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Brain and peripheral organ toxicity of “ecstasy” in adolescent rats in human relevant doses

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ABSTRACT

3,4-Methylenedioxymethamphetamine (MDMA or “ecstasy”) is a well-known psychoactive drug, with acknowledged neurotoxic properties. It is commonly used in recreational settings by many teenagers and young adults. Despite the great deal of study on MDMA neurotoxicity, the consequences of MDMA exposure during the adolescent period require additional research. Moreover, MDMA effects on the cerebellum and peripheral organs have been scarcely studied. The susceptibility of different aged groups is also poorly known.

This dissertation aimed to assess the acute toxicity of a MDMA binge dose regimen comparable to human usage in four different brain areas (cerebellum, hippocampus, cortex and striatum), and in three peripheral organs (liver, heart and kidneys) in an adolescent rat, 24 h after MDMA binge administration. Another aim was to evaluate the long-term neurotoxicity of the binge MDMA dose regimen in the cerebellum, by assessing its energetic and oxidative stress status in adolescent and old rats, seven days after MDMA administration.

To achieve the aims of the present dissertation, two experiments were conducted. In experiment one, adolescent male Wistar rats (postnatal day 40) were divided in two groups and treated: control saline (three doses of NaCl 0.9%, intraperitoneally, every 2 h, n=7) and MDMA-treated (three doses of MDMA 5 mg/Kg, intraperitoneally, every 2 h, n=7). The temperature of each animal was monitored for 7 h after the first injection. Twenty-four hours after the MDMA administration, brain areas were dissected and the peripheral organs were collected. A significant hyperthermia in the adolescent rats was observed after MDMA exposure. No differences were found between controls and MDMA-treated groups in body weight gain, as well as in food or water consumption, within the experimental period. Adenosine triphosphate (ATP) content was significantly decreased in the cortex of MDMA-treated rats, but not in the other areas. In all brain areas, no alterations were found in glutathione, quinoproteins and protein carbonylation levels in MDMA-treated animals. In liver, heart and kidneys no differences were found in glutathione, protein carbonylation or ATP levels, after MDMA exposure. However, the formation of protein-bound quinones was significantly increased in the liver of MDMA-treated rats, but not in the other two peripheral organs. The histological hepatic examination of adolescent MDMA-treated rats revealed a marked cellular vacuolization in the periportal regions, and sinusoidal dilatation with periportal and centrilobular vascular congestion. In the heart, MDMA promoted punctual signs of cardomyocyte oedema, particularly in the sub-endocardic region. In the kidneys of MDMA-treated adolescent rats,

scattered interstitial oedema, with dilatation between the nefrotic tubular structures, and signs of vascular congestion were found. The lack of severe cellular damage and/or necrosis in MDMA-treated rats is corroborated by the lack of increase of caspase-3, -8 and -9 activities or changes in the plasma biomarkers of liver or heart injury.

Experiment two was conducted with adolescent (postnatal day 40) and aged (18 to 22 months old) male Wistar rats. Animals were divided in two groups: control saline (n=5) and MDMA group (n=5) that were treated as described for experiment one. Seven days later, animals were sacrificed and the cerebellum was collected. Aged animals did not receive the third dose of MDMA given the high risk of mortality, due to the hyperthermic response verified after the second MDMA administration. Until sacrifice, animal weight and food/water consumption were registered daily. Despite the exposure to a lower cumulative dose the hyperthermic response was more pronounced in aged rats than in adolescents, and the temperature of aged rats remained significantly elevated 24 h after drug exposure. MDMA promoted decreases in adolescent animals' body weight at 24 h post-MDMA administration. In aged animals, the food intake suffered a biphasic response after MDMA exposure: at 24 h a decrease was observed, while at 48 h an increase in consumption occurred in MDMA-treated animals, when compared to aged controls. The water intake was significantly increased in aged animals, 24 and 48 h after MDMA exposure. In the cerebellum, all the oxidative stress parameters remained unaltered in both adolescent and aged MDMA-treated rats, whereas decreases in the ATP content were seen in MDMA-treated aged rats.

Altogether, these results suggest that moderate MDMA doses do not promote any significant alterations in the oxidative stress parameters in the adolescents' brain areas, 24 h after the exposure. Importantly, the MDMA binge scheme used in adolescent animals promoted morphological tissue alterations to the peripheral organs. Regarding the age factor, a long-term ATP decrease was seen in the cerebellum of aged rats, indicating a higher susceptibility of this group to MDMA. Further investigation is needed to evaluate the impact of moderate MDMA doses either in the brain as in peripheral organs of adolescent animals, and to explore the dissimilar effects of MDMA to different aged animals.

Keywords: Amphetamines, "Ecstasy", Adolescence, Rats, Brain, Peripheral organs, Oxidative stress, Toxicity.

RESUMO

A 3,4-metilenodioximetanfetamina (MDMA ou “ecstasy”) é uma substância psicoativa bastante conhecida, com propriedades neurotóxicas bem documentadas. É habitualmente consumida em contextos recreativos, principalmente entre os adolescentes e jovens adultos. Apesar de existirem imensos estudos acerca da neurotoxicidade da MDMA, as consequências da exposição a esta substância durante a adolescência requerem, ainda, mais investigação. Para além disso, os efeitos da MDMA no cerebelo e nos órgãos periféricos têm sido alvo de poucos estudos. Acrescente-se que pouco se sabe sobre a suscetibilidade de diferentes faixas etárias a esta droga de abuso.

Esta dissertação teve como objetivo avaliar a toxicidade aguda decorrente de um regime de MDMA, comparável ao utilizado pelos humanos, em quatro áreas do cérebro (cerebelo, hipocampo, córtex e corpo estriado), e três órgãos periféricos (fígado, coração e rins) em ratos adolescentes, 24 h após a administração de MDMA. Outro objetivo centrou-se na avaliação da toxicidade a longo prazo a nível do cerebelo, decorrente da administração repetida de MDMA. Para atingir os objectivos propostos foram avaliados quer parâmetros de stress oxidativo, quer do estado energético, em ratos adolescentes e idosos, sete dias após a administração de MDMA.

De forma a atingir os objetivos traçados nesta dissertação, foram levados a cabo dois trabalhos experimentais distintos. No primeiro, ratos Wistar macho (40 dias de idade) foram divididos em dois grupos e tratados: controlo salino (três doses de NaCl 0.9 %, intraperitonealmente, a cada 2 h, n=7) e tratados com MDMA (três doses de MDMA 5 mg/kg, intraperitonealmente, a cada 2 h, n=7). A temperatura de cada animal foi monitorizada durante 7 h após a primeira injeção. As áreas cerebrais foram dissecadas e os órgãos periféricos recolhidos 24 h após a administração de MDMA. Após a exposição à MDMA foi observada hipertermia nos ratos adolescentes. Não foram encontradas diferenças entre o grupo controlo e o grupo tratado com MDMA no que diz respeito ao ganho de peso, consumo de água e ingestão de alimentos, durante o período experimental. O conteúdo de adenosina trifosfato (ATP) diminuiu significativamente no córtex dos ratos tratados com MDMA, mas não em outras áreas cerebrais. Nos animais tratados com MDMA não foram observadas alterações nos níveis de glutationa, quinoproteínas e carbonilação proteica, em todas as áreas cerebrais estudadas. No fígado, coração e rins dos animais não se encontraram alterações nos níveis de glutationa, carbonilação proteica e ATP, após a exposição à MDMA. No entanto, houve um aumento significativo da formação de quinoproteínas no fígado dos ratos tratados com MDMA, mas não nos restantes órgãos estudados. A observação histológica do

fígado dos ratos adolescentes tratados com MDMA, revelou vacuolização celular nas regiões periportais e um alargamento dos espaços sinusoidais com congestão sanguínea periportal e centrilobular. No coração dos animais tratados com MDMA, foram observados pontualmente cardiomiócitos edemaciados, particularmente na região sub-endocárdica. Nos rins de ratos adolescentes tratados com MDMA foram encontrados sinais de edema intersticial disperso com alargamento do espaço entre as estruturas tubulares dos nefrónios, assim como sinais de congestão sanguínea. A ausência de dano celular acentuado e/ou necrose em ratos adolescentes tratados com MDMA é corroborada pelo facto de não se ter verificado aumento nas atividades das caspases 3, 8 e 9, ou alteração nos níveis plasmáticos dos biomarcadores de lesão cardíaca ou hepática.

O segundo trabalho experimental foi executado com ratos Wistar macho adolescentes (40 dias de idade) e ratos idosos (18 a 22 meses de idade). Os animais foram, mais uma vez, divididos em dois grupos: controlo salino (n=5) e grupo tratado com MDMA (n=5), administrados como descrito no primeiro trabalho experimental. Sete dias depois, os animais foram sacrificados e o cerebelo foi recolhido. Os animais idosos não receberam a terceira dose de MDMA, dado o elevado risco de mortalidade decorrente da resposta hipertérmica verificada após administração da segunda dose da droga. Até ao sacrifício dos animais, o seu peso, ingestão de água e alimentos foram registados diariamente. Apesar dos ratos idosos terem sido expostos a uma dose total de MDMA menor que a dos ratos adolescentes, a resposta hipertérmica dos ratos idosos foi mais pronunciada e a temperatura dos mesmos manteve-se significativamente elevada 24 h após a exposição à droga. A MDMA levou à perda de peso nos ratos adolescentes, 24 h após a sua administração. Relativamente aos animais idosos, na ingestão de alimentos verificou-se uma resposta bifásica após a exposição à MDMA: foi observada uma diminuição às 24 h, enquanto que às 48 h houve um aumento no grupo de animais tratados com a droga em comparação com os animais controlo. A ingestão de água aumentou significativamente nos animais idosos, 24 e 48 h após a exposição à MDMA. Ao nível do cerebelo, todos os parâmetros de stress oxidativo se mantiveram inalterados nos animais adolescentes e idosos tratados com MDMA, enquanto que houve uma diminuição do conteúdo de ATP nos animais idosos expostos à droga.

Analisando todos os resultados, estes parecem sugerir que doses moderadas de MDMA não levam a alterações notórias nos parâmetros de stress oxidativo nas áreas cerebrais dos ratos adolescentes, 24 h após a exposição à droga. É importante referir que o regime de administração de MDMA usado nos animais adolescentes levou ao aparecimento de alterações morfológicas nos tecidos dos órgãos periféricos. Tendo em conta o fator idade, foi observada uma diminuição dos níveis de ATP, a longo prazo, no

cerebelo de ratos idosos, indicando uma maior suscetibilidade deste grupo à MDMA. Será necessária uma investigação mais aprofundada para avaliar o impacto da exposição a doses moderadas de MDMA, quer no cérebro, quer nos órgãos periféricos, de animais adolescentes e explorar os efeitos da MDMA em animais de diferentes faixas etárias.

Palavras-chave: Anfetaminas, "Ecstasy", Adolescência, Ratos, Cérebro, Órgãos Periféricos, Stress Oxidativo, Toxicidade.

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ABBREVIATIONS

[^{123}I]-β-CIT	^{123}I-labelled 2b-carbomethoxy-3b-(4-iodophenyl)tropane
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine, serotonin
5-HTT	Serotonin transporter
ADHD	Attention deficit hyperactivity disorder
ALT	Alanine aminotransferase
AMPH	Amphetamine
AST	Aspartate aminotransferase
ATP	Adenosine 5'-triphosphate
ATS	Amphetamine-type psychostimulants
AUC	Area under the plasma concentration-time curve
BSA	Bovine serum albumin
CK-MB	Creatine-kinase MB
CK-R	Total creatine kinase
C_{max}	Peak plasma concentration
CNS	Central nervous system
COMT	Catechol-*O*-methyltransferase
$CuSO_4$	Cupper (II) sulphate
CYP	Cytochrome P450
DA	Dopamine
DAT	Dopamine transporter
DIC	Disseminated intravascular coagulation
DMSO	Dimethyl sulfoxide
DOI	1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
DOPAC	Dihydroxiphenylacetic acid
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
fMRI	Functional magnetic resonance imaging
GFAP	Glial fibrillary acidic protein
GSH	Reduced glutathione
GSHt	Total glutathione
GSSG	Oxidized glutathione
h	Hour

HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HMA	4-hydroxy-3-methoxyamphetamine
HMMA	4-hydroxy-3-methoxymethamphetamine
HVA	Homovanillic acid
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
KH_2PO_4	Potassium dihydrogen phosphate
$KHCO_3$	Potassium bicarbonate
MAO	Monoamine oxidase
MDA	3,4-methylenedioxyamphetamine
MDMA	3,4-methylenedioxymethamphetamine
α-MeDA	α-methyldopamine, 3,4-dihydroxyamphetamine, HHA
METH	Methamphetamine
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
min	Minutes
NA	Noradrenaline
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Disodium phosphate
NAC	N-acetylcysteine
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaF	Sodium fluoride
$NaHPO_4$	Sodium phosphate monobasic
NaOH	Sodium hydroxide
$NaVO_3$	Sodium metavanadate
NBT	Nitrotetrazolium blue chloride
NET	Noradrenaline transporter
N-Me-α-MeDA	N-methyl-α-methyldopamine, 3,4-dihydroxymethamphetamine, HHMA
PBS	Phosphate buffered saline
PK	Pharmacokinetic
PMSF	Phenylmethanesulfonyl fluoride
PND	Postnatal day
p.o.	*Per os*

ROS	Reactive oxygen species
s.c.	Subcutaneous
SDS	Sodium dodecyl sulphate
SICAD	General-Directorate for Intervention on Addictive Behaviours and Dependencies
SPECT	Single photon emission computed tomography
TH	Tyrosine hydroxylase
TPH	Tryptophan hydroxylase
TUNEL	Terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick-end labelling
UNODC	United Nations Office of Drugs and Crime
Vd	Volume of distribution
VMAT	Vesicular monoamine transporter

Part I

Introduction

1. INTRODUCTION

1.1. An historical overview of amphetamines

Amphetamines are psychoactive substances and members of the phenylethylamine family, which include a broad range of substances that may be stimulant, euphoric, anorectic, entactogenic or hallucinogenic agents (Carvalho et al 2012). Amphetamine (AMPH) has a phenyl ring, a two carbon side chain between the phenyl ring and nitrogen, an α-methyl group, and a primary amino group (Figure 1). This basic structural feature is shared by other amphetamine-type psychostimulants (ATS) that enable their typical pharmacological actions (Sulzer et al 2005). AMPH, methamphetamine (METH), and 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") are widely abused amphetamine-like synthetic drugs, with the basic chemical structure of phenylethylamine. AMPH, METH, and MDMA may be ingested, snorted, and less frequently, injected, and they can be taken in form of tablet, powder, or capsule, and in METH's case the crystalline form can also be smoked (EMCDDA 2011).

Their history starts at 1887 in Germany, where AMPH was synthesized for the first time (Greene et al 2008). For thousands of years, humans have used natural amphetamines-like compounds through the consumption of plants, namely *Catha edulis* (Khat) and various plants of the genus Ephedra (family *Ephedraceae*), including *Ephedra sinica* (má huáng). Chewing Khat's fresh leaves is a widespread habit in the local populations of East Africa and Arabian Peninsula, and the users report increased levels of energy, alertness, self-esteem, a sensation of elation, enhanced imaginative ability, and a higher capacity to associate ideas. Plant-derived stimulant Ephedra (má huáng), is one of the oldest known medicinal herbs, and has been used in Traditional Chinese Medicine for over 5000 years to treat asthma and the common cold (Kalix 1996).

Over the past decades, MDMA, also known as "ecstasy", "XTC," "E," and "Adam," has become widely used as a recreational drug by young people around the world. It was first synthesized in 1912 by Merck, and patented in 1914, but it was never produced commercially, nor did it achieve clinical use (Burgess et al 2000). MDMA has often been said to have been originally patented for use as an appetite suppressant, but it was actually first patented as a precursor agent for therapeutically active compounds, and was never intended for use as an anorectic drug (Freudenmann et al 2006).

The toxicology of MDMA was examined in the year 1953 for the first time, together with other similar compounds, in a classified research program sponsored by the USA military, presumably as part of a chemical warfare program. In 1976, researchers

suggested that it might be useful as an adjuvant for psychiatric treatment (Green et al 2003). Recreational use of “ecstasy” began in United States in the late 1960s, and 20 years later in Europe (Montoya et al 2002).

The typical recreational use of AMPH, MDMA or METH is often characterized by a repeated pattern of frequent administrations of the drug during a short time period, also known as a binge administration (Badon et al 2002).

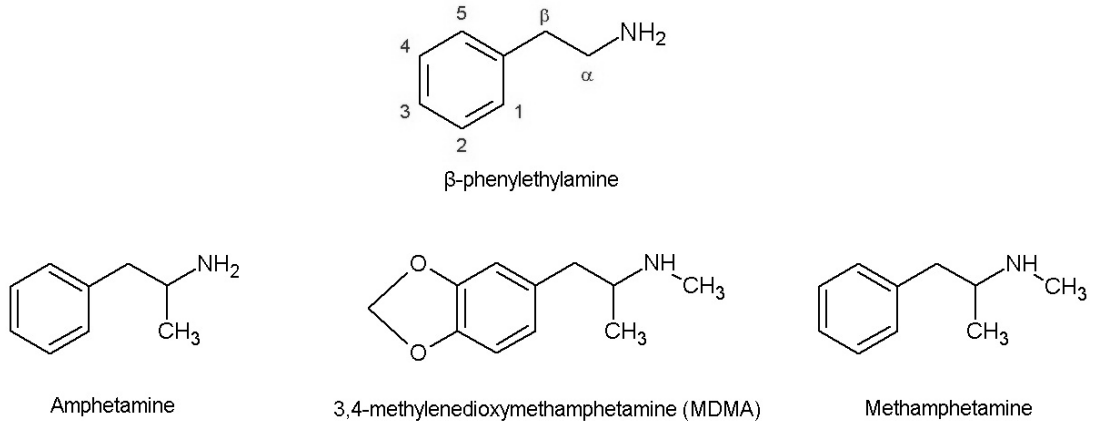

Figure 1 – Chemical structures of β-phenylethylamine (numbered), amphetamine (AMPH), 3,4-methylenedioxymethamphetamine (MDMA or “Ecstasy”) and methamphetamine (Meth, “Ice”).

1.2. Epidemiology

According to the World Drug Report 2014 of the United Nations Office of Drugs and Crime (UNODC), amphetamines (AMPH and METH) or “ecstasy” are the second most commonly used illicit substances. The illicit drug abuse is commonly related with nightlife, which is more frequently attended by young people, but can also be associated with some specific social contexts and cultural groups (EMCDDA 2014, UNODC 2014). Among ATS, AMPH and MDMA are the most available in Europe (EMCDDA 2014, UNODC 2014).

The 2013 annual report of the General-Directorate for Intervention on Addictive Behaviours and Dependencies (SICAD) states that the use of amphetamines by the Portuguese population became wider after 2007, being more frequent among the young population. Besides, “ecstasy” is the third illegal drug most commonly used in Portugal, after cannabis and cocaine (SICAD 2013).

1.3. Pharmacology

ATS are psychostimulants known to interact with monoamine transporter sites in the central nervous system (CNS). Amphetamines act as substrates for the membrane transporters of noradrenaline (NET), dopamine (DAT), and serotonin (5-HTT), due to the structural similarity with monoamine neurotransmitters, noradrenaline (NA), dopamine (DA) and serotonin (5-hydroxytryptamine; 5-HT) (Berger et al 1992, Crespi et al 1997, Jones et al 1998, Kegeles et al 1999, Rothman et al 2000, Silvia et al 1996). Thereby, they reduce the uptake of endogenous neurotransmitters to the cytoplasm and favour the reverse transport of endogenous neurotransmitters into the synaptic cleft, resulting in non-exocytotic neurotransmitter release (Berger et al 1992, Crespi et al 1997, Sulzer et al 1995). Amphetamines can also elevate cytoplasmic transmitter concentrations by promoting DA and 5-HT release from storage vesicles via vesicular monoamine transporter (VMAT), while preventing the uptake into vesicles, making neurotransmitters more readily available for reverse transport (Jones et al 1998, Partilla et al 2006, Rothman & Baumann 2003, Sulzer et al 1995).

Although AMPH and its analogues present similar actions at several transporters, its principal mechanism of action is on DAT by increasing release of DA from the presynaptic nerve terminals (Jones et al 1998, Kegeles et al 1999, Silvia et al 1996). Thus, AMPH enters the cell by acting on DA terminals. This entry can occur via DAT, which is the most common route, or, it also can happen by AMPH lipophilic diffusion through the plasma membrane, which is only efficient at higher AMPH concentrations. The presence of the α-methyl group leads to an increased ability to cross membranes due to its amphipathic nature. Moreover, AMPH structure prevents monoamine oxidase (MAO) enzyme ability to oxidize its amine group by the presence of the α-methyl group in its chemical structure (Figure 1) (Carvalho et al 2012, Jones et al 1998). In general, DA is mostly increased by AMPH in the synaptic cleft, by the mechanisms of reverse transport of VMAT and DAT, as referred above (Jones et al 1998, Partilla et al 2006, Sulzer et al 1995).

In normal conditions, DA is metabolized to dihydroxiphenylacetic acid (DOPAC) by MAO (Davidson et al 2001). AMPH also influences DA-metabolic pathways, decreasing the levels of DOPAC, as a result of AMPH-induced MAO inhibition, thus prolonging the monoaminergic transmission (Jones et al 1998, Miller et al 1980, Taylor et al 2013).

Likewise, METH is also an indirect monoaminergic agonist. It is a substrate of NET, DAT and 5-HTT and increases the levels of NA, 5-HT and DA in the synaptic cleft, by acting on their reuptake and also on storage vesicles (Brown et al 2000, Cruickshank & Dyer 2009, Rothman et al 2000). METH also prolongs monoaminergic transmission by

inhibiting MAO activity, which results in an additional increase of cytosolic DA (Larsen et al 2002).

Table 1 summarizes some of the major findings regarding the pharmacodynamics of AMPH, MDMA, and METH.

Table 1 – Relevant findings related with amphetamines pharmacological mechanism of action.

Findings	References
In vivo studies in rats demonstrate that AMPH acts as a DA releaser.	(Chiueh & Moore 1975, Sulzer & Rayport 1990, Von Voigtlander & Moore 1973)
Studies in the mouse brain indicated that MDMA causes an efflux of 5-HT, DA, and NA.	(Johnson et al 1986, Nichols et al 1982)
It was shown that MDMA interacts with monoamine carriers, leading to a non-exocytic release of 5-HT, DA and NA, in the mouse brain.	(Berger et al 1992, Crespi et al 1997)
In vivo microdialysis showed that MDMA induces DA and 5-HT release in the rat brain, and the effects of the drug on 5-HT release were predominant.	(Baumann et al 2005, Yamamoto & Spanos 1988)
Experiments *in vitro* using *Planorbis corneus* giant dopamine cells indicated that AMPH decreases vesicular DA content, and redistributes the neurotransmitter to the cytosol, promoting reverse transport, and DA release.	(Sulzer et al 1995)
Experiments in human embryonic kidney 293 cells indicated that AMPH is a substrate of the human DAT.	(Sitte et al 1998)
Studies with DAT knockout (DAT - / -) mice, after AMPH administration, have shown that despite increased cytosolic DA concentrations through vesicular depletion, the synaptic levels of DA remained unchanged, since no DAT is available for transporter-mediated release.	(Jones et al 1998)
In vitro studies in rats demonstrated that the 5-HTT uptake blocker fluoxetine inhibited the calcium-independent release of 5-HT after MDMA/METH, demonstrating that MDMA and METH induce release of cytoplasmic 5-HT via 5-HTT.	(Berger et al 1992, Wichems et al 1995)
Studies with rats demonstrated that the ability of AMPH and METH to release DA is directly proportional to the dose.	(Kuczenski et al 1995)
In rats, METH causes dose-dependent release of DA, NA and 5-HT, and is more potent in the release than in the reuptake inhibition assay.	(Rothman et al 2000)

MDMA interaction with the monoaminergic system is similar to that of AMPH and METH, but it has more significant effects on the 5-HT system, namely, inducing the reverse transport of 5-HT in 5-HTT and VMAT (Baumann et al 2005, Berger et al 1992, Crespi et al 1997, Green et al 2003, Partilla et al 2006, Wichems et al 1995). Moreover, it was shown that MDMA also inhibits MAO, mainly MAO type A, the enzyme that preferentially degrades 5-HT. The cytoplasmatic non-vesicular stored 5-HT should, in normal conditions, be degraded by MAO. Therefore, as MDMA is a competitive inhibitor of MAO-A activity, it will contribute to the accumulation of more extracellular 5-HT (Leonardi & Azmitia 1994).

In summary, amphetamines differ in their affinities for monoamine transporters, therefore resulting in the disruption of pathways related to different neurotransmitters. AMPH and METH have more potent actions on DA release rather than in 5-HT release, while MDMA shows greater affinity for 5-HTT over DAT. Accordingly, MDMA causes a higher release of 5-HT instead of DA. Importantly, AMPH, METH, and MDMA are more potent NA releasers, rather than DA and 5-HT (Rothman & Baumann 2003).

1.4. Pharmacokinetics

Amphetamines are weak bases with a pka around 9.9, a low molecular weight, with good oral bioavailability, a high volume of distribution (Vd) (around 4 L/kg), and a low plasma protein binding (ordinarily less than 20%). These properties enable an easy diffusion of these drugs across cellular membranes and lipid layers, reaching high levels in tissues and biological matrices with a pH lower than blood (Carvalho et al 2012, de la Torre et al 2004).

1.4.1. Absorption

AMPH is commonly consumed orally and available either as S-(+)-enantiomer (d-AMPH) or as the racemic mixture. Pizarro and co-workers performed a study in 17 healthy volunteers, in which four different doses of dl-AMPH (25, 30, 35 and 40 mg) were tested. After AMPH administration, they found that the peak plasma concentration (C_{max}) was attained between 2 to 3 hours (h). Moreover, when comparing area under the plasma concentration-time curve (AUC) over 24 h and C_{max} in the range of doses administrated, investigators were able to conclude that plasma levels can be easily predicted, since variations behave in a linear mode (Pizarro et al 1999). In general, C_{max} are reached

within 4 h after ingestion, and both AUC over 24 h and C_{max} are proportional to the dose administered, increasing in a dose-dependent manner. Besides, it has not been reported significant differences of pharmacokinetic (PK) parameters between the two enantiomers (Carvalho et al 2012, de la Torre et al 2004).

PK data reported for MDMA are commonly based on oral and parental administration. Studies showed that after a single *per os* (p.o.) administration of MDMA (50, 75, 100, 125 or 150 mg) in humans, the C_{max} is reached between 1.5 to 3 h. They also showed that for doses of 50, 75, 100 and 125 mg, AUC over 24 h and C_{max} increased in a dose-dependent manner (de la Torre et al 2000, Mas et al 1999). Even so and according to de la Torre and co-workers, for the higher 150 mg dose, the increase in MDMA kinetic parameters was not proportional to the dose, suggesting a non-linear PK (de la Torre et al 2000). The non-linear MDMA PK has been confirmed in recent studies, in humans and squirrel monkeys (Mueller et al 2009).

Farré and co-workers studied MDMA PK with the successive administration of this drug in nine healthy male subjects, by administrating 100 mg in two doses, separated by an interval of 24 h. The repeated administration of MDMA led to a disproportionate increase in plasma concentrations, being superior than expected by simple accumulation, which indicated metabolic inhibition (Farré et al 2004). These observations can be related with the non-linear PK of MDMA in humans described above, and both can be explained by a possible saturation of MDMA metabolism, as well as by the inhibitory interaction of metabolites with some MDMA metabolizing enzymes. *In vitro* studies indicate that MDMA can act as an inhibitor of cytochrome P450 (CYP) 2D6 through a competitive interaction mechanism and/or by forming an enzyme metabolite complex (Carmo et al 2006, Delaforge et al 1999, Heydari et al 2004, Wu et al 1997).

MDMA is commonly consumed orally and available as a racemic mixture. An MDMA enantioselective disposition has been reported in humans. After an oral administration of 40 mg of MDMA racemic mixture, it was observed that the C_{max} of (R)-(-)-MDMA enantiomer was significantly higher than the C_{max} of (S)-(+)-MDMA, and the more pharmacologically active (S)-(+)-MDMA enantiomer was more rapidly eliminated (de la Torre et al 2004, Fallon et al 1999, Pizarro et al 2004).

METH can be used p.o., intravenously (i.v.), snorted, or inhaled either through vapour or by the smoking of the (S)-(+)-METH hydrochloride salt. Hart *et al*. were able to conclude that after administration of intranasal METH doses, (0, 12, 25, and 50 mg / 70 kg) the C_{max} is observed after 4 h (Hart et al 2007). Schepers and co-workers performed a study of METH PK, administrating oral doses of 10 and 20 mg of (S)-(+)-METH to individuals. They conclude that the C_{max} was reached between 5 to 8 h after administration, and that the 24 h AUC and C_{max} were significantly dose-dependent

(Schepers et al 2003). Previous studies in humans, also showed that C_{max} is attained after 3 h by oral administration of d-METH hydrochloride (Shappell et al 1996). On the other hand, after i.v. administration in humans of 15 and 30 mg doses of METH, was reported that the C_{max} was reached in 0.53 and 0.51 h (Newton et al 2005). Besides, the bioavailability of smoked METH (90%) is significantly superior when compared to the bioavailability of 67% after oral ingestion (Cook et al 1993).

1.4.2. Distribution

As said before, amphetamines in general have low protein binding (< 20%), which means that they are able to diffuse rapidly to the extracellular compartment and to every body tissues (de la Torre et al 2004).

Amphetamine-dependent individuals appear to have larger Vd (6 L/kg) than drug-naive individuals (4 L/kg). They have higher tissue affinity for the drug that leads to AMPH tissue sequestration, as a consequence of the development of tolerance. Protein binding of the two enantiomers, (S)-(+)-AMPH and (R)-(-)-AMPH, is similar, as well as their distribution volumes (de la Torre et al 2004).

There is a lack of clinical studies regarding the MDMA distribution in humans after a controlled administration, in contrast to the various numbers of reported studies in fatal intoxications. It has been observed that the highest MDMA concentrations are found in the brain and liver, suggesting that these drugs preferentially accumulate in the referred organs (De Letter et al 2004, De Letter et al 2006, García-Repetto et al 2003). From the data reported by de la Torre *et al*., they established a Vd of 6.4 L/kg after the oral administration of 100 mg for MDMA (de la Torre et al 2004). Regarding enantiomers of MDMA, it was also indicated that this drug undergoes an enantioselective disposition in humans, since it was observed that the more pharmacologically active (S)-(+)-MDMA enantiomer had a higher Vd (Vd = 595 ± 204 L) than (R)-(-)-MDMA enantiomer (Vd = 383 ± 97 L) (Fallon et al 1999).

The METH Vd is similar to that of AMPH (4 L/kg) and, according to the literature, is not affected by the time or route of administration (Shappell et al 1996). Volkow and co-workers measured the whole-body distribution and bioavailability of METH in 19 healthy participants (nine Caucasians and ten African Americans), showing that the drug was distributed through several organs. METH appears to be highly accumulated in liver, lungs, and, in a smaller extension, in brain and kidneys. In this study the lung accumulation of METH was reported to be 30% higher for African Americans than Caucasians, though no difference was observed for the other organs (Volkow et al 2010). Another study showed that METH has a fast brain uptake [peak uptake at 9 minutes

(min)], a widespread (accumulating in cortical and subcortical areas and in white matter), and a long-lasting (slow clearance from brain) distribution in human brain. Besides, no difference among Caucasians and African Americans in METH PK and bioavailability was found in that study (Fowler et al 2008). Moreover, METH accumulates in saliva, hair and nails of drug abusers, which also has clinic and forensic interest (Cook et al 1993, Suzuki et al 1989).

1.4.3. Metabolism

Amphetamines are mainly metabolized in the liver by several enzymes. The main metabolic pathways of AMPH are: (1) N-deamination to phenylacetone, which is converted to benzoic acid by oxidation, followed by conjugation with glycine generating hippuric acid, capable of being excreted; and (2) aromatic hydroxylation in position four of the ring, resulting in 4-hydroxyamphetamine, and further conjugation of the phenol group with sulfate or glucuronic acid (Kraemer & Maurer 2002, Musshoff 2000) (Figure 2). Carvalho and co-workers reported that in the hydroxylation step, AMPH can also be converted to a reactive intermediate that would react with glutathione generating the (glutathion-*S*-yl)-*p*-hydroxyamphetamine adduct (Carvalho et al 1996). *In vitro* studies indicate that N-deamination appears to be catalysed by CYP isoenzymes of the CYP2C subfamily (Yamada et al 1997). On the other hand, hydroxylation of the aromatic ring seems to be catalysed by CYP2D6. Oxidation at the β-carbon of the side chain (aliphatic hydroxylation) is the minor metabolic pathway that, regarding to AMPH, leads to the formation of norephedrine (stereoisomer of phenylpropanolamine), followed by oxidation, in the aromatic ring, to hydroxynorephedrine, a compound that is implicated in the tolerance to peripheral actions of the drug (Lin et al 1997, Musshoff 2000) (Figure 2).

METH is mainly metabolized through N-demethylation into AMPH, and by 4-hydroxylation of the aromatic ring resulting in 4-hydroxymethamphetamine, that can undergo a further conjugation of the phenol group with sulphate or glucuronic acid (Figure 2). Both of the metabolic pathways are catalysed by CYP2D6. Besides, the involvement of the genetic polymorphism associated with CYP2D6 may result in a interindividual variability in metabolism (Lin et al 1997). METH is also metabolized to norephedrine, and hydronorepherine (Caldwell et al 1972, Lin et al 1997, Musshoff 2000). Urinary levels of unchanged METH are much higher than of the methylenedioxy derivatives, like MDMA, suggesting that METH is metabolized in a lower extent (de la Torre et al 2004). According to Cruickshank and Dyer, METH metabolites are formed at low levels and at times where the acute effects are minimal, resulting in a poor contribution of metabolites to the clinical effects of the drug (Cruickshank & Dyer 2009) (Figure 2).

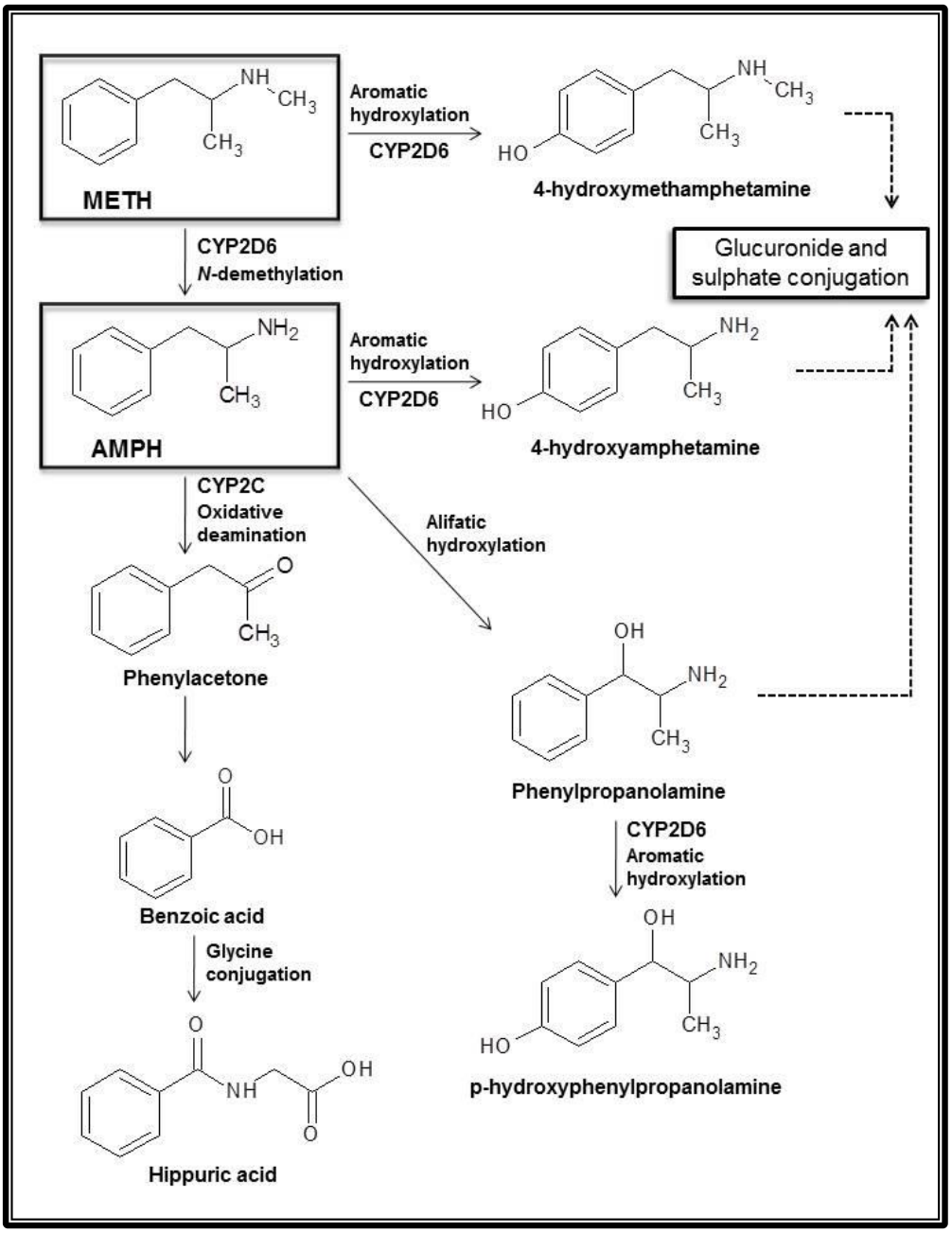

Figure 2 – Major metabolic pathways of amphetamine (AMPH) and methamphetamine (METH).

MDMA is mainly metabolized by two pathways. Firstly, *O*-demethylenation of the methylenedioxy group occurs, resulting into a catechol N-methyl-α-methyldopamine (N-Me-α-MeDA, 3,4-dihydroxymethamphetamine, HHMA). This step is catalysed by CYP2D6 and, also, by CYP3A4 and CYP1A2 in humans, and by isoenzymes CYP2D1 and CYP3A2 in rats. That demethylenation is followed by *O*-methylation of one of the hydroxyl groups to form 4-hydroxy-3-methoxymethamphetamine (HMMA), catalysed by catechol-*O*-methyltransferase (COMT). Both can be possibly conjugated with glucuronide or sulphate. Secondly, MDMA can be N-demethylated into 3,4-methylenedioxyamphetamine

(MDA), mainly mediated by CYP2B6 and also by CYP2D6, CYP1A2, and, CYP3A4 in humans and by CYP1A2 and CYP2D1 in rats. This step is followed by *O*-demethylenation to the catechol α-methyldopamine (α-MeDA, 3,4-dihydroxyamphetamine, HHA), and is catalysed by CYP2D6 and CYP3A4 in humans, and by CYP2D1 and CYP3A2 in rats. Lastly, α-MeDA is methylated into 4-hydroxy-3-methoxyamphetamine (HMA) by COMT. Both, α-MeDA and HMA can be possibly conjugated with glucuronide or sulphate (de la Torre et al 2005, de la Torre et al 2000, Kraemer & Maurer 2002, Segura et al 2001) (Figure 3).

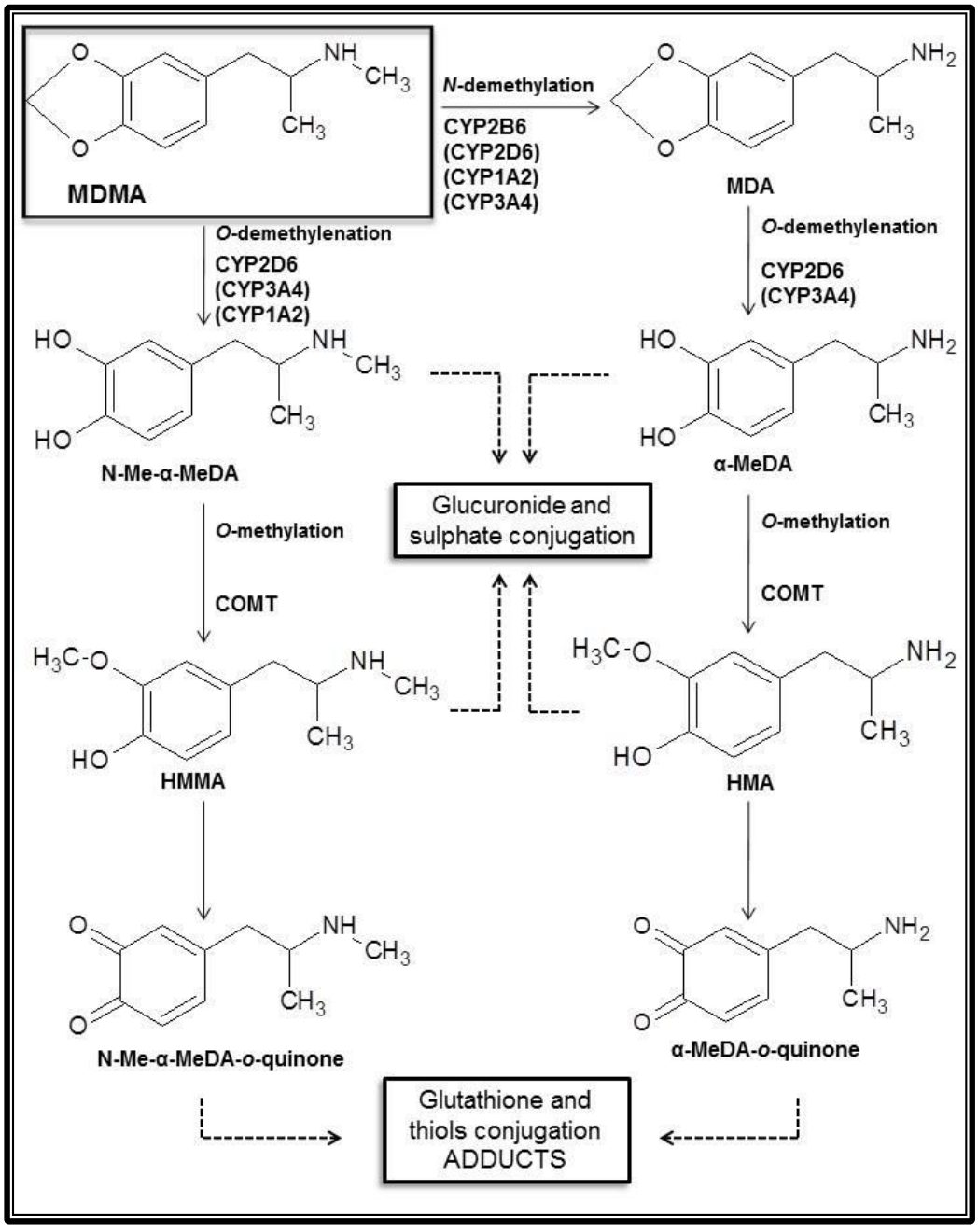

Figure 3 – Major 3,4-methylenedioxymethamphetamine (MDMA or "Ecstasy") metabolic pathways.

Studies demonstrated that both catechol metabolites N-Me-α-MeDA and α-MeDA can be oxidized into *ortho*-quinones, and can conjugate with thiols and glutathione originating adducts (Cho et al 1999). These conjugates formation was also demonstrated *in vitro* in human liver microsomes, by Easton *et al.* (Easton et al 2003). Conjugates are formed in the liver, but can be distributed to other organs, namely, to the brain. That was demonstrated by an *in vivo* study where metabolites were measured in the rat brain (Jones et al 2005).

1.4.4. Excretion

Amphetamines plasma half-life is highly dependent on the urine pH, since the major elimination route is the renal excretion. As weak bases, amphetamines renal excretion is increased by urinary acidification and decreased by urinary alkalinisation. For these reason, some amphetamines consumers ingest bicarbonate, so they can increase the amphetamines plasma half-life, prolonging the drug's effect (Cook et al 1993, Quinn et al 1997). The elimination half-life tends to be longer in users dependent of AMPH and METH and in individuals with alkaline urine (de la Torre et al 2004). Data obtained by Poklis *et al.* demonstrated that about 35 to 44% of AMPH is excreted over 24 h. They also reported that elimination of AMPH increases with increasing urine flow and decreasing urine pH (Poklis et al 1998).

MDMA elimination half-life is between 6 to 9 h, and the majority of the dose is excreted in the 24 h following administration. Most MDMA (80%) is metabolized through the liver, and around 20% is excreted with no changes in urine. Besides, the urinary elimination is independent of the doses administrated, while non-renal elimination appears to be dose-dependent (de la Torre et al 2004, Fallon et al 1999).

Segura *et al*. reported that the major MDMA metabolite excreted in urine is HMMA (22.7% of the dose), and N-Me-α-MeDA the second with a 17.7% of the total MDMA dose recovery (Segura et al 2001). MDMA elimination in humans is stereoselective, and the more pharmacologically active (S)-(+)-MDMA enantiomer has a shorter half-life (3.6 ± 0.9 h) than the less active (R)-(-)-MDMA enantiomer (5.8 ± 2.2 h) (Fallon et al 1999).

METH is mainly excreted (70% of METH dose) in urine within 24 h after administration. Also, 50% of the METH dose is eliminated unchanged, 15% as the 4-hydroxymethamphetamine, and 10% as AMPH. The percentage of METH excreted unchanged in urine decreases with increasing doses, which can be explained by a lower elimination rate or an increasing in non-renal elimination for higher doses (Carvalho et al 2012, Cook et al 1993).

1.5. Acute effects in laboratory animals and humans

1.5.1. Laboratory animals

The acute effects of amphetamines in laboratory animals include mainly hyperthermia and hyperlocomotion. These effects are due to the high release of monoaminergic neurotransmitters and the interaction with the specific pre- and postsynaptic receptors (Carvalho et al 2012).

Randrup and Munkvad reported the behaviour of rats, mice, guinea-pigs, cats and squirrel monkeys after the subcutaneous (s.c.) administration of AMPH. All animals performed stereotyped activity. Rats, mice and guinea-pigs presented similar stereotyped sniffing, licking, and biting activity, and in the initial and final phase after the injection was also reported increased locomotion. Cats presented stereotyped head movements, and the squirrel monkeys all performed stereotyped activity, but with individual differences. The behaviour reported for rats was also produced after METH administration (Randrup & Munkvad 1967).

Researchers have studied the DA neurotransmission influence on amphetamine actions, due to its involvement in locomotion, cognition, affect, and neuroendocrine functions. Giros and co-workers showed that AMPH promotes locomotor activity due to its action on DAT, once in mice lacking this transporter, the drug had no effect on locomotor activity or DA release and uptake. They also demonstrated that DAT is the main transporter that mediates hyperlocomotor effects of AMPH (Giros et al 1996). Kuczenski and Segal studied in rats the temporal and dose-related behavioural and striatal monoamine response to AMPH and observed that: (1) a significant relationship between the magnitude and duration of DA release and the increases in behavioural response occurred, (2) the presence of stereotyped movements was not dependent on the quantity of striatal DA, and (3) some of the behavioural responses were related to changes in striatal 5-HT concentrations. These data suggested that the behavioural response to AMPH might be related with levels of both DA and 5-HT release (Kuczenski & Segal 1989). Studies in rats also showed that METH is capable of increasing locomotor activity due to the dopaminergic transmission (Wallace et al 1999).

Several studies in rats showed that MDMA also produces an hyperlocomotor response due to the release of 5-HT in several brain regions, in a dose-dependent manner (McNamara et al 1995, Shankaran & Gudelsky 1999, Souza et al 1997, Yamamoto & Spanos 1988). In addition to the hyperlocomotion, it was also reported the 5-HT syndrome, as a result of excessive 5-HT release. The criteria used to determine the

presence of the 5-HT syndrome in rats is usually based in stereotyped motor behaviours as low body posture, forepaw treading, head weaving, hind limb abduction, piloerection, ejaculation, salivation, followed by dose-dependent convulsions, and possible death (Morley et al 2005, Piper et al 2005, Shankaran & Gudelsky 1999, Souza et al 1997, Spanos & Yamamoto 1989). Callaway and colleagues demonstrated that MDMA produced a dose-dependent increase in rat locomotor activity, due to 5-HT release. They stated that pre-treatment with fluoxetine (a 5-HT uptake inhibitor) inhibited MDMA-induced locomotor hyperactivity, whereas it failed to affect the AMPH-induced hyperactivity. These data suggested that AMPH and MDMA produced locomotor activity by different mechanisms, possible due to the affinity of the drugs to the different drug transporters (Callaway et al 1990).

One of the most dangerous acute effects of amphetamines is hyperthermia, and the most studied amphetamine in this subject is MDMA. Several studies demonstrated that MDMA causes a dose-dependent hyperthermia in various laboratory animals, such as mice, rats, rabbits, guinea pigs, and pigs (Capela et al 2009). Colado and co-workers demonstrated in Lister Hooded rats that an intraperitoneal (i.p.) administration of MDMA (20 mg/kg) induced hyperthermia with a temperature increase of approximately 2.5ºC, that continued for 3 h (Colado et al 1993). Carvalho and colleagues reported an hyperthermic response in mice, after an i.p. injection of MDMA (5, 10, or 20 mg/kg), potentiated by high ambient temperature, since at an ambient temperature of 30 ± 2ºC, the maximum increases were higher than the maximum temperature reached at an ambient temperature of 20 ± 2ºC. MDMA increased mice body temperature, with a maximum at approximately 30 min after injection, and remained for more than 4 h. Besides, the hyperthermic response induced was dependent on MDMA doses, with a largest change of approximately 2ºC at the highest dose (20 mg/kg) (Carvalho et al 2002). Researchers also demonstrated that MDMA administration in rats produced hypothermia at an ambient temperature of 15ºC, and hyperthermia at 19ºC or 30ºC (Green et al 2005, Green et al 2004b). Gordon and co-workers studied the MDMA effects on temperature regulation, and observed that the metabolic rate, evaporative water loss, motor activity, and colonic temperature were augmented and were related to ambient temperature (Gordon et al 1991). Yehuda and Wurtman also reported hyperthermia after AMPH administration in rats kept at ambient temperature of 20ºC and 37ºC and hypothermia in a ambient temperature range of 4 – 15ºC (Yehuda & Wurtman 1972).

The increase in temperature produced by MDMA has been attributed to 5-HT, although, recent studies showed that hyperthermia may be related with the activation of postsynaptic DA D_1 receptors that occurs after DA release (Baumann et al 2007). Studies in mice and rats, regarding the hyperthermia induced by amphetamines (MDMA and *p*-

cloroamphetamine), and using 5-$HT_{1/2}$, 5-HT_{2A}, and 5-HT_{2C} antagonists, and 5-HT uptake inhibitors, demonstrated that treatment with these agents failed to alter the course of the hyperthermia promoted by amphetamines. Moreover, the DA D_1 receptors antagonists were able to antagonize hyperthermia, contrarily to the DA D_2 receptor antagonist. These results suggested that the DA release and activation of DA D_1 receptor are important to hyperthermia induced by those amphetamines (Mechan et al 2002b, Sugimoto et al 2001).

After administration of METH (2.5, 5.0, 7.5 mg/kg) to rats at an ambient temperature of 21 ± 1ºC, it has been reported a significant increase in body temperature at 21ºC, in which the maximum core temperature increase ranged from 0.92 to 1.51ºC. They also reported a decrease in body temperature at an ambient temperature of 7ºC, in which the obtained decrease ranged from 2.22 to 4.15ºC lower than the initial body temperature. These results suggest that METH is also capable of inducing hyperthermia, and at low environmental temperature may generate hypothermia (Mohaghegh et al 2002).

1.5.2. Humans

Amphetamines are able to generate a large variety of acute clinical effects by modulating peripheral and central monoamine neurotransmitter function. Amphetamines cause CNS stimulation, producing effects as euphoria, arousal, enhanced mood, well-being, increased alertness, wakefulness and concentration, reduced appetite and fatigue, insomnia, behavioural disinhibition, and MDMA users particularly report feelings of increased closeness to others, greater sociability, and heightened sensory perception (Cretzmeyer et al 2003, Cruickshank & Dyer 2009, Derlet et al 1989, Hart et al 2001, Johanson & Uhlenhuth 1980, Liechti et al 2000, Vollenweider et al 1998).

The peripheral effects of amphetamines seem to be caused by indirect sympathomimetic stimulation, resulting in an increased energy, psychomotor activation, restlessness, elevated body temperature (hyperthermia), increased blood pressure (hypertension), increased heart rate (tachycardia), increased respiration rate, palpitations, pupil dilatation (mydriasis), tremor, sweating (diaphoresis), jaw clenching (bruxism), dry mouth (xerostomia) and hyperreflexia (Cohen & Cocores 1997, Cruickshank & Dyer 2009, Derlet et al 1989, Greene et al 2008, Liechti et al 2000, Vollenweider et al 1998).

A study on the effects of intranasal METH administration in humans showed that the cardiovascular effects (heart rate and blood pressure) and the subjective effects described by the users, like “good drug effects” and “stimulated”, increased in a dose-dependent manner (Hart et al 2007). Mas and co-workers reported that AMPH and MDMA were able

to increase blood pressure and heart rate, when compared with placebo, after administration in humans (Mas et al 1999).

One of the most dangerous acute effects of amphetamines is hyperthermia. Both METH and MDMA have been implicated in fatalities related with this physiological effect. As already mentioned, MDMA is the most studied drug, and there have been reported body temperatures of about 43ºC (Green et al 2004a, Jaehne et al 2007). This drug is frequently used recreationally in popular “rave” parties or nightclubs where elevated ambient temperature, crowded rooms, physical exertion (dancing), and dehydration are common and can contribute to an elevated body temperature (Henry 1992, Irvine et al 2005, Patel et al 2005). Accordingly, factors as repetitive physical activity, peripheral vasoconstriction, loss of thermoregulatory mechanisms at the CNS, loss of body signal perception (like thirst and exhaustion), euphoria, increased muscle tone, and heat production are the factors that mainly contribute to hyperthermic effects associated with MDMA use (Capela et al 2009).

A study under two different ambient temperatures, 30ºC and 18ºC, in ten healthy recreational MDMA users, reported that this drug produced increases in body temperature, blood pressure, heart rate, and subjective effects. After MDMA, the increased body temperature was significant in either warm or cold environments, but the temperatures reached were higher in the warm environment. The increase in body temperature was higher under warm condition but it did not reach statistically significance, once under the placebo conditions an elevation in temperature under the warm environment was also reported (Freedman et al 2005). Another study in individuals who participated in “raves” and were MDMA users evaluated their physiological parameters before and after a dance party. Those with the highest MDMA plasma concentrations reported moderate increases in blood pressure, heart rate and body temperature after drug use at the dance party (Irvine et al 2005).

Furthermore, hyperthermia induced by amphetamines can lead to fatal consequences, as rhabdomyolysis, acute renal failure, disseminated intravascular coagulation (DIC), and multiple organ failure (Henry 1992, Kalant 2001, Kendrick et al 1977).

Amphetamines can also induce unpleasant CNS symptoms. It has been reported in several studies symptoms such as anxiety, insomnia, hallucinations (sound and visual hallucinations), irritability, mental confusion and panic attacks. The psychological side effects related with the use of these drugs can also lead to depression, paranoia, violent behaviour, memory lapses, flashbacks, cognitive deficits, and suicidal ideation (Cole & Sumnall 2003, Davison & Parrott 1997, Degenhardt & Topp 2003, Derlet et al 1989, Sommers et al 2006). When comparing the psychological side effects of crystal METH

with those of AMPH, researchers observed that the prevalence of panic attacks among crystal METH users was higher than in AMPH users (20% vs. 9%), although the prevalence in terms of violent behaviour (43% vs. 24%) and hallucinations (46% vs. 20%) was respectively higher among AMPH users than in METH (Degenhardt & Topp 2003). According to the Davinson and Parrot study, the duration of effects like insomnia, irritability, depression and paranoia varied, depending on the individuals, from a few hours to over a day (Davison & Parrott 1997). Moreover, AMPH use can lead to delirium, convulsions, acute panic disorder, psychosis, arrhythmia, ischemic and haemorrhagic stroke, acute renal failure, rhabdomyolysis, DIC, hepatic necrosis, hyponatremia, multi-organ failure, coma, and, in a worst case scenario, it can lead to sudden death (de la Torre et al 2004, Hall & Henry 2006, Henry 1992, Kendrick et al 1977). In MDMA use commonly emerges another acute effect named 5-HT syndrome. This syndrome shows similarity to the acute hyperthermia and multi-organ failure events, and it's characterized by having a rapid onset, associated with confusion, diaphoresis, diarrhoea, cardiovascular instability, and increased muscle tone and rigidity that can lead to dead (Hall & Henry 2006).

Table 2 presented below summarizes the desired effects of consuming amphetamines, as well as the most relevant adverse effects and toxicity observed.

Table 2 – Desired, adverse effects, and acute toxicity of amphetamines use.

Desired effects	Adverse physiological effects	Adverse psychological effects	Acute toxicity
• Euphoria • Energy • Arousal • Well-being • Behavioural disinhibition • Concentration • Greater sociability • Increased closeness to others	• Hyperthermia • Hypertension • Tachycardia • Mydriasis • Diaphoresis • Bruxism • Xerostomia • Headaches • Ataxia	• Anxiety • Insomnia • Panic attacks • Mental confusion • Irritability • Hallucinations • Paranoia • Psychosis • Suicidal ideation	• Rhabdomyolysis • DIC • Hepatotoxicity • Cardiotoxicity • Nephrotoxicity • Teratogenic effects

1.6. Evidences of neurotoxicity

1.6.1. Laboratory animals in general

The potential neurotoxic effects of amphetamines have been immensely studied proving their ability to damage brain monoaminergic cells, namely by causing long-term deficits in dopaminergic and serotoninergic systems in several brain areas (Commins et al 1987, Gibb et al 1997, Ricaurte et al 1982, Schmidt 1987, Sonsalla et al 1996, Villemagne et al 1998). Importantly, the neurotoxic actions of amphetamines have been evaluated by biochemical (decreased levels of monoamines and their major metabolites, decrease of monoamine transporter binding sites, and lower expression and/or activity of enzymes involved in synthesis and metabolism of neurotransmitters), histological, and immunocytochemical techniques (Carvalho et al 2012).

One of the major neurotoxic actions of amphetamines seen in laboratory animals is the sustained depletion of monoamine brain levels. A study in mice clearly proved this neurotoxic action, after the administration of (+)-AMPH (6.3, 12.5, 25, 37 or 62 µg/h) by means of osmotic mini pumps, placed subcutaneously, for two to seven days, where animals were sacrificed eight days after starting the treatment. It was described a reduction of NA levels for AMPH infusion of 62 µg/h in several brain areas, and, also, depletion of DA levels for AMPH infusions of 25, 37 and 62 µg/h in the olfactory tubercle and striatum, in a dose-dependent manner (Jonsson & Nwanze 1982).

MDMA also exhibits the ability of depleting monoamine neurotransmitter content in the rat's brain. Several studies reported depletion in brain 5-HT levels after MDMA administration (Aguirre et al 1998a, Colado et al 1993, Colado et al 1995, Commins et al 1987, O'Shea et al 1998, Schmidt 1987, Shankaran & Gudelsky 1999). In fact, Schmidt demonstrated that the 5-HT depletion caused by 10 mg/kg, s.c. administration, of MDMA has two distinguishable phases. The first one appears to produce a reversible depletion of 5-HT, in which 5-HT concentrations declined to 16% of control, 3 h after administration. Following this initial decline, occurred a recovery of 5-HT levels, returning to basal levels, between 6 and 24 h. The second phase is characterized by another 5-HT depletion in the days following MDMA administration: after a week the 5-HT levels declined to 74% of control (Schmidt 1987).

METH also causes severe depletion in brain monoamine levels. The repeated s.c. administration of high doses of METH (50 and 100 mg/kg, twice daily, for four days) to rats resulted in a dose-related decrease in DA levels and its uptake sites in the striatum, two weeks after the last administration period. No changes in brain NA levels were

reported after this regimen of repeated METH administration when compared to controls (Wagner et al 1980).

Relevant studies regarding the ability of AMPH, MDMA, and METH to deplete monoamine neurotransmitter content, after administration to laboratory animals, are summarized in Tables 3, 4, and 5, respectively.

The neurotoxic action of amphetamines can also be related with the degeneration of neuronal fibres. A study in mice demonstrated, histochemically, that the continuous infusion of large doses of (+)-AMPH (25 or 37 µg/h) for seven days, by means of osmotic minipumps, led to a dose-dependent and long-lasting reduction of DA nerve terminals until eight weeks after the last (+)-AMPH infusion. The results also suggest that with time there was a slow regeneration of DA nerve terminals (Jonsson & Nwanze 1982). MDMA was also shown, in several studies, to be able to promote damage to the nerve terminals (Commins et al 1987, O'Hearn et al 1988, O'Shea et al 1998, Schmidt 1987). By using an immunocytochemical technique, O'Hearn and colleagues were able to demonstrate the structural damage to the terminal portions of axons, promoted by MDMA, in rats. Two weeks after the administration of MDMA (20 mg/Kg, s.c., twice a day for four days) fragmented 5-HT axons were observed in the forebrain. These fragmented axons are an anatomic evidence for the degeneration of 5-HT projections (O'Hearn et al 1988). METH was also shown to induce terminal degeneration in laboratory animals. Ricaurte and co-workers reported that METH (3 x 50 mg/kg, s.c., every 8 h) induced destruction of DA terminals, along with correlative DA neurochemical deficits in the striatum and nucleus accumbens of rats, even three weeks after the administration (Ricaurte et al 1982). Several relevant studies related with the degeneration of neuronal fibres induced by AMPH and MDMA are summarized in Tables 3 and 4, respectively.

Table 3 – Relevant studies related to the neurotoxicity of AMPH to laboratory animals.

Dosage Regimen	Relevant studies	Reference
17 mg/kg, s.c., twice daily	Repeated injections of d-AMPH to rats resulted in the decrease of brain NA levels. These effects persisted for several days after the last injection of the drug.	(McLean & McCartney 1961)
10 mg/kg, s.c., twice daily, 2 h interdose interval	A study in rabbits demonstrated a reduction in NA levels in the superior cervical ganglia and in the brain, 4 h after the first administration of d-AMPH to the referred animals.	(Sanan & Vogt 1962)
9.2 mg/kg, i.p., single dose	A single injection of d-AMPH administered to rats pre-treated with iprindole (interferes with the metabolism of AMPH and prolongs its half-life) resulted in the destruction of DA nerve terminals, two weeks after the treatment.	(Ricaurte et al 1984)
0.1 or 0.25 mg/kg, s.c., twice daily, for 5 days	Prolonged exposure of rats to AMPH at doses near threshold for locomotor activation, and within the therapeutic range for attention deficit hyperactivity disorder (ADHD) treatment, can produce sensitization-like effects on the locomotor response to a subsequent exposure. Four days after the last treatment, animals received another 0.5 mg/kg of AMPH, which promoted a marked increase in locomotor response.	(Kuczenski & Segal 2001)
1, 2 or 3 mg/kg, every other day	A study on the performance of rats with a repeated intermittent administration of AMPH showed that the effects of repeated administration of the drug may interfere with cognitive processes, once a psychostimulant-sensitization can lead to attentional consequences. There were increases in the number of false alarms, like “claims” for hits in non-signal trials, suggesting possible hyper attentional dysfunctions that may contribute to the development of psychotic symptoms.	(Deller & Sarter 1998)
0.1-1.0 mg/kg, intramuscular (i.m.), twice daily, for 6 or 12 weeks	A research in rhesus monkeys reported that the AMPH-sensitized monkeys were deeply impaired in their ability to acquire cognitive tasks, since several months after the treatment with AMPH, the monkeys had profound and enduring deficits in the acquisition of a spatial working memory task, and spatial delayed response.	(Castner et al 2005)

Table 4 – Relevant studies related to the neurotoxicity of MDMA to laboratory animals.

Dosage Regimen	Relevant studies	Reference
10, 15 or 20 mg/kg, i.p., single dose	A single dose of MDMA resulted in significant loss of 5-HT and decreases in the 5-HT major metabolite, 5-hydroxyindoleacetic acid (5-HIAA) in rats' brain, seven days after the administration of 10 or 15 mg/kg, or four days after 20 mg/kg.	(Colado et al 1993, Colado et al 1995, O'Shea et al 1998).
10 mg/kg x 4, i.p., every 2 h	A study in rats reported a 45% depletion of 5-HT levels one week after several doses of MDMA.	(Shankaran & Gudelsky 1999)
10, 20 or 40 mg/kg, s.c., twice daily, for 4 days; or 40 mg/kg, s.c., single dose	Commins and co-workers reported in rats depletions of 5-HT after repeated injections of MDMA and, also, after a single administration of a higher dose of the drug, two weeks after exposure to MDMA. It was also observed nerve terminal degeneration in the striatum.	(Commins et al 1987)
5 mg/kg, s.c., twice daily for 4 days	In a study performed in squirrel monkeys, researchers injected the animals with MDMA and observed the reduction of brain serotonergic innervations, and 5-HT levels. The serotonergic innervations still remained reduced seven years after exposure to the drug, showing that the damage to serotonergic system is, probably, irreversible.	(Hatzidimitriou et al 1999)
10, 15, 20 mg/kg, i.p., twice daily, for 3 days	After a neurotoxic exposure of rats to MDMA, it was reported a long lasting cognitive impairment over the 16 days following the exposure.	(Marston et al 1999)

Table 5 – Relevant studies related to the neurotoxicity of METH to laboratory animals.

Dosage Regimen	Relevant studies	Reference
10 mg/kg x 4, i.p., very 2 h	Rats treated with a neurotoxic regimen of METH, presented a reduction of DA contents in caudate nucleus (56%) and nucleus accumbens (30%) one week later. The levels of 5-HT were also decreased in caudate nucleus (50%) and nucleus accumbens (63%).	(Wallace et al 1999)
10 or 20 mg/kg x 4, i.p., every 2 h	A study in mice demonstrated a marked depletion of striatal DA (≥ 90%) seven to eight days after METH administration.	(Sonsalla et al 1996)
1.25 mg/kg, p.o., given twice, 4 h apart	A study in squirrel monkeys treated with METH reported decreases in striatal dopaminergic markers one week after treatment. Moreover, the study was conducted in two different AT (26 and 33ºC), and the ones treated at the highest AT had the largest dopaminergic deficits.	(Yuan et al 2006)
0.2 mg/kg or 2 x 2 mg/kg, i.m., 24 h apart	Studies with vervet monkeys demonstrated that after a long-term exposure to METH there is a decrease in phenotypic markers of the DA system. Decreases in striatal DA content were reported, as well as decreases in DAT, TH, and VMAT.	(Harvey et al 2000, Melega et al 2007)

In addition to the damage to dopaminergic and serotoninergic neuronal systems, amphetamines can also induce neuronal death. Using a terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick-end labelling (TUNEL) histochemical method to verify DNA fragmentation, Krasnova and co-workers reported that the i.p. administration of AMPH (10 mg/kg, four times, every 2 h) in mice resulted in the death of non-dopaminergic cells in the striatum, the maximal cell death occurring four days after AMPH injections (Krasnova et al 2005). In several studies, MDMA administration to mice and rats produced neuronal death in several brain areas including the cortex, hippocampus, amygdala, ventromedial/ventrolateral thalamus, and tenia tecta (Armstrong & Noguchi 2004, Commins et al 1987, Meyer et al 2004, Schmued 2003, Tamburini et al 2006, Warren et al 2007). METH-induced neuronal death was also investigated in the brain of mice and rats, and it was observed in several areas, namely the striatum, cortex, hippocampus, indusium griseum and medial habenular nucleus (Armstrong & Noguchi 2004, Deng et al 2001, Warren et al 2007, Zhu et al 2006). Corroborating these *in vivo* findings, several *in vitro* studies demonstrated that AMPH, METH and MDMA induced neuronal apoptosis in

cultured neurons of the rat cortex and in cerebellar granule neurons (Capela et al 2007b, Capela et al 2006a, Capela et al 2006b, Jiménez et al 2004, Stumm et al 1999).

The neurotoxic actions of amphetamines were also reported in non-human primates. One example of a study that report these findings was conducted by Scheffel and co-workers, in which 5 mg/kg MDMA was injected to baboons, s.c., twice a day, for four days, and animals were sacrificed 13, 19 and 40 days, or 9 and 13 months after the last dose of the drug. In experiments conducted for short periods after the treatment with MDMA (13 to 40 days) a substantial loss of 5-HTT was observed in all areas of the brain examined. Meanwhile, 9 and 13 months after administration, this loss was only reported in neocortical areas. In fact, no reductions of 5-HTT were observed in the hypothalamus and midbrain, between 9 and 13 months after MDMA, and there was a recovery and an increase in the binding sites when compared to controls (Scheffel et al 1998). Other relevant studies related with the neurotoxic actions of AMPH, MDMA and METH in non-human primates are summarized in Tables 3, 4 and 5, respectively.

Regarding long-term behavioural changes, several reports were published in ATS. Segal and Kuczenski observed that a repeated exposure to AMPH [2.5 or 4.0 mg/kg, s.c., multiple runs (four injections a day at 2 h intervals)] resulted in an increase in the magnitude of post-stereotypy locomotor activation with a continuous state of extreme agitation, as seen by the locomotor response pattern to the 4^{th} injection of the 15^{th} run. Additionally, after a challenge with 2.5 mg/kg of AMPH, three days after the last injection of 15^{th} run of the drug, a persistent MDMA-induced behavioural pattern (like nose-poking) occurred, and the post-stereotypy locomotion remained elevated (Segal & Kuczenski 1997).

MDMA can also affect the experimental animal's cognitive function. Spanos and Yamamoto reported that after repeated injections of MDMA (2.5, 5.0, and 7.5 mg/kg, i.p.) to rats, the stereotypic locomotor behaviour and the serotonin syndrome were augmented, which suggest that, like AMPH, MDMA can produce behavioural sensitization (Spanos & Yamamoto 1989). A long-term effect of MDMA exposure (three to four weeks after the administration) is the decrease in social interaction, contrarily to MDMA acute effects that induce increase sociability (Bull et al 2003, Clemens et al 2004, Fone et al 2002). Other studies in rats also report that MDMA induces long-term and persistent anxiety-like behaviours (Baumann et al 2007, Bull et al 2004, Clemens et al 2004).

METH is also known to interfere with animal behaviour. Segal and Kuczenski observed that a subsequent repeated exposure to METH (2.5 or 4.0 mg/kg, s.c., four daily injections, 2 h apart) led to an increase in the magnitude of post-stereotypy locomotor activation, and that the METH response seems to be markedly longer than the AMPH response in male Sprague-Dawley rats (Segal & Kuczenski 1997). Besides, a neurotoxic

regimen with METH can also induce impairment of working memory, both spatial and non-spatial working memory in rats (Nagai et al 2007, Schröder et al 2003). Decreased social interaction and increased anxiety were reported after METH exposure in either Wistar rats and Vervet Monkeys (Clemens et al 2004, Melega et al 2007). In Tables 3 and 4 are presented relevant studies related with changes in animal's behaviour and cognitive function induced by AMPH and MDMA, respectively.

1.6.2. Adolescent animals

Amphetamines are commonly used as illicit recreational drugs by many teenagers and young adults and several studies showed that it can greatly affect their cognitive skills, during their lifetime (King et al 2010, Kish et al 2010, McCann et al 1999). The prevalence of amphetamines usage is higher in adolescents and young adults (EMCDDA 2014), and therefore there is an increased need for performing more studies regarding the consequences of exposure to these drugs during the adolescent period (Spear 2000). For that reason, researchers mimic the exposure of human adolescents by conducting experiments with laboratory animals from that developmental period.

Adolescence is characterized by neurobiological processes that influence behaviour and abilities during adulthood. The use of amphetamines sometimes starts in this developmental period, and the early use of these drugs has been found to predict the development of drug abuse issues and mood disorders in adulthood (Chambers et al 2003, Chen et al 2009, Laviola et al 1999, Spear 2000). The neurological alterations after ATS misuse in adolescence have been proven in animal studies, as it will be detailed bellow.

AMPH is commonly used in humans in the therapy for attention deficit hyperactivity disorder (ADHD), a disease that specially affects children and adolescents (Kutcher et al 2004). Despite many years of therapeutic experience with this drug, concerns are still raised regarding the possible neurotoxic effects that could occur when it is chronically used. Soto and co-workers performed a study in peri-adolescent rhesus monkeys and the cognitive, behavioural, physiological and *in vivo* neurochemical parameters were assessed. For 18 months, the juvenile male monkeys (24 months of age) received a methylphenidate solution (12 to 16 mg/kg) or a dl-AMPH mixture (0.7 to 0.8 mg/kg), with doses comparable to the therapeutic levels used in children, twice daily, seven days a week. Researchers found no differences to control animals concluding that methylphenidate and AMPH have a reduced effect on cognitive, behavioural or physiological development on peri-adolescent non-human primates, when administered in a therapeutic range (Soto et al 2012). Importantly, the animal model used in that study,

the juvenile male monkeys, had no ADHD contrarily to the children and young adults that need this therapy. Nonetheless, this laboratory study argues for the safety of these drugs in the clinical treatment of ADHD.

AMPH doses (0.5, 1.5, or 5.0 mg/kg, i.p.) were given daily to adolescent Sprague-Dawley rats models from postnatal day (PND) 30 to PND 50 and the behavioural profile and electrophysiological properties of midbrain monoaminergic neurons were assessed later during adulthood (between PND 71 and PND 85) under drug-free conditions (Labonte et al 2011). The middle dose of AMPH (1.5 mg/kg) produced an increase in extracellular levels of 5-HT and DA in adulthood, in contrast to the highest AMPH dose (5.0 mg/kg) that augmented the extracellular levels of NA, but not 5-HT or DA levels, when evaluated between PND 71 and PND 85. In addition, the adolescent AMPH exposure also induced a hyperlocomotion response between PND 71 and PND 85 in rats treated with 1.5 mg/kg dose, and all the three doses promoted more risk in drug-taking behaviours during adulthood. These results suggest that AMPH exposure during adolescence may promote long-lasting neurophysiological alterations that influence their future behaviour (Labonte et al 2011).

Adolescents are particularly susceptible to drug-induced neuroadaptations and cognitive changes, since their brain is still under development and undergoing anatomic and functional changes (Andersen et al 2002, Smith 2003). A recent study performed the administration of AMPH (1 or 3 mg/kg, i.p., one injection every other day) to adolescent rats from PND 37 to PND 55 and to adult rats from PND 98 to PND 116. This regimen induced long-lasting consequences on drug sensitivity and cognitive functions to adolescent rats (Sherrill et al 2013). Adolescents were less sensitive to psychomotor effects of AMPH and more vulnerable to exposure-induced cognitive impairments, when compared to adult rats. The results demonstrated that the effects of AMPH in cognitive function depend on the age of exposure, and suggest that adolescent rats are more susceptible to amphetamine-induced neurobehavioral deficits (Sherrill et al 2013).

A study examined the reinforcing properties of AMPH (0, 1, 3.3, or 10 mg/kg, i.p.) in developing mice on PND 14 to 17, 21 to 24, or 28 to 31 (Cirulli & Laviola 2000). The results indicated that AMPH-induced conditioned place preference was developed early, considering that mice were able to acquire a place preference that relied on adult-like sensory, motor and associative capabilities. Moreover, this conditioned-place preference appeared earlier in females than in males. Also, AMPH increased, in a dose-dependent fashion, the locomotor activity and females were also more sensitive when compared to males. Females increased sensitivity to the locomotor activity effects of AMPH occurred between PND 14 and 21, with no significant changes at PND 21 and 28. The results

suggest that AMPH-induced changes in adolescent mice seem to depend on gender and age of exposure (Cirulli & Laviola 2000).

McPherson and Lawrence performed a study in adolescent (PND 33 to 41) Sprague-Dawley rats, which were exposed to i.p. injections of 2 mg/kg or 10 mg/kg of AMPH, once daily, for ten days, followed by a four week period of abstinence and a subsequent rechallenge with AMPH (1.5 mg/kg, i.p.) (McPherson & Lawrence 2006). The exposure to AMPH during the adolescence period promoted behavioural sensitization to an AMPH rechallenge in adulthood. The rechallenge with AMPH promoted a higher neuronal activation in rats that were exposed previously to 10 mg/kg of AMPH when comparing to those exposed previously to 2 mg/kg. Nevertheless, the sensitized locomotor activity was similar between the two groups. The researchers concluded that the adolescent exposure to AMPH led to sensitization in adulthood accompanied by a widespread neuronal activation (McPherson & Lawrence 2006).

In summary, it may be postulated that, in healthy animal models, AMPH impairs neurological functions. With that being said, it would be extremely useful to ascertain whether in a juvenile animal model of ADHD, the AMPH neurotoxicity would occur in the same fashion as observed in healthy adolescent animals.

As mentioned previously in this thesis, MDMA induces neurotoxicity in adult animals, with reductions in the biochemical 5-HT markers, including depletion on the levels of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), lower 5-HTT density and decreased activity of tryptophan hydroxylase (TPH), the 5-HT rate-limiting synthesis enzyme (Capela et al 2009, Lyles & Cadet 2003, Ricaurte et al 2000).

Several studies indicate that perinatal or neonatal exposure to MDMA causes less neurotoxic effects than in adult animals (Aguirre et al 1998b, Colado et al 1997, Kelly et al 2002, Klomp et al 2012, Meyer & Ali 2002, Meyer et al 2004). A neurotoxic dose of MDMA (4 x 10 mg/kg, i.p., every 2 h) given to adolescent rats on PND 45, induced hyperthermia in the day of the experiment and, two weeks after the exposure, researchers observed lipid and protein peroxidation, mitochondrial DNA deletion and subsequent impaired expression of subunits of the mitochondrial complexes I (NADH dehydrogenase) and IV (cytochrome c oxidase). These are essential complexes of the mitochondrial respiratory system required for energy production, and therefore these alterations may cause long-term impairment in brain's mitochondria (Alves et al 2007). Rats pre-treated with selegiline (2 mg/kg, i.p.), a MAO-B inhibitor, had no changes in the hyperthermic response promoted by MDMA, but reversed the oxidative stress effects to mitochondria evoked by that same MDMA regimen. Authors suggested that monoamine oxidation by MAO-B may play an important role in the neurotoxicity induced by this amphetamine (Alves et al 2007).

Kelly and co-workers examined the toxicity induced by MDMA in the perinatal rat brain and its relation to the normal development of 5-HTT sites. They also determined whether early exposure to MDMA can alter brain function in future adult rats (Kelly et al 2002). In this report, researchers evaluated perinatal development of 5-HTT sites by quantification of [^{3}H]-paroxetine binding autoradiography. Time-mated female Sprague-Dawley rats were injected s.c. with MDMA (20 mg/kg, twice daily) for four consecutive days, starting on gestational day 15 (E15), and neonatal male rats (from untreated dams) were also injected with MDMA (20 mg/kg, s.c., twice daily, for four consecutive days) on PND 10, 15, 20, 25, or 30. All animals groups mentioned before were sacrificed on PND 40. Subsequently, another offspring group was injected with MDMA (20 mg/kg, s.c., twice daily, in four consecutive days) at PND 90 and, in this case, animals were sacrificed ten days after the drug treatment. The results showed no difference in the density of [^{3}H]-paroxetine binding sites, measured at PND 40, in brains of rats treated with MDMA from E15 to PND 20, when compared to controls. However, treatments with MDMA started on PND 25 or later resulted in significant reductions in [3H]-paroxetine binding, with decreases of 46% at PND 25 and 63% at PND 30, when compared to controls. These decreased levels were not as significant as those found in rats treated with MDMA at PND 90, in which the density of [3H]-paroxetine binding sites decreased by 90%. These results suggest that the susceptibility of serotonergic terminals to MDMA-induced neurotoxicity is absent in the immediate perinatal period; however it is markedly increased when the treatment started on PND 25 and further, although not reaching the same extent as what is found in the adult rat brain (Kelly et al 2002). In another paper, the lack of sensitivity of perinatal rats to the neurotoxic effects of MDMA was also reported. MDMA (20 mg/kg, s.c.) was given to pregnant female Wistar rats from E6 to E20, and the rat pups were sacrificed at PND 15 (Aguirre et al 1998b). Other group of rats received a single dose of MDMA (20 mg/kg, s.c.) at PND 14, 21, 28, and 35, meanwhile adult (3-month-old) rats received the same regimen, and both groups were sacrificed seven days after MDMA treatment. The researchers observed that MDMA did not alter the density of [^{3}H]-paroxetine binding sites when MDMA was administered repeatedly during gestation, or as a single dose at postnatal ages prior to PND 28. But, when MDMA was administered at PND 35, 5-HTT density was significantly decreased in frontal cortex, seven days after the treatment. When exposure to MDMA occurred during the gestation period or at PND 14 and 21, it did not cause any significant reduction in 5-HT and 5-HIAA levels. However, at PND 28 MDMA induced a significant decrease in 5-HT and 5-HIAA in the hippocampus, seven days after the administration. Also, at PND 35, MDMA-induced long-term reduction of 5-HT and 5-HIAA levels in several brain areas. The reported data indicated that MDMA exposure during gestational periods or during early postnatal ages did not produce

neurotoxicity to the serotonergic system. Moreover authors concluded that the onset of susceptibility to this drug is placed between PND 28 and PND 35, in this animal model exposed to this MDMA regimen (Aguirre et al 1998b).

Furthermore, other studies reported that exposure to MDMA during the adolescent period results in long-term reductions in 5-HT levels, as well as decreases in its major metabolite, 5-HIAA, and reduced 5-HTT binding sites (Bull et al 2003, Bull et al 2004, Morley-Fletcher et al 2002, Piper et al 2005, Piper & Meyer 2004). A dose of MDMA (5 mg/kg, i.p.) was given hourly for 4 h on two consecutive days, to young Wistar rats at PND 28, and the involvement of the 5-HT_{2A} receptor in the long-term anxiogenic effect was evaluated (Bull et al 2004). On PND 84, the rats pre-treated with MDMA showed a 27% decrease on social interaction, when compared to controls. Sixty days after the last MDMA injection, corresponding to PND 92, researchers observed significant reductions in hippocampal 5-HT and 5-HIAA concentrations, and 5-HT levels were also depleted in the frontal cortex and in the striatum. Moreover, the changes in anxiety-related behaviour was also examined as a possible result of modifications in the 5-HT_{2A} receptor function, by assessing the behavioural response evoked by the 5-HT_{2A} receptor agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), to MDMA (5 mg/kg exposure was followed by 1 mg/kg i.p. of DOI on PND 86). The results showed that the 5-HT and 5-HIAA depletions and the anxiogenic effect, after the repeated MDMA treatment, were accompanied by an attenuation of the anxiogenic response to DOI. Therefore the short-term exposure to MDMA might cause a long-lasting reduction in specific 5-HT_{2A} receptor mediated behaviour (Bull et al 2004). In fact, serotonergic neurotoxicity accompanied by an increased anxiety in the social interaction test has been reported by other authors in rats, 20 to 29 days after MDMA exposure (Bull et al 2003, Fone et al 2002). Other studies reported that intermittent MDMA administration throughout adolescence in rats can elicit tolerance to temperature dysregulation and 5-HT syndrome responses to the drug, and also produce later changes in performance of working memory and anxiety tests (Piper et al 2005, Piper & Meyer 2004). A study conducted by Piper and Meyer used adolescent rats that received MDMA (10 mg/kg, s.c.), given twice daily with an interdose interval of 4 h, starting on PND 35 to PND 60 (Piper & Meyer 2004). Researchers observed that the repeated MDMA treatment led to an anorectic effect in rats, and at PND 65, rats showed impairments in non-spatial working memory and decreased anxiety-like behaviour. The results suggest that MDMA exposure during adolescence can alter subsequent cognitive and affective function in the absence of severe damage to the serotonergic system, since only mild decreases were found in 5-HT binding at PND 70 (Piper & Meyer 2004). Administration of MDMA also showed the ability to produce deficits in working memory and promote anxiety-related responses in adult rats (Gurtman et al 2002, Mechan et al

2002a, Morley et al 2001). Exposure to MDMA (5, 10, or 20 mg/kg, s.c., twice daily) also promoted changes in the learning and memory ability, as rats exposed during the period PND 11 to 20 revealed impairments in the sequential learning on PND 59 to 68, spatial learning and memory on PND 73 to 82, although rats exposed on the period PND 1-10 presented no significant changes in the adulthood (Broening et al 2001).

The differences in the administration scheme strongly influence the effects observed after MDMA exposure during adolescence. A study examined in adolescent rats the influence of intermittent administration of MDMA (10 mg/kg x 2, s.c., every fifth day from PND 35 to 60, with each dose separated by 4 h) on their behavioural, physiological, and neurochemical responses to a further, at PND 67, MDMA binge (5 or 10 mg/kg x 4, hourly for 4 h) rechallenge or a 5-HT_{1A} receptor agonist challenge [a single dose of 0.1 or 0.5 mg/kg of 8-hydroxy-2-(di-n-propylamino)tetralin] (Piper et al 2006). The intermittent exposure to MDMA during adolescence attenuated or even prevented some physiological behaviours and neurotoxic responses to the MDMA binge exposure at PND 67. The hyperthermic effects were attenuated and the locomotor hypoactivity was blocked, which was also accompanied by ablation of 5-HT neurotoxicity. MDMA-treated animals also showed an attenuation of 5-HT syndrome response promoted by a high dose of the 5-HT_{1A} receptor agonist. The results suggest that chronic intermittent MDMA exposure during adolescence may induce 5-HT_{1A} receptor desensitization, as well as neuroadaptive changes that can protect against the adverse consequences of a later high-dose MDMA binge rechallenge (Piper et al 2006).

Repeated intermittent MDMA binges (3 x 5 mg/kg, i.p., given 3 h apart, every 7^{th} day for four weeks) were administered to adolescent rats and mice aged seven weeks (Kindlundh-Högberg et al 2007). In rats and mice, the total horizontal activity was significantly increased after the first and third weeks of MDMA binge exposure. The repeated intermittent administration of MDMA to rats promoted a significant down-regulation of 5-HTT density in the nucleus accumbens' shell. In contrast, in mice the same treatment produced a significant down-regulation of DAT in the caudate putamen and nucleus accumbens' shell, and no changes in 5-HTT density. These results showed that the long-term intermittent administration of MDMA to adolescent rats and mice produced differential regulation of 5-HTT and DAT densities (Kindlundh-Högberg et al 2007).

Repeated and intermittent administration of MDMA (5 or 10 mg/kg, i.p. for three days) was given to mice at different periods of development, particularly at early (28 days old), middle (38 days old), or late (52 days old) adolescence and the carryover effects of this treatment were investigated in the adulthood (80 days old) (Morley-Fletcher et al 2002). The referred treatment with MDMA produced, mainly at early and middle adolescence, long-term increases in social interaction and environment exploratory activity, and no

changes in the animals' aggressive behaviour. MDMA administration also produced long-term reduction in hypothalamic 5-HT levels, with a specific marked reduction on the 5-HT concentration in mice treated at early and middle adolescence. Moreover, a rechallenge with MDMA (5 mg/kg, i.p.) at adulthood, after a previous exposure to MDMA during adolescence, induced hyperactivity in all groups, and increased hypothalamic levels of 5-HT and reduced hypothalamic levels of 5-HIAA in all groups. These results suggest that the long-lasting behavioural and neurotoxic effects promoted by MDMA are dependent on the developmental stage at exposure to the drug (Morley-Fletcher et al 2002).

A recent study examined the effects of repeated administration of MDMA (5 or 10 mg/kg, i.p., once daily for four days) to rats during late adolescence (PND 38 to 41) on place conditioning, anxiety behaviour, and monoamine levels (Cox et al 2014). The treatment with 5 mg/kg of MDMA had no effect on any of the studied parameters. However, the 10 mg/kg MDMA treatment during adolescence caused place aversion one day after the last exposure to the drug, avoidant behaviours in the light-dark box, and also increased anxiety-like behaviours in the open field five days after cessation of MDMA. On the other hand, the same dose of 10 mg/kg produced no changes in monoamine levels in the hippocampus, decreased 5-HT levels in the dorsal raphe, and increased 5-HT and 5-HIAA in the amygdala, five days after the last MDMA administration. These data imply that the production of anxiety-like behaviours is related with a more complex mechanism associated with regionally-distinct dysregulation of the 5-HT system. In addition, data also suggest that more studies are necessary to assess the mechanism that leads to this regionally-distinct neuroplastic changes in the monoamine system that are accompanied with altered behaviour (Cox et al 2014).

A recent study was performed in rats to evaluate the effects of MDMA on 5-HTT densities in the frontal cortex and midbrain using single photon emission computed tomography (SPECT) with the 5-HTT ligand, ^{123}I-labelled 2b-carbomethoxy-3b-(4-iodophenyl) tropane ([^{123}I]-β-CIT) (Klomp et al 2012). Authors aimed to access whether MDMA effects in 5-HTT densities were dependent on the age of first exposure. Therefore, they administered MDMA (10 mg/kg, s.c., twice daily for four days) to adolescent rats at PND 27 and to adult rats at PND 63 (+/- five days). The 5-HTT densities were evaluated seven days after the last MDMA treatment, at PND 38 in early-exposed (adolescent) rats and at PND 74 (+/- five days) in late-exposed (adult) rats. This MDMA treatment produced significant reductions in 5-HTT binding in several brain regions, which were less pronounced in adolescent rats (ranging from 20% to 69%) when compared to adult rats (ranging from 35% to 75%). The effect of age and treatment was observed in the frontal cortex of rats and not in the midbrain (probably due to an early maturation of the midbrain in rats). The degree of 5-HTT loss in adolescent rats (35%) was less extensive when

compared to adult rats (49%) after MDMA treatment, presumably because 5-HTT increases with the increasing age ([^{123}I]-β-CIT binding ratios in the prefrontal cortex of control adult rats were 21% higher when compared to adolescent control rats). Researchers concluded that the differences on MDMA effects on the developing and mature brain might be due to differential maturational stages of the 5-HT projections at age of first exposure (Klomp et al 2012).

In order to assess the importance of MDMA chirality in its behavioural effects, researchers treated adolescent (32 days old) rats with 5 or 10 mg/kg of RS-MDMA or with 5 mg/kg of each one of its enantiomers, R- and S-MDMA, during two treatment stages (stage one: days 1 to 10; stage two: days 15, 17, 19, s.c., once *per* day. All the animals were also rechallenged with 5 mg/kg of S-MDMA on day 31 and with 10 mg/kg of RS-MDMA on day 33 (Von Ameln & Ameln-Mayerhofer 2010). The treatment with R-MDMA failed to produce hyperactivating effects, but instead decreased the locomotor behaviour. On the other hand, RS-MDMA or S-MDMA generated a massive hyperlocomotion and led to the development of behavioural sensitization. When R- and S-MDMA were administered together, researchers observed an increasing in hyperactivity and behavioural sensitization induced by S-MDMA, and the animals pre-treated with R-MDMA showed a sensitized response after a rechallenge with RS-MDMA in adulthood. Taken together, these findings suggest that both MDMA enantiomers can induce behavioural changes after repeated administration during adolescence, and that the sensitization development is more pronounced with S- and RS-MDMA (Von Ameln & Ameln-Mayerhofer 2010).

Altogether, these studies may suggest that young animals are more resistant to MDMA immediate neurological damage, although long-term effects are still scarcely investigated. Furthermore, adolescent animals are vulnerable to MDMA neurotoxicity and the use of this amphetamine can severely influence their normal neurotransmitter functions, and consequently induce changes in behaviour and neurotoxicity markers. At this point, mainly the serotoninergic function has been deeply addressed, meaning that further study on MDMA-induced neurotoxicity is needed.

Like other amphetamines, METH is also widely used by adolescents, and therefore is extremely important to understand its neurotoxic potential during this developmental period. METH administration might result in neurotoxicity, which can be characterized by depletion of DA, 5-HT, along with an increase in the expression of glial fibrillary acidic protein (GFAP). Moreover, neurotoxicity seems to be facilitated by a hyperthermic response (Cappon et al 1997). A single METH dose (10 mg/kg, s.c.) was given to developing rats at PND 20, 40, or 60 at ambient temperatures of 22ºC or 30ºC. The drug administration to PND 60 rats at 22ºC induced animal hyperthermia and resulted in a 47%

decrease of neostriatal DA, and 49% increase in the GFAP content, while administration to PND 40 rats at the same ambient temperature failed to induce a hyperthermic response and no changes in DA or GFAP were reported. Interestingly, when administered to PND 40 rats at an ambient temperature of 30ºC, METH induced hyperthermia, and resulted in a 54% reduction of neostriatal DA and a 70% increase in GFAP. Moreover, the METH administration at PND 20 did not cause DA depletion or increases in GFAP, at either ambient temperature. These data showed that hyperthermia is necessary to develop neurotoxicity at PND 40, while at PND 20 the rats are resistant to the neurotoxic effects induced by METH. Altogether, these data suggest that the rat neostriatal susceptibility to this drug may start at approximately PND 40 and the younger animals (20-day-old) are less susceptible to the neurotoxic effects of METH (Cappon et al 1997).

Another study performed in rats examined the response of the monoaminergic system in adolescent (PND 40) and adult (PND 90) animals when subjected to a high dose regimen of METH (10 mg/kg, s.c., four injections, 2 h apart) (Kokoshka et al 2000). This treatment did not evoke death in younger rats, but led to a 20% mortality rate within the older group at PND 90. The treatment with METH produced long-term (seven days after the treatment) decreases of 33-53% in DAT activity, tyrosine hydroxylase (TH) activity, and DAT ligand binding in the striatum of PND 90 rats when compared to control rats, although these deficits were absent in PND 40 animals. In fact, 1 h after the treatment, DAT activity was already decreased at PND 90. In contrast, in PND 40 only showed a transient decrease in striatal DAT activity at 1 h, which returned to the control levels at seven days. Moreover, to examine the effects of METH to the serotonergic systems, researchers also measured the long-term (seven days) and acute (1 h) responses induced by this drug to TPH activity, and observed reduced striatal TPH activity in both PND 40 and 90 animals. There was also an age-dependent difference in the concentration of METH in the striatum and plasma 1 h after the drug administrations, with PND 90 having the double concentration of METH when compared to PND 40 rats. These data clearly showed an age-dependent difference in the long-term monoaminergic responses to METH exposure, which might also be related to the age differences in METH pharmacokinetics. However, in the acute settings, serotoninergic system seems equally sensitive in both populations (Kokoshka et al 2000).

In mice, METH also induces age-related different responses in terms of dopaminergic neurotoxicity. The neurotoxic regimen of METH (4 x 10 mg/kg, s.c., every 2 h) produced minimal and non-persistent depletion of DA and its metabolites in 1-month-old mice, while mice with 12 months of age suffered large and persistent depletions of DA (87%), DOPAC (71%), and homovanillic acid (HVA) (94%) in the striatum (Miller et al 2000). This METH regimen was also minimally effective in inducing elevations of GFAP in 1-month-old mice,

when compared to the large elevations in striatal GFAP in mice of 2, 5, 12 or 23 months of age. Moreover, with increasing dosages of METH (4 x 20, 40 or 80 mg/kg, s.c., every 2 h) the response of GFAP increased by 100% over control in one-month-old mice, but it still remained extremely below the levels of increase reported in mice with 12 and 23 months of age (300-400% over control). These results also suggest that younger animals are less susceptible to the neurotoxic damage induced by METH, and this neurotoxicity might be dependent on the maturity of the striatal DA systems (Miller et al 2000).

In another study, METH (0, 5, 10, 20, and 40 mg/kg, i.p., single dose) was administered to rats with 1, 6, or 12 months. The administration of 40 mg/kg METH resulted on 100% mortality in 12-month-old animals (Imam & Ali 2001). In the striatum of 1-month-old rats after the 5 mg/kg METH dose there was no formation of 3-nitrotyrosine, indicating no formation of peroxynitrite, and no dopaminergic alterations. However, at this age the administration of 10, 20, and 40 mg/kg METH produced a significant dose-dependent increase in 3-nitrotyrosine and dose-dependent depletions of DA and its metabolites, DOPAC and HVA, when compared to the paired control group. In the 6-month-old rats, all METH doses (5, 10, 20, or 40 mg/kg, i.p.) produced a significant increase in 3-nitrotyrosine formation in a dose-dependent manner, and the levels were also higher when compared to those of 1-month-old rats. In this age, rats also reported dose-dependent depletions in DA and its metabolites, in all dosages used. Moreover, a single injection of METH (5, 10 or 20 mg/kg, i.p.) also resulted in a significant dose-dependent formation of striatal 3-nitrotyrosine on 12-month-old rats, which was significantly higher when compared to the other two younger groups tested. In this group also occurred a significant dose-dependent depletion of DA, DOPAC, and HVA levels, and dopaminergic neurotoxicity was more pronounced when comparing to the 1-month-old or 6-month-old METH-treated rats. Moreover, an age-dependent increase in the hyperthermia was also reported after METH administration. Hence, these results suggest, once again, that aging increases the susceptibility of animals to neurotoxic doses of METH (Imam & Ali 2001).

A study performed in rats used a biweekly treatment with METH (7.5 mg/kg on consecutive days, s.c., for six weeks) that started in adolescence (PND 40) and a binge treatment (4 x 7.5 mg/kg, 2 h apart, s.c.) in adulthood (PND 90) (Mcfadden et al 2011). The drug treatment during adolescence had no effects, in contrast to the binge treatment at PND 90 that caused acute and persistent deficits in VMAT2 function. However, the biweekly treatment during adolescence prevented the acute and persistent deficits in VMAT2 function and the acute hyperthermic response caused by the subsequent METH rechallenge in adulthood. Nevertheless, when the rechallenge was administered at a higher ambient temperature, the hyperthermia was maintained and the referred protection

produced by the biweekly treatment during adolescence was abolished. These results demonstrate that the administration of METH during development prevents against the hyperthermia and dopaminergic toxicity promoted by a later exposure in the adulthood (Mcfadden et al 2011).

Another study tested the dose-response to successive ten-day intervals of METH exposure in rats within the periods PND 21 to PND 30, PND 31 to PND 40, PND 41 to PND 50, or PND 51 to PND 60 (Vorhees et al 2005). Several METH doses were tested in each group of animals. Some METH regimens failed to produce changes in the spatial learning/reference memory and sequential learning, namely on PND 21-30 (2.5, 5, or 10 mg/kg/dose x 4 dose/day, s.c.), PND 31-40 (1.25, 2.5, 5 or 7.5 mg/kg/dose x 4 dose/day, s.c.), or PND 51-60 (1.25, 2.5, 3.75 or 5 mg/kg/dose x 4 dose/day, s.c.). But impairments in spatial learning/reference memory and sequential learning were seen after treatment with the highest dose (6.25 mg/kg/dose, s.c.) on PND 41-50 (1.25, 2.5, 5 or 6.25 mg/kg/dose x 4 dose/day, s.c.). The effects observed at PND 41-50, which is in the adolescent stage of brain development in rodents, suggests that, at this age, there is a superior susceptibility of rats to cognitive deficits induced by METH when compared to juvenile (PND 21-30, PND 31-40) or adult rats (PND 51-60) (Vorhees et al 2005).

A study in adolescent mice (28-42 days old) with exposure to a repeated neurotoxic regimen of METH (24 mg/kg, i.p., once daily, for 14 days) was conducted to ascertain if METH exposure promoted changes in hippocampal plasticity or short-term memory (North et al 2013). Researchers observed that after 14 days of METH exposure there were no deleterious consequences on short-term memory or hippocampal neurotransmission. However, after a period of 21 days of drug abstinence they found deficits in spatial memory and decreases in hippocampal plasticity. Authors concluded that the deleterious consequences on short-term memory and hippocampal neurotransmission induced by a neurotoxic regimen of METH may manifest and persist after an abstinence period (North et al 2013).

To sum up, the developing animals appear to be protected, at least in part, against METH neurotoxicity, hence the susceptibility of animals to the neurotoxic effects increased with the animal age. Besides, PND 40 might be the halfway age between the developmental and adult response patterns to this drug.

1.6.3. Humans

Studying the long-term consequences of amphetamines use by humans is extremely important, for an adequate awareness of the neurotoxic effects in drug users. However, it is difficult to extrapolate the results obtained in animal experimentation to humans, being

increasing relevant to confirm the ATS neurotoxicity demonstrated in laboratory animals with studies conducted in human subjects. Importantly, studies performed in young adults are necessary to assess the effects of amphetamines at this developmental stage, since these psychoactive drugs are extensively consumed by young people. Also, the assessment of the neurotoxicity in previous consumers in adolescence can warrant important data.

As previously mentioned, AMPH is not only a recreational drug, since it is also used for ADHD therapy in children and adolescents. For that reason, researchers have shown concerns due to the possible neurotoxic effects of this drug. Unfortunately, the adolescent human population is understudied. Studies with human adolescents have some ethical barriers, due to their age. Thus, they are frequently not enrolled in studies to evaluate the drug abuse consequences. Several investigators evaluate the later consequences of possible exposure to the drugs during adolescence in people with more than 18 years old (King et al 2010). Below are reviewed the few studies conducted mostly in young adults to evaluated the neurotoxic consequences of the drug.

Reske and co-workers performed a study in non-dependent stimulant users, with ages between 18 and 25 years that used AMPH and methylphenidate at least for the past six months, to assess the behaviour and brain functioning under the occasional use of these drugs. It was observed that an increased use of AMPH and methylphenidate was associated with strong verbal memory and learning deficits. Also, the learning and memory problems were present in individuals with a minimal use of stimulants, leading to possible pre-existing neurocognitive impairments in the studied population. Even so, they concluded that the prescription of AMPH and methylphenidate may lead to an intensification of these deficits (Reske et al 2010).

A paper reported the AMPH effects on motor and verbal skills, memory, and spatial attention task in 18 healthy volunteers (with an average age of 25.4 ± 6.51 years) with the help of functional magnetic resonance imaging (fMRI) (Willson et al 2004). In that study, a single oral dose of 25 mg d-AMPH caused decreases in brain activity in several regions during cognitive tasks. These effects may be linked to the behavioural changes observed after the AMPH administration, and are possibly mediated by alterations in dopaminergic activation (Willson et al 2004).

“Poly-drug-but-no-ecstasy users” (extensive use of AMPH and/or cocaine who consumed AMPH, on average, for 78 times), with ages between 18 and 37 years, showed high impairment in memory performance, which can be related to different mechanisms of the drugs, due to the different affinities to the neurotransmitter systems by these drugs (Jager et al 2007).

"Ecstasy" is also widely used by young humans, being extremely important to study the neurotoxic effects in MDMA users. A fMRI study on 33 heavy MDMA users (mean use of 322 pills) with a mean age of 23 ± 3.8 years, evaluated the effects of "ecstasy" on working memory, attention, and associative memory. The results showed that the use of this drug had no effects on working memory and attention, but was associated with reduced memory performance. Memory performance is apparently more affected by AMPH than MDMA in humans (Jager et al 2007). Several studies reported a decreased density in 5-HT neurons, with reduced 5-HTT binding sites in young adult MDMA users (McCann et al 1998, McCann et al 2005, McCann et al 2008). Brain 5-HT density is related with memory performance, suggesting that the observed deficits in 5-HTT will be related with the deficits in memory seen in humans with an history of MDMA abuse (McCann et al 2008). An investigation conducted in 49 chronic "ecstasy" users (mean four years, typically one to two tablets bi-monthly), with an average age of 25.9 ± 0.8 years, reported a significant decrease in 5-HTT binding in cerebral cortex (19-46% loss of 5-HTT), and hippocampus (21% loss of 5-HTT) (Kish et al 2010). Those effects were related with the years of drug use. Moreover, the MDMA users reported subnormal mood and deficits in some attention tests, executive function and memory, and the memory deficits were correlated with the decreased 5-HTT binding sites (Kish et al 2010), which corroborates the previous study. In another study performed in MDMA users (with an average age of 26.23 ± 1.99 years and an average MDMA use of 4.52 ± 0.71 years), it was observed that this drug decreased the levels of 5-HT metabolite, 5-HIAA, in the cerebrospinal fluid. Moreover, it was stated that the brain 5-HT injury might be related with cognitive deficits, since the MDMA users revealed performance deficits on several tasks, namely sustained attention task requiring arithmetic calculations, a task that required complex attention and incidental learning, a task requiring short-term memory, and a task of semantic recognition and verbal reasoning (McCann et al 1999). Other studies also reported possible 5-HT neurotoxicity, which was related with evidences of cortical hyper-excitability and chronic alterations in cortical 5-HT signalling in MDMA users, aged between 18 to 35 years old (Bauernfeind et al 2011, Di Iorio et al 2012).

A recent study was performed in adolescents (14-18 years) and young adults (18-36 years) to evaluate the effects of MDMA on brain 5-HTT densities in the frontal cortex and midbrain using SPECT and the 5-HTT ligand, [^{123}I]-β-CIT (Klomp et al 2012). The MDMA users were stratified in two different groups: group one with ages between 14-18 years, representing the early-exposed group (developing brain), and group two with ages between 18-36 years, representing the late-exposed users (mature brain). On average, five years after the first exposure, researchers reported that early age of first exposure accounted a notable 79% of midbrain 5-HTT density variability in the developing human

brain, in contrast to 0.3% variability in late-exposed users. No relationship between age at first MDMA use and 5-HTT binding was observed in the frontal cortex. It was concluded that the differential effects of MDMA on the developing and mature brain might be due to differential maturational stages of the 5-HT projections at age of first exposure (Klomp et al 2012).

A study conducted with 31 polydrug users that consume MDMA (average age of 21.7 ± 3.3 years old and abstinent from MDMA at least three weeks before starting the study) *versus* 29 non-MDMA users with history of abuse of other substances (average age of 24.3 ± 3.5 years old) compared their brain grey and white matter concentration. The researchers observed that MDMA users had decreased grey matter concentration in several brain regions (neocortical, bilateral cerebellum, and midline brainstem) (Cowan et al 2003). With the aim of assessing the sustained effects of "ecstasy" on the brain, researchers performed a study in abstinent novel "ecstasy" users (low-dose MDMA users) with a combination of neuroimaging techniques. The researchers used young adults with a mean age of 21.7 ± 3.0 years that consumed, on average, six tablets in 20.3 ± 23.8 weeks. They observed that novel users, even in low doses of the drug, showed signs of damage in the brain, such as vasoconstriction and axonal damage (de Win et al 2008).

Flavel and co-workers performed a study in MDMA abstinent users, with an average age of 22 ± 3 years old, to assess the late effects of this stimulant on human tremor during rest and movement. The MDMA users surprisingly presented an abnormal large tremor during movement, since the subjects had a minimal to moderate lifetime use of MDMA (subjects that used MDMA on less than 20 occasions) and had been abstinent for an average of three months. These data suggests no associations with an acute mechanism of action of the drug. The authors stated that these abnormalities may account for a possible risk for movement disorders in MDMA users (Flavel et al 2012). The same group reported abnormal substancia nigra morphology in abstinent illicit stimulant (AMPH, MDMA, METH and cocaine) users with an average age of 31 ± 9 years. These deficits in nigro-striatal system raised some concerns, giving the high risk for a later development of Parkinson's disease (Todd et al 2013).

The evaluation of former (even abstinent) MDMA use during adolescence demonstrates that the use of this drug may affect the neurotransmitter function and reduce memory performance. Moreover, researchers state that the distinct maturational brain stages may have an important role in the long-term consequences of "ecstasy" use.

Like other amphetamines, METH is also widely abused by humans and it's extremely important to assess the neurotoxic actions of METH in humans' brain. McKetin and co-workers presented a paper with 309 METH users with ages ranging from 16 to 60 years-old, being the median age of first use of the drug at 17 years old. They observed that 23%

of the participants had experienced clinically significant suspiciousness and 30% of the participants screened positive for psychosis in that year. That leads to the thought that chronic use of METH might be associated with the development of paranoid psychosis (McKetin et al 2006).

A study involving 54 adolescent METH users and 74 control subjects, with ages ranging from 12 to 23 years old, assessed the neuropsychological performance of these adolescents after exposure to METH (an average use of 0.58 ± 0.08 g *per* day in males and 1.02 ± 0.22 g *per* day in females). All subjects were submitted to several neuropsychological tests and the METH users presented impairments in memory and executive function. Moreover, these impairments appear to be attenuated by a prolonged abstinence to the drug. Researchers concluded that the use of METH is associated with cognitive deficits (King et al 2010).

A study with 34 METH-dependent adults with an average age of 33.1 ± 8.9 years (population generally used the drug for ten years, in a frequency superior to five times *per* week, and abstinent for 18 days) and 31 healthy non-METH user subjects with an average age of 35.7 ± 8.4 years, measured the grey matter volumes in the both groups. METH users showed an age-dependent loss of cortical grey matter in frontal, occipital, temporal, and the insular lobes when compared to control subjects, and smaller grey matter volumes in several brain subregions. The authors concluded that METH users increased their grey matter loss with age, raising the possibility of accelerated decline in mental function (Nakama et al 2011). As reported for MDMA, METH abusers also presented grey matter deficits, a marker of neurotoxicity (Schwartz et al 2010, Thompson et al 2004). Researchers pointed that METH users, which used the drug for an average of 10.5 years starting at their mid-twenties, had 7.8% smaller hippocampal volumes than control subjects and that data was associated with impaired memory performance. They also reported a significant white matter hypertrophy of 7.0%, accompanied with damage in the medial temporal lobe and in the cingulated-limbic cortex in METH users. Altogether, these indications of cerebral deterioration caused by chronic METH use can lead to impairments of memory performance (Thompson et al 2004). Other authors reported that the use of drugs in a juvenile age (subjects that initiated the drug use before 21 years old) was related with smaller intracranial volume (Schwartz et al 2010).

Regarding METH use in adolescence, studies show evidences of long-term neurotoxicity, such as, grey matter loss, development of paranoid psychosis, cognitive deficits and impaired memory performance.

Part II

Aims

2. AIMS

"Ecstasy" neurotoxic properties have been studied in laboratory animals and humans. Given the fact that MDMA is a widespread illicit recreational drug frequently used by many teenagers and young adults, there is a need to understand the consequences of exposure to this drug during adolescence. In particular, the adolescent period is frequently neglected by investigators, as well as the potential differences among different aged groups. Specifically, it is extremely important to clarify the actions of MDMA to the adolescent developing brain. Moreover, in this context, the cerebellum was never approached.

The main aim of this dissertation was to assess the acute toxicity and oxidative stress of this drug in an adolescent rat model (postnatal day 40) promoted by a binge administration scheme comparable to humans. MDMA abusers frequently intake several doses of "ecstasy" on the same session, using the binge scheme of administration. The acute toxicity of this drug to the adolescent rat brain was evaluated in four different brain areas (cerebellum, hippocampus, cortex and striatum) and in three peripheral organs (liver, heart and kidneys) 24 h after the MDMA administration. Another aim of this thesis was to evaluate the long-term effect of age in the neurotoxicity of MDMA using adolescent (postnatal day 40) and aged rats (18 to 22 months old). In particular, long-term effects in the cerebellum were evaluated in both adolescent and aged rats, seven days after the MDMA administration, since the effects in this brain area are scarcely studied, and little is known about the susceptibility of different aged groups. To achieve the referred aims, several techniques were performed being the redox status, energetic content, protein-bound quinones formation and protein carbonylation evaluated.

Part III

Materials and methods

3. MATERIALS AND METHODS

3.1. Materials

Dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), Folin–Ciocalteu reagent, perchloric acid, sodium hydroxide (NaOH), cupper (II) sulphate ($CuSO_4$), sodium carbonate (Na_2CO_3), potassium bicarbonate ($KHCO_3$), magnesium chloride ($MgCl_2$), magnesium sulphate ($MgSO_4$), potassium dihydrogen phosphate (KH_2PO_4) and disodium phosphate (Na_2HPO_4) were purchased from Merck (Darmstadt, Germany). Sodium phosphate monobasic ($NaHPO_4$) was purchased from Panreac (Barcelona, Spain), potassium sodium tartrate from Fluka (Buchs SG, Switzerland), phosphate buffered saline solution (PBS) from Biochrom (Berlin, Germany), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), methanol, xylene from Fisher Scientific (Loughborough, UK), sodium chloride (NaCl), and sodium dodecyl sulphate (SDS) from VWR (Leuven, Belgium). EMLA® Lidocaine 25 mg/g + Prilocaine 25 mg/g was purchased from AstraZeneca (London, UK). Isoflurane (Isoflo® 100% p/p) was obtained from Abbott Animal Health (North Chicago, IL, USA). The fluorescent peptide substrates for caspase-3 (Ac-DMQD-AMC), the peptide substrate for caspase-8 (Ac-IETD-AMC), and for the peptide substrate for caspase-9 (Ac-LEHD-AMC) were obtained from Peptanova (Sandhausen, Germany). ABX Pentra reagents were purchased from HORIBA (Kyoto, Japan). Bio-Rad DC protein assay kit and the Clarity Western ECL Substrate were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Horseradish peroxidase (HRP) conjugated anti-rabbit antibody, and 0.45 µm Amersham Protran nitrocellulose blotting membrane was purchased from GE Healthcare Bio-Sciences (Pittsburgh, PA, USA). Dinitropenhyl-KLH rabbit IgG antibody was purchased from Invitrogen/Life Technologies (Grand Island, NY, USA). Eosin 1% aqueous was obtained from Biostain (Traralgon, Australia), Harris hematoxylin was from Harris Surgipath (Richmond, IL, USA) and Histofluid from Marienfeld (Lauda-Königshofen, Germany). MDMA (HCl salt) was extracted and purified from high purity MDMA tablets, which were provided by the Portuguese Criminal Police Department. The extracted salt was fully characterized by nuclear magnetic resonance and mass spectrometry (Capela et al 2007a, Capela et al 2007b). All the other reagents used were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2. Animals

All procedures were carried out to provide an appropriate animal care, minimizing their suffering. Housing and experimental treatment of the animals were in accordance with the guidelines defined by the European Council Directive (2010/63/EU). Moreover, the experiments were performed with the approval of the Portuguese National Authority for Animal Health (General Directory of Veterinary Medicine) and the Ethical Committee of the Faculty of Pharmacy, University of Porto (opinion nº 17/03/2014).

In this study, two different populations were used, adolescent and aged male Wistar rats born in the Institute for Biomedical Sciences Abel Salazar, University of Porto (ICBAS-UP) vivarium. The animals were maintained under a controlled ambient (temperature 22.0 ± 2.0ºC, 40% humidity, 12 h light/dark cycles) and given *ad libitum* access to food and water. In the adolescent population group, 24 rats at PND 40, weighing an average 136 g were used. In the aged population, 11 rats with ages between 18 to 22 months and an average body weight of 505 g were used.

3.3. Experimental protocol

Two experiments were conducted in order to assess the susceptibility of different populations of rats to MDMA, one adolescent and the other aged, in an administration schedule that mimics human MDMA-pattern of use (binge use) and uses realistic human doses (Capela et al 2009, Goni-Allo et al 2008). Two different MDMA solutions in sterile NaCl 0.9% were prepared: 2.5 mg/mL for the adolescent rats, and 5.0 mg/mL for the aged rats. Three days before both experiments, animals were trichotomised in the lumbar region and anesthetized locally with a local anaesthetic (EMLA ® Lidocaine 25 mg/g + Prilocaine 25 mg/g). Each animal was also anesthetized with isoflurane before inserting a s.c. temperature transponder (BioMedic Data Systems Inc., Seaford, DE), in the lumbar region, as to allow core body temperature measurement (Alves et al 2007). In the days prior to MDMA administration, they were housed in groups allowing socialization, except for the aged rats, which given their size were individually caged to maintain animal comfort. In the day of the experiment, animals were caged individually.

In experiment one, adolescent animals were exposed to an MDMA binge regimen (3 x 5 mg/kg) and sacrificed 24 h after the beginning of treatment. Regarding experiment two, two different populations were used: adolescent and aged animals, which also received via i.p. route the MDMA binge administration (3 x 5 mg/kg and 2 x 5 mg/kg,

respectively), but were sacrificed seven days after exposure to the drug. Four different brain areas (cerebellum, hippocampus, cortex and striatum), and three peripheral organs, liver, heart and kidneys were collected in the first experiment. In experiment two only the cerebellum brain area was studied.

3.3.1. Experiment 1

In experiment one, 14 adolescent male Wistar rats were divided in two groups: control (n=7) and MDMA-treated (n=7). The MDMA-treated group received three doses of 5 mg/kg MDMA, i.p., every 2 h, for a total dose of 15 mg/kg. To control animals was given NaCl 0.9% (saline solution) in the same schedule and equivalent injection volume of MDMA-treated conditions. During administration, the temperature of each animal was monitored and registered every 15 min for a total of 7 h. The weight of the animals, and their food and water intake were assessed before the first injection and at the next day, approximately at the same hour of the first administration. Twenty-four hours after the MDMA administration, animals were prepared to leave the vivarium and housed in groups for transportation. The adolescent rats were sacrificed, approximately, 24 h after the first dose administration (Figure 4). This binge dosing regimen (three doses of 5 mg/kg MDMA, i.p., every 2 h) used was previously described in adult ten week old Wistar rats, with an average weight of 300 g, to promote serotonergic neurotoxicity (Goni-Allo et al 2008). This regimen of MDMA in adolescent Wistar rats PND 40 was proven to not promote serotonergic toxicity seven days after MDMA exposure, as no depletion of 5-HT could be seen in any of the brain areas analysed in the present study (Feio-Azevedo et al Submitted). Regarding the equivalence of doses to humans, the equivalence was based on the allometric scaling principles using the formula: *dose Human (mg/kg) = dose animal (mg/kg) x (animal weight/human weight)*$^{1/4}$ (Hayes 2001). According to this correlation, the dose used in adolescent rats ($MDMA_{total\ dose}$= 15 mg/kg of body weight) is approximately equivalent to 170 mg in a 50 kg adolescent human. The majority of human abusers usually take more than one tablet *per* session, normally from two to four tablets using the binge dosing pattern (Morgan 1998, Scholey et al 2004). The latest European Union report on drugs mentioned that the seized MDMA pills during 2012 had a mean range of 57 to 102 mg with high purity (EMCDDA 2014), which means that the dose used in our protocol can be equivalent to the intake of two to three pills by human adolescents. Therefore, the present paradigm of exposure to animals can mimic the dose schedule used by human adolescents.

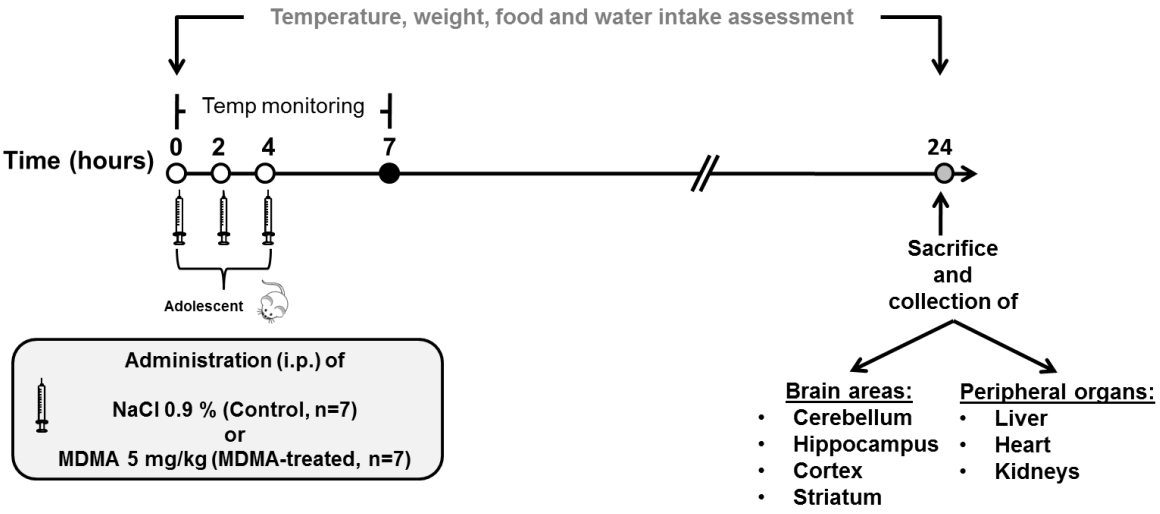

Figure 4 – Experiment one was conducted with 14 adolescent rats. Three doses of NaCl 0.9% or three doses of MDMA 5 mg/kg were administrated intraperitoneally (i.p.), every 2 h, to controls (n=7) or to MDMA-treated animals (n=7), respectively. MDMA total dose was 15 mg/kg. Temperature was monitored for 7 h, starting at the first administration. Temperature, animal weight, food and water intake were assessed before and 24 h after MDMA exposure. The animals were sacrificed 24 h after the administration and four different brain areas (cerebellum, hippocampus, cortex and striatum), and three peripheral organs (liver, heart and kidneys) were collected.

3.3.2. Experiment 2

Two different populations of male Wistar rats were used in this experiment: 10 adolescent (PND 40) and 11 aged (18 to 22 months old) animals.

The adolescent animals were divided in control (n=5) and MDMA-treated (n=5) groups. The MDMA-treated group received three doses of 5 mg/kg MDMA (i.p.), every 2 h, ($MDMA_{total\ dose}$= 15 mg/kg of body weight), the same regimen used in experiment one. Control animals received NaCl 0.9% (saline solution) in the same schedule and equivalent injection volume of MDMA-treated conditions.

Concerning the aged population, on the day of the experiment, two groups were formed: control (n=5) and MDMA treated (n=6). Given the high risk of mortality due to the high temperature reached after the second MDMA dose, and given the fact that one animal dyed of extreme hyperthermia several hours following the last injection (in this animal the core temperature surpassed 41ºC), it was decided, to assure animal survival and welfare, that aged rats would not receive the third dose of MDMA. Of note, that no data from the deceased animal were included in the results of aged rats. Accordingly, in the aged population, MDMA-treated rats only received two doses of 5.0 mg/kg MDMA i.p., every 2 h ($MDMA_{total\ dose}$= 10 mg/Kg of body weight), and two doses of saline solution were given to controls. According to the same allometric principles mentioned above for

experiment one, the total dose used in aged rats ($MDMA_{total\ dose}$= 10 mg/Kg of body weight) is equivalent to 200 mg in a 70 Kg human. Therefore, by the same rationale mentioned above, the present dose given to aged animals can be equivalent to the intake of two to three pills and mimic the dose schedule used by humans.

As previously said, the regimen of MDMA used in this experiment was shown not to promote serotonergic toxicity seven days after MDMA exposure in adolescent rats. However, the MDMA regimen used in aged rats (2 x 5 mg/kg, i.p., 2 h apart) was shown to promote serotonergic depletions in the hippocampus, but not in the cerebellum (Feio-Azevedo et al Submitted). In both populations, the temperature of each animal was measured and registered for 6 to 7 h, every 15 min, starting at the first dose administration. For the next seven days, the temperature and weight of the animal, food and water intake were registered approximately at the same hour of the first injection. At the seventh day, animals were prepared to leave the vivarium and housed in groups for transportation. Both adolescent and aged rats were sacrificed seven days after the exposure to MDMA (Figure 5).

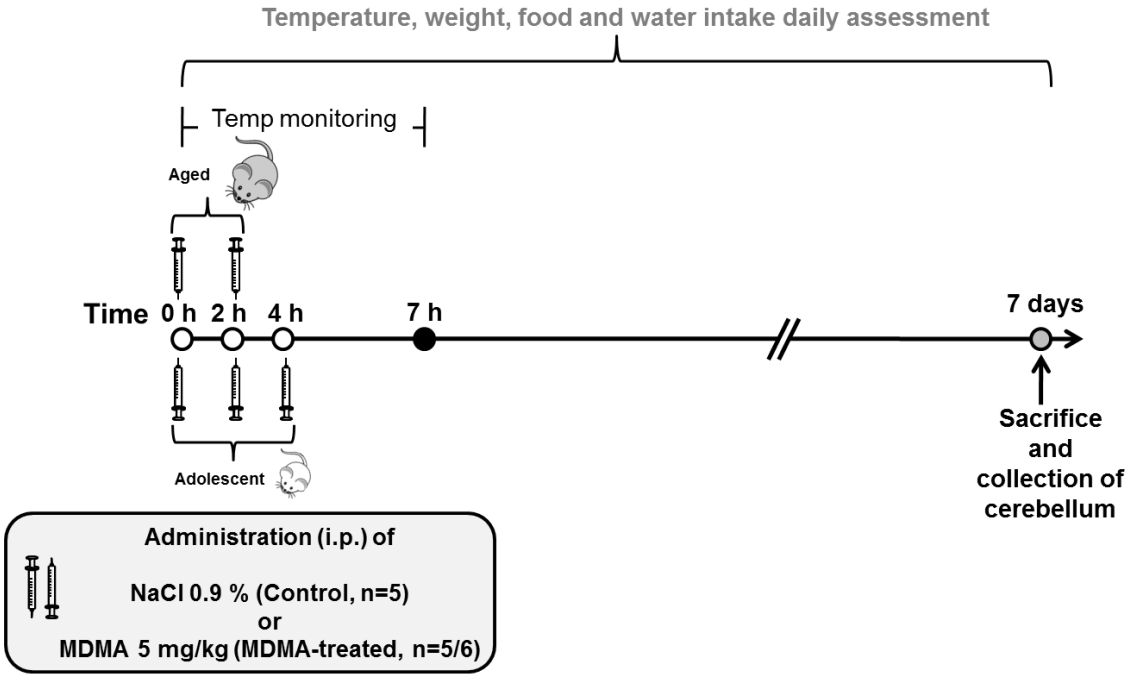

Figure 5 – Experiment two was conducted with 10 adolescent rats and 11 aged rats. The adolescent rats received three doses of NaCl 0.9% (control group, n=5) or MDMA 5 mg/kg (MDMA-treated group, n=5) intraperitoneally (i.p.), every 2 h. MDMA total dose administered to adolescents was 15 mg/kg. The aged rats received two doses of NaCl 0.9% (control group, n=5) or MDMA 5 mg/kg (MDMA-treated group, n=6), i.p., every 2 h. MDMA total dose administered to aged rats was 10 mg/kg. Temperature was monitored for 6 to 7 h, starting at the first administration. Temperature, animal weight, food and water intake were assessed before and during the seven days following MDMA exposure. The animals were sacrificed seven days after the administration and the cerebellum was collected.

3.4. Blood and tissue collection

In both experiments, animals were anesthetized and euthanized in a chamber with the general anaesthetic isoflurane. Blood was, immediately, collected from the inferior vena cava, and decapitation followed. Blood was used to determine the levels of total creatine kinase (CK-R), creatine-kinase MB (CK-MB), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). In experiment one, immediately after the sacrifice, the brain was weighted and then dissected into four brain areas (cerebellum, hippocampus, frontal cortex and striatum). Three peripheral organs were collected (liver, heart and kidneys) and weighted. In experiment two, only the cerebellum was removed, seven days after MDMA administration, and handled as referred below for the brain areas of experiment one.

The four brain areas dissected from the left side of each hemisphere were homogenized in perchloric acid solution 5% using a sonicator (20 seconds, continuously) being the tubes maintained on ice. Homogenates were then centrifuged at 13,000 rpm, for 10 min at 4ºC, and the supernatants were collected for total glutathione (GSHt) or oxidized glutathione (GSSG), and adenosine 5'-triphosphate (ATP) determination. The pellet was kept for posterior protein determination. The areas from the right side of the hemisphere were collected in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, 0.25 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM sodium fluoride (NaF), 1 mM sodium metavanadate ($NaVO_3$), proteases inhibitor cocktail from Sigma, pH=8.0). The samples were also homogenized using a sonicator (20 seconds, continuously), while tubes were maintained on ice. The resultant homogenates were centrifuged at 13,000 rpm, for 15 min at 4ºC, and the supernatants were separated to the quinoprotein and protein carbonylation assays.

A 2 mm section of liver, heart and kidneys was collected into a 4% paraformaldehyde solution in PBS, and kept on ice for further treatment. Another section of liver, heart and kidneys was collected in complete caspase lysis buffer (25 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA, 0.5% Triton X-100, 1 mM PMSF and 5 mM dithiothreitol (DTT), pH=7.4) for caspases activity determination and was immediately stored at -80ºC. Another section was collected in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, 0.25 mM PMSF, 1 mM NaF, 1 mM $NaVO_3$, proteases inhibitor cocktail from Sigma, pH=8.0) and frozen at -80ºC. The samples stored in RIPA buffer were homogenized using a sonicator (30 seconds, continuously) while the tubes were maintained on ice. Homogenates were then centrifuged at 5,000 rpm, for 10 min at 4ºC, and the supernatant was separated to clean tubes for the quinoprotein and protein

carbonylation assays. The remaining of each organ was homogenized in a 0.1M KH_2PO_4 solution (pH=7.4) using an Ultra-Turrax. Following homogenization, one aliquot was added to perchloric acid (final concentration 5%). The resultant mixture was centrifuged at 13,000 rpm, for 10 min at 4ºC, and the supernatant was collected and separated to clean tubes for GSHt or GSSG, and ATP determination. The samples concerning the GSH/GSSG analysis were stored at -20ºC, and the samples for ATP determination were frozen at -80ºC. The remaining tissue homogenate was also stored at -20ºC for protein quantification. All these procedures were performed on ice.

3.5. Measurement of CK-R, CK-MB, AST and ALT

The blood was collected in EDTA-containing tubes and centrifuged at 920*g* for 10 min, in order to separate the plasma. The plasma was frozen at -20ºC until determination of CK-R, CK-MB, AST and ALT. These parameters were determined using enzymatic assays in the apparatus ABX Pentra 400 with ABX Pentra reagents (HORIBA, Kyoto, Japan), according to the manufacturer's instructions. The determinations were performed by Drª Laura Pereira, at clinical analysis laboratory of the Faculty of Pharmacy University of Porto.

3.6. Measurement of GSHt, GSH and GSSG

The GSHt and GSSG levels were evaluated in the four brain areas and peripheral organs of rats in experiment one, and also in the cerebellum for experiment two, by the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)-GSSG reductase recycling assay, as described in previous works (Capela et al 2007a, Costa et al 2007). The quantification of GSHt levels in the brain areas and organs was initially performed by neutralization of the sample homogenates and standards with 0.76 M $KHCO_3$, followed by centrifugation for 2 min at 13,000 rpm (4ºC). Before the measurements, the reagent solution containing 0.68 mM nicotinamide adenine dinucleotide phosphate (NADPH) and 3.96 mM DTNB was prepared in a phosphate buffer (71.5 mM $NaHPO_4$, 71.5 mM Na_2HPO_4 and 0.63 mM EDTA, pH 7.0), and was kept protected from light. In a 96-well plate (Biotek PowerWaveX plate reader, VT, USA), 100 µL of neutralized samples, standards or blanks, was added in triplicates, and mixed with 65 µL of the reagent solution. An incubation at 30ºC for 15 min followed. Afterwards, 40 µL of freshly reconstituted glutathione reductase solution (10

U/mL in phosphate buffer) was added to each well. The 5-thio-2-nitrobenzoic acid formation was monitored for 3 min in 10 seconds intervals, at 415 nm, and compared with the standard GSHt curve. Standards were made in perchloric acid solution 5%, being the range of concentrations used between 0.5 to 15 µM.

To determine GSSG levels, before the neutralization 10 µL of 2-vinylpyridine was added to the samples homogenates, standards and blanks and mixed for 1 h, on ice, to block the reduced glutathione (GSH). The determination of GSSG was then performed as described for GSHt. The GSSG standard solutions were also made in 5% perchloric acid with concentrations that ranged between 0.25 and 8.00 µM. The levels of GSH were calculated using the next formula: GSH = GSHt – 2 x GSSG. The levels of GSHt, GSSG and GSH were normalized to the protein content, and the results were expressed in nmol of GSH or GSSG *per* mg of protein.

3.7. Assessment of ATP levels

ATP levels of the different brain areas, liver, heart and kidneys in experiment one, and of the cerebellum in experiment two were determined using a bioluminescent assay, based on the generation of light using the luciferin-luciferase system (Capela et al 2007a, Costa et al 2007). Aliquots of D-luciferin 90.9 mg/L stock reagent and luciferase from *Photinus pyralis* (firefly) (concentration of 3,000,000 U/mL) were prepared in a luciferin-luciferase buffer [50 mM glycine, 100 mM $MgSO_4$, 1 mM Tris-base, 0.55 mM EDTA and 0.1% bovine serum albumin (BSA), pH=7.6] and kept, until use, at -20ºC, protected from light. An ATP standard curve was prepared in perchloric acid 5% solution with concentrations ranging from 0.625 to 10 µM. At the day of the assay, the stock solutions of luciferase and luciferin were mixed and maintained at room temperature. The samples and standards were neutralized using 0.76 M $KHCO_3$, and then centrifuged at 13,000 rpm for 10 min, at 4ºC. After centrifugation, 100 µL of the samples or standards supernatants was added to a 96-well white microplate. Then, 100 µL of luciferin-luciferase reagent solution was added to each well and bioluminescence was immediately read in a microplate reader Biotech Synergy HT (VT, USA). The results were expressed in nmol of ATP *per* mg of protein.

3.8. Determination of protein-bound quinones (quinoproteins)

The nitrotetrazolium blue chloride (NBT)/glycinate calorimetric assay was performed for the detection of protein-bound quinones (quinoproteins) in the brain and peripheral organs of rats in experiment one and in the cerebellum in experiment two, as previously described (Capela et al 2007a). Lysates in RIPA buffer with 50 µg of protein in the case of brain areas, or 25 µg of protein for the three peripheral organs, was added to 2 M potassium glycinate (pH=10.0) solution (resulting in a final volume of 250 µL). A NBT reagent solution (0.24 mM NBT in 2 M potassium glycinate, pH=10.0) was prepared and 500 µL was added to the protein containing solution. This was followed by an incubation under agitation at room temperature, for 3 h protected from light. The reaction between quinoproteins and NBT formed a blue-purple coloured complex, and its absorbance was measured at 530 nm in a 96-well microplate reader (Biotek PowerWaveX plate reader, VT, USA). The results were expressed in OD *per* mg of protein.

3.9. Assessment of protein carbonylation

Protein carbonyls, an index of protein oxidation, were determined, as previously described (Barbosa et al 2012). After protein determination all samples were diluted down to a final protein concentration of 0.1 mg/mL in complete RIPA. In tubes, 200 µL of 12% SDS (w/v) was added to 200 µL of samples containing 20 µg of protein. Samples were then incubated with 400 µL of a freshly made solution of 20 mM 2,4-dinitrophenylhydrazine [DNPH, in 10% trifluroacetic acid (v/v)] for 30 min, at room temperature protected from the light. Afterwards, the samples were neutralized with 300 µL of neutralization solution [18% β-mercaptoethanol (v/v) in 2 M Tris, pH=7.0] followed by centrifugation for 2 min at 13,000 rpm (4ºC). The supernatants were diluted down to a final protein concentration of 2 µg/mL using PBS as a diluent solution. Before sample loading, the nitrocellulose membrane was hydrated in 10% methanol for a few seconds. A volume of 100 µL of the derivatized proteins (0.2 µg) was loaded into nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech), under vacuum, using a slot blot apparatus. Next, the membranes were washed in TBS-T [20 mM Tris base, 300 mM NaCl and 0.05% Tween 20 (v/v), pH=8.0], followed by blocking in the blocking buffer solution [5% milk (w/v) in TBS-T] for 1 h, under agitation, at room temperature. The membranes were then incubated with the primary antibody (rabbit polyclonal anti-DNP, 1:1000, Molecular Probes A-6430) overnight, at 4ºC. After washing, five times under

agitation for 10 min each, with TBS-T, the membranes were incubated with the secondary antibody (anti-rabbit IgG-peroxidase, 1:2000, Amersham Pharmacia Biotech) for 1 h, at room temperature, under agitation. Antibodies were diluted in blocking buffer. After three washes (10 min each) with TBS-T, immunoreactive bands were visualized using the Clarity™ Western ECL Substrate, according to the supplier's instructions. Digital images were acquired using a Molecular Imager® ChemiDocTM XRS+ System (Bio-Rad Laboratories, CA, USA) and analysed with Image LabTM Software (Bio-Rad Laboratories, CA, USA). Optical density results were expressed as % of control values.

3.10. Histology treatment and optical microscopy analysis

The sections of circa 2 mm of heart, kidney and liver were kept on ice in a 4% paraformaldehyde solution in PBS for fixation. The dehydration, clearing and paraffin embedding were next performed, and the obtained samples were sectioned with a 5 µm thickness using a manual rotator microtome Leica RM2125 (Wetzlar, Germany). In these sections the hematoxylin/eosin staining was done as follows: 5 min xylene (2x), 5 min ethanol 100%, 5 min ethanol 95%, 5 min ethanol 80%, 5 min ethanol 75%, 5 min H_2O, 8-10 min hematoxylin, 5 min running water, 5 min eosin, 5 min ethanol 95%, 5 min ethanol 100%, 5 min xylene and, finally, mounted in HistoFluid. All preparations were analysed and photographed using a Carl Zeiss Imager A1 light microscope equipped with AxioCam MRc 5 digital camera (Oberkochen, Germany).

3.11. Determination of caspases-3, -8 and -9 activities

The assessment of each caspase activity in the liver, heart and kidneys of the animals in experiment one was made by a fluorescent assay for tissues as previously described (Maianski et al 2004), with some modifications. The tissues were thawed and then homogenized with a Potter A561 in complete caspase lysis buffer. The samples were centrifuged at 13,000 rpm, for 30 min at 4ºC, and the supernatant was collected to new tubes placed on ice. In a black 96-well plate, 50 µL of cell lysates and 200 µL of assay buffer (100 mM NaCl, 50 mM HEPES, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, 10 mM DTT, pH=7.4) were added. For negative control, 50 µL of assay buffer was added to the plate, instead of the 50 µL of cell lysates. To each well 10 µL of each substrate was added. The substrate for caspase-3 was Ac-DMQD-AMC, the substrate for caspase-8

was Ac-IETD-AMC, and the substrate for caspase-9 was Ac-LEHD-AMC. All substrates were used in a final concentration *per* well of 100 µM. The plates were incubated at 37ºC, protected from the light for 24 h and the fluorescence (excitation 360 nm / emission 460 nm) was read using a microplate reader Biotech Synergy HT (VT, USA). Results were normalized to the amount of protein of each sample added to the well.

3.12. Protein quantification

The protein content in the collected samples was measured according to a method previously described by Lowry (Lowry et al 1951). Protein standards were prepared with BSA in 0.3 M NaOH, and the calibration curve ranged from 25 to 250 µg/mL. To a 96-well microtiter plate was added, in triplicates, 50 µL of samples, standards or blanks, followed by the addition of 100 µL of freshly prepared reagent (reagent solution: 9.8 mL of 2% Na_2CO_3, 100 µL of 2% sodium potassium tartrate and 100 µL of 1% $CuSO_4$). After 10 min in the dark, 100 µL of extemporaneously prepared Folin–Ciocalteu reagent [Folin–Ciocalteu in H_2O (1:14)] was added to each well. The plate was kept protected from light for 20 min and the absorbance was measured in a 96-well microplate reader (Biotek PowerWaveX plate reader, VT, USA) at 750 nm.

Regarding the samples containing high detergent concentrations or reducing agents (namely complete RIPA buffer or caspase lysis buffer), the DC™ Protein Assay kit from Bio-Rad (CA, USA) was used, according to the manufacturer's instructions.

3.13. Statistical analysis

Results are presented as mean ± standard deviation. When two groups were compared, the t-test was used for data that followed a normal distribution or the Mann-Whitney Rank Sum test, if data did not pass the normality tests. Regarding the statistical analysis of the temperature, as well as animal weight, food and water intake of experiment two, the two-way analysis of variance (ANOVA) with repeated measurements was used, followed by Bonferroni post-hoc test, once a significant *p* was achieved. Statistical significance was accepted at *p* values less than 0.05.

Part IV

Results

4. RESULTS

4.1. Experiment 1

4.1.1 MDMA binge administration led to hyperthermia in adolescent rats and had no effect on body weight gain and food or water intake

Three MDMA doses were given to the animals (each dose 5 mg/kg i.p., every 2 h) and the first dose did not cause significant changes in body temperature (Figure 6). After the second dose of MDMA, the MDMA-treated adolescent rats had a significantly higher body temperature when compared to the control group (p<0.0001). Temperature persisted significantly higher for almost 2 h after the third dose in treated animals. The temperature of MDMA-treated animals never surpassed 39ºC even after the third MDMA dose (Figure 6). The temperature was also measured 24 h post-MDMA binge administration and no differences in the temperature between controls and MDMA-treated rats were observed (data not shown).

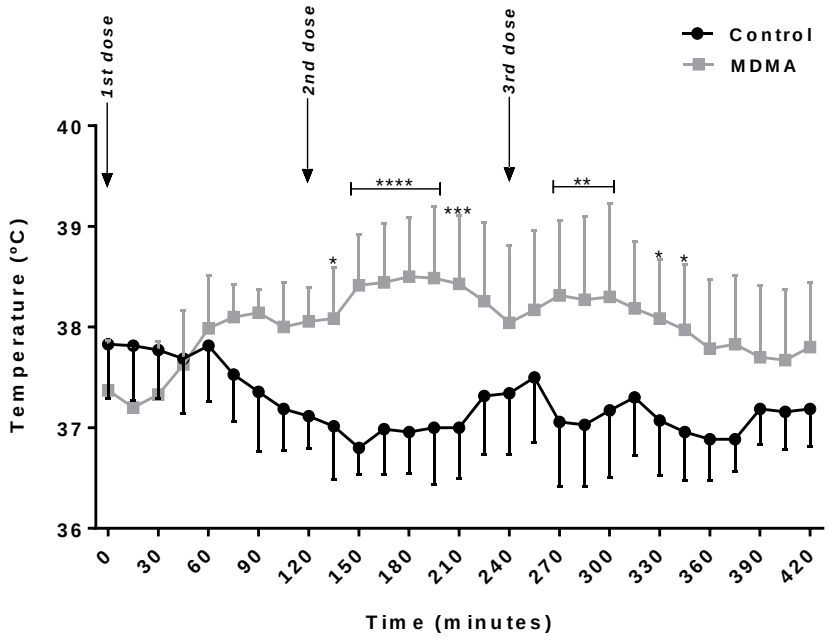

Figure 6 – Temperature monitoring during 7 h of adolescent rats treated with three administrations of NaCl 0.9% i.p. (control) or 5 mg/kg MDMA i.p. (MDMA-treated). Results in degrees Celsius (ºC) are presented as means ± standard deviation, from seven animals in each group. Statistical comparisons were made using two-way ANOVA repeated measurements followed by the Bonferroni post hoc test (*p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 treatment vs. control).

Regarding the body weight gain *per* day, food or water intake (*per* day and *per* weight) no differences were observed between control and MDMA-treated rats. In fact, there was a decrease in the body weight of animals in both groups (Figure 7A), possibly as a result of the animal stress due to the manipulation. The food consumption was similar in both control and MDMA-treated animals (Figure 7B). However, data showed a tendency for an increased water intake in animals that received the three MDMA doses, but it did not reach statistical significance (p=0.057) (Figure 7C).

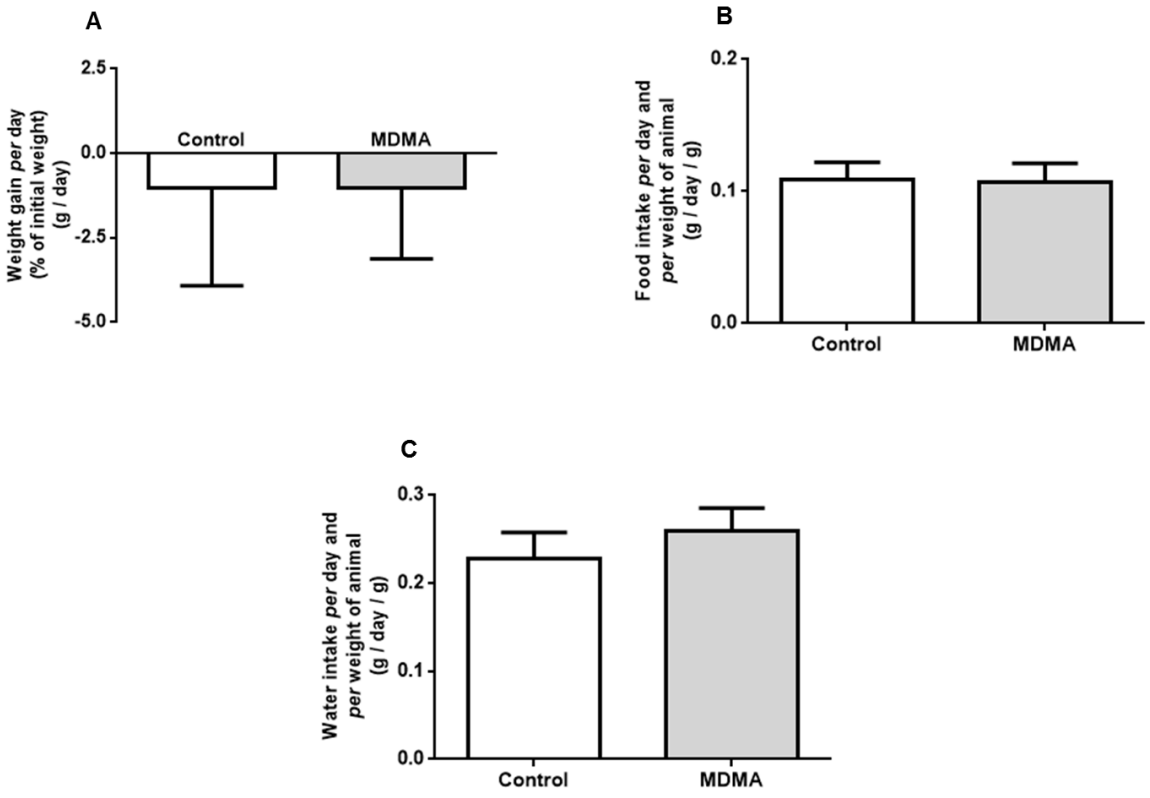

Figure 7 – Body weight gain *per* day (A), food (B) or water (C) intake *per* day and *per* weight of animal of control and MDMA-treated rats. Results expressed in g / day or g / day / g are presented as means ± standard deviation from seven animals in each group. Statistical comparisons were made using the t-test (p > 0.05 treatment vs. control).

4.1.2. The weight of the organs liver, heart and kidneys was not altered by MDMA administration

Each core organ (kidney, heart, liver and brain) had their weight registered and the weight ratio of each organ was taken to brain weight. No significant differences were

observed between MDMA-treated rats and the control group concerning the liver weight / brain weight ratio, heart weight / brain weight ratio and kidneys weight / brain weight ratio (Table 6).

Table 6 – Liver weight / brain weight ratio, heart weight / brain weight ratio, and kidney weight / brain weight ratio of control and MDMA-treated rats.

	Control	MDMA
Liver weight / Brain weight ratio	3.42 ± 0.17	3.15 ± 0.36
Heart weight / Brain weight ratio	0.33 ± 0.03	0.32 ± 0.05
Kidneys weight / Brain weight ratio	0.69 ± 0.03	0.66 ± 0.07

Data are presented as means ± standard deviation, and were obtained from seven animals in each group. The mean brain weight of control animals was 1.57 ± 0.04, and of MDMA-treated was 1.63 ± 0.05. Statistical comparisons were made using the t-test for the heart weight / brain weight ratio levels and the Mann-Whitney Rank Sum test for the liver weight / brain weight ratio and kidney weight / brain weight ratio levels ($p > 0.05$ treatment vs. control).

4.1.3. MDMA binge administration to adolescent rats did not promote alterations on levels of GSHt, GSSG, GSH and GSH/GSSG ratio in the brain areas

In table 7, the levels of GSHt, GSSG, GSH and GSH/GSSG ratio in the cerebellum, hippocampus, cortex, and striatum of the animals can be observed. There were no significant alterations of all these parameters in the referred brain areas of MDMA-treated rats when compared to controls.

Table 7 – Total glutathione (GSHt), oxidized glutathione (GSSG), reduced glutathione (GSH) and GSH/GSSG ratio levels in cerebellum, hippocampus, cortex, and striatum of adolescent rats.

CEREBELLUM		
	Control	**MDMA**
GSHt	13.32 ± 0.69	12.34 ± 2.80
GSSG	0.51 ± 0.12	0.47 ± 0.21
GSH	12.29 ± 0.80	11.40 ± 2.67
GSH/GSSG ratio	25.20 ± 6.52	28.77 ± 12.21

HIPPOCAMPUS		
	Control	**MDMA**
GSHt	21.17 ± 1.22	22.87 ± 3.46
GSSG	0.42 ± 0.09	0.41 ± 0.18
GSH	20.33 ± 1.37	22.05 ± 3.72
GSH/GSSG ratio	50.30 ± 12.72	50.26 ± 19.37

CORTEX		
	Control	**MDMA**
GSHt	18.60 ± 1.78	17.93 ± 1.70
GSSG	0.30 ± 0.05	0.27 ± 0.07
GSH	18.00 ± 1.77	17.39 ± 1.67
GSH/GSSG ratio	61.44 ± 10.67	68.60 ± 18.71

STRIATUM		
	Control	**MDMA**
GSHt	20.70 ± 6.14	20.49 ± 4.68
GSSG	0.45 ± 0.22	0.35 ± 0.04
GSH	19.79 ± 5.88	19.78 ± 4.69
GSH/GSSG ratio	47.49 ± 16.60	56.76 ± 14.98

Data of GSHt, GSSG and GSH levels, in nanomol *per* mg of protein (nmol / mg protein), and the GSH/GSSG ratio are presented as means ± standard deviation. Data were obtained from six to seven animals in each group. Statistical comparisons were made using the t-test for GSHt and GSH levels in hippocampus, cortex and striatum, GSSG levels in cerebellum, hippocampus and cortex and GSH/GSSG ratio levels in cortex and

striatum; the Mann-Whitney Rank Sum test was used for GSHt and GSH levels in cerebellum, GSSG levels in striatum and GSH/GSSG ratio levels in cerebellum and hippocampus ($p > 0.05$ treatment vs. control).

4.1.4. ATP content in cortex was decreased 24 hours after the binge MDMA administration to adolescent rats

Twenty-four hours after a MDMA binge administration, the ATP levels presented no significant changes in cerebellum, hippocampus and striatum, as can be seen in Figure 8A, B and D, respectively. Importantly, there were significant decreases in the cortex ATP content of the MDMA-treated group (p=0.007) (Figure 8C).

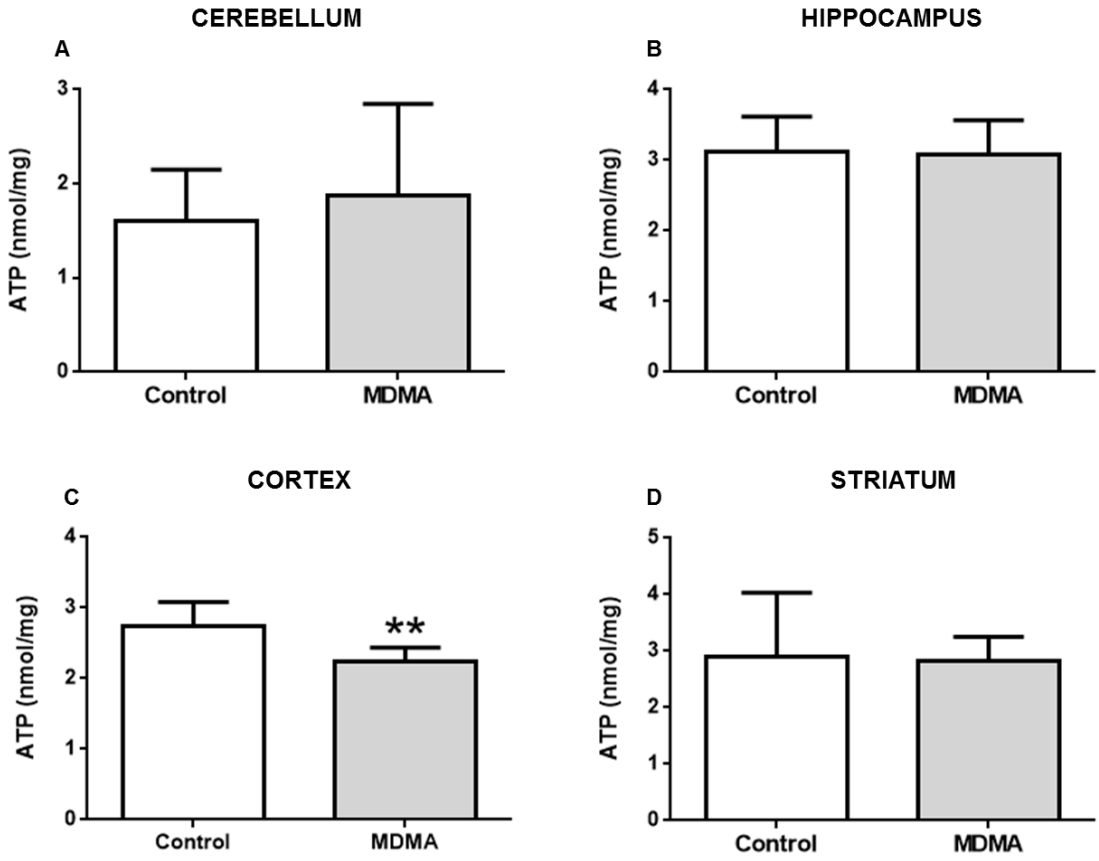

Figure 8 – ATP content in the cerebellum (A), hippocampus (B), cortex (C) and striatum (D) after MDMA administration to adolescent rats. Results, in nanomol *per* mg of protein (nmol / mg protein), are presented as means ± standard deviation, and were obtained from seven animals in each group. Statistical comparisons were made using the t-test (**$p < 0.01$ treatment vs. control).

4.1.5. MDMA administration to adolescent rats had no influence on quinoprotein levels in the brain areas

In cerebellum, hippocampus, cortex and striatum no differences were found between controls and MDMA-treated animals regarding quinoprotein levels (Table 8).

Table 8 – Quinoprotein levels in the cerebellum, hippocampus, cortex and striatum of controls and MDMA-treated adolescent rats.

	QUINOPROTEIN (OD / mg protein)	
	Control	**MDMA**
Cerebellum	5.93 ± 0.42	5.95 ± 0.29
Hippocampus	6.01 ± 0.78	6.09 ± 0.71
Cortex	5.54 ± 0.70	5.73 ± 0.50
Striatum	5.76 ± 0.20	5.78 ± 0.15

Data of quinoprotein levels, in optical density per mg of protein (OD / mg protein), are presented as means ± standard deviation, and were obtained from seven animals in each group. Statistical comparisons were made using t-test for the quinoprotein levels in cerebellum and striatum, and Mann-Whitney Rank Sum test for quinoprotein levels in hippocampus and cortex ($p > 0.05$ treatment vs. control).

4.1.6. Protein carbonylation was not altered in the brain areas by MDMA binge administration

The levels of protein carbonylation in the cerebellum, hippocampus, cortex and striatum of adolescent rats were also determined. As presented in Table 9, there were no significant alterations in the protein carbonyl levels 24 h after MDMA treatment, in the four brain areas, when comparing controls.

Table 9 – Protein carbonylation in the cerebellum, hippocampus, cortex and striatum of controls and MDMA-treated adolescent rats.

PROTEIN CARBONYLATION (% of control)		
	Control	**MDMA**
Cerebellum	100 ± 28	95 ± 20
Hippocampus	100 ± 12	101 ± 10
Cortex	100 ± 29	88 ± 13
Striatum	100 ± 54	109 ± 46

Data of protein carbonylation, in percentage of controls (% of control), are presented as means ± standard deviation, and was obtained from seven animals in each group. Statistical comparisons were made using t-test for protein carbonylation in cerebellum, hippocampus and cortex, and Mann-Whitney Rank Sum test for protein carbonylation in striatum ($p > 0.05$ treatment vs. control).

4.1.7. MDMA administration to adolescent rats had no effect on plasma AST, ALT, CK-MB and CK-R levels

The plasma levels of CK-R, CK-MB, AST and ALT-R were measured as biomarkers of liver or heart integrity (Table 10). The administration of MDMA to adolescent rats did not promote significant changes in these parameters when compared to the control group. The ratio AST/ALT was also calculated, but no significant differences among groups were found (data not shown).

Table 10 – Plasma levels of creatine kinase (CK-R), creatine kinase-MB (CK-MB), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of control and MDMA-treated rats.

	Control	**MDMA**
CK-R (U/L)	203 ± 77	200 ± 76
CK-MB (U/L)	340 ± 139	306 ± 133
AST (U/L)	39 ± 18	46 ± 19
ALT (U/L)	34 ± 25	29 ± 4

Data of CK-R, CK-MB, AST and ALT plasma levels are presented as means ± standard deviation, and were obtained from seven animals in each group. Statistical comparisons were made using the t-test for CK-R, CK-MB and AST levels, and the Mann-Whitney Rank Sum test for ALT levels ($p > 0.05$ treatment vs. control).

4.1.8. MDMA induced vacuolization, oedema and vascular congestion in the peripheral organs, 24 hours after the exposure

The histologic examination of peripheral organs (liver, heart and kidneys) of control and MDMA-treated rats was performed by means of optical microscopy. The representative structural alterations can be observed in Figures 9, 10 and 11.

The control group showed a preserved liver tissue structure, despite the slight cellular vacuolization observed in the periportal regions (Figure 9A and B). The MDMA-treated rats presented a marked cellular vacuolization observed in the periportal regions, and sinusoidal dilatation with periportal and centrilobular vascular congestion (Figure 9C and D). No necrotic zones or interstitial inflammatory cell infiltration were observed in both groups.

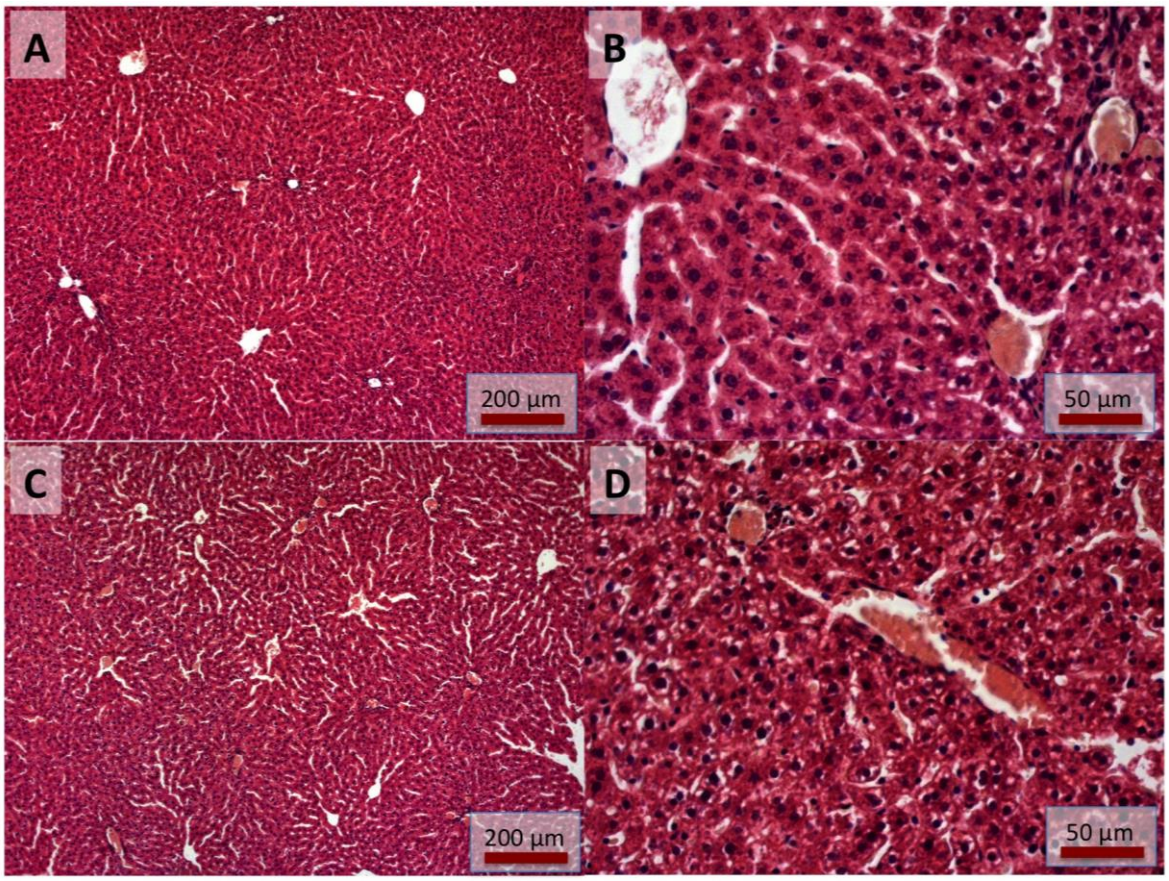

Figure 9 – Optical micrographs of liver sections from control (A and B) and MDMA-treated rats (C and D) stained with hematoxylin/eosin. C and D showed sinusoidal dilatation with a marked cellular vacuolization in the periportal regions.

In the histological analysis of the heart, both controls (Figure 10A and B) and MDMA-treated (Figure 10C and D) animals revealed no signs of necrosis or interstitial

inflammatory cell infiltration, presenting both groups a normal tissue organization. In the experimental group, animals presented random signs of cardiomyocyte oedema, particularly in the sub-endocardic region (Figure 10C and D).

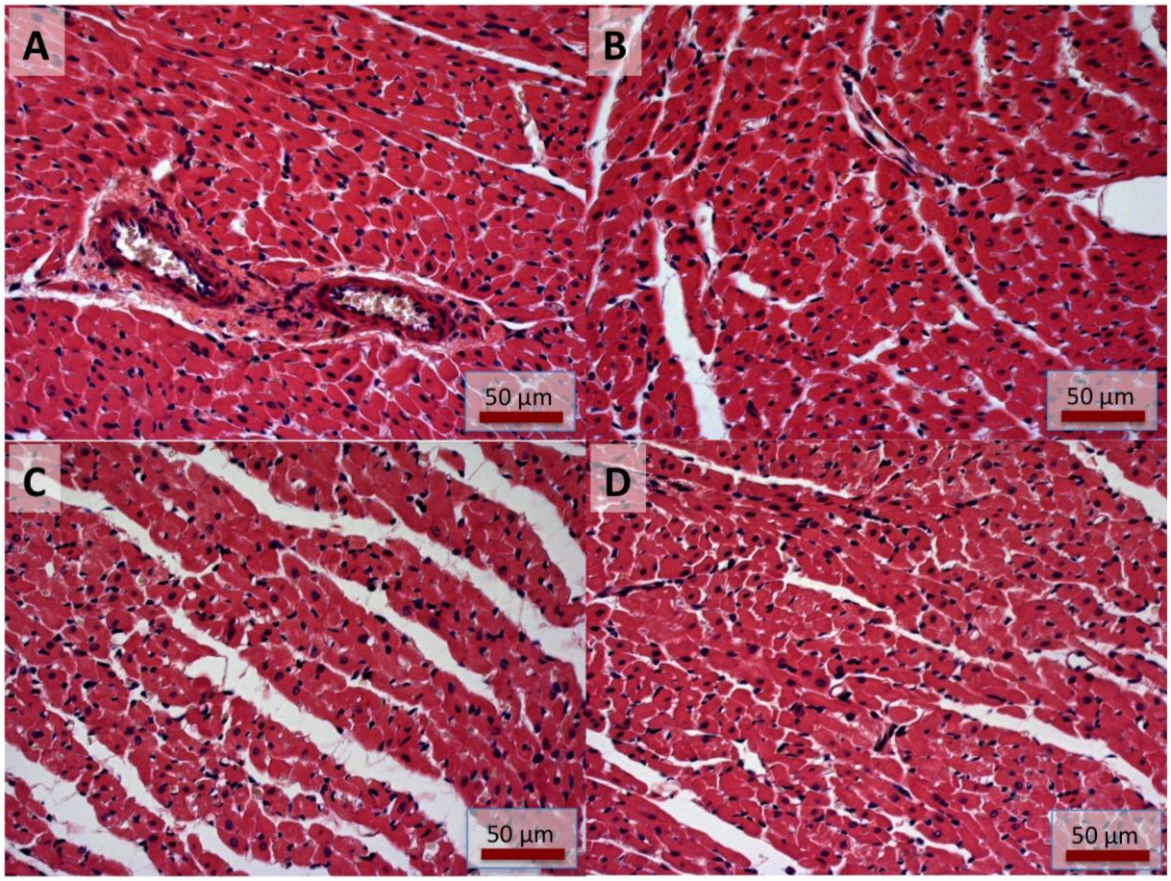

Figure 10 – Optical micrographs from heart sections of controls (A and B) and MDMA-treated rats (C and D) stained with hematoxylin/eosin. In C and D scattered cardiomyocytes with signs of intracellular oedema can be observed, as identified by the reduced cytoplasmic staining.

The renal tissue organization remained preserved in the control group, without signs of cellular damage (Figure 11A and B). However, the MDMA-treated group presented scattered interstitial oedema, detected by the enlarged space between the tubular structures, and signs of vascular congestion (Figure 11C and D). No necrotic zones or interstitial inflammatory cell infiltration were observed in both groups.

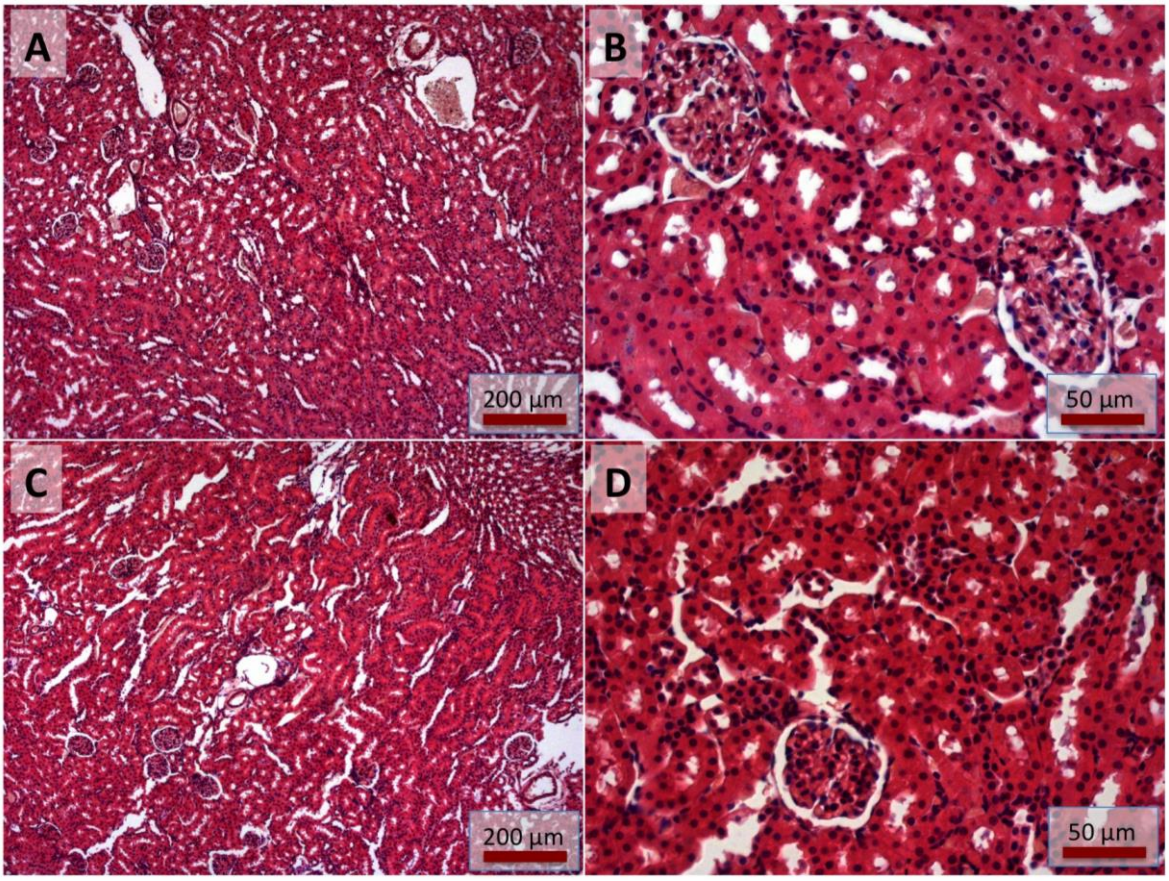

Figure 11 – Optical micrographs of kidney sections from controls (A and B) and MDMA-treated rats (C and D) stained with hematoxylin/eosin. In C and D a slight interstitial oedema, detected by the enlarged space between the tubular structures, can be observed.

4.1.9. GSHt, GSSG, GSH and GSH/GSSG ratio remained unchanged in liver, heart, and kidneys of adolescent rats after MDMA administration

The levels of GSHt, GSSG, GSH and GSH/GSSG ratio in the liver, heart and kidneys in control and MDMA-treated animals can be observed in Table 11. No differences were found for all these parameters in the three organs, between the group that received the binge MDMA administration and the control group.

Table 11 – Total glutathione (GSHt), oxidized glutathione (GSSG), reduced glutathione (GSH) and GSH/GSSG ratio in liver, heart, and kidneys of control and MDMA-treated adolescent rats.

LIVER		
	Control	**MDMA**
GSHt	19.31 ± 1.96	18.51 ± 2.61
GSSG	0.60 ± 0.13	0.62 ± 0.14
GSH	18.10 ± 2.04	17.28 ± 2.38
GSH/GSSG ratio	31.46 ± 9.22	28.68 ± 4.46
HEART		
	Control	**MDMA**
GSHt	9.66 ± 1.30	9.53 ± 1.70
GSSG	0.53 ± 0.14	0.59 ± 0.23
GSH	8.60 ± 1.43	8.35 ± 1.46
GSH/GSSG ratio	17.62 ± 7.38	15.86 ± 5.93
KIDNEYS		
	Control	**MDMA**
GSHt	2.25 ± 0.38	1.93 ± 0.60
GSSG	0.06 ± 0.02	0.06 ± 0.01
GSH	2.12 ± 0.34	1.82 ± 0.60
GSH/GSSG ratio	37.58 ± 11.26	32.39 ± 11.29

Data of GSHt, GSSG, and GSH levels, in nanomol *per* mg of protein (nmol / mg protein), and the GSH/GSSG ratio are presented as means ± standard deviation, and were obtained from six to seven animals. Statistical comparisons were made using the t-test for the GSHt, GSSG, GSH levels in the liver, heart, and kidneys, and the GSH/GSSG ratio in the liver, and the Mann-Whitney Rank Sum test for the GSH/GSSG ratio levels in the heart and kidneys ($p > 0.05$ treatment vs. control).

4.1.10. ATP content remained unchanged in the liver, heart, and kidneys of rats 24 hours after the MDMA administration

ATP levels were measured in the liver, heart and kidneys of adolescent rats that received saline or three doses of MDMA. No significant differences in the ATP content

were observed between the two groups in the three referred organs, as can be seen in Table 12.

Table 12 – ATP levels in liver, heart, and kidneys of the control and exposed to MDMA groups.

ATP (nmol / mg protein)		
	Control	**MDMA**
Liver	1.60 ± 0.84	1.69 ± 0.38
Heart	0.63 ± 0.20	0.60 ± 0.27
Kidneys	1.75 ± 0.62	1.43 ± 0.57

Results, in nmol *per* mg of protein (nmol / mg protein), are presented as means ± standard deviation, and were obtained from seven animals in each group. Statistical comparisons were made using the t-test for the ATP levels in the heart and kidneys, and the Mann-Whitney Rank Sum test for the ATP levels in the liver ($p > 0.05$ treatment vs. control).

4.1.11. Protein-bound quinones formation in the liver were increased 24 hours after MDMA administration to adolescent rats

In Figure 12, the measurement of protein-bound quinones (quinoproteins) formation in the liver, heart and kidneys of control and MDMA-treated animals can be observed. There was a significant increase in the hepatic quinoprotein levels in the MDMA-treated group (p=0.039) (Figure 12A). However, no significant alterations were found regarding this parameter in the heart or kidneys when comparing both groups (Figure 12B and C).

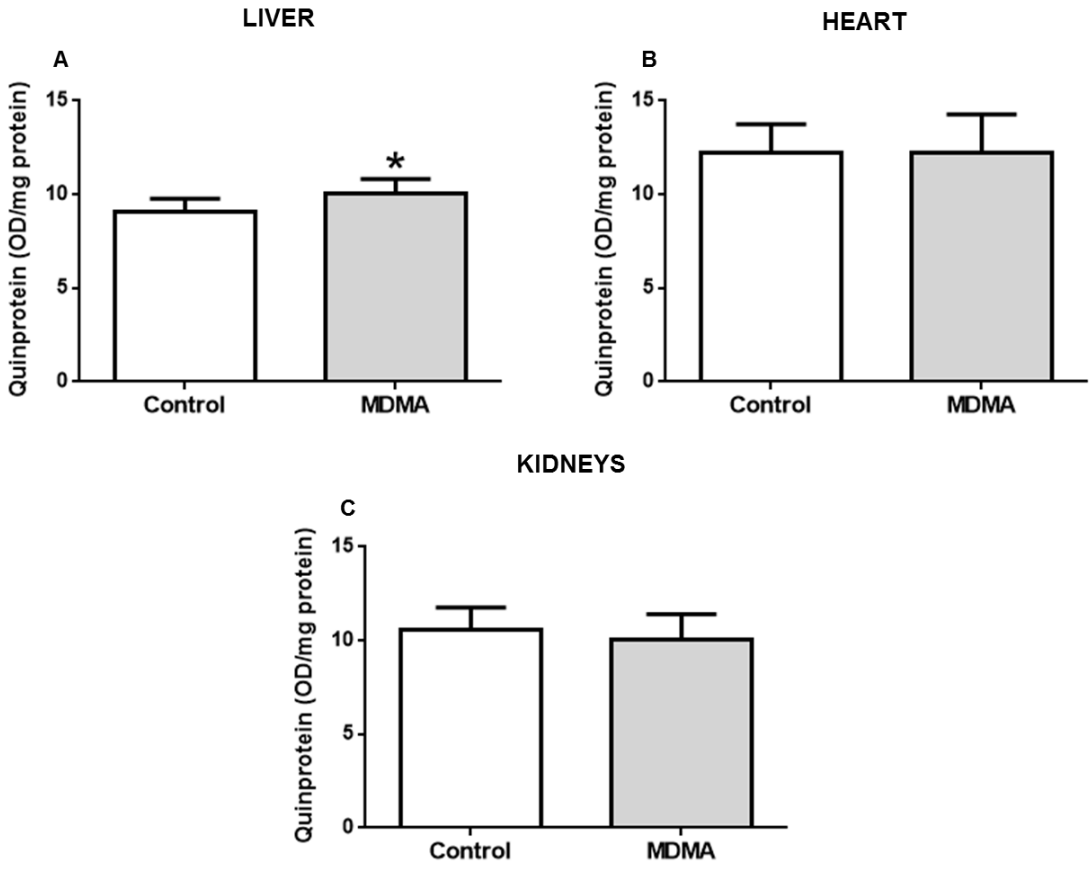

Figure 12 – Quinoprotein levels in liver (A), heart (B) and kidneys (C) of control and MDMA-treated adolescent rats. Results, in optical density *per* mg of protein (OD / mg protein), are presented as means ± standard deviation, and were obtained from seven animals in each group. Statistical comparisons were made using the t-test for the quinoprotein levels in the liver and kidneys and the Mann-Whitney Rank Sum test for the quinoprotein levels in the heart (*$p < 0.05$ treatment vs. control).

4.1.12. Protein carbonylation showed a tendency to increase in the adolescent rat kidneys after MDMA administration

In Figure 13, can be observed the results of protein carbonylation in the liver (Figure 13A), heart (Figure 13B) and kidneys (Figure 13C) of adolescent rats after an i.p. administration of three doses of NaCl 0.9% or 5 mg/kg of MDMA. No significant differences were found in the levels of protein carbonyls in the liver and heart between controls and the MDMA group. However, data showed a tendency for an increase (p=0.051) in protein carbonylation in the kidneys, 24 h after the MDMA administration.

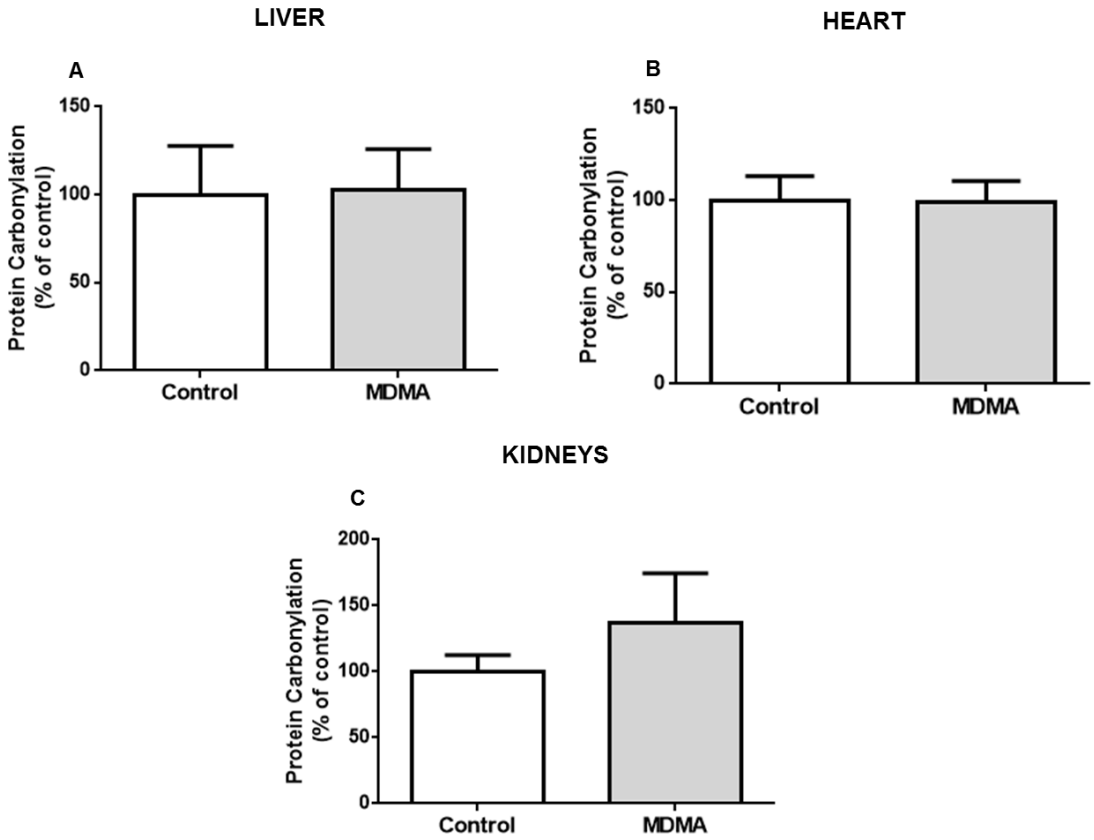

Figure 13 – Protein carbonylation in liver (A), heart (B) and kidneys (C) of control and MDMA-treated adolescent rats. Results, in percentage of controls (% of control), are presented as means ± standard deviation, and were obtained from six to seven animals in each group. Statistical comparisons were made using the t-test for the protein carbonylation in the liver and heart, and the Mann-Whitney Rank Sum test for the protein carbonylation in the kidneys ($p > 0.05$ treatment vs. control).

4.1.13. MDMA administration promoted a decrease in caspase-8 activity in rats' liver, while caspase-3 and -9 activities remained unaltered

The data concerning the activities of caspase-3, -8 and -9 in liver, heart, and kidneys of adolescent rats after administration of MDMA are presented in Table 13. Caspase-8 activity significantly decreased in the liver of the MDMA-treated rats when compared to controls (p=0.038). No differences were found in the activity of this protease either in heart or kidneys. The activities of caspase-3 and caspase-9 had no significant alterations in all three organs following MDMA administration when compared to the control group.

Table 13 – Caspase-3, -8 and -9 activities in liver, heart and kidneys of adolescent rats that received either saline or MDMA.

LIVER		
FU / μg protein	**Control**	**MDMA**
Caspase-3	1.19 ± 0.31	1.12 ± 0.31
Caspase-8	1.00 ± 0.06	0.93 ± 0.20*
Caspase-9	0.90 ± 0.07	0.86 ± 0.13

HEART		
FU / μg protein	**Control**	**MDMA**
Caspase-3	0.44 ± 0.05	0.42 ± 0.06
Caspase-8	0.48 ± 0.08	0.47 ± 0.11
Caspase-9	0.09 ± 0.02	0.11 ± 0.05

KIDNEYS		
FU / μg protein	**Control**	**MDMA**
Caspase-3	0.59 ± 0.08	0.56 ± 0.06
Caspase-8	0.66 ± 0.09	0.62 ± 0.07
Caspase-9	0.90 ± 0.18	0.79 ± 0.11

Data of caspase-3, -8 and -9 activities, in fluorescent units *per* mg of protein (FU / mg protein), are presented as means ± standard deviation, and were obtained from seven animals in each group. Statistical comparisons were made using the t-test for the caspase-3 activity in the liver, heart and kidneys and caspase-8 and -9 activities in the heart and kidneys, and the Mann-Whitney Rank Sum test for the caspase-8 and -9 activities in the liver (*$p < 0.05$ treatment vs. control).

4.2. Experiment 2

4.2.1. MDMA binge administration led to a higher hyperthermic response in aged than adolescent rats

On experiment two, two different populations were used: adolescents and aged animals. To adolescent animals were i.p. administered three doses of either MDMA 5 mg/kg or NaCl 0.9%, and sacrifice was performed seven days after MDMA exposure. In adolescent rats, after the first dose of MDMA the increase in temperature was significantly higher when compared to controls ($p<0.05$), being higher in magnitude following the second dose ($p<0.001$), and the temperature persisted high for more than 3 h after the third dose (Figure 14A). To aged rats were i.p. administered two doses of either MDMA 5 mg/kg or NaCl 0.9%, and sacrifice was performed seven days after MDMA exposure. In aged rats, a significant increase in temperature occurred only after the second dose ($p<0.0001$) of MDMA that persisted for the following couple of hours (Figure 14B). In particular, in aged animals the temperature reached a peak mean near 41ºC after the second MDMA dose (Figure 14B), while adolescent animals never surpassed the mean of 39ºC even after the third MDMA dose (Figure 14A).

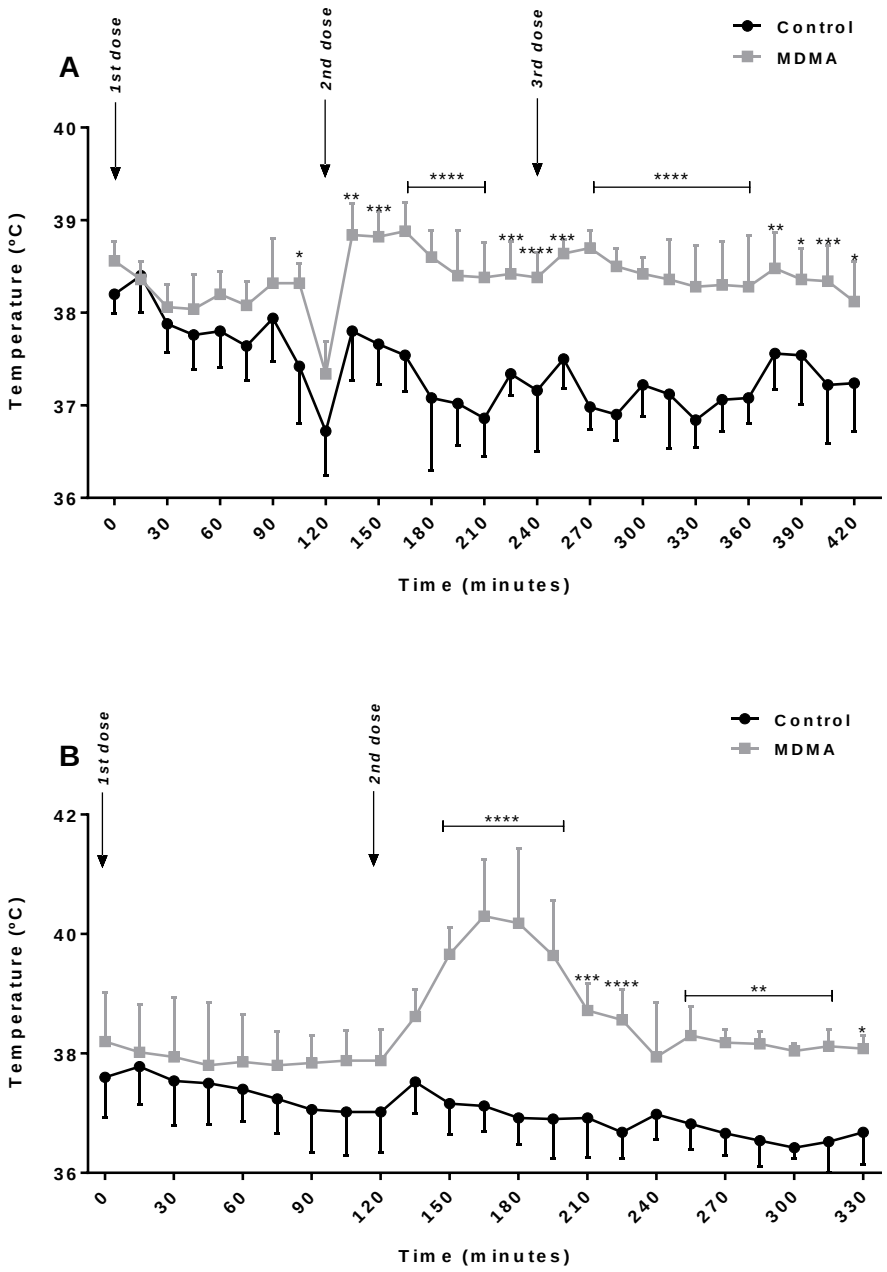

Figure 14 – Temperature monitoring of adolescent (A) and aged (B) rats for 6 to 7 h after the administration of either saline (control) or MDMA (MDMA-treated). Results in degrees Celsius (ºC) are presented as means ± standard deviation, from five animals in each group. Statistical comparisons were made using two-way ANOVA followed by the Bonferroni post hoc test (*p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 treatment vs. control).

In Figure 15, the results of the daily temperature of adolescent (Figure 15A) and aged (Figure 15B) rats for the following seven days after MDMA exposure are presented. Following the day of exposure to MDMA the animal body temperature was monitored daily at the same hour of the first administration. No significant differences were observed in MDMA-treated adolescent animals when compared to the control group. In aged rats, the

temperature was significantly elevated in the MDMA-treated animals (p<0.001), 24 h post-MDMA binge administration.

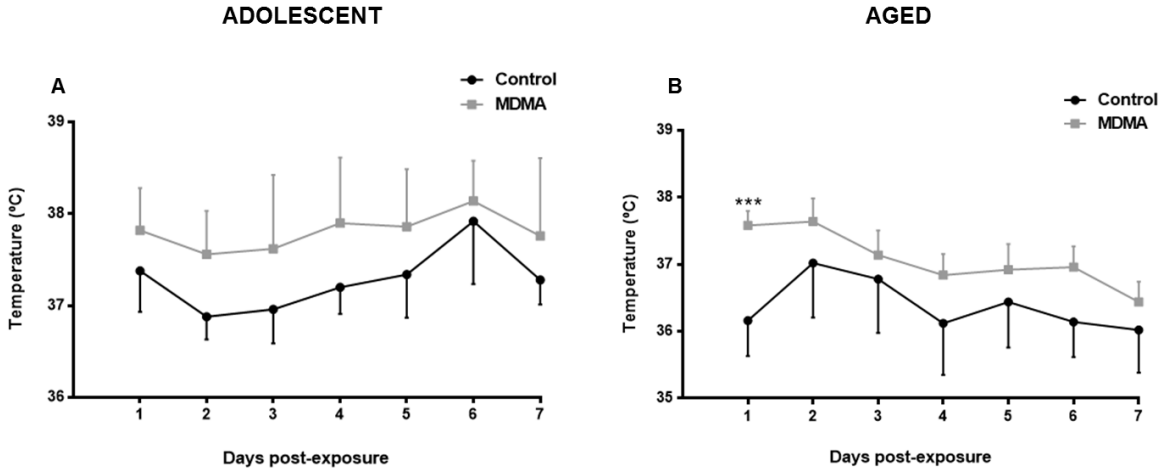

Figure 15 – Temperature monitoring of adolescent (A) and aged (B) rats for the seven days after the administration of either saline (control) or MDMA (MDMA-treated). Results in degrees Celsius (ºC) are presented as means ± standard deviation, from five animals in each group. Statistical comparisons were made using two-way ANOVA followed by the Bonferroni post hoc test (***p < 0.001 treatment vs. control).

4.2.2. MDMA treatment induced alterations in the body weight of adolescent animals and food and water intake of aged animals

Data of animal body weight gain *per* day (Figure 16A and B), and food (Figure 16C and D) or water (Figure 16E and F) intake, *per* day and *per* weight, during the seven days post-treatment in adolescent and aged rats, are presented in Figure 16. In the adolescent population, there was a significant decrease on the body weight of the MDMA group on the first day post-MDMA administration (p<0.05) (Figure 16A). However, no differences were observed in this population for both food and water consumption (Figure 16C and E, respectively). In contrast to the adolescent rats, in the aged animals there were no significant differences in body weight between treated and respective controls (Figure 16B). However, in the MDMA-treated aged rats, food intake significantly decreased on the first day post-MDMA administration (p<0.05), while it significantly increased on the second day post-MDMA administration (p<0.01) (Figure 16D), when compared to controls. Moreover, in aged rats, the water intake was significantly higher in the MDMA group, when compared to controls, in the two consecutive days following the MDMA binge administration (p<0.001) (Figure 16F).

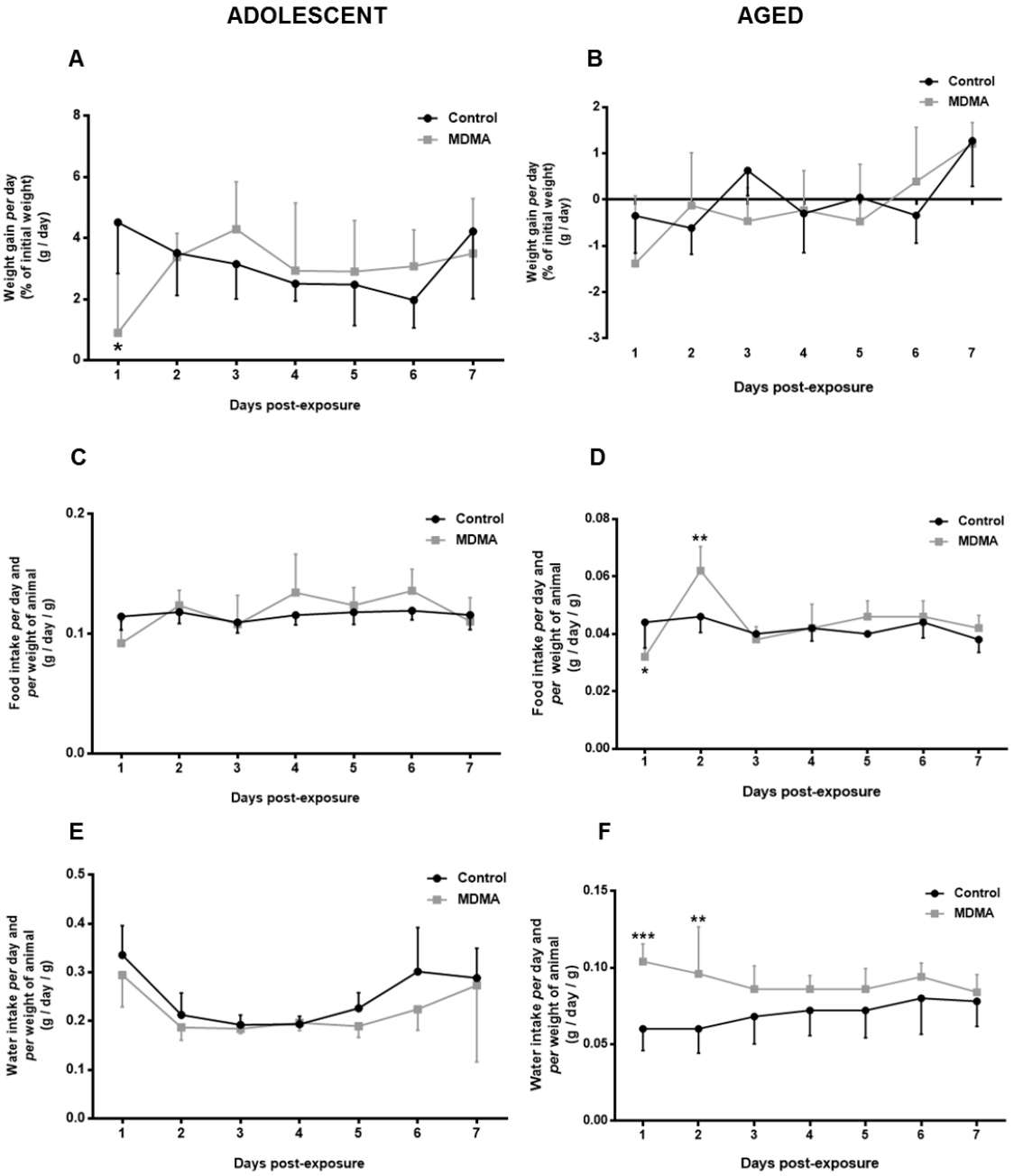

Figure 16 – (A and B) Body weight gain *per* day of adolescent (A) and aged (B) rats, respectively, for seven days after the treatment day. (C and D) Food intake *per* day and *per* weight of animal of adolescent (C) and aged (D) rats, respectively, for seven days after the treatment day. (E and F) Water intake *per* day and *per* weight of animal of adolescent (E) and aged (F) rats, respectively, for seven days after the treatment day. Results in g / day or g / day / g are presented as means ± standard deviation, from five animals in each group. Statistical comparisons were made using two-way ANOVA followed by the Bonferroni post hoc test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ treatment vs. control).

4.2.3. GSHt, GSSG, GSH and GSH/GSSG ratio levels in the cerebellum of adolescent and aged rats remained unaltered seven days after the MDMA administration

In the cerebellum, no differences were found between controls and MDMA-treated groups concerning the levels of GSHt (Figure 17A and B), GSSG (Figure 17C and D), GSH (Figure 17E and F) and GSH/GSSG ratio (Figure 17G and H) in both adolescent and aged rats, one week after the MDMA administration. Moreover, the levels of GSHt and of the GSH/GSSG ratio were very similar in the control groups of aged and adolescent rats.

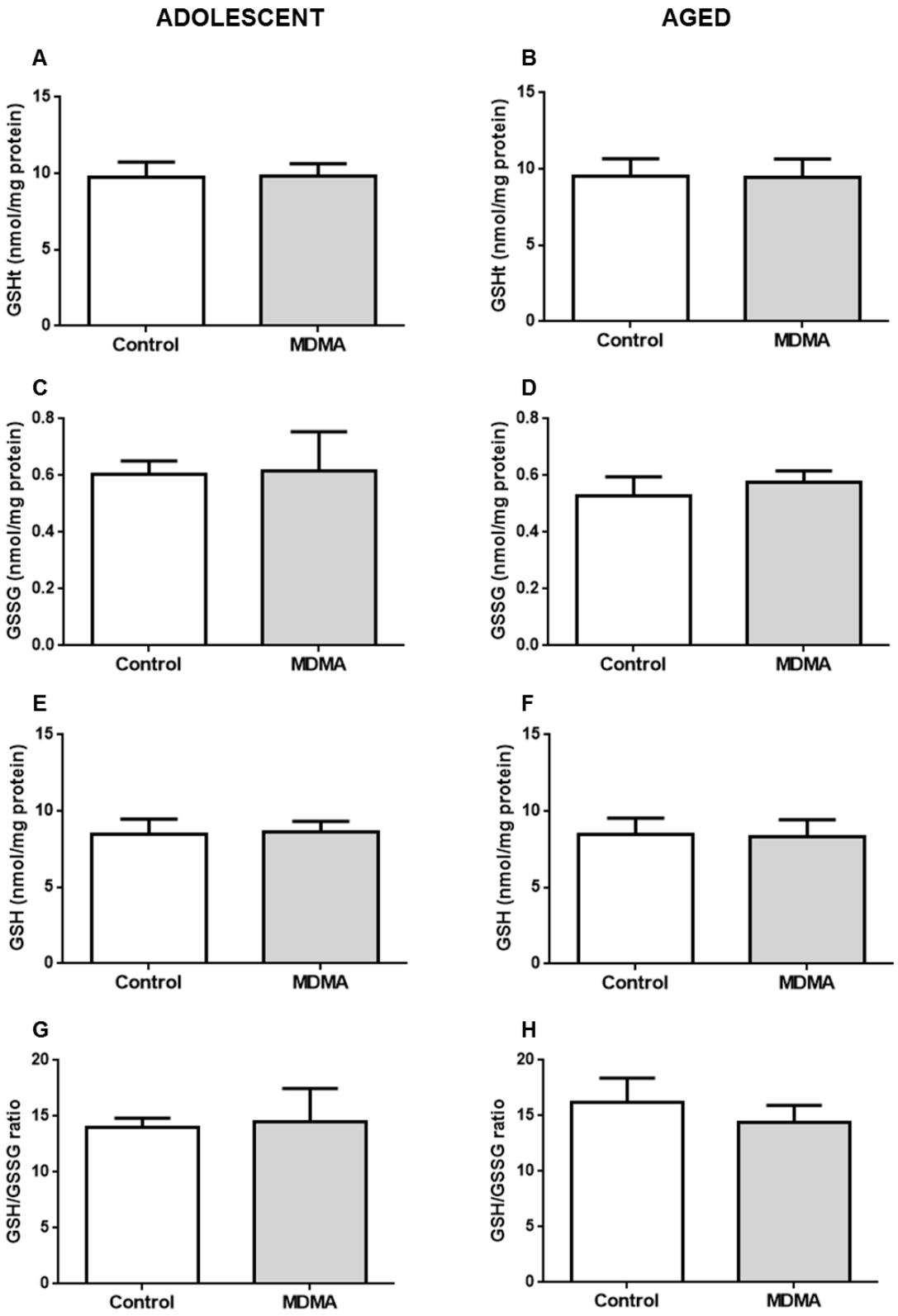

Figure 17 – (A and B) GSHt levels, in nanomol *per* mg of protein (nmol / mg protein), in cerebellum of adolescent (A) and aged (B) rats, respectively, seven days after treatment. (C and D) GSSG levels, in nmol / mg protein, in the cerebellum of adolescent (C) and aged (D) rats, respectively, seven days after treatment. (E and F) GSH levels, in nmol / mg protein, in the cerebellum of adolescent (E) and aged (F) rats, respectively, seven days after treatment. (G and H) GSH/GSSG ratio levels in the cerebellum of adolescent (G) and aged

(H) rats, respectively, seven days after treatment. Results are presented as means ± standard deviation, and were obtained from five animals in each group. Statistical comparisons were made using the t-test for GSHt, GSH and GSH/GSSG ratio levels in adolescent and aged rats and for GSSG levels in aged rats, and the Mann-Whitney Rank Sum test for GSSG levels in adolescent rats ($p > 0.05$ treatment vs. control).

4.2.4. One week after the MDMA administration aged animals presented significant decreases in the cerebellum ATP content

In Figure 18, are presented the ATP levels in the cerebellum of adolescent (Figure 18A) and aged (Figure 18B) rats, one week after the MDMA administration. No differences were observed between controls and MDMA-treated animals in the cerebellum ATP levels of adolescent rats (Figure 18A). However, in aged animals there was a significant decrease of the cerebellum ATP content in the MDMA group (p=0.015) (Figure 13B).

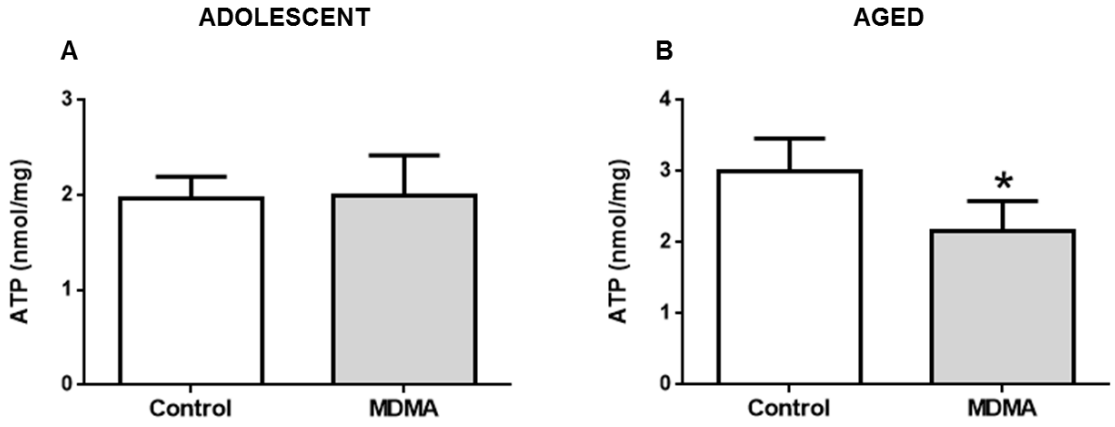

Figure 18 – ATP content in the cerebellum, seven days after treatment, in adolescent (A) and aged (B) rats. Results, in nanomol *per* mg of protein (nmol / mg protein), are presented as means ± standard deviation, and were obtained from five animals in each group. Statistical comparisons were made using the t-test (*$p < 0.05$ treatment vs. control).

4.2.5. Quinoprotein levels were unaltered in the cerebellum of rats one week after MDMA administration

One week after the MDMA binge administration, there were no significant differences between controls and MDMA-treated animals in the protein-bound quinones (quinoproteins) in the cerebellum of either adolescent or aged rats. The data regarding the measurement of quinoproteins is presented in Figure 19.

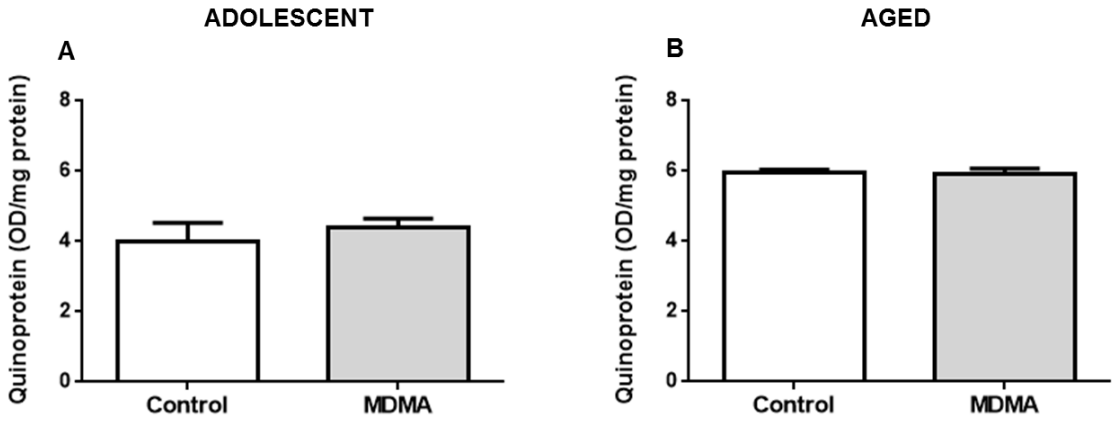

Figure 19 – Quinoprotein levels in the cerebellum, seven days after treatment, in adolescent (A) and aged (B) rats. Results, in optical density *per* mg of protein (OD / mg protein), are presented as means ± standard deviation, and were obtained from five animals in each group. Statistical comparisons were made using t-test for quinoprotein levels in adolescent rats, and Mann-Whitney Rank Sum test for quinoprotein levels in aged rats ($p > 0.05$ treatment vs. control).

4.2.6. Seven days after MDMA administration no significant differences were found in cerebellum protein carbonyl levels of rats

In Figure 20, are presented the results concerning the protein carbonylation in the cerebellum of adolescent (Figure 20A) and aged (Figure 20B) rats. Seven days after the binge administration of MDMA, no significant differences were found between the control and the MDMA-treated group in either adolescent or aged rats.

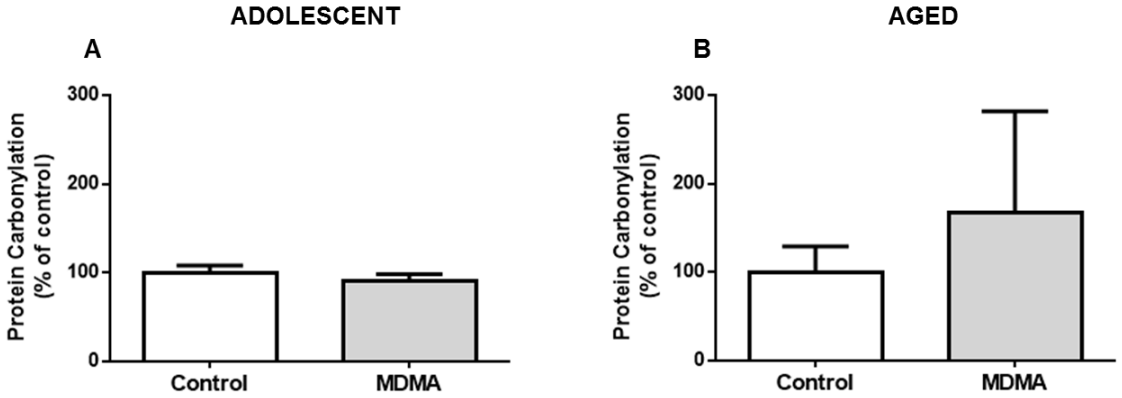

Figure 20 – Protein carbonylation in the cerebellum, seven days after treatment, in adolescent (A) and aged (B) rats. Results, in percentage of control (% of control), are presented as means ± standard deviation, and were obtained from five animals in each group. Statistical comparisons were made using t-test for protein

carbonylation in adolescent rats, and Mann-Whitney Rank Sum test for protein carbonylation in aged rats ($p > 0.05$ treatment vs. control).

Part V

Discussion and conclusions

5. DISCUSSION AND CONCLUSIONS

5.1. Experiment 1

5.1.1. MDMA-induced hyperthermia in adolescent animals

Hyperthermia is an important sign following MDMA administration, therefore we controlled the body temperature of rats in both experiments. In experiment one, MDMA administration promoted a significant increase in the rats' body temperature, as expected. The MDMA use, particularly, the hyperthermic response may lead to several physiological changes, and, for that reason, we also assessed the body weight gain, as well as the food and water consumption. The adolescent animals' body weight decreased in both controls and MDMA-treated animals in experiment one, probably as a result of the stress induced by the manipulation during the experiment, and no differences were found between the groups. No significant differences were also found in terms of food or water consumption in the adolescent animals, 24 h after the MDMA exposure.

Hyperthermia is commonly associated with recreational use of MDMA and body temperatures of about 43ºC in humans and several associated fatalities have been reported (Green et al 2004a, Henry 1992, Jaehne et al 2007). As previously referred, this drug is often used in parties with elevated ambient temperature and crowded rooms, where physical exertion (dancing) and dehydration are common. These aspects can contribute to an elevated body temperature of consumers (Henry 1992, Irvine et al 2005, Patel et al 2005). Physical activity, peripheral vasoconstriction, loss of thermoregulatory mechanisms at the CNS, loss of body signal perception (like thirst and exhaustion), euphoria, increased muscle tone, and heat production are the aspects that mainly contribute to the hyperthermic effects associated with MDMA use (Capela et al 2009). Studies in laboratory animals reproduce the hyperthermic response seen in humans, and several studies reported that MDMA evokes an hyperthermic response in rats and mice (Alves et al 2007, Carvalho et al 2002, Colado et al 1993). Furthermore, hyperthermia is also a factor that contributes for the neurotoxic effects of MDMA in rats (Malberg & Seiden 1998). Several studies reported that the prevention of the MDMA-induced hyperthermia resulted in a reduction of the serotonergic neurotoxicity promoted by this drug (Colado et al 1999, Farfel & Seiden 1995, Malberg et al 1996). Moreover, *in vitro* studies using neuronal cultures demonstrated that the neurotoxic actions induced by MDMA are potentiated by hyperthermia (Barbosa et al 2014, Capela et al 2007a, Capela et al 2006a, Capela et al 2006b). A study conducted with adult male Wistar rats (ten week old), treated

with the same MDMA binge regimen (5 mg/kg x 3, i.p., every 2 h) used in the experiments performed within this dissertation, showed that the administration of the drug in reduced ambient temperature (15ºC) blocked the hyperthermic response and the long-term depletion of 5-HT, found at normal environmental temperature (21.5ºC). These data confirm the existence of a relation between the body temperature and MDMA serotonergic neurotoxicity (Goni-Allo et al 2008). Herein, this binge regimen of MDMA in adolescent Wistar rats (PND 40) did not promote serotonergic toxicity (Feio-Azevedo et al Submitted), ruling that hyperthermia *per se* is not a triggering factor for neurotoxic events. Nonetheless, MDMA-induced hyperthermia certainly potentiates the toxicity observed in the brain areas and also the peripheral organs.

5.1.2. Acute brain changes induced by MDMA in adolescent animals

Though the MDMA binge scheme used in this study was previously shown to be non-neurotoxic to the serotonergic and dopaminergic system of PND 40 adolescent rats (Feio-Azevedo et al Submitted), we evaluated several other brain changes. We aimed to verify if this MDMA regimen could promote oxidative stress or ATP decreases in the brain, despite not promoting neurotoxicity to the monoaminergic system.

In this dissertation, no differences were found in glutathione levels in the brain areas of the rat, namely cerebellum, hippocampus, cortex and striatum, 24 h after MDMA binge administration. To the best of our knowledge, the glutathione levels presented in this thesis were for the first time obtained in adolescent rats, 24 h after a non-neurotoxic MDMA regimen to the serotonergic system. Other studies have evaluated the glutathione brain levels following neurotoxic regimens of amphetamines at different time-points. Riezzo and co-workers demonstrated that a single dose of MDMA (20 mg/kg, i.p.) to Wistar rats (200-250 g), promoted significant decreases of GSH/GSSG ratio, 3 and 6 h after the MDMA exposure, in several brain areas (frontal cortex, hippocampus and striatum) (Riezzo et al 2010). A study in nine week old Swiss Webster mice showed that METH (5 mg/kg x 3, i.p., 3 h apart) promoted decreases in the GSH levels 5 to 13 days following METH exposure, in the frontal cortex, striatum, amygdala and hippocampus (Achat-Mendes et al 2007). Another study in mice assessed the GSH levels after MDMA administration. Male C57 black mice, with ages from nine to ten weeks old, received 2.5 mg/kg of MDMA (i.p., every day, for five days) and levels of GSH were measured immediately after the last exposure to the drug. The GSH content was significantly decreased in the hippocampus (Frenzilli et al 2007). Major differences from the three reports mentioned above to the present study are the use of older animals and the

administration of different doses. A threshold of MDMA might be necessary to promote GSH decrease or the effect at this marker might be transient. On the other hand, adolescent animals might be less susceptible to changes in glutathione redox status.

In this dissertation, no differences were found in protein-bound quinone formation, between controls and MDMA-treated groups in the rat brain areas (cerebellum, hippocampus, cortex and striatum) 24 h after MDMA binge administration. No previous studies *in vivo* were found that evaluate this parameter after MDMA administration. Nine weeks-old BALB/c mice received four injections of 4 mg/kg of METH (i.p., 2 h apart) and, 3 and 14 days after the repeated administration of METH, elevated quinoprotein levels were observed in the striatum along with decreases in DAT immunoreactivity (Miyazaki et al 2006). That paper also reported that METH promoted in dopaminergic CATH.a cells neuronal cell death and increased quinoprotein levels, in a dose-dependent fashion. Altogether, the results observed confirm the importance of DA-dependent quinone formation in the neurotoxicity of abuse drugs, namely METH (Miyazaki et al 2006). Two studies from our group, conducted *in vitro*, showed that MDMA or its metabolites were able to increase quinoprotein formation in the brain (Barbosa et al 2012, Capela et al 2007a). In this thesis it became demonstrated that MDMA in rats was not able to increase protein-bound quinone formation in the brain, up to 24 h after its administration, in a regimen that does not promote monoaminergic neurotoxicity.

MDMA metabolites can oxidize into ortho-quinones that may produce reactive oxygen species (ROS) and cause oxidative stress through the formation of oxidized cellular macromolecules, leading to structural and functional modifications such as protein carbonylation (Carvalho et al 2004a, Carvalho et al 2004b, Carvalho et al 2004c). The MDMA dosage regimen used in this thesis was not able to promote any alterations in protein carbonylation. Alves and co-workers evaluated the MDMA-induced neurotoxicity in mitochondria isolated from several brain regions of adolescent male Wistar rats at PND 45. The administration of a neurotoxic dose of MDMA (10 mg/kg x 4, i.p., every 2 h) promoted increases in the levels of protein carbonyls in brain mitochondria, two weeks after MDMA exposure (Alves et al 2007). We used a moderate dosage regimen of MDMA and the protein carbonylation levels were measured 24 h after the exposure to the drug, so it remains to be verified if later time-points would be crucial in this biochemical marker. Another study conducted with mouse brain synoptosomes found that exposure to MDMA metabolites was able to increase protein carbonyl levels, but not MDMA itself (Barbosa et al 2012).

The present study did not find any significant role for oxidative stress related changes in the rat adolescent brain, 24 h following administration of MDMA in human relevant doses.

MDMA hepatic metabolism plays an important role in the toxicity of this drug, since it can result in the formation of several highly reactive metabolites, like quinone intermediates, that can subsequently produce ROS and lead to oxidative stress (Capela et al 2009). Several studies reported that MDMA metabolites produce neurotoxicity in laboratory animals (Escobedo et al 2005, Jones et al 2005, Miller et al 1996). As previously mentioned, the MDMA metabolites N-Me-α-MeDA and α-MeDA can be oxidize into ortho-quinones, that can retain the ability to redox cycle and induce oxidative stress (Monks & Lau 1997). These metabolites can conjugate with thiols and glutathione originating adducts. Jones and colleagues showed that glutathione and N-acetylcysteine (NAC) conjugates of N-Me-α-MeDA are present in striatum of rats after an injection of MDMA (20 mg/kg, s.c.). The mentioned NAC conjugates produce serotonergic neurotoxicity when injected directly into the striatum, suggesting that these metabolites are capable of causing neurotoxic damage (Jones et al 2005). A study in cultured rat cortical neurons tested the neurotoxicity of MDMA metabolites, N-Me-α-MeDA, α-MeDA, and their glutathione and NAC conjugates. The MDMA thioether metabolites increased production of reactive species in a time-dependent manner, depleted intracellular GSHt in a concentration-dependent manner, and increased protein-bound quinones. NAC, an antioxidant, was also able to prevent neuronal death and oxidative stress in that model (Capela et al 2007a).

In this thesis, a significant depletion of the ATP levels in the cortex of the MDMA-treated rats, 24 h after MDMA binge administration, was found. No alterations were found in the other three brain areas (cerebellum, hippocampus and striatum) tested. A study with Sprague-Dawley rats (200-275 g), which received four i.p. administrations of 10 mg/kg MDMA, 2 h apart, showed decreased ATP content in the striatum 1, 12 and 24 h after the exposure, as well as in the hippocampus 12 h after MDMA administration (Darvesh & Gudelsky 2005). In another study, a single administration of METH (5 mg/kg, i.p.) to adolescent (4 weeks old) Wistar rats, promoted decreases in ATP levels in the brain, 30 min after the drug administration (Shiba et al 2011). Interestingly, the same study showed that 24 h after the exposure to the drug, the ATP levels were normalized to the control levels (Shiba et al 2011). In C57BL/6 mice, with ages ranging from eight to ten weeks old, showed decreases in striatal ATP levels 1.5 h after multiple METH administration (10 mg/kg x 4, i.p., 2 h apart). ATP content returned, approximately, to the control levels 24 h after the exposure and no decreases were found in the cerebellar cortex and hippocampus (Chan et al 1994). When comparing the studies described with the results obtained in this thesis, it can be seen that: Darvesh & Gudelsky found differences in the ATP content of striatum and hippocampus in older animals and with higher MDMA doses than the ones used herein (Darvesh & Gudelsky 2005); meanwhile Shiba and co-workers

found decreases in the brain ATP levels 30 min after METH administration, but not 24 h after the exposure (Shiba et al 2011), as it was the case for Chan and co-workers that exposed mice to METH and changes were found 24 h after the administration (Chan et al 1994). The drug used, the dosage regimen, the animal model, the animals' age, and the time elapsed until the measurements seem to be important factors regarding the impairment in the brains' energetic functions. The time-frame for ATP content analysis may reveal that at earlier times of exposure a decrease in ATP levels occurs, but at later periods no changes are found, perhaps ruling for a transitory effect at least in adolescent animals.

5.1.3. Organs-related changes promoted by acute exposure to MDMA in adolescent animals

In experiment one was observed an increase in protein-bound quinone formation in the liver of MDMA-treated rats, 24 h after MDMA exposure. No differences in quinoprotein levels in the heart and kidneys of the exposed rats were observed. These results might be related to the fact that the metabolism of this drug is primarily hepatic, which generates catechol metabolites that can promote the formation of protein-bound quinones. *In vitro* studies in rat cardiomyocytes and hepatocytes showed that the MDMA metabolism can result in ortho-quinones, and subsequent oxidative stress (Carvalho et al 2004a, Carvalho et al 2004b, Carvalho et al 2004c). The increased protein-bound quinone formation in the liver of rats is an indicator of possible oxidative stress in this organ, 24 h after MDMA, possibly caused by the catechol MDMA metabolites, and possibly a sign of higher susceptibility towards MDMA toxicity.

Glutathione is an important antioxidant that plays a major role in protecting against oxidative stress. In adolescent rats, no differences were found neither in the GSHt levels nor in the GSH/GSSG ratio in the liver, heart and kidneys. An *in vivo* study in rats reported decreases in hepatic GSH content after MDMA repeated exposure (Beitia et al 2000). Acute administration of a single dose of MDMA (20 mg/kg, i.p.) had no significant effect in hepatic GSH levels of Wistar rats (200-220 g), 3 or 6 h after the MDMA administration. However, with a repeated MDMA administration (20 mg/kg x 2, i.p., for four days), GSH content in the liver of the MDMA-treated rats was significantly decreased, 3 or 6 hours after the last MDMA exposure. Seven days later, the GSH content returned to the control levels (Beitia et al 2000). Another study in rats (200-250 g) reported decreases in cardiac GSHt levels after a single MDMA administration (20 mg/kg, i.p.), 3 and 6 h after the exposure. No differences were found in GSSG levels (Cerretani et al 2008). In fact, the

heart has unique features that render it a target organ for oxidative stress-related injuries, including those related to drug exposure (Costa et al 2013). Several studies conducted *in vitro* with rat and mice hepatocytes demonstrated that one of the toxic events observed after exposure to MDMA or MDMA metabolites is the depletion of GSH (Carvalho et al 2001, Carvalho et al 2004a, Carvalho et al 2004b). N-Me-α-MeDA and α-MeDA incubation also resulted in the GSH depletion in cardiomyocytes of rats (Carvalho et al 2004c). In this thesis, no differences were found in the GSH levels of the peripheral organs studied, but the doses of MDMA were lower than those reported by the previous *in vivo* studies, and the GSH levels were assessed 24 h after the MDMA administration, contrarily to the 3 and 6 h reported in the referred studies. Additionally, it is possible that at earlier time-points, there would be changes in the GSH levels that were transient. In fact, the Beitia and colleagues argues, even with high MDMA dose (total cumulative dose of 160 mg/kg) that GSH levels changes in the liver might be transient (Beitia et al 2000).

Herein, a tendency to an increase in protein carbonylation in kidneys was observed, but it did not reach statistically significance. The liver and heart presented no alterations in this oxidative stress parameter. Protein carbonylation degree is an indicator of protein oxidation. An *in vivo* study reported increases in protein carbonyls in the liver of rats treated with MDMA (10 mg/kg, p.o., twice daily, for two days), 12 h after the exposure to the drug (Upreti et al 2011). Another study reported increases in protein carbonylation in liver and kidneys of mice (7 weeks old). METH administration (10 mg/kg, i.p., every 2 h, for 8 h) promoted significant increases in protein carbonyls in liver and kidneys of mice, 24 h after the last administration (Zhang et al 2012). Although these studies were performed in different animals using a different dose regimen and not only with MDMA, they corroborate the tendency of increased protein carbonylation in peripheral organs after administration of amphetamines.

Using an MDMA regimen comparable to human adolescent doses, and using adolescent rats, the present study only found augmented quinoprotein levels in the liver of MDMA-treated rats, 24 h following administration of MDMA. No changes could be seen in other parameters, such as glutathione levels and protein carbonylation in the peripheral organs of adolescent rats. Altogether these results imply that a threshold MDMA dose exists, possibly above the one herein used, to promote very significant oxidative stress-related damage. Additionally, adolescent animals could be less susceptible to oxidative stress related changes than adult animals. The time-point for oxidative stress related parameters evaluation seems also to be important.

In the peripheral organs, we were not able to find alterations in the ATP content of the MDMA-treated rats. *In vitro* studies in freshly isolated rat hepatocytes reported decreases of the ATP content induced by high concentrations of MDMA and its metabolites in a

concentration- and time-dependent manner (Beitia et al 1999, Carvalho et al 2004b, Nakagawa et al 2009). A study in isolated adult rat cardiomyocytes also reported ATP depletions after exposure to high concentrations of MDMA metabolites (Carvalho et al 2004c). No *in vivo* studies were found in the literature, reporting the ATP levels in the peripheral organs of laboratory animals following MDMA exposure. The present results imply that MDMA moderate doses, which can be comparable to those used by humans, do not acutely impair the energetic status of peripheral organs of adolescent rats.

In this thesis, the histological examination of the liver of MDMA-treated rats found marked cellular vacuolization in the periportal regions, and sinusoidal dilatation with periportal and centrilobular vascular congestion. In the heart, punctual signs of cardomyocyte oedema, particularly in the sub-endocardic region, were found after MDMA exposure. The kidneys of MDMA-treated adolescent rats presented scattered interstitial oedema, with dilatation between the nefrotic tubular structures, and signs of vascular congestion. In the three organs studied there were no signs of necrosis or interstitial inflammatory cell infiltration induced by the drug.

In experiment one, we found no alterations in the plasma levels of CK-R, CK-MB, AST and ALT following MDMA administration to the adolescent rats. Since these parameters are biomarkers of liver or heart integrity, the absence of necrosis in the histological examination corroborates the data of these plasmatic parameters. Beitia and co-workers reported changes in AST and ALT serum activities after MDMA exposure to Wistar rats, using higher MDMA doses than our study. AST activity significantly increased 6 h after a single MDMA dose (20 mg/kg, i.p.) and 3 and 6 h after a repeated MDMA administration (20 mg/kg x 2, i.p., for four days), remaining significantly elevated for seven days after the last drug administration. The single MDMA dose (20 mg/kg, i.p.) also promoted increases in ALT activity 6 h after the administration, while the repeated MDMA treatment (20 mg/kg x 2, i.p., for four days) had no effect on ALT activity (Beitia et al 2000). The intracellular enzymes leakage to extracellular medium is suggestive of irreversible damage, being AST and ALT serum activities markers of liver injury, and indicators of important cell necrosis. For that reason, in the previous study mentioned, Beitia and co-workers also made the histological hepatic examination of the animals. Six hours after the acute MDMA administration, researchers observed necrosis on portal areas with inflammatory infiltration, and the repeated administration also promoted hepatic necrosis and inflammatory infiltrate around the hepatic vein, being more marked 6 h after the last administration and being consistent with drug-induced hepatitis. Seven days later, no liver changes were reported, raising the possibility that these changes might be transient (Beitia et al 2000). A study in humans, also reported a case with a 19 years old male that presented morphological signs of acute hepatitis, after the ingestion of two

"ecstasy" tablets, with a full recovery after two months (Brncic et al 2006). In another study in rats, researchers reported alterations in cardiac morphology with a single MDMA administration (20 mg/kg, i.p.). Six hours after MDMA administration, myocardial necrosis was observed, and 16 h after the MDMA administration the heart presented macrophagic monocytes around the necrotic myocardial cells. Twenty-four hours later, the referred infiltrate became more intense and widespread, and the removal of the necrotic material had already begun (Cerretani et al 2008). Other studies in humans reported several histopathological evidences of MDMA toxicity to the kidneys of "ecstasy" consumers. These case reports demonstrated that MDMA induced tubular degeneration and necrosis and inflammatory infiltrates (Bingham et al 1998, Dar & McBrien 1996, Fineschi et al 1999).

The absence of severe tissue damage promoted by MDMA to adolescent rats was also confirmed by the inexistence of increases in caspases activity in every peripheral organ studied, since they are crucial components in apoptotic pathways (Parrish et al 2013). We found no alterations in caspase-3 and -9 activities, in the three organs, after MDMA exposure. The caspase-8 activity remained unaltered in the heart and kidneys of the MDMA-treated animals, but the drug promoted decreases in this caspase activity in the liver of the treated rats. The caspase-8 activity decrease probably occurred due to an attenuation of a natural on-going degenerative process (Kudryashova et al 2012). Tiangco and co-workers reported that MDMA exposure to cultured rat striated cardiac myocytes caused a repression of caspase-1 and caspase-8 genes, being the adverse effects of MDMA not caused by an activation in the apoptotic mechanism (Tiangco et al 2005).

5.2. Experiment 2

5.2.1. Age related differences in MDMA-induced hyperthermia and in weight, food and water intake

As in experiment one, significant increases in the rats' body temperature were also observed after the MDMA administration to either adolescent or aged rats. There were also differences in the hyperthermic profile of aged and adolescent rats. In the case of aged rats only after the second MDMA dose the increase in body temperature could be significant, meanwhile in adolescent rats temperature was already significantly higher following the first dose. The hyperthermia induced by the MDMA binge administration had a higher magnitude in aged rats than in the adolescents.

The rats' body temperature was also evaluated for the following seven days after the exposure, and 24 h post-MDMA administration the temperature of the aged animals was significantly elevated, leading to the belief that the animals body temperature possibly remained elevated for 24 h after MDMA exposure. In contrast, adolescent rats, in both experiments, presented no alterations in body temperature 24 h after MDMA exposure, in comparison to controls. This certainly argues for a higher susceptibility of aged rats towards MDMA-induced impaired thermoregulation.

In fact, as previously reported, in experiment two, one of the aged animals didn't resist to the elevated temperatures that surpassed 41ºC, possibly as a consequence of the extreme hyperthermia. As previously reported by Goni-Allo and co-workers, it was observed that the same MDMA binge regimen of administration used in our study (5 mg/kg x 3, i.p., 2 h apart) promoted in adult Wistar rats a death rate of 5% at an environmental temperature of 21ºC. Meanwhile if MDMA administration was conducted with a single injection of 15 mg/kg (i.p) to the same rat strain, the mortality rate would reach 20% at an environmental temperature of 21ºC (Goni-Allo et al 2008). In our study conducted with adolescent PND 40 rats, no signs of severe hyperthermia could be seen and the MDMA regimen did not cause any death to this population. Thus, it is reasonable to assume that animal aging increases the susceptibility for severe MDMA-induced hyperthermia. The susceptibility to hyperthermia and death events is certainly dependent on the MDMA dose regimen, but also the animal age and strain (Capela et al 2009).

In experiment two, MDMA promoted a decrease in the body weight of adolescent animals, in comparison to controls. Meanwhile, in experiment one the animals' body weight decreased in both controls and MDMA-treated animals. That probably happened due to the fact that the experiments were conducted in different vivarium conditions. In experiment one, animals were housed in individually ventilated cages, creating a more stressful procedure, meanwhile in experiment two they were housed in normal cages. The decrease in body weight is probably related with the anorectic properties of MDMA (Cohen 1995). Piper and Meyer showed that the repeated MDMA treatment (10 mg/kg, s.c., twice daily, 4 h apart, for 25 days) produced an anorectic effect in adolescent rats (Piper & Meyer 2004), corroborating the findings in this thesis. Looking at other parameters, no significant differences were found in food or water consumption for the adolescent animals in both experiments. Importantly, aged animals of experiment two, presented decreases in food consumption 24 h after the MDMA administration and increases 48 h post-MDMA administration. Moreover, the aged animals of the experiment two, presented increases in water intake in the next 24 and 48 h, after the MDMA administration. Aged animals appear to be more susceptible to the MDMA-induced physiological changes than the adolescent animals. The higher magnitude of the

hyperthermic response in aged animals also supports for the need of these animals to increase their water intake.

5.2.2. Age-related changes in cerebellum

MDMA effects in the cerebellum have been scarcely studied and little is known about the susceptibility of different aged groups. Thus, in this thesis the neurotoxicity of MDMA in cerebellum was assessed, by evaluating its energetic and oxidative stress status, in adolescent and old rat animals after MDMA-binge administration. In humans, MDMA neurotoxic effects were associated to decreased grey matter in the cerebellum (Cowan et al 2003). Cowan and co-workers performed a study with 31 MDMA polydrug human users (average age of 21.7 ± 3.3 years old and abstinent from MDMA at least three weeks before starting the study) versus 29 non-MDMA users (average age of 24.3 ± 3.5 years old) and they compared their brain grey and white matter concentration. They found decreases in grey matter concentration in several brain areas, including the cerebellum (Cowan et al 2003). Meanwhile, in an experiment conducted with six weeks-old Sprague-Dawley rats revealed significant decreases in 5-HT and 5-HIAA levels in the rats cerebellum, seven days after the exposure to a single MDMA dose (40 mg/kg, i.p.) (Zheng & Laverty 1998). Another study in Sprague-Dawley rats (175–200 g) observed significant decreases in 5-HT uptake sites, in several brain regions, 18 h and two weeks, following the last MDMA administration (20 mg/kg, twice daily, 12 h apart, for four consecutive days). In cerebellum, no differences were found in the 5-HT uptake sites with that dose regimen (Battaglia et al 1991). Unfortunately, none of the previous mentioned studies evaluated the oxidative stress parameters in the cerebellum.

In the cerebellum, we observed depletion of the ATP content in aged rats, seven days after the exposure to MDMA. A study from Shiba and co-workers reported decreases in ATP levels in the brain of adolescent (4 weeks old) Wistar rats 30 min after a single METH administration (5 mg/kg, i.p.). The same study also showed that 24 h after the exposure, ATP brain levels were normalized to control levels (Shiba et al 2011). The results presented in this thesis with aged animals reveal that the decrease in the ATP levels in the cerebellum seem to be more long lasting, in opposite to the effects in adolescent animals described by Shiba and co-workers. As mentioned above, the ATS used, the dosage regimen, the animals' age, and the time elapsed until the measurements seem to be important factors regarding the impairment in the brains' energetic functions. All the oxidative stress parameters remained unaltered in the cerebellum of both adolescent and aged MDMA-treated rats. As previously described for experiment one, several studies reported depletions of GSH (Achat-Mendes et al 2007, Frenzilli et al 2007)

and ATP (Chan et al 1994, Darvesh & Gudelsky 2005, Shiba et al 2011), and increases in quinoproteins (Miyazaki et al 2006) and protein carbonylation (Alves et al 2007), in the brain, following the exposure to MDMA or other ATS. However, these studies used different dose regimens and the time elapsed until the measurements, as well as the brain areas assessed were diverse. Besides, none of the referred studies evaluated the oxidative stress related changes in the cerebellum.

No studies were found regarding the age-related neurotoxicity induced by MDMA in cerebellum, although some were found in other brain areas. Some studies suggest that young animals are more resistant to immediate neurological damages promoted by MDMA. Nevertheless, the long-term effects are scarcely investigated. Klomp and co-workers administered MDMA (10 mg/kg, s.c., twice daily for four days) to adolescent rats at PND 27 and to adult rats at PND 63 (+/- five days), and observed significant reductions in 5-HTT binding in several brain regions seven days after the last MDMA administration. The reductions were less pronounced in adolescent rats (ranging from 20% to 69%) when compared to adult rats (ranging from 35% to 75%). Besides, the degree of 5-HTT loss in adolescent rats (35%) was, also, less extensive when compared to adult rats (49%) after MDMA exposure (Klomp et al 2012). In another study, Sprague-Dawley rats were injected with MDMA (20 mg/kg, s.c., twice daily, in four consecutive days) starting on embryonic day 15, or PND 10, 15, 20, 25, or 30 and were sacrificed on PND 40. Other group was also injected, with the same dose regimen, on PND 90, being sacrificed ten days after the drug treatment (Kelly et al 2002). The researchers observed no differences in the 5-HTT sites, on PND 40, in the brain of rats treated with MDMA at the development stages from E15 to PND 20, when compared to controls. However when MDMA treatment started on PND 25 or later, it resulted in significant reductions in the density of [3H]-paroxetine binding sites, with decreases of 46% at PND 25, and 63% at PND 30 compared to control, being the referred reductions not as intense as in animals treated at PND 90 (density of [3H]-paroxetine binding sites decreased by 90%) (Kelly et al 2002). These studies confirm that young animals are, apparently, less susceptible to MDMA than adults.

In summary, the results in experiment two show that MDMA binge administration leads to hyperthermia in adolescent and old rats, with a higher magnitude in aged animals. One week after the MDMA administration, aged animals presented significant decreases in the cerebellum ATP content, indicating a higher susceptibility of this group to the neurotoxic effects of MDMA. Altogether, these findings confirm that aged animals are more susceptible to MDMA toxic actions than adolescents.

5.3. Conclusions

In conclusion, the moderate binge dosage regimen of MDMA used, which was non-neurotoxic to the serotonergic system and equivalent to the ones used by adolescent humans, did not promote any significant changes in oxidative stress related parameters in the brain areas of adolescent rats. Moreover, the same dose regimen acutely promoted a decrease in the ATP cortex content. Though frequently not evaluated in most studies, in the cerebellum, MDMA promoted neurotoxic effects in aged rats. Unfortunately, studies are mainly focused in the neurotoxicity of MDMA and neglect the toxicity promoted by this drug to the peripheral organs. The MDMA binge scheme used in this study promoted morphological tissue alterations to the peripheral organs of adolescent animals and increases in protein-bound quinone formation in the liver. More studies are needed to evaluate the impact of moderate MDMA doses in the brain and peripheral organs of adolescent animals and explore the different effects of MDMA to animals in dissimilar age stages.

Part VI

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