vesicles (Green et al., 1995; Sulzer et al., 2005). The acute increase of 5-HT after drug exposure inside nerve terminals leads to an excess of 5-HT in the synaptic cleft that tends to accumulate due to the competitive binding of MDMA to SERT, thus leading to the overstimulation of the post-synaptic receptors. On the other hand, the increased release of 5-HT from storage vesicles

leads to very high concentrations of this neurotransmitter inside nerve terminals. Increased turnover of 5-HT by MAO-B leads to increased formation of H_2O_2, which is able to diffuse across mitochondrial membranes and there, by means of the Fenton reaction, is converted into the highly reactive radical $HO^{\cdot}$. Besides MAO-B activity and direct related factors, some other indirect consequences could come upon the neuronal hyperactivity developed after drug exposure. Among these indirect related responses is the excess of Ca^{2+} that enters mitochondria and activates cell death related mechanisms and the increased production of ROS that follows hyperthermia (Broening et al., 1995; Globus et al., 1995). All these conditions justify and help to explain the overall results presented throughout the dissertation, mainly those concerning increased oxidative damage to macromolecular mitochondrial constituents, and support MDMA-induced neurotoxicity as being, at least in part, related to a sustained overproduction of ROS (Esteban et al., 2001; Shankaran et al., 2001).

The most direct evidences of oxidative damage obtained in the present study were protein carbonylation and lipid peroxidation. Noteworthy, the influence of lipid moiety in the activity of some mitochondrial proteins such as MAO, cytochrome c oxidase and NADH dehydrogenase are well characterized (Daum, 1985; Dean et al., 1997). Indeed, an adequate performance of these proteins is quite dependent on a closely associated lipid moiety, thus supporting the requirement of lipid-protein interactions to occur (Dean et al., 1997) [(e.g. the enzymatic activity of NADH dehydrogenase depends on cardiolipin, phophatidylcholine and phosphatidylethanolamine (Daum, 1985)]. If lipids are modified, the protein activity could be compromised or completely lost, and thus, considering NADH dehydrogenase and cytochrome c oxidase as essential components of the ETC, hampering of its activity by lipid peroxidation, could result in decrease of energy supply. In the case of MAO, damage inflicted could compromise the correct metabolism of brain monoamine neurotransmitters. In either of the cases, the overall brain cellular function could be compromised with serious adverse consequences.

Here, we verified that MDMA is able to induce alterations in both lipids and proteins in mitochondria. Thus, as above described, both primary and secondary oxidative modification of mitochondrial protein constituents probably occurred and accounted for the overall toxicity induced by the drug.

The MDMA-induced mtDNA deletion produced in the specific brain areas analysed in this dissertation, encompasses coding regions for NDI, NDII and COXI constituents of the ETC, and tRNA 16S and rRNA for six aminoacids. This specific deletion was already referred as accompanied by the accumulation of substantial 8-OHdG (Suliman et al., 2002) that is known to be the best indicator of oxidative injury to DNA bases

(Richter, 1992, 1995) and thus implies oxidative damage as a plausible explanation for the considerable deletion observed in animals exposed to MDMA.

A vicious circle among mtDNA deletions, lipid peroxidation and protein carbonylation could have therefore occurred and accounted for the high extension of damage observed 14 days after exposure. Inefficient repair mechanisms against mtDNA damage comparatively to nuclear DNA could have contributed to a cumulative damage of brain mitochondria. Furthermore, impaired entry of reducing equivalents into the respiratory chain at complex I, with associated increases in $O_2^{\cdot -}$ production and functional alterations in molecular O_2 reduction at complex IV could have also resulted, thus giving an additional support to the overall results obtained.
MAO enzymes are active intervenients in MDMA-induced 5-HT and DA depletion (Fornai et al., 2001). In the present study, we observed that inhibition of MAO-B with selegiline was able to protect all the MDMA-induced mitochondrial neurotoxic effects long term after drug exposure with exception of hyperthermia. Inhibition of MAO-A presented opposite effects and the overall damage was increased.

Our hypothesis on selegiline neuroprotection hinges on the fact that, as a potent inhibitor of MAO-B, is able to prevent part of the increased production of ROS after MDMA exposure, namely that related to the increased 5-HT catabolism. Although MAO-B is more specific for DA, excess 5-HT in the pre-synaptic cell leads to an extension of its metabolic capability also for 5-HT. MAO-B inhibition decreases H_2O_2 overproduction derived from increased 5-HT catabolism thus lessening the oxidative effects observed in mitochondria. Support on our hypothesis arise from a previous investigation that found MDMA-induced chronic 5-HT loss as dependent on MAO-B activity (Fornai et al., 2001).
An additional explanation, although controversial, surrounds excess DA uptaken by 5-HT terminals (Schmidt and Lovenberg, 1985; Waldmeier, 1985; Faraj et al., 1994; Sprague and Nichols, 1995b). If this is also the case, MAO-B inhibition could also have resulted in the same mechanism described for 5-HT by means of decreasing H_2O_2 overproduction caused by DA catabolism, and thus giving emphasis to the extensive protection achieved.
Besides these protective mechanisms, inhibition of MAO-B is also associated with the treatment and prevention of several neurodegenerative states associated with increased ROS overproduction, namely PD and AD (Portin and Rinne, 1983; Piccinin et al., 1990), and aging (Knoll, 1988, 1989; Freisleben et al., 1994) where increased MAO activity with associated increased ROS production inside mitochondria occurs.

MAO-A inhibition lead to an aggravation of MDMA-mediated toxicity that was traduced in increased body temperature and oxidative stress in whole brain mitochondria. Both MDMA and clorgyline are inhibitors of MAO-A activity, thus accounting for increased serotonergic neurotoxicity when taken together, which, as already mentioned, accounts for MDMA-induced hyperthermia and increased ROS formation (Broening et al., 1995; Globus et al., 1995). High rates of mortality as well as behavioural alterations characteristically attributed to the SS (Parrott, 2002) were observed when we administered clorgyline, previously to MDMA, to adolescent male Wistar rats, corroborating previous findings observed in humans exposed concomitantly to MDMA and MAO-A inhibitors (Vuori et al., 2003), thus reinforcing the importance of the serotonergic neurotransmission for the mitochondrial damage observed after drug exposure.

Carnitines are compounds whose relevant antioxidant action and prevention of altered neurological states related with mitochondrial dysfunctions is extensively reported and accepted by the scientific community.
ALC administration, previously to MDMA, lead to a significant prevention of MDMA-induced injury of brain mitochondria, lessening the majority of the effects observed, although to a lower extent when compared with selegiline.
The ability of carnitines to act synergically with some antioxidants, such as GSH, selenium and Vitamin E and reports on carnitine prevention against METH induced damage to CNS further support its protective properties (Arockia Rani and Panneerselvam, 2001; Virmani et al., 2003) and accounts for other tools apart from MAO-B inhibition as alternative ways to prevent the increase of oxidative damage in mitochondria after drug exposure.

Mitochondria, besides being the major intracellular source of ROS due to spontaneous electron leakage under basal metabolic conditions, are also the main energy suppliers to neurons, by coupling of the respiration-generated proton gradient with the proton-driven phosphorylation of ADP by F_0F_1ATPase. Increased accumulation of ROS inside mitochondria accounts for many pathological states including those ones associated to aging processes (Harman, 1956). MDMA exposure, as previously referred, leads to increased ROS formation inside mitochondria in such a manner that the endogenous defences (antioxidant enzymes) could no longer counteract the damage inflicted and mitochondrial dysfunction may result. Our measurements on ATP levels of different brain areas revealed an overall decrease in energy levels on animals exposed to

MDMA. These results, once again, are in agreement with those previously obtained concerning an evident uncoupling of mitochondrial function after drug exposure. The most plausible reason for this effect seems to be the abnormal transfer of electrons across the ETC due to the diminished expression of important protein subunits of the mitochondrial inner membrane (western blot analysis) together with the diminished reductive capacity to supply equivalent reducers for the respiratory pool (measured by TTC staining) and the proper loss of function of the constitutive elements of the ETC, due to oxidative phenomena's (measured by the assessment of lipid peroxidation and protein carbonylation), all converging to a loss of function of the ATPase. Thus, it seems quite reasonable to accept as an evident consequence of the high degree of mitochondrial injury observed, the depletion of ATP reservoirs on the harvested areas 14 days after exposure.

The apparent lack of protective effect observed for selegiline, concerning ATP levels, is still not clear from the present findings. Nevertheless, considering that ATP levels reflect not only ATP synthesis but also its consumption for the various cellular energy-dependent processes, it seems that other factors are contributing for MDMA-mediated neurotoxicity and allow us to infer that MAO-B inhibition won't be a panacea for all toxic effects.

Altered ETC electron flow due to deficiencies of its constituting subunits lead, as already referred, to altered energy supply and metabolic performance. TTC staining allowed to observe macroscopically the extent of the damage produced along with the specific localization of the injured areas. The techniques previously used concerning PCRs of mtDNA and the analysis of the expression of NDII and COXI proteins permitted us to infer about a deficient functioning of these important constitutive subunits of the ETC after drug exposure. TTC analysis of different brain slices, as a test that permits to infer about the activity of mitochondrial dehydrogenases, namely of succinate dehydrogenase, allowed us to extend the results achieved and to characterize the general activity of mitochondrial dehydrogenases. As expected, MDMA was able to diminish the overall mitochondrial metabolic performance in the brain areas analysed with the exception of RN and SN thus giving further support to our hypothesis of oxidative damage to mitochondria as one of the events importantly related with MDMA-induced neurotoxicity and emphasizing the previous results obtained. TTC staining showed to be a powerful tool to complete the biochemical approaches achieved from where we could infer about a deregulation of the mitochondrial metabolism.

The overview of all the results achieved suggest a proximity of the mechanisms involved with MDMA-induced mitochondrial neurotoxicity and those ones that imply aging. The bioenergetic function of mitochondria declines with age and there are at least four lines of research that lead us to the same conclusion. The aging tissues are characterized by a decreased activity of cytochrome c oxidase (Muller-Hocker, 1989; Muller-Hocker et al., 1993) that could probably result from the same effects we've observed after drug exposure concerning the deletion of the subunit I of cytochrome c oxidase from the mitochondrial genome and the decreased expression of the correspondent protein of the ETC. Aging is accompanied by a decline of the respiratory function and electron transfer activities (Trounce et al., 1989; Yen et al., 1989; Cooper et al., 1992) and by a decrease in the catalytic activity of the ADP/ATP translocator, ATP synthase (Hoch, 1988). In this study, the diminished capacity of mitochondrial dehydrogenases to supply equivalent reducers and the decreased levels of the brain energy stores converge to a similar result. An increased rate of mutation of NADH dehydrogenase genes and a close correlation between the extent of this mtDNA mutation and the decrease of cytochrome c oxidase activity (Muller-Hocker et al., 1993) are also deleterious consequences that accumulate and extend with age. In the present work, some proximal related evidences were disclosed: the mtDNA deletion detected accompanied a fragment that encompasses both NADH dehydrogenase and cytochrome c oxidase and, besides cytochrome c oxidase, also a diminution of the expression of NADH dehydrogenase was encountered. Finally, the age-related accumulation of oxidized lipids and proteins (Shigenaga et al., 1994; Sohal and Dubey, 1994) were also verified in our model as observed by the increased accumulation of oxidized lipids and proteins in the animals exposed to the drug.

A progressive increase of MAO-B activity (Fowler et al., 1980) is another of the demarked characteristics that accompanies age related alterations. This feature makes selegiline a good tool to prevent some of the mitochondrial alterations associated with age as proved by several researchers that showed its efficacy in increasing the life span of laboratory animals (Milgram et al., 1990; Yen and Knoll, 1992; Kitani et al., 1993; Freisleben et al., 1994).

In our experiments, ALC was also shown to be efficient in preventing the majority of MDMA-induced damaging effects in mitochondria. This type of effect has also been shown to extensively attenuate the age-induced alterations produced in mitochondria (Paradies et al., 1992; Petruzzella et al., 1992; Paradies et al., 1994; Paradies et al., 1997).

It is plausible to suggest, therefore, that the effects produced by MDMA at the mitochondrial level might also be able to induce an acceleration of aging and age-

related disorders such as PD and AD. In this way, the mechanisms of toxicity and prevention here presented constitute an undoubtedly important new insight that could be also extended for a better knowledge of the consequences of increased ROS production inside mitochondria in several pathological states.

The results achieved in the different parts of the present work corroborate each of the individual conclusions. ALC protection against MDMA-induced neurotoxic effects corroborate our hypothesis on increased ROS formation inside mitochondria as being the main mechanism involved in the overall response after exposure. Clorgyline administration supports serotonergic injury as being the major and primordial pathway that underlies drug induced neurotoxic effects and MAO-B inhibition by selegiline reinforced MAO activity inside serotonergic terminals and the subjacent increased ROS formation as phenomena's of primordial interest to the understanding of MDMA mechanistic action inside nerve endings.

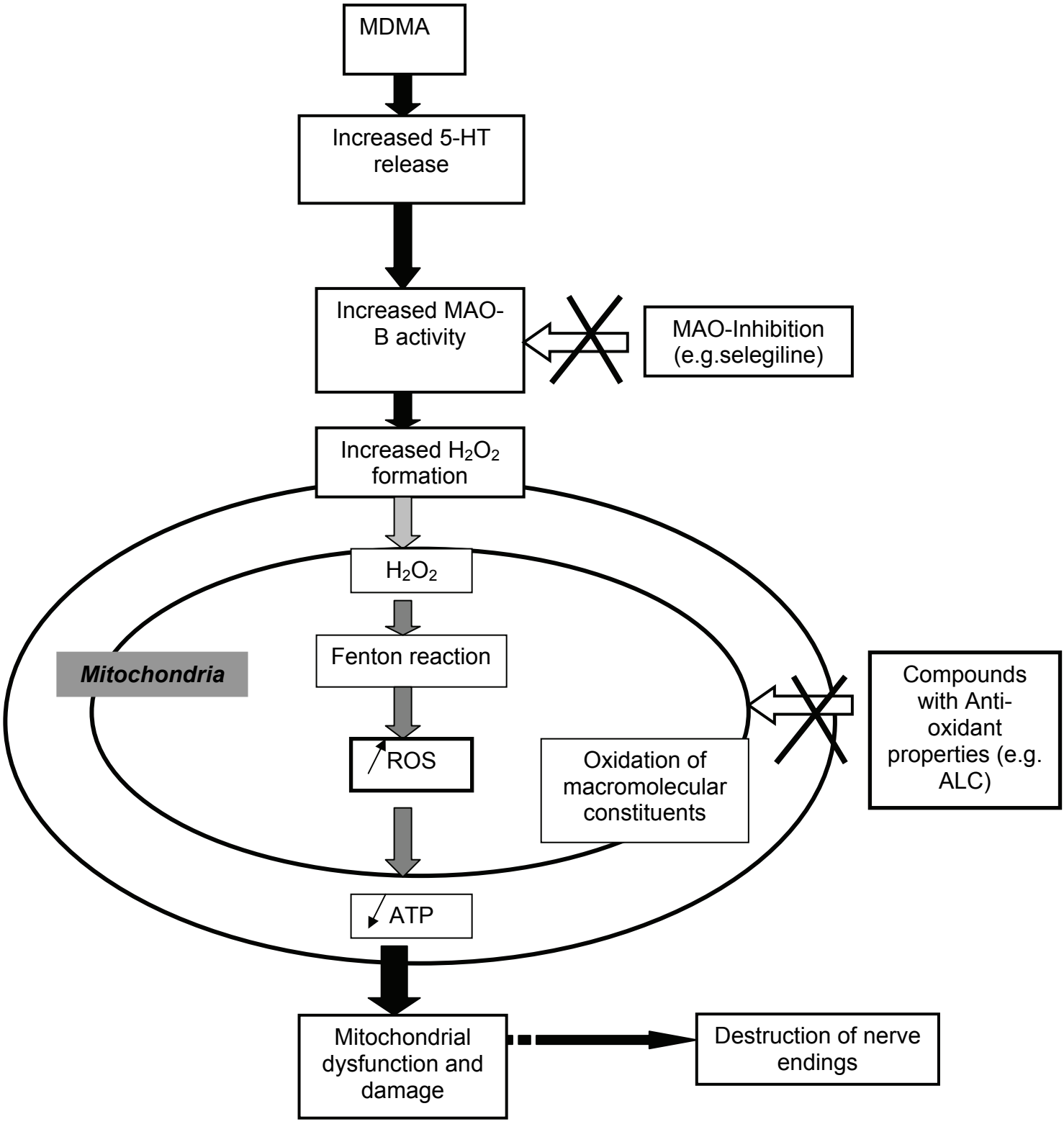

Figure 1: MDMA, the serotonergic sytem and ROS formation. MDMA leads to increased release of vesicular 5-HT that could be further metabolized by MAO-B. H_2O_2, a secondary by-product of MAO-B activity could easly diffuse across mitochondrial membranes and there give rise to increased ROS formation. Inside the organelle, oxidation of macromolecular constituents, namely by interfering with ETC,

leads to a decrease of brain energy stores that could further account for mitochondrial damage, and, ultimately, to the destruction of nerve endings. Prevention could be achieved by inhibiting MAO-B activity in the mitochondrial membrane or by acting directly in mitochondrial matrix by the use of compounds that present anti-oxidant properties like ALC.

2. Conclusions

A global analysis of the data described in the present dissertation, concerning the evaluation of the deleterious effects resulting from the exposure of adolescent rats to a binge neurotoxic dose of MDMA, at the mitochondrial level, and the investigation about the possible mechanisms involved, allows us to draw the following conclusions:

● MDMA administration induced an increased oxidation of lipids and proteins of brain mitochondria, observable at long-term after exposure. This effect was extensively prevented by MAO-B but not by MAO-A inhibition. ALC supplementation prevented this effect only at the level of protein oxidation

● MDMA administration induced a deletion of the mitochondrial genome in a region that encompasses NDI, NDII and COXI mitochondrial genes and decreased the expression of the correspondent protein subunits NDII and COXI in several areas of the rat brain. MAO-B inhibition prevented this effect but, MAO-A inhibition, contrarily, decreased even more the expression of COXI. ALC supplementation was able to diminish the MDMA-induced effects but its protective activity is less pronounced than that observed with MAO-B inhibition. This was the first time that the effects of MDMA on mitochondrial gene deletion were verified and described.

● MDMA administration induced dual effects in the mitochondrial metabolic performance, as evaluated by the TTC assay, depending on the studied brain regions. Brain regions like CPU, NAccb, VTA, Hip and cerebellum had its mitochondrial metabolism diminished while others, namely SN and RN, presented increased metabolic activity. MAO-B inhibition tendencially afforded protection against this effect in the majority of the brain regions analysed. The effect of ALC supplementation was less clear. In some of the regions, namely CPU, Naccb and cerebellum a protective effect was achieved, but in other (VTA, RN, SN and hip), an increased metabolic dysfunction was observed.

● MDMA administration induced an overall decrease in ATP production in brain areas analysed. Selegiline was able to prevent this effect in RN and cerebellum, but not in the remaining regions.

● MDMA administration induced a hyperthermic response on the day of exposure and abnormal thermoregulation thereafter. This effect was not prevented by MAO inhibition or ALC supplementation.

●MDMA administration was followed by loss of body weight gain throughout the period of experiment. MAO-B inhibition was not able to prevent this effect. ALC administration, conversely, aggravated this effect.

●A general overview of all the results allows one to conclude that MDMA-induced neurotoxic effects are associated with oxidative damage to brain mitochondria and that these effects are quite dependent on MAO-B activity. MAO-A is also related with MDMA inflicted damage, but, in contrary, its inhibition is not able to prevent the toxic effects thus accounting for a specific action of the drug at MAO-B rich structures (serotonergic nerve endings) and discarding MAO-A inhibition as a protective tool. ALC prevention, afforded for great part of MDMA-induced damage, gives emphasis on the central role played by ROS in the overall process and evidences the importance of antioxidant neuroprotection for the prevention of the oxidative damage observed long-term after acute exposure.

Taken all the results together, this dissertation represents an important contribution for the understanding of the mechanisms involved in MDMA-induced neurotoxicity at long-term after exposure, clarifying the important role of mitochondria in the overall process and emphasizing the relevance of MAO-B inhibition in preventing a great part of the toxic effects produced.

Chapter V
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