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Neuronal dysfunction induced by drugs of abuse

Disfunção neuronal induzida por drogas de abuso

Universidade de Coimbra

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Ao meu filhote Miguel

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ABBREVIATIONS

8OHdG	8-hydroxy-2-deoxyguanosine
$\Delta\Psi_m$	Mitochondrial membrane potential
A1	Bcl-2-Related Protein A1
Ac-DEVD-pNA	N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide
Ac-IEPD-pNA	N-Acetyl-Ile-Glu-Pro-Asp p-nitroanilide
Ac-LEHD-pNA	N-Acetyl-Leu-Glu-His-Asp p-nitroanilide
Ac-VDVAD-pNA	N-Acetyl-Val-Asp-Val-Ala-Asp p-nitroanilide
Ac-VEID-pNA	N-Acetyl-Val-Glu-Ile-Asp p-nitroanilide
ACTH	Adrenocorticotropic hormone
ADHD	Attention-Deficit Hyperactivity Disorder
ADP	Adenosine Diphosphate
AIF	Apoptosis Inducing Factor
APAF-1	Apoptotic Protease Activating Factor
AMP	Adenosine Monophosphate
ANT	Adenine Nucleotide Translocator
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
Bad	Bcl-2-antagonist of cell death
Bak	Bcl-2 antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell leukemia/lymphoma 2
Bcl w	Bcl-2-Like 2 Protein
Bcl-X_L	Bcl-2-Like 1 Protein large
BDNF	Brain Derived Neurotrophic Factor
BH	Bcl-2 homology
Bid	BH3 interacting domain death agonist
Bim	Bcl-2-interacting mediator of cell death
Bik	Bcl-2-interacting killer
Boo	Bcl-2-like 10 protein
Bok	Bcl-2-related ovarian killer
BSA	Bovine Seric Albumin
cAMP	Cyclic Adenosine Monophosphate
CD95	Cluster of Differentiation 95
CG	Cingulate Gyrus
CNS	Central Nervous System
COMT	Catechol-O-Methyl Transferase
CREB	cAMP response element binding
CRF	Corticotropin-releasing factor
CTOP	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2
CypD	Cyclophylin D
dAP5	D-2-amino-5-phosphonopentanoate
DAT	Dopamine Transporter
dATP	Deoxy-ATP
DFF/CAD	DNA fragmentation factor/caspase-associated DNAse
DG	Dentate Gyrus

DHBA	Di-hydroxy-benzylamine
DMSO	Dimethyl sulfoxide
DOPAC	3,4-dihydroxyphenylacetic acid
ECF	Enhanced Chemofluorescence
EndoG	Endonuclease G
ER	Endoplasmic Reticulum
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
fMRI	Functional Magnetic Ressonance Imaging
GABA	Gamma aminobutyric acid
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GPx	Glutathione peroxidase
GRK	G-protein receptor kinases
GRed	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione-S-Transferase
H_2O_2	Hydrogen peroxide
HEK	Human Embryonic Kidney
HNE	Hydroxy-nonenal
HPA	Hypothalamic-pituitary-adrenal axis
HPLC	High Performance Liquid Chromatography
ICS	Intracristal space
IDT	Instituto da Droga e da Toxicodependência
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
LAAM	Levo-alpha-acetylmethadol
LDH	Lactate dehydrogenase
L-DOPA	L-3,4-dihydroxyphenylalanine
LSD	Lysergic acid
6-MAM	6-Monoacetylmorphine
MAO	Monoamine Oxidase
MAP-2	Microtubule Associated Protein 2
MAPK	Mitogen-Activated Protein Kinase
Mcl1	Myeloid Cell Leukemia-1
MDA	Malondialdehyde
MDMA	Methylenedioxymethamphetamine
Meth	Methamphetamine
MK-801	Dizocilpine maleate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
NAc	Nucleus Accumbens
NADH	Nicotinamide Adenine Dinucleotide
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NBT	Nitroblue-Tetrazolium
NET	Norepinephrine Transporter
NMDA	N-methyl-D-aspartate
Noxa	From the latin word for damage (*noxa*)
NR	NMDA receptor
NT2	Neuroteratocarcinoma cells

OFC	Orbitofrontal Cortex
˙OH	Hydroxyl radical
Omi/HtrA2	Omi protease/High temperature requirement protease A2
OMM	Outer mitochondrial membrane
Opa-1	Optic Atrophy 1
PARL	Presenilin-associated rhomboid like
PARP	Poly (ADP-ribose) polymerase
PBR	Peripheral Benzodiazepine Receptor
PBS	Phosphate Buffered Saline
PC12	Pheochromocytoma 12
PET	Positron Emission Tomography
Pi	Inorganic Phosphate
PKA	Protein kinase A
PKC	Protein kinase C
POMC	Proopiomelanocortin
PTP	Permeability Transition Pore
PUMA	P53 Upregulated Modulator of Apoptosis
PVDF	Poly(vinylidene fluoride)
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute medium
SBTI	Soybean Trypsin Inhibitor
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS-Polyacrilamide Gel Electrophoresis
SERT	Serotonin Transporter
SGZ	Subgranular Zone
Smac/DIABLO	Second Mitochondria-Derived Activator of Caspases/ Direct IAP-Binding Protein with Low pI
SOD	Superoxide Dismutase
SVZ	Subventricular Zone
SY5Y	Human bone marrow neuroblastoma cells
TBARS	Thio-Barbituric Acid Reactive Substances
TH	Tyrosine Hydroxylase
TNF	Tumor Necrosis Factor
TRAIL	TNF-related apoptosis inducing ligand
TUNEL	Terminal dUTP nick-end labeling
VDAC	Voltage Dependent Anion Channel
Vit E	Vitamin E
VMAT	Vesicular Monoamine Transporter
VTA	Ventral Tegmental Area
z-VAD-fmk	Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone

RESUMO

O abuso de substâncias e a toxicodependência estão entre as mais dispendiosas desordens neuropsiquiátricas. O abuso repetido de drogas está associado a alterações da actividade neuronal. Neste trabalho estudámos a disfunção neuronal induzida pelas drogas de abuso cocaína, anfetamina e heroína de rua, usando modelos *in vitro*. O trabalho experimental foi dividido em dois capítulos. No primeiro capítulo estudámos os efeitos crónicos das drogas de abuso estimulantes, cocaína e da anfetamina e no segundo capítulo estudámos os efeitos crónicos e agudos da heroína de rua.

No primeiro capítulo, os efeitos da cocaína e da anfetamina foram avaliados em células PC12, uma linha celular catecolaminérgica, uma vez que os principais alvos dos estimulantes são os sistemas monoaminérgicos. A dopamina, em particular, está associada aos efeitos de recompensa induzidos por todas as drogas de abuso, e é um possível mediador dos efeitos neurotóxicos das drogas de abuso devido ao seu metabolismo oxidativo, que leva à produção de H_2O_2. Observámos que o tratamento crónico com cocaína (7-12 meses) sensibilizou as células PC12 para a toxicidade aguda da cocaína, o que poderá estar relacionado com o aumento da acumulação extracelular de dopamina induzido por um estímulo agudo com cocaína. A exposição crónica à anfetamina induziu deplecção da dopamina, embora a toxicidade aguda da anfetamina se tivesse mantido nestas células, sugerindo que a dopamina não é necessária para a citotoxicidade aguda da anfetamina. Contudo, as células PC12 tratadas cronicamente com H_2O_2 apresentaram-se totalmente resistentes à citotoxicidade aguda do H_2O_2, mas não à citotoxicidade aguda da cocaína ou da anfetamina, sugerindo que a toxicidade aguda destas drogas é independente da adaptação ao stresse oxidativo. Em contraste, as células cronicamente expostas à cocaína ou à anfetamina apresentaram-se parcialmente resistentes ao H_2O_2, sugerindo que a exposição crónica a estas drogas envolve adaptação ao stresse oxidativo. Assim, estudámos a actividade de enzimas antioxidantes nas etapas iniciais

da exposição crónica à cocaína, anfetamina ou H_2O_2. A cocaína e a anfetamina afectaram de um modo diferente a actividade das enzimas antioxidantes. A cocaína induziu um aumento da actividade da glutationa peroxidase (GPx) após 4 semanas de exposição. A anfetamina apresentou um efeito difásico, com um aumento inicial da actividade da GPx e uma diminuição das actividades da glutationa redutase (GRed) e da superóxido dismutase (SOD), após 3 semanas de exposição. Após 4 semanas de exposição à anfetamina a actividade das enzimas antioxidantes regressou a valores semelhantes ao controlo. A exposição crónica ao H_2O_2 induziu um aumento gradual da actividade da GPx e uma diminuição da actividade da SOD. A actividade da GRed aumentou após 1 semana de exposição ao H_2O_2, regressando a valores semelhantes ao controlo após 2 semanas de exposição ao H_2O_2. Estes resultados indicam que as primeiras etapas de adaptação à cocaína e à anfetamina envolvem alterações da actividade das enzimas antioxidantes, sugerindo uma adaptação ao stresse oxidativo, após exposição crónica a estas drogas de abuso.

No segundo capítulo, estudámos os efeitos neurotóxicos da heroína de rua. Os efeitos crónicos da heroína de rua foram avaliados em células PC12. Apesar de os opiáceos não interagirem directamente com os sistemas monoaminérgicos, foi anteriormente demonstrado que as células PC12 apresentam um decréscimo dos níveis intracelulares de dopamina e um aumento dos níveis intracelulares de ácido dihidroxifenilacético (DOPAC), quando expostas agudamente à heroína de rua. A exposição crónica à heroína de rua sensibilizou as células PC12 para a toxicidade aguda desta droga de abuso, o que pode estar relacionado com um aumento da acumulação extracelular de dopamina induzido pela exposição aguda à heroína de rua nestas células. As células cronicamente expostas à heroína de rua apresentaram um aumento da resistência à toxicidade aguda do H_2O_2, que se relacionou com a manutenção dos níveis de ATP nestas condições. Contudo, as células cronicamente expostas à heroína de rua apresentaram níveis intracelulares de ATP e ADP mais baixos, o que pode explicar o maior grau de sensibilização destas células a uma exposição aguda à heroína de rua, quando comparadas com as células cronicamente expostas ao H_2O_2.

A neurotransmissão glutamatérgica envolvendo o receptor N-metil-D-aspartato (NMDA) contribui para a dependência de opiáceos em humanos. Por outro lado, a sobre-activação dos receptores ionotrópicos do glutamato é um dos mecanismos envolvidos na neurotoxicidade e na morte celular. De modo a avaliar a importância do receptor NMDA na citotoxicidade aguda da heroína de rua, utilizaram-se células HEK293 transfectadas com diferentes subunidades do receptor NMDA e neurónios corticais. As células HEK293 foram transfectadas com as subunidades NR1-GFP como controlo, ou NR1/NR2A ou NR1/NR2B. A heroína de rua induziu perda da integridade membranar em células transfectadas com as subunidades NR1/NR2B, e este efeito foi bloqueado pelo maleato de dizocilpina (MK-801), um antagonista dos receptores NMDA, indicando que os receptores NMDA compostos pelas subunidades NR1/NR2B estão envolvidos na neurotoxicidade da heroína de rua.

A disfunção do córtex pré-frontal é uma característica importante da toxicodependência, contribuindo para a perda do controlo da impulsividade observada em toxicodependentes. Neste trabalho, determinou-se a composição química da amostra de heroína de rua e estudaram-se as vias apoptóticas envolvidas na neurotoxicidade desta droga de abuso, usada numa concentração que induziu um decréscimo moderado da viabilidade celular. A composição da heroína de rua apresentou 62% de heroína, 12% de 6-monoacetilmorfina e 1% de morfina. Quando usada numa concentração que induziu 10% de perda de viabilidade celular sem perda de integridade membranar, a heroína de rua induziu a activação de caspases da via apoptótica mitocondrial. Foi também observada fragmentação do ADN, que foi prevenida pelo inibidor não-selectivo de caspases z-VAD-fmk. A activação da caspase-3 não foi prevenida por antagonistas dos receptores opióides, antagonistas dos receptores ionotrópicos do glutamato nem por antioxidantes. A activação das caspases pareceu ter sido mediada pela disfunção mitocondrial envolvendo a libertação do citocromo c, a perda de potencial da mitocôndria e um decréscimo da razão Bcl-2/Bax. A heroína pura, na mesma concentração presente na heroína de rua, induziu apenas um pequeno aumento da activação da caspase-3, sugerindo que a activação da caspase-3 é potenciada pela presença da mistura de compostos

presentes na heroína de rua. Este resultado indica também que o abuso de heroína impura representa um risco neurotóxico superior.

Estes resultados demonstram que as drogas de abuso induzem neurotoxicidade através de vários mecanismos celulares, culminando em stresse oxidativo e disfunção mitocondrial, que poderão representar importantes alvos para futuras estratégias terapêuticas.

ABSTRACT

Substance abuse and addiction are the most costly of all the neuropsychiatric disorders. The repeated abuse of drugs is associated with changes in neuronal activity. In this work, we studied the neuronal dysfunction induced by the drugs of abuse cocaine, amphetamine and *street* heroin, using *in vitro* models. The experimental work was divided into two chapters. In the first chapter we studied the chronic effects of the psychostimulant drugs cocaine and amphetamine, and in the second chapter we studied the chronic and acute effects of *street* heroin.

In first chapter, chronic effects of cocaine and amphetamine were evaluated in PC12 cells, a catecholaminergic cell line, since the main targets of the stimulants are the monoaminergic systems. Dopamine, in particular, is associated with the rewarding effects of all the drugs of abuse, and is a candidate mediator of the neurotoxic effects of the drugs due to its oxidative metabolism, which produces hydrogen peroxide (H_2O_2). We observed that chronic cocaine treatment (7-12 months) sensitizes PC12 cells to the acute toxicity of cocaine, which may be related to increased cocaine-evoked extracellular dopamine accumulation in these cells. Chronic exposure to amphetamine induced dopamine depletion, but amphetamine maintained its acute toxicity in these cells, suggesting that dopamine is not required for acute amphetamine cytotoxicity. However, PC12 cells chronically treated with H_2O_2 were totally resistant to acute H_2O_2, but not to acute cocaine or amphetamine exposure, implicating that the acute toxicity induced by these stimulant drugs is unrelated to adaptation to oxidative stress. In contrast, cells chronically exposed to cocaine or amphetamine were partially resistant to H_2O_2, suggesting that chronic exposure to these drugs involves adaptation to oxidative stress. Therefore, we studied the activity of antioxidant enzymes in the early stages of exposure to cocaine, amphetamine and H_2O_2. Cocaine and amphetamine differentially affected the activity of the antioxidant enzymes. Cocaine induced an increase in the activity of glutathione peroxidase (GPx) at 4 weeks of exposure. Amphetamine seemed to have a biphasic effect, with an initial increase in GPx activity and a decrease in glutathione reductase (GRed) and superoxide

dismutase (SOD) activities (upon 3 weeks of exposure). One week later the activities returned to control levels. Chronic exposure to H_2O_2 induced a gradual increase in GPx activity and a decrease in SOD activity. GRed activity was increased upon 1 week exposure to H_2O_2, and returned to control levels after 2 weeks of H_2O_2 exposure. These data show that changes in the activity of antioxidant enzymes are involved in the chronic effects of cocaine and amphetamine, suggesting that oxidative stress is involved in the chronic effects of these drugs of abuse.

In the second chapter we studied the neurotoxic effects of *street* heroin. Chronic effects of *street* heroin were also evaluated in PC12 cells. Although opiates do not directly interact with monoaminergic systems, PC12 cells were previously shown to have decreased intracellular dopamine levels and increased intracellular dihydroxyphenylacetic acid (DOPAC) levels, when acutely exposed to *street* heroin. Chronic exposure to *street* heroin or H_2O_2 sensitized PC12 cells to acute *street* heroin cytotoxicity, which may be related to increased extracellular dopamine accumulation induced by acute *street* heroin in these cells. Cells chronically exposed to *street* heroin presented increased resistance to acute H_2O_2 toxicity, which was correlated with the maintenance of ATP levels in these conditions. However, cells chronically exposed to *street* heroin presented lower intracellular levels of ATP and ADP, which may explain the higher degree of sensitization of these cells to acute *street* heroin exposure, when compared to cells chronically exposed to H_2O_2.

Glutamatergic neurotransmission involving the N-methyl-D-aspartate (NMDA) receptor has been suggested to contribute to opiate dependence in humans. Moreover, hyperactivation of ionotropic glutamate receptors is one of the mechanisms involved in neurotoxicity and cell death. Taking this into account, we analysed the contribution of NMDA receptor subunits to acute neurotoxicity of *street* heroin n HEK293 cells, transfected with different subunits of the NMDA receptor, and in cortical neurons. HEK293 cells were transfected with NR1-GFP, as a control, or with NR1/NR2A or NR1/NR2B subunits. *Street* heroin induced loss of membrane integrity in NR1/NR2B transfected cells, and this effect was blocked by the NMDA receptor antagonist dizocilpine maleate (MK-801), indicating that NR1/NR2B composed receptors are involved in *street* heroin neurotoxicity.

Prefrontal cortex dysfunction is an important event in drug addiction, contributing to the loss of impulse control observed in drug addicts. The chemical composition of the *street* heroin sample was determined and the apoptotic mechanisms involved in the neurotoxicity of a concentration of *street* heroin that induced a moderate decrease in cell viability were studied. *Street* heroin was composed by 62% heroin, 12% 6-monoacetylmorphine (6-MAM) and 1% morphine. In a concentration that induced 10% loss in cell viability without causing loss of membrane integrity, *street* heroin was shown to activate caspases through the mitochondrial apoptotic pathway. DNA fragmentation was also observed, and was prevented by the non-selective caspase inhibitor z-VAD-fmk. Caspase-3 activation was not prevented by μ-opioid receptor antagonists, ionotropic glutamate receptor antagonists or by antioxidants. The activation of caspases seemed to be mediated by mitochondrial dysfunction involving cytochrome c release, loss of mitochondrial potential and a decrease in Bcl-2/Bax ratio. Pure heroin, in the same concentration found in *street* heroin, only induced a small increase in caspase-3 activation, suggesting that caspase-3 activation is enhanced by the presence of the cocktail of compounds found in the *street* heroin sample. This also indicates that the use of impure heroin represents an increased neurotoxic risk.

These results show that the drugs of abuse cause neurotoxicity by several intracellular pathways, culminating in oxidative stress and mitochondrial dysfunction, which may represent important targets for future therapeutic strategies for drug addiction.

OUTLINE

This thesis is divided into the following sections:

- **Part I – Introduction**

This section reviews the most relevant literature related with neuronal dysfunction in drug addiction. It also provides the rationale for the objectives of this thesis.

- **Part II – Material and Methods**

This section contains the descriprion of the materials and methods used to perform the studies presented in Part III

- **Part III – Results and Discussion**

This section includes the experimental data and the discussion of the results.

- **Part IV - Conclusions**

This section contains the general conclusions of the work presented in Part III.

PART I - INTRODUCTION

1.1 Historical and geographical perspective of drug abuse

Drugs with psychoactive properties have been used by humans for centuries. The use of plants containing hallucinogenic substances is thought to have played an important role in the development of philosophy and religious thought in early human cultures (Nichols, 2004). Plant derivatives have also been used medicinally in various cultures, as is the case of opium, which has been used for centuries to treat pain and other symptoms (von Zastrow and Evans, 2006).

Cocaine was first isolated in 1855 by the german chemist Friedrich Gaedcke. This alkaloid is extracted from the plant *Erythroxylum coca,* which is cultivated in the South American countries Bolivia, Colombia and Peru. The natives of these countries chew the coca leaves in magical cerimonies and initiation rites. Cocaine was present in coca-cola, which was invented in 1886 and was removed from its formula in 1903.

Cocaine may be processed in water soluble or insoluble forms. Water soluble forms include cocaine sulphate and cocaine hydrochloride. These forms are active by oral, intranasal and intravenous administrations. In contrast, water insoluble forms such as free base cocaine or crack are mainly active through smoking, a route of administration that enables immediate absorption into the blood and a rapid, short, but very intense euphoric effect.

According to the United Nations Office on Drugs and Crime (2006), cocaine is mainly processed in its countries of origin, from where it is illegally transported to the rest of the world.

Amphetamine was first synthesized in 1887 by Lazar Edeleano at the University of Berlin. This drug is a synthetic derivative of the plant alkaloid ephedrine, extracted from plants in the genus *Ephedra*. *Ephedra sinica,* also known as Ma Huang,

has been used in traditional Chinese medicine for 5000 years to treat several diseases, such as asthma and common cold.

Amphetamines are still used medicinally to treat narcolepsy and attention-deficit hyperactivity disorder (ADHD). Amphetamine production occurs mainly in North America, East and South-East Asia and in Europe.

Opium is extracted from the opium poppy (*Papaver somniferum*). Historically there are reports of cultivation of this plant in the Mesopotamia since 3400 BC (see: http://www.pbs.org/wgbh/pages/frontline/shows/heroin/etc/history.html).

Nowadays, the opium poppy is mainly cultivated in Asia, and the countries responsible for the majority of opium production are Afghanistan, Union of Myanmar and Lao People's Democratic Republic. More recently, since 1980, the opium poppy is also cultivated in the South American countries Colombia, Mexico and Peru. In 2004 most of the laboratories of opium processing were dismantled in Russia, Moldova and Afghanistan, from where opium was distributed to the rest of the world.

Opium contains about 40 alkaloids that make up 10-20% of total opium substances. The most abundant opium alkaloids are morphine (8-17%), codeine (0.7-5%), thebaine (0.1-1.5%), papaverine (0.5-1.5%) and noscapine (narcotine, 1-10%) (Schiff, 2002).

Morphine is purified from opium extracts and converted into heroin by acetylation. Heroin is more lipid soluble than morphine and is easily transported across the blood brain barrier, being 2-4 times more potent than morphine to treat acute pain (Sawynok, 1986).

Heroin was first synthesized in 1874 by C.R. Alder Wright in England but it was only discovered by the medical community when it was independently re-synthesized, 23 years later, by Felix Hoffmann, who worked for Bayer. From 1898 to 1910 heroin was commercialized by Bayer as a non-addictive morphine substitute and cough medicine for children (Figure 1.1.A). The use of heroin was thought to be a potential cure for morphine addiction until it was found that heroin is converted into morphine, when metabolized in the liver (Wikipedia, 2007).

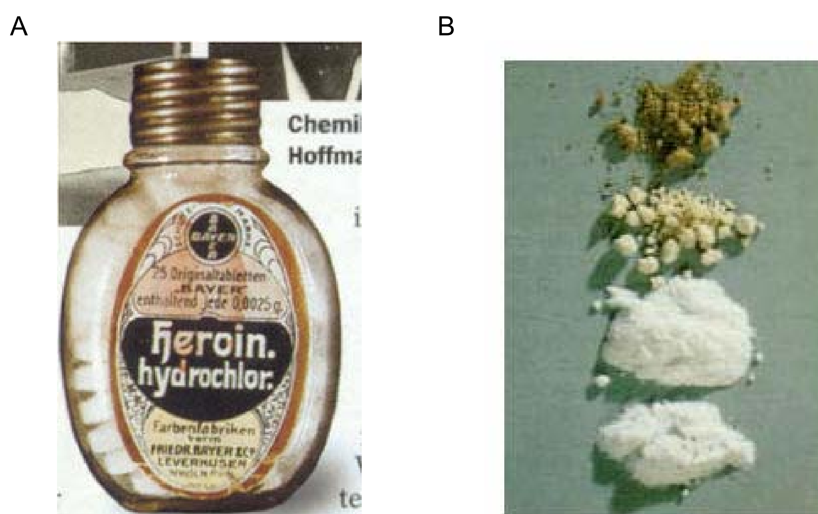


Figure 1.1: Presentations of heroin. **A**) Heroin commercialized by Bayer in the beginning of the 20th century. **B**) Heroin in different purification forms (images from http://heroin.know-library.net/ (A) and http://www.druginfo.adf.org.au/article.asp?ContentID=heroin (B)).

1.1.1 Present situation

Nowadays, the use of drugs has become a complex issue, namely due to the development of purification procedures that enable an increase in the effective quantities of the active compounds consumed. Another factor that contributed to the increase in the deleterious effects of drug abuse was the invention of the hypodermic syringe, in the mid 19th century, which allowed the direct injection of purified active compounds into the blood stream. This also contributed to increase infections among drug addicts.

Presently, drug addiction seriously affects public health and society worldwide. The Annual Report of the European Monitoring Centre for Drugs and Drug Addiction (2006) refers that the most abused drugs in Europe are cannabis, cocaine, ecstasy, amphetamines and opiates. Worldwide, cannabis is used by 3.9% of the world population, followed by the amphetamines (0.6%) cocaine (0.3%) and heroin (0.3%) (Table 1.1).

Although less consumed, opiates are the drugs that lead more people to seek treatment, due to their severe withdrawal effects and the increased risk for infections and, therefore, opiates have a strong negative economic impact.

Table 1.1: Percentage of drug users in the last 12 months

	% population (age 15-64)			
	Heroin	Cocaine	Amphetamines	Cannabis
Europe	0.6	0.7	0.5	5.6
West and Central Europe	0.5	1.1	0.7	7.4
South East Europe	0.2	0.1	0.2	2.3
East Europe	1.2	0.1	0.2	3.8
Americas	0.3	1.5	0.8	6.4
North America	0.4	2.3	1.1	10.3
South America	0.1	0.7	0.4	2.6
Asia	0.2	0.1	0.6	2.1
Oceania	0.2	0.9	3	15.3
Africa	0.2	0.2	0.4	8.1
Global	**0.3**	**0.3**	**0.6**	**3.9**
Global users	11,250,000	13,358,000	24,880,000	162,400,000
Source: **United Nations Office on Drugs and Crime, World Drug Report, 2006**				

1.2 Polydrug use

Polydrug use is an increasingly important issue in Europe (European Monitoring Centre for Drugs and Drug Addiction, 2006). A relatively common combination of drugs is the *speedball*, which consists in concurrent administration (by injection) of cocaine and heroin. *Speedball* has been reported to cause more rewarding effects in rats than cocaine or heroin alone (Ranaldi and Munn, 1998). This may be explained by the reduction of the unwanted side-effects of one drug by the other, which has different mechanisms of action, or by the fact that one drug enhances the effect of the other (Leri et al., 2003). When injected together, the different effects of the combination of drugs, when compared to the drugs alone, can be also due to chemical interactions between the molecules of the two different drugs (Garrido J. *et al.*, unpublished results). If the molecules of cocaine and heroin (or morphine) interact, this may confer different behavioural properties to the *speedball*, when compared to the drugs alone.

Ethanol is frequently combined with other drugs of abuse. When ethanol and cocaine are co-consumed, the euphoric effects of cocaine are enhanced. However, this combination also increases the toxic effects of both drugs, because the drugs are combined in the liver to form a very toxic metabolite – cocaethylene (see Figure 1.7). Cocaethylene is a very lipophilic compound and is able to cross the blood brain barrier. The effects of cocaethylene are similar to those of cocaine but the metabolite has a longer half live, prolonging the acute effects of cocaine (Henry, 2007).

1.3 Adulterants and contaminants

A critical issue associated with drug abuse is the fact that the drugs available in the streets are illegally synthesized, usually under poor conditions. Deficient purification and low quality of the reagents used often leave some impurities in the final products. Frequently, adulterants are also intentionally added to the drugs to increase profit.

Heroin is a semi-synthetic drug, obtained from acetylation of morphine. Heroin possesses little or no opioid activity (White and Irvine, 1999) but its metabolism, which may occur *in vivo* and *in vitro* (Hutchinson and Somogyi, 2002), generates 6-monoacetylmorphine (6-MAM) and morphine, two µ-opioid receptor agonists (White and Irvine, 1999) – see section 1.5.2.2.2 (Opiates and reward).

Street heroin may contain different quantities of heroin and other components depending on its origin and on the method of illicit synthesis. *Street* heroin is illegally synthesized from morphine purified from opium extracts (Figure 1.1.B). Upon illicit purification, morphine is often contaminated with other alkaloids, which may also suffer synthetic acetylation during heroin manufacture.

Depending on the purification procedure, *street* heroin may contain some impurities (Moore et al., 1984), namely morphine and 6-MAM (heroin metabolites), codeine and acetylcodeine (Soine, 1986, for review). Heroin in seized samples may also contain various inert diluents (starch, lactose, fructose, sucrose, mannitol, powdered milk) and active adulterants (caffeine, paracetamol, strychnine, acetylsalicylic acid, barbiturates, quinine and amphetamines) (Chiarotti et al., 1991;

Sharma et al., 2005). These and other frequent additives are presented in Table 1.2. Common additives of cocaine are shown in Table 1.3.

Table 1.2: Frequent heroin additives (adapted from Roth et al., 1998; Shesser et al., 1991)

Opium alkaloids	Other pharmacologically active compounds	Inert	Volatile
Thebaine	Tolemectin	Starch	Rosin
Codeine	Quinine	Sugar	Toluene
Acetylcodeine	Phenobarbital	Calcium tartarate	Methanol
Papaverine	Methaqualone	Calcium carbonate	Acetaldehyde
Noscapine	Lidocaine	Sodium carbonate	Ethanol
Narceine	Phenolphtalein	Sucrose	Acetone
	Caffeine	Dextrine	Diethyl ether
	Dextromoramide	Magnesium sulphate	Chloroform
	Chloroquine	Dextrose	Acetic acid
	Diazepan	Lactose	
	Nicotinamide	Barium sulphate	
	N-phenyl-2-naphtylamine	Silicon dioxide	
	Phenacetin	Vitamin C	
	Acetaminophen		
	Fentanyl		
	Doxepin		
	Naproxen		
	Promazine		
	Piracetam		
	Procaine		
	Diphenhydramine		
	Aminopyrine		
	Allobarbital		
	Indomethacin		
	Glutethimide		
	Scoopolamine		
	Sulfonamide		
	Arsenic		
	Strychnin		
	Cocaine		
	Amphetamine		
	Methamphetamine		

Table 1.3: Frequent cocaine additives (adapted from Roth et al., 1998; Shesser et al., 1991).

Pharmacologically active compounds	Inert	Volatile
Lidocaine	Inositol	Benzene
Cyproheptidine	Mannitol	Methyl ethyl ketone
Diphenhydramine	Lactose	Ether
Benzocaine	Dextrose	Acetone
Mepivacaine	Starch	
Aminopyrine	Sucrose	
Methapyrilene	Sodium bicarbonate	
Tetracaine	Barium carbonate	
Nicotinamide	Mannose	
Ephedrine		
Phenylpropanolamine		
Acetaminophen		
Procaine base		
Caffeine		
Acetophenetidin		
1-(1 Phenylcyclohexyl)pirrolidine		
Methaqualone		
Dyclonine		
Pyridoxin		
Codeine		
Stearic acid		
Piracetam		
Rosin (colophonum)		
Fencafamine		
Benzoic acid		
Phenothiazines		
L-Threonin		
Boric acid		
Aspirin		
Dibucaine		
Propoxyphene		
Heroin		
Amphetamine		
Methamphetamine		

1.4 Drug addiction

There are several reasons that lead teenagers to experiment drugs. In Portugal about one half of the teenagers that experimented drugs started using them at the age of 12-13 (Matos et al., 2007).

Drug use usually starts due to curiosity or by the need to have fun, becoming part of a group or to escape from physical and/or psychological problems. The acute effects of drugs induce pleasurable feelings that lead the individuals to take drugs again. Although some regular users are able to cease drug use, about 8-32% of them lose control over drug consumption, becoming drug addicts (Madras, 2006). The factors that trigger the transition from controlled drug use to addiction are drug-specific and are not completely understood.

The main characteristic of drug addiction is the compulsive drug use despite serious negative consequences. Drugs become more important than other goals and all activities are directed towards obtaining and consuming the drugs (Hyman et al., 2006). Drug addiction is associated with a reduction in life-sustaining activities that has both medical and social consequences.

When addiction develops, changes in the brain occur in order to adapt to the presence of the drug. Abstinence from the drug triggers physiological and psychological withdrawal, which are negative consequences of drug use. During withdrawal the brain is hyper-reactive and requires the drug for its proper function. Withdrawal symptoms include anxiety, stress, irritability, insomnia or hypersomnia, aches, craving, among other effects. Upon prolonged abstinence, intense craving can develop, suppressing the individual's will to control the compulsive drug-seeking, and often leading to relapse. The transition between these states is cyclic, as illustrated in Figure 1.2.

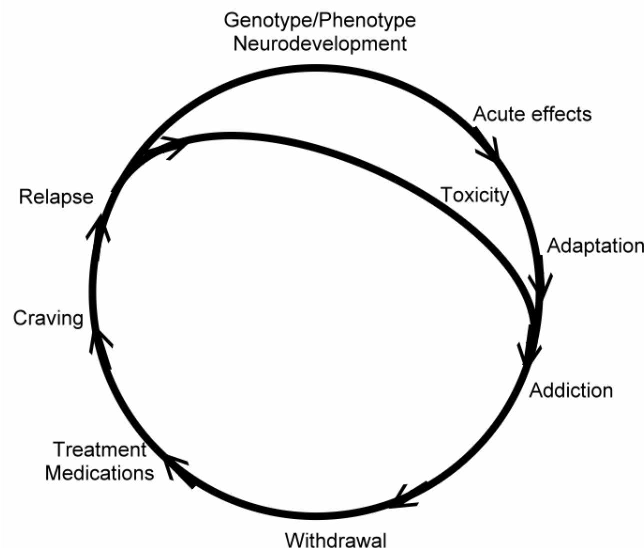


Figure 1.2: Cycle of addiction, as described by Madras (2006). Genotype, phenotype and neurodevelopment contribute to the first experience with drugs. The acute effects of the drugs lead to toxicity and adaptations that induce drug addiction and physical dependence. When the drug is absent, withdrawal symptoms appear leading to craving and relapse. During withdrawal, medications may be useful to prevent relapse.

Addiction is considered a chronic disease (Leshner, 1997), often accompanied by cognitive dysfunction with loss of impulse control towards drug consumption (Pau et al., 2002).

1.4.1 Characteristics of psychoactive drugs

Drugs with psychoactive effects can be divided into several groups, according to their specific actions. The most common illicit drugs of abuse are the **psychostimulants** (include the psychomotor stimulants amphetamines and cocaine), **depressants** (include the opiate narcotic analgesics) and **hallucinogens** (include mescaline and lysergic acid - LSD). The properties of these groups of drugs are summarized in Table 1.4.

Table 1.4: Principal effects of the main illicit drugs-of-abuse (based on Roth et al., 1998).

Effects	Acute	Chronic	Withdrawal	Drugs
Psychostimulants	Euphoria, tachycardia, hypertension, hyperthermia, increased mental alertness, seizures.	Psychosis, paranoia, reduced appetite, weight loss, heart failure, nervousness, insomnia	Severe depression (sometimes), headache	Cocaine, amphetamine, ecstasy
Depressants	Pain relief, euphoria, drowsiness/nausea, constipation, confusion, sedation, respiratory depression and arrest, hypothermia, unconsciousness, seizures, coma, death.	Depressed sexual drive, lethargy, general physical debilitation, infections, hepatitis, tolerance, addiction.	Anxiety, Insomnia, nausea, vomiting, diarrhea, anorexia, tachycardia, lacrimation, sweating, severe back pains, stomach cramps, muscle spasms.	Opium, morphine, heroin
Hallucinogens	Altered states of perception and feeling.	Persisting perception disorder (flashbacks)	No typical symptoms	Mescaline, LSD, psilocybin, ecstasy

Some drugs of abuse have effects that are common to more than one group. For example, ecstasy (or 3,4-methylenedioxymethamphetamine - MDMA) belongs to the class of psychedelic drugs, which share stimulant and hallucinogenic effects. These drugs are also known by empathogens or entactogens, because they induce feelings of empathy (Riedlinger and Riedlinger, 1994). Another example is cannabis, which shares properties of all the groups described (Murray, 1986).

1.5 Brain circuits affected by the drugs of abuse

Drugs of abuse interact with the brain, affecting brain systems that respond to physiological stimuli such as food, water and social interaction, which are critical to survival. Imaging studies, such as positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), have implicated the involvement of different brain circuits in drug addiction (reviewed by Volkow et al., 2003). The interaction of the drugs with these circuits may be different in the different phases of drug addiction: intoxication, withdrawal and craving.

The main brain circuits implicated in the effects of the drugs are the ones that mediate reward, memory, motivation/drive and control, and are mainly composed by the following brain areas:

(a) **reward**- nucleus *accumbens* (NAc) and ventral *pallidum*;
(b) **motivation/drive**- orbitofrontal cortex (OFC) and subcallosal cortex;
(c) **memory and learning**- amygdala and hippocampus;
(d) **control**- the prefrontal cortex and anterior cingulate gyrus (CG)

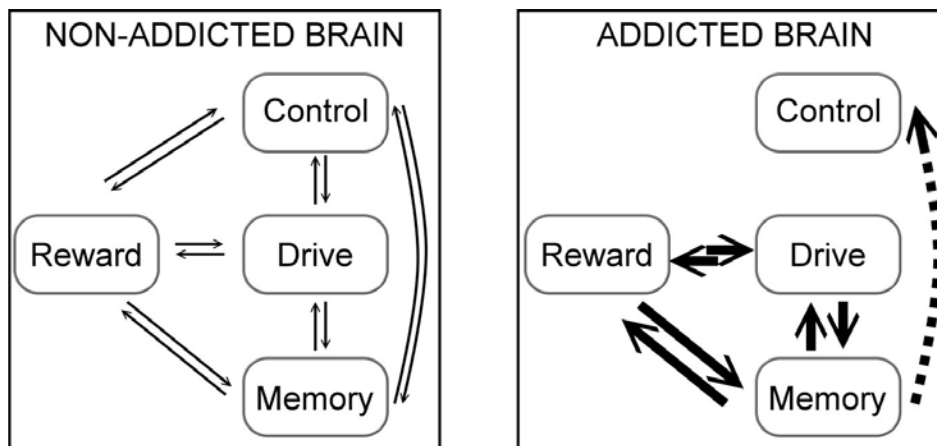


Figure 1.3: Interaction between brain circuits in addiction. The brain circuits that mediate reward, motivation/drive, learning/memory and control are interconnected and the interactions between these circuits change with experience. In drug addiction, the drug over-activates the reward, motivation and memory circuits, overcoming the inhibitory control exerted by the prefrontal cortex (based on Volkow et al., 2003).

For drug-addicted individuals, reward, motivation and memory circuits are over-activated by the drug. This over-activation is accompanied by the loss of inhibitory control of the prefrontal cortex, which no longer regulates the activation of the other circuits, perpetuating the cycle of addiction (Figure 1.3).

Imaging studies (PET and fMRI) have shown that drug-associated cues activate cortical regions (such as the anterior cingulate cortex and the orbitofrontal cortex) and the insula. The function of the insula, which is involved in conscious emotional feelings, seems to be essential to mediate relapse (Naqvi et al., 2007).

The brain areas involved in the circuits affected by drug addiction are innervated by dopaminergic and glutamatergic projections, and modifications in these projections mediate many of the adaptations involved in drug addiction. The reward pathway is an important dopaminergic circuit involved in the reinforcing effects of the drugs of abuse.

1.5.1 The reward pathway

The reward pathway in the brain is acutely activated by all the drugs of abuse. This pathway consists in the projection of dopaminergic neurons in the ventral tegmental area (VTA) to several structures involved in emotions, thoughts, memories and planning and executing behaviours (Figure 1.4). One of these structures, implicated in the addictive effects of the drugs, is the NAc, in the ventral striatum. This structure is involved in reward and addiction, but also in motivation and learning (Di Chiara et al., 1999). Another brain structure receiving projections from VTA neurons is the prefrontal cortex, which coordinates executive functions.

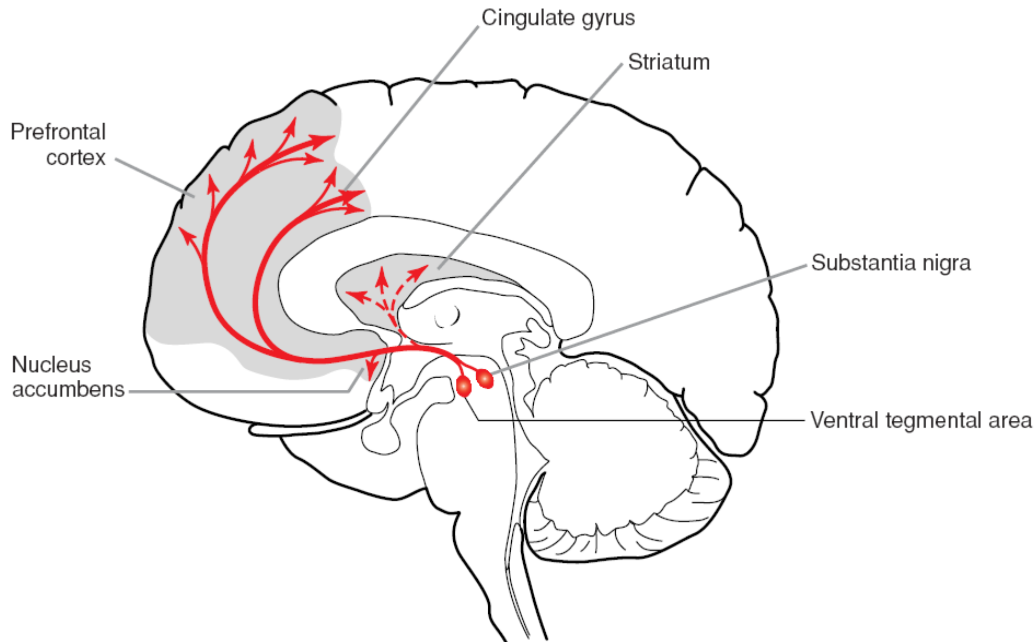


Figure 1.4: Representation of the reward pathway in the human brain. This pathway is composed by neurons that project from the VTA to several structures, including the NAc, in the ventral striatum and the prefrontal cortex. Reprinted, with permission, from the Annual Review of Neuroscience, Volume 29 © 2006 by Annual Reviews www.annualreviews.org" (Hyman et al., 2006).

1.5.2 Molecular mechanisms involved in the effects of the drugs of abuse in the brain

Drugs of abuse affect brain functions mainly due to structural similarities with neurotransmitters (Figure 1.5). Cocaine, amphetamine and ecstasy have structures that are similar to the monoamine neurotransmitters dopamine and noradrenaline.

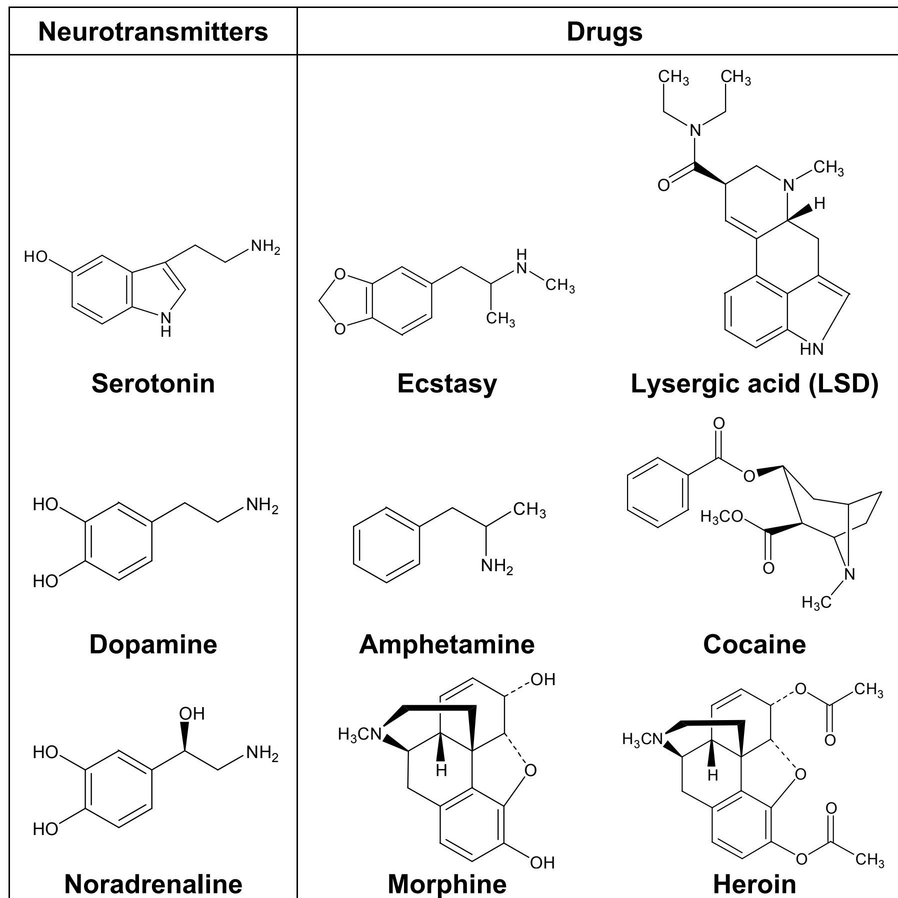


Figure 1.5: Chemical structures of some neurotransmitters and psychoactive drugs. Ecstasy and LSD resemble the neurotransmitter serotonin. Amphetamine and cocaine are similar to dopamine and noradrenaline.

The structures of ecstasy and LSD resemble the neurotransmitter serotonin (Figure 1.5), whereas morphine and heroin have some structure overlap with the neuropeptides enkephalins and endorphins (Figure 1.6).

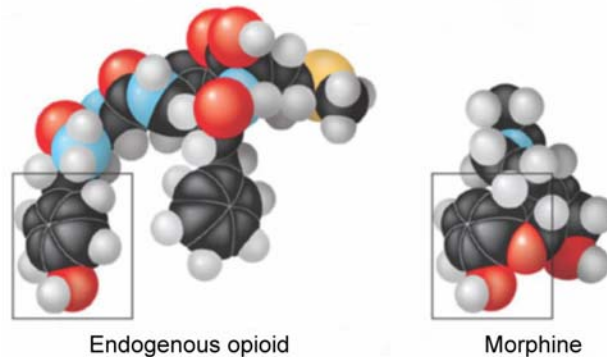


Figure 1.6: Structural similarities between endogenous opioids and morphine (adapted from Romero, 2005).

The drugs of abuse may be administered in several ways, depending on the drug and on the desired effect. Upon administration, the drugs are usually metabolized and therefore the metabolites can contribute to the effects of the drugs.

Cocaine is usually self-administered by nasal insuflation (snorting), smoking, genital application and injection (Jenkins and Cone, 1998). Cocaine may be metabolized by several pathways (Figure 1.7):

- Plasma and liver cholinesterases produce the inactive metabolite **ecgonine methyl ester**
- The second major metabolite, **benzoylecgonine**, is spontaneously produced at physiological pH.
- N-demethylation of cocaine in the liver produces the active metabolite **norcocaine**.
- When cocaine and ethanol are co-administered, the toxic metabolite **cocaethylene** is produced in the liver by transesterification. Cocaethylene is a lipid soluble molecule and crosses the blood brain barrier.
- Smoking of cocaine may produce the pyrolysis compound anhydroecgonine methyl ester, which can be used as a marker of this route of administration.

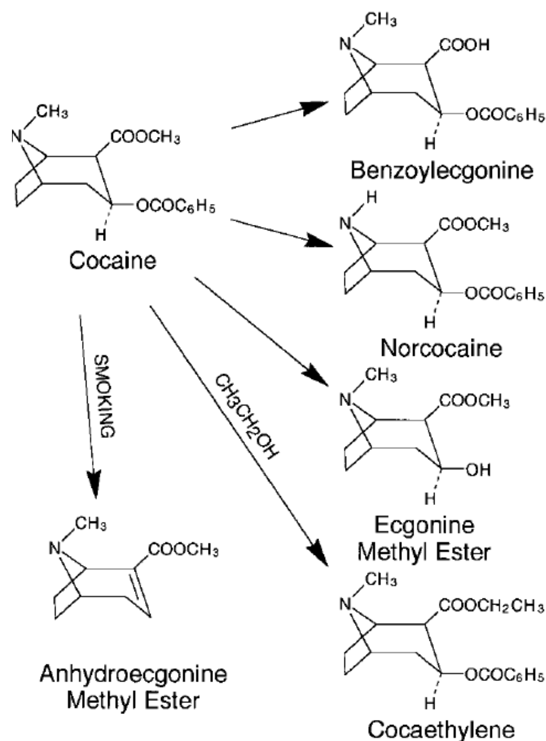


Figure 1.7 Metabolism of cocaine. Plasma and liver cholinesterases convert cocaine in ecgonine methyl ester. Benzoylecgonine is spontaneously produced at physiological pH. N-demethylation of cocaine in the liver produces norcocaine. Transesterification of cocaine and ethanol produces cocaethylene. Smoking of cocaine results in the pyrolysis product anhydroecgonine methyl ester (adapted from Jenkins and Cone, 1998).

Heroin is normally administered by intramuscular or intravenous injection, snorting or smoking. Heroin is rapidly deacetylated to the active metabolite **6-MAM** (Figure 1.8), which is then converted into **morphine**. The formation of 6-MAM may occur spontaneously in aqueous media. Heroin may be considered as a pro-drug, because it does not have intrinsic activity, but, due to its high lipophilicity it facilitates the distribution of the active metabolites, 6-MAM and morphine (Sawynok, 1986).

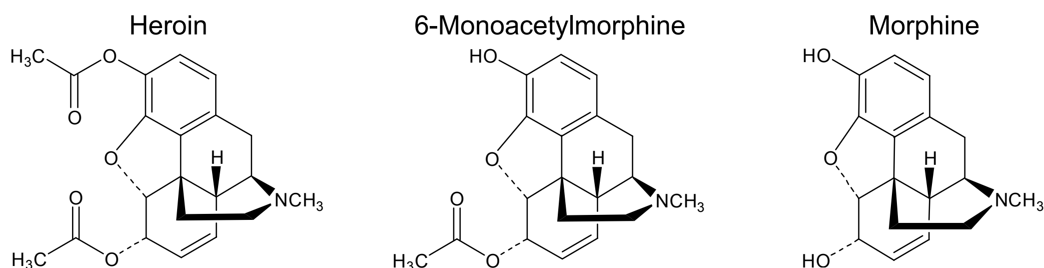


Figure 1.8 Heroin and its main metabolites

Amphetamines may be self-administered by oral, intravenous or smoked routes. d-Amphetamine is metabolized by deamination, oxidation and hydroxylation (Figure 1.9). Deamination produces the inactive metabolite **phenylacetone**, which is oxidized to **benzoic acid** and excreted as **hippuric acid**. Oxidation of amphetamine produces **norephedrine**, which is pharmacologically active. Hydroxylation of amphetamine and norephedrine respectively produces **hydroxyamphetamine** and **hydroxynorephedrine**, which have pharmacologic activity. Methamphetamine may be hydroxylated to hydroxymethamphetamine or N-demethylated to amphetamine (Jenkins and Cone, 1998).

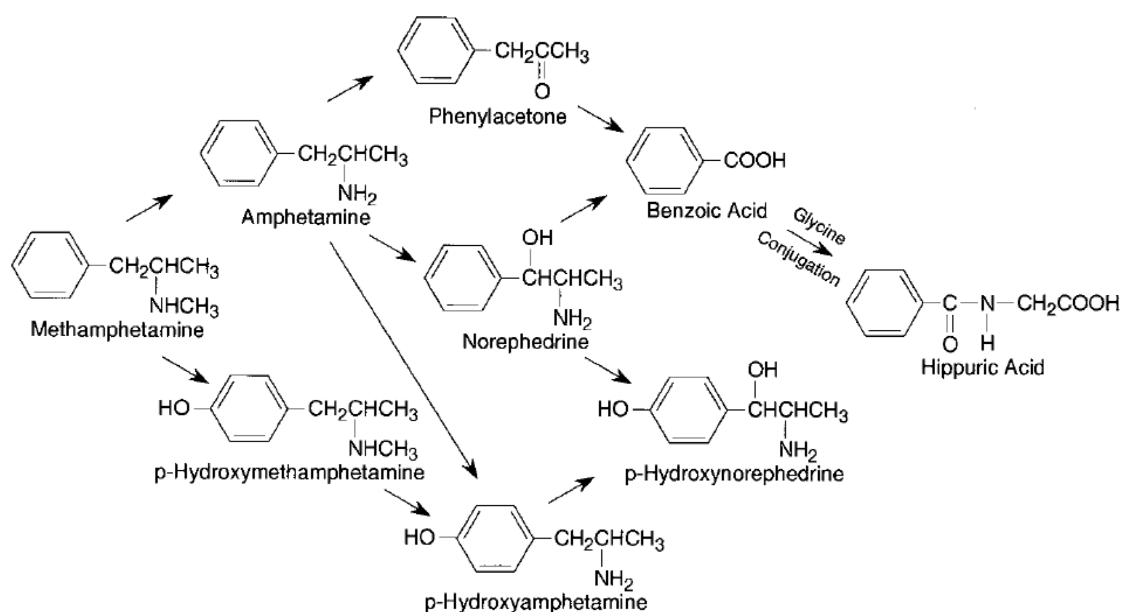


Figure 1.9 Metabolism of amphetamine and methamphetamine. Deamination of amphetamine produces phenylacetone, which is further oxidized to benzoic acid and excreted as hippuric acid. Oxidation of amphetamine produces norephedrine. Hydroxylation of amphetamine and norephedrine produces hydroxyamphetamine and hydroxynorephedrine, respectively. Methamphetamine may also be hydroxylated to hydroxymethamphetamine or N-demethylated to amphetamine (adapted from Jenkins and Cone, 1998).

1.5.2.1 Neurotransmitter systems affected in drug addiction

1.5.2.1.1 Monoamine neurotransmitters

Psychostimulant drugs share structural similarities with monoamines (Figure 1.5) and thereby interfere with the activity of these neurotransmitters.

Monoamine neurotransmitters include the catecholamines dopamine and noradrenaline and also serotonin. **Dopamine** and **noradrenaline** are synthesized from tyrosine by the highly regulated enzyme tyrosine hydroxylase (TH), giving rise to L-dihydroxyphenylalanine (L-DOPA). L-DOPA is converted into dopamine by the enzyme L-aminoacid decarboxylase. In noradrenergic neurons, dopamine is converted into noradrenaline by the enzyme dopamine β-hydroxylase. **Serotonin** is synthesized from tryptophan by the enzyme tryptophan hydroxylase, originating 5-hydroxytryptophan, which is then converted into serotonin by the enzyme L-aminoacid decarboxylase. The monoamines are actively transported by vesicular monoamine transporters (VMATs) into synaptic vesicles where they are stored. When the neurons depolarize, the synaptic vesicles fuse with the plasma membrane releasing the neurotransmitters into the synaptic cleft, where they interact with pre- and post-synaptic receptors. The neurotransmitters are then reuptaken by the pre-synaptic neuron for further release, or metabolized, in order to terminate their synaptic activity. Reuptake is performed by specific transporters in the plasma membrane of the synaptic terminal. Dopamine transporter (DAT), noradrenaline transporter (NET) and serotonin transporter (SERT) are the specific proteins involved in the reuptake of monoamines.

Enzymatic metabolism of monoamines is mainly mediated by the enzymes monoamine oxidases (MAO_A and MAO_B) and catechol-O-methyl-transferase (COMT). Monoamine oxidases are mitochondrial enzymes that catalyse the oxidative deamination of biogenic amines in the brain and in peripheral tissues (see also section 1.6.2.1). The two isoforms of MAO have different substrate specificities. MAO_A preferentially oxidizes serotonin and noradrenaline and MAO_B has a preference for

phenylethylamine and benzylamine. Both isoforms oxidize dopamine but in humans dopamine is preferentially oxidized by MAO_B, whereas in rodents it is predominantly oxidized by MAO_A (Shih et al., 1999). The other enzyme involved in catecholamine inactivation, COMT, may be either cytoplamatic or in a membrane-bound form present in the synaptic cleft and may inactivate cytoplasmic or synaptic catecholamines.

1.5.2.1.1.1 Dopaminergic pathways

The reward circuit (Figure 1.4) is composed by dopaminergic pathways, and is a key mediator of the reinforcing effects of the drugs. All the drugs of abuse acutely induce large and fast increases in dopamine in this and other brain circuits. Dopamine is synthesized by a subset of neurons that express tyrosine hydroxylase. These neurons are present in specific regions in the brain, namely in the VTA, the *substantia nigra* and the *arcuate* and periventricular nuclei of the hypothalamus (Venero et al., 2002).

Dopamine mediates physiologic processes including reward, movement and lactation. There are four dopaminergic pathways: the mesolimbic, mesocortical, nigro-striatal and tuberoinfundibular pathways (Figure 1.10). The first two of these pathways are part of the reward circuit activated by virtually all the drugs of abuse (Di Chiara and Imperato, 1988). The mesolimbic pathway is mainly involved in motivated behavior, whereas the mesocortical pathway, which projects to the prefrontal cortex, is involved in learning and memory. The nigrostriatal pathway is involved in the control of movement and the tuberoinfundibular pathway is involved in the stimulation of milk production. Blocking of dopamine neurotransmission in the tuberoinfundibular pathway may cause increases in blood prolactin levels, causing abnormal lactation, disruption of the menstrual cycle, and sexual dysfunction.

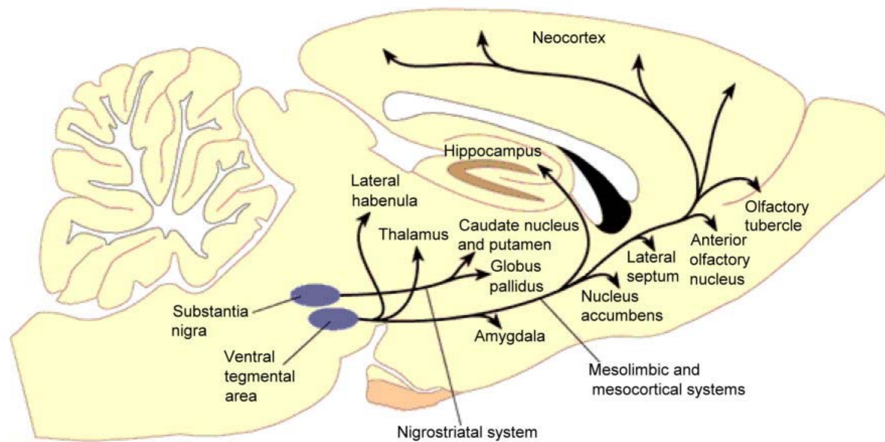


Figure 1.10: Dopaminergic pathways in the rat brain. There are four dopaminergic pathways in the brain. The mesolimbic pathway is composed by neurons projecting from the VTA to the NAc, olfactory tubercle and parts of the limbic system. The mesocortical pathway originates in the VTA and projects to the prefrontal cortex. The nigrostriatal pathway projects from the substantia nigra to the caudate-putamen and globus *pallidus.* The tuberoinfundibular pathway (not represented) originates in the periventricular and *arcuate* nuclei of the hypothalamus and projects to the capillary plexus of the hypothalamic-hypophyseal portal system (Vallone et al., 2000) (image adapted from Salinas, 2006).

As it was described in the previous sections, drugs of abuse interfere with the molecular and cellular pathways involving some neurotransmitters that are structurally similar to the drugs. The molecular targets of the drugs are the transporters or receptors that mediate the physiological actions of the neurotransmitters, activating specific intracellular signalling pathways. However, the drugs do not completely mimic the action of the neurotransmitters because the molecular machinery involved in the removal of the neurotransmitters from the synapse does not recognize the drugs, which are able to interfere with neurotransmission for a longer period of time (Madras, 2006).

Specific interactions of these drugs of abuse with the neurotransmitter receptors/transporters are described in section 1.5.2.2.

1.5.2.1.2 Opioid neuropeptides

The prediction that opioid receptors would be the target of opiate drugs of abuse led to the search of a natural agonist of the receptors. Enkephalins (Hughes et al., 1975), β-endorphin (Li and Chung, 1976) and dynorphin (Goldstein et al., 1979) were the first opioid neuropeptides discovered. Endomorphins were later discovered in the search for a more selective μ-opioid receptor agonist (see Figure 1.14) (Zadina et al., 1999, for review). The aminoacid sequences of the main endogenous opioids are represented in Table 1.5.

Table 1.5: Aminoacid sequences of endogenous opioids

Name	Amino acid sequence
Leucine-enkephalin	*Tyr-Gly-Gly-Phe*-Leu-OH
Methionine-enkephalin	*Tyr-Gly-Gly-Phe*-Met-OH
β-Endorphin	*Tyr-Gly-Gly-Phe*-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Val-Lys-Asn-Ala-His-Lys-Gly-Gln-OH
α-Neoendorphin	*Tyr-Gly-Gly-Phe*-Leu-Arg-Lys-Tyr-Pro-Lys
Dynorphin	*Tyr-Gly-Gly-Phe*-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH
Endomorphin-1	Tyr-Pro-Trp-Phe-NH_2
Endomorphin-2	Tyr-Pro-Phe-Phe-NH_2

β-Endorphin is a cleavage product of the proopiomelanocortin peptide (POMC) (Figure 1.11.A) and is expressed in the pituitary gland and in the *arcuate* nucleus of the hypothalamus. Enkephalins are cleavage products of the proenkephalin A peptide (Figure 1.11.B) and are the most widely distributed opioids in the central nervous system (CNS), with the highest concentration found in the *globus pallidus*.

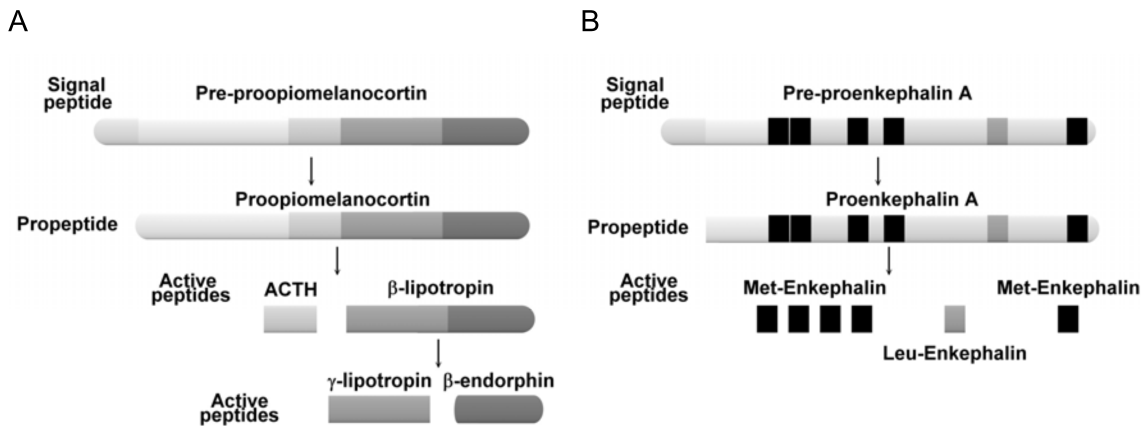


Figure 1.11: Proteolytic processing of the pre-propeptides (**A**) pre-proopiomelanocortin and (**B**) pre-proenkephalin A. The maturation of the pre-propeptides involves cleaving the signal sequence and other proteolytic processing. This results in the production of several neuroactive peptides such as adrenocorticotropic hormone (ACTH), γ-lipotropin, and β-endorphin (A), or multiple copies of the same peptide, such as met-enkephalin (B) (based on Purves et al., 2001).

The distribution of opioid neuropeptides in the brain depends on the expression of the precursor proteins and on the distribution of specific proteases responsible for cleavage of the precursors.

1.5.2.2 Short-term (acute) effects

1.5.2.2.1 Psychostimulants and reward

The stimulant drugs amphetamine and cocaine directly increase extraneuronal dopamine levels through different mechanisms (Figure 1.12).

Amphetamine induces an increase in extracellular monoamines, by interacting directly with monoaminergic cells. Although amphetamine has comparable effects in neurons containing serotonin, noradrenaline and dopamine, the effects implicated in its reinforcing properties are mainly mediated by dopamine (Fleckenstein et al., 2007).

Due to its structural similarity with dopamine, amphetamine is a substrate for the DAT (Sitte et al., 1998). In low concentrations amphetamine is transported by the DAT to the cytosol and increases the intracellular binding sites of the DAT for dopamine, resulting in the exchange of extracellular amphetamine by intracellular

dopamine, and leading to an increase in extracellular dopamine (Jones et al., 1999). When present in higher extracellular concentrations, amphetamine may diffuse into the cell, due to its lipophilicity (Sulzer et al., 1995; Kahlig et al., 2005).

Intracellular amphetamine also induces reverse transport by the DAT because it contributes to increase the intracellular dopamine concentration. Amphetamine interferes with VMAT-2 function, impairing the active transport of the monoamines into synaptic vesicles, where they are stored. In addition, amphetamine may enter in the vesicles by diffusion, due to its weak base properties (Sulzer et al., 2005, for review). Since amphetamine is a weak base, at acidic pH it accepts protons leading to alkalinization inside the vesicles. A low pH inside the vesicles is essential to maintain the proton gradient used by VMAT-2 for active transport of monoamines into the vesicles. Therefore, amphetamine induces the release of vesicular dopamine to the cytosol and impairs the storage of dopamine in the vesicles. Cytosolic dopamine is then released to the extracellular space via reverse-transport by the DAT.

Amphetamine also interferes with dopamine synthesis by inhibiting tyrosine hydroxylase (TH) (Ellison et al., 1978) and with dopamine metabolism, by inhibiting MAO (Ramsay and Hunter, 2002).

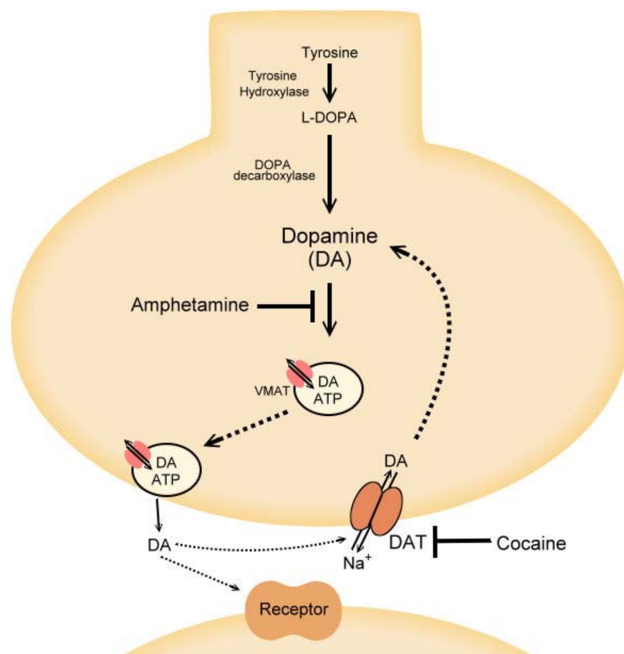


Figure 1.12: Direct effects of amphetamine and cocaine in dopaminergic nerve terminals. Both drugs increase extracellular dopamine accumulation by different mechanisms. Briefly, amphetamines inhibit the storage of dopamine in synaptic vesicles, inducing an increase in cytosolic dopamine concentration and reversal of the DAT. Cocaine blocks the reuptake of dopamine by the DAT.

Cocaine inhibits the DAT (Brown et al., 2001), preventing the reuptake of dopamine into the nerve terminal. The inhibition of the DAT results in increased levels of dopamine in the synaptic cleft. Cocaine also inhibits the monoamine transporters NET and SERT (Gatley et al., 1998). However, as it happens with amphetamine, reinforcing effects of cocaine are largely dependent on its effect in dopaminergic neurotransmission (Ritz et al., 1987).

Cocaine also interacts with the VMAT-2, favoring the storage of catecolamines inside synaptic vesicles (Brown et al., 2001). It was suggested that cocaine-induced inhibition of the DAT and increased vesicular sequestration of dopamine, causes a shift in the ratio of cytoplasmic to vesicular dopamine increasing the amount of neurotransmitter packaged in each vesicle before its release. This effect on the VMAT would contribute to further increase in synaptic dopamine, upon a depolarizing stimulus (Brown et al., 2001).

1.5.2.2.2 Opiates and reward

In contrast with the stimulant drugs, opiates induce an increase in dopamine in the NAc by an indirect mechanism.

The major targets of opiates are the opioid receptors, which mediate the endogenous effects of the opioid neuropeptides. The drug of abuse morphine shares structural similarities with the opioid peptides and, therefore, is recognized by the opioid receptors, activating them (Figure 1.13).

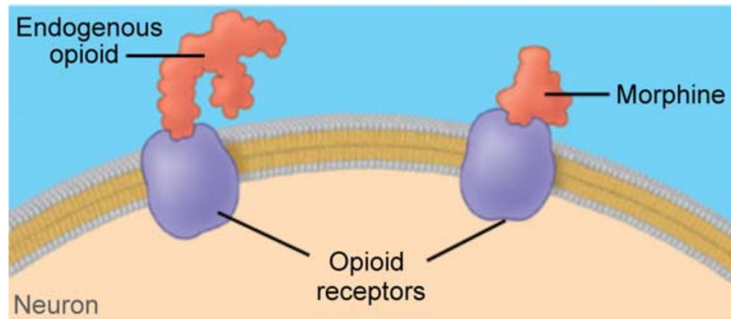


Figure 1.13. Functional similarity between morphine and endogenous opioids. Due to its structural similarities with endogenous opioids, morphine is recognized by the opioid receptors and activates them (adapted from Romero, 2005).

Opioid receptors are G-protein-coupled receptors that respond to endogenous opioids. There are 3 subtypes of opioid receptors, with different pharmacological selectivities (Figure 1.14): mu (μ), delta (δ) and kappa (κ) receptors. Morphine is a specific agonist of μ-opioid receptors, with binding potencies (Ki) of 14 nM for μ-opioid receptors, 538 nM for κ –receptors and above 1000 nM for δ –receptors (Figure 1.14) (Raynor et al., 1994).

Binding of the specific ligands to any of the opioid receptors induces the activation of $G_{i/o}$ proteins, resulting in the inhibition of adenylyl cyclase, activation of potassium conductance, inhibition of calcium conductance and the inhibition of neurotransmitter release (Williams et al., 2001).

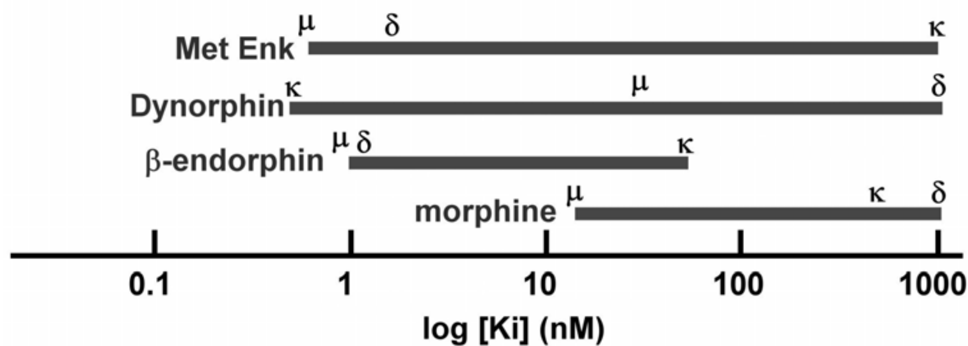


Figure 1.14: Selectivity windows of morphine and endogenous opioid peptides for the subtypes of opioid receptors. Met-enkephalin (Met Enk) and β endorphin preferentially bind to μ and δ opioid receptors. Dynorphin is selective for κ opioid receptors. Morphine selectively binds to μ opioid receptors (adapted from Williams et al., 2001).

Upon the activation of the opioid receptor, the alpha subunit of the G protein inhibits the enzyme adenylyl cyclase (Figure 1.15), which is responsible by the production of cyclic AMP (cAMP). cAMP normally activates protein kinase A (PKA), which is responsible for the phosphorylation of several proteins, involved in numerous cellular processes. One of these proteins is the transcription factor cAMP response element-binding protein (CREB).

It has also been reported that acute exposure to opiates may lead to the activation of protein kinase (PKC), release of calcium from intracellular stores and activation of mitogen-activated protein kinase (MAPK) cascade (Williams et al., 2001). These effects seem to be mediated by the beta/gamma subunits of the G protein.

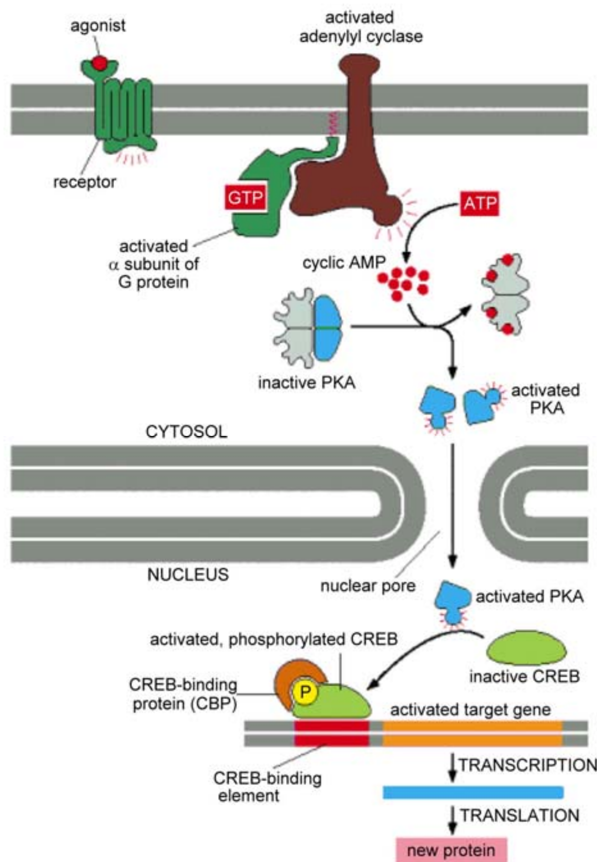


Figure 1.15: Intracellular signalling mediated by G-protein coupled receptors. Binding of the agonist to the receptor activates heterotrimeric G proteins composed of α,β and γ subunits. Stimulatory α subunits activate the enzyme adenylyl cyclase, which produces cAMP. Inhibitory α subunits inhibit this pathway. cAMP activates PKA that is translocated to the nucleus. PKA is active in the cytosol and in the nucleus, phosphorylating several proteins such as the transcription factor CREB, which is activated by this phosphorylation. Activated CREB binds to CREB-binding protein (CBP) and activates transcription of its target genes, leading to the expression of new proteins (adapted from Alberts et al., 2002).

Figure 1.16 summarizes the actions of opiates and other reinforcing drugs of abuse in the neurons of the reward pathway, which culminate in an increase in dopamine in dopaminergic synapses of the NAc.

VTA neurons (represented in the bottom left of Figure 1.16) project to the NAc (bottom right). The activity of these projections is regulated by interneurons, which interact with VTA or NAc neurons.

Due to the inhibitory actions of opiates described above, opiate drugs of abuse bind to μ-opioid receptors present in gamma aminobutyric acid (GABA)-releasing interneurons in the VTA, inhibiting the release of GABA, an inhibitory neurotransmitter that acts on dopaminergic neurons to inhibit dopamine release (Figure 1.16). Thus, the action of the opiate drugs in the GABAergic interneurons results in an increase of dopamine release by the dopaminergic neurons of the VTA. Opiates also interact directly with NAc neurons and produce reward in a dopamine-independent manner.

Other drugs induce dopamine release in the NAc by different mechanisms. Nicotine causes dopamine release by interacting with nicotinic acetylcholine receptors in the VTA. Ethanol interacts with $GABA_A$ receptors in the VTA. Some drugs of abuse may also interact directly with NAc neurons.

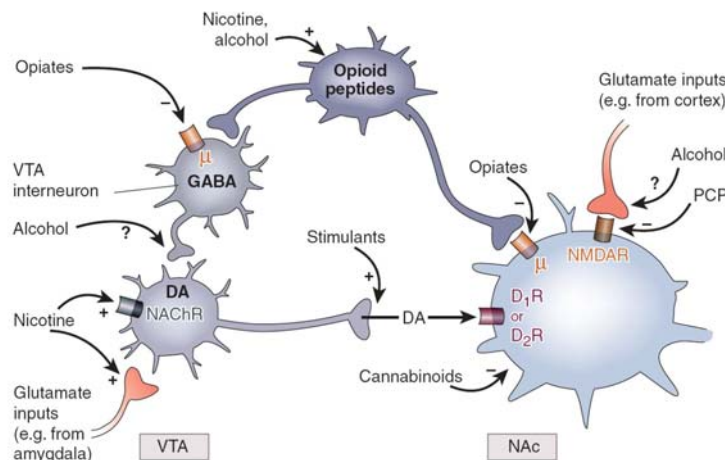


Figure 1.16: Effects of psychoactive drugs in the reward pathway. All the drugs of abuse induce an increase in the synaptic concentration of dopamine (DA) in the NAc. Stimulants act directly in the synaptic terminals of dopaminergic neurons projecting from the VTA. Opiates, nicotine and alcohol affect the activity of interneurons that regulate this pathway. Opiates, alcohol, cannabinoids and phenylcyclidine (PCP) may also directly affect NAc neurons. Reprinted, with permission, from the Annual Review of Neuroscience, Volume 29 © 2006 by Annual Reviews www.annualreviews.org" (Hyman et al., 2006).

1.5.2.3 Long-term homeostatic adaptations

Upon chronic drug abuse and during withdrawal, dopamine function in the brain is markedly decreased. Several adaptations to the drugs occur, leading to the development of drug addiction. Chronic exposure to the drugs of abuse in humans induces long-lasting changes in gene expression, and some of these changes may be correlated with the development of the compulsive behaviour associated with drug addiction (Rhodes and Crabbe, 2005, for review). Changes in gene expression induced by chronic drug exposure may also underlie changes in the cellular responses to stress. These alterations are in the basis for the development of sensitization or tolerance, in human drug abusers, in which the response to the same dose of a drug is increased or decreased, respectively. These processes are responsible for the withdrawal symptoms and increased motivation to drug abuse (Nestler, 2004b).

Opiates have the most evident addictive effects, involving tolerance, withdrawal and a high rate of relapse. Binding of opioids to their receptors activates intracellular signalling pathways that regulate gene expression.

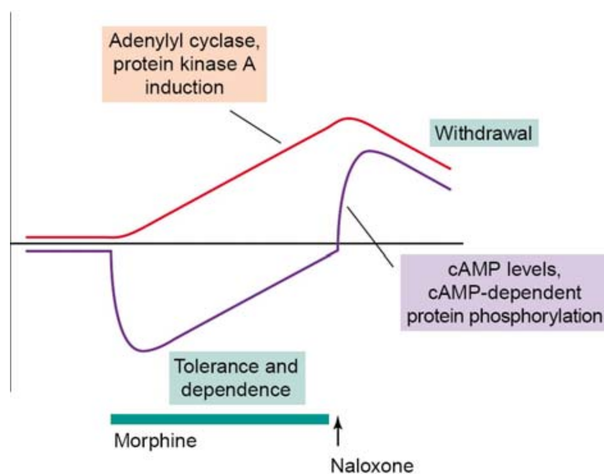


Figure 1.17: Activity of the cAMP pathway upon acute or chronic exposure to opiates. Acute exposure to opiates inhibits the cAMP pathway (lower line: cAMP and phosphorylated PKA levels). Upon repeated exposure to opiates the activity of the cAMP pathway recovers to normal levels. When an antagonist of the opiate receptors is present (withdrawal) the activity of the cAMP pathway increases far above control levels. These changes are due to induction of adenylyl cyclase and PKA expression during chronic exposure to the opiates (upper line: adenylyl cyclase and PKA protein levels) (adapted from Nestler, 2004a)

Physical dependence develops when the physiology of cells, and circuits, is changed by the drugs in such a way that, when the drug is absent, withdrawal symptoms appear. It was demonstrated that acute morphine exposure decreases the levels of cAMP in the *locus coeruleus*, the major brain noradrenergic nucleus.

However, upon continued exposure to morphine, compensatory upregulation of the cAMP pathway occurs, with increase in adenylyl cyclase expression and cAMP recovery to normal levels (Figure 1.17 and Figure 1.18). During withdrawal or in the presence of an opioid receptor antagonist (such as naloxone), the upregulation of the cAMP pathway results in the increase in the levels of cAMP, which can rise several times above normal levels. The expression of other signalling molecules is also affected upon chronic exposure to morphine. These phenomena were also demonstrated to occur in NAc neurons (Chieng and Williams, 1998), which are implicated in the motivational aspects of drug addiction.

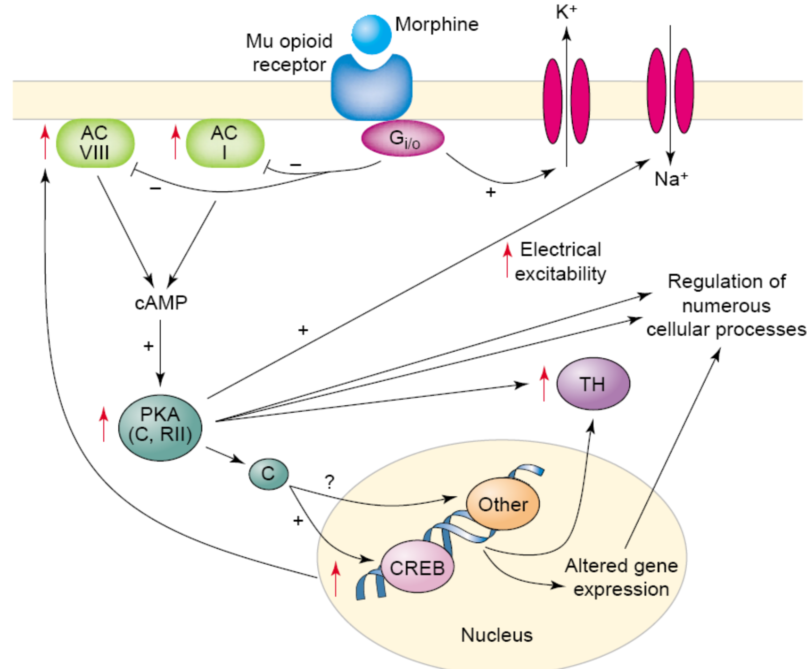


Figure 1.18: Long-term effects of opiate drugs of abuse due to changes in gene expression. Intracellular signalling mediated by the activation of opioid receptors is changed upon chronic exposure to morphine. Several components of the cAMP pathway are upregulated, such as adenylyl cyclase (AC), PKA, TH and CREB, contributing to increased electrical excitability which underlies tolerance, dependence and withdrawal observed upon chronic exposure to opiates. C- catalytic subunit of PKA (adapted from Nestler, 2004a). Reprinted with permission from AAAS.

The molecular mechanisms involved in the upregulation of the cAMP pathway seem to be partially mediated by the transcription factor CREB. Chronic opiate exposure induces CREB expression and activity, which is thought to mediate the expression of some of the components of the cAMP pathway. Cocaine and

amphetamine also induce CREB activation due to increase in the stimulation of dopamine (D1) receptors (Hyman et al., 2006, for review).

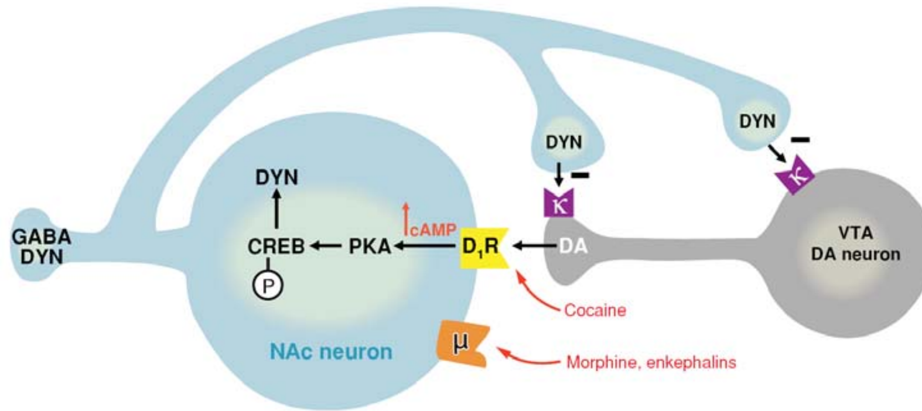


Figure 1.19: Induction of dynorphin peptides by dopamine. One of the target genes of CREB is dynorphin, which is expressed in NAc neurons and regulates the activity of VTA neurons, decreasing dopamine release. Over-activation of CREB induced by chronic exposure to the drugs of abuse increases the expression of dynorphin, representing a negative-feedback loop. Reprinted, with permission, from the Annual Review of Neuroscience, Volume 29 © 2006 by Annual Reviews www.annualreviews.org" (Hyman et al., 2006).

CREB has many target genes, such as cFos, corticotropin-releasing factor (CRF), TH, brain derived neurotrophic factor (BDNF), adenylyl cyclase (isoform VIIII), enkephalins and dynorphin (Carlezon, Jr. et al., 2005). The upregulation of dynorphin seems to mediate the decrease in the rewarding properties of the drugs, mediated by CREB (Cole et al., 1995). Dynorphin acts on k-opioid receptors present on VTA neurons to decrease dopamine release (Figure 1.19) and represents a negative-feedback loop.

Upregulation of cAMP pathway by the opiates leads to decreased dopamine release in the VTA when opiates are present. When opiates are no longer present, the decrease in dopamine release mediated by dynorphin may contribute to anhedonia and dysphoria that characterize the early phases of opiate withdrawal.

Another transcription factor involved in the chronic effects of drugs of abuse is **ΔFosB**, a member of the Fos family of transcription factors (McClung et al., 2004). Acute exposure to drugs of abuse transiently induces the expression of several members of the Fos family. However, chronic exposure to the drugs results in increasing accumulation of stable isoforms of ΔFosB, which persist in NAc neurons for

a long time after cessation of drug use (Nestler, 2004b). ΔFosB may be viewed as a "molecular switch", initiating and maintaining long-lived adaptations associated with drug addiction.

Opiates also induce changes in the synaptic activity of opiate receptors, by inducing desensitization, internalization and intracellular trafficking of these receptors, leading to decreased receptor availability or response (Figure 1.20).

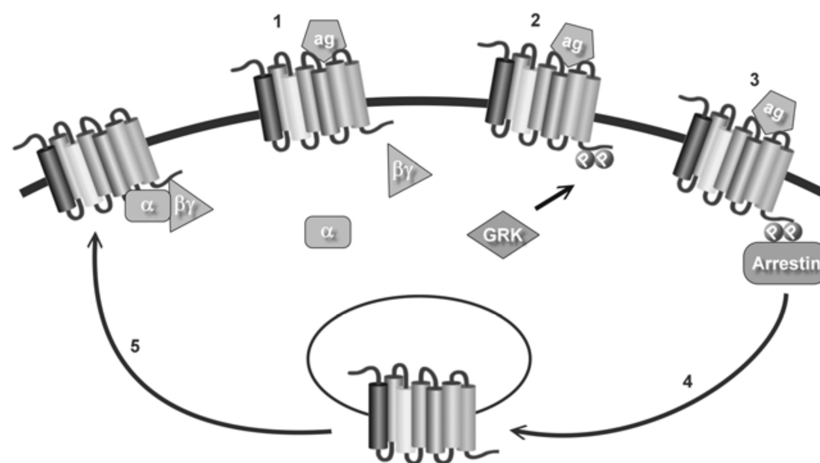


Figure 1.20: Effects of acute and prolonged activation of opioid receptors by opioid receptor agonists. Binding of the agonist (ag) to the G protein-coupled receptor triggers G protein activation (1) and phosphorylation of the receptor by G protein-coupled receptor kinases (GRK) (1), allowing the binding of arrestin (3), which targets the receptor to endocytosis (4). The receptor may be recycled to the cell membrane (5) or degraded in the lysosome (not shown) (based on Bailey, 2007).

As discussed in section 1.5.2.2.2, opioids are recognized by specific receptors, triggering an intracellular cascade of events. Acute exposure to opiate drugs activates the opioid receptors, inducing the activation of heterotrimeric G-proteins that mediate signal transduction events. However, upon prolonged exposure to endogenous opioids, the receptors are desensitized due to phosphorylation by G protein-coupled receptor kinases (GRKs) (Figure 1.20). This allows the binding of arrestin molecules, preventing the activation of G proteins, due to uncoupling of the receptor from the G protein. Arrestin-bound receptors are internalized by endocytosis in clatrin-coated vesicles, reducing the number of receptors available in the cell membrane. The internalized receptors may be either dephosphorylated and transported back to the plasma

membrane, inducing resensitization, or degradated by proteolysis in the lysosomes, inducing downregulation of the receptors, a permanent decrease in the number of receptors in the cell membrane.

Studies performed in human embryonic kidney (HEK293) cells (Keith et al., 1996), gut neurons (Sternini et al., 1996), cortical neurons (Keith et al., 1998) and spinal cord neurons (Trafton et al., 2000), showed that morphine was much less effective in inducing μ-opioid receptor internalization, when compared to endogenous opioids. However, in cultures of striatal neurons, representative of the GABAergic neurons of the NAc, μ-opioid receptors were recently shown to be internalized upon exposure to morphine, probably due to the presence of different isoforms of signalling molecules in these neurons (Haberstock-Debic et al., 2005). Two explanations for the tolerance induced by morphine have been reported. One possibility is that morphine does not trigger significant receptor internalization and the receptor remains coupled to the intracellular signalling systems for a long period of time, inducing tolerance due to adaptations in the intracellular signalling machinery (Finn and Whistler, 2001). Another possibility is that morphine-desensitized receptors accumulate at the cell surface and, in this case, tolerance may be caused by the fact that morphine-desensitized receptors are not efficiently internalized and resensitized (Schulz et al., 2004).

The effect of morphine seems to be cell-specific but the end-result seems to be common to all systems - the cells decrease the number or the activity of receptors that respond to the opioids, in order to decrease the deleterious effects of the chronic presence of the drug. During abstinence, the amount of opiates decreases and the system becomes unbalanced due to adaptation to the excessive inhibitory signals, when the drugs are present. This may explain some of the withdrawal symptoms observed in opiate addicts.

1.5.2.4 Learning and memory

Long-term adaptations to drugs explain the withdrawal symptoms observed upon abstinence and the dependence on drugs, but do not completely explain the

persistence of relapse risk observed long time after the individuals stop consuming. It was suggested that the life-long risk of relapse may be explained by long-term associative memory processes triggered by the drugs (Hyman et al., 2006). This is highly suggested by the observation that most relapses occur after exposure to cues associated with previous drug use. These cues may be external, associated with sensory stimuli (persons, objects or places previously associated with drug use) or internal, as for example the withdrawal symptoms. Stress can also induce relapse, probably due to the activation of the reward pathway, resembling drug-exposure (Hyman et al., 2006).

In relapse, drug-seeking is facilitated by impairment in the impulse control mediated by the prefrontal cortex, which is responsible for inhibiting harmful behaviours in non-addicts.

An increase in synaptic dopamine induced by the drugs of abuse may be responsible for the activation of circuits involved in processing long-term associative memories because dopaminergic neurons project to several brain areas and innervate multiple targets (Hyman et al., 2006).

1.5.2.5 Stress and drug addiction

Stress also plays an important role in relapse to drug-taking behavior. The drugs of abuse activate the hypothalamic-pituitary-adrenal (HPA) axis, which mediates the stress response (Figure 1.21). The release of stress hormones begins in the brain, by the release of the hypothalamic neuropeptide CRF. Upon a stressful event CRF is released from the hypothalamus and acts in the pituitary gland, stimulating the release of β-endorphin and ACTH, which are derived from POMC (see section 1.5.2.1.2 and Figure 1.11). ACTH is secreted into the blood and triggers the release glucocorticoids, namely cortisol, from the adrenal glands. Cortisol acts in a negative feedback manner in the hypothalamus and in the pituitary to inhibit the production of CRF, ACTH and β-endorphin. Endogenous opioids may be involved in the regulation of this axis.

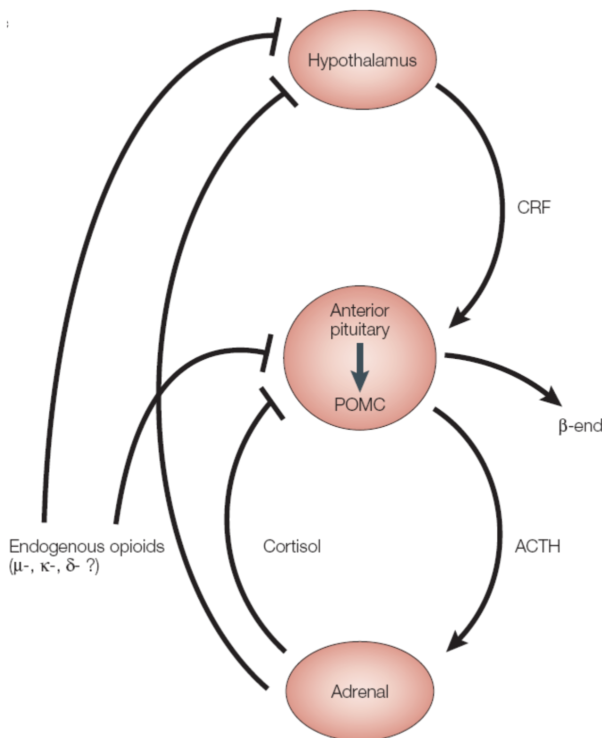


Figure 1.21: Components of the stress-response axis. The release of stress hormones begins in the brain, with the release of CRF to the anterior pituitary, stimulating the release of β-endorphin (β-end) and ACTH, which are derived from POMC. ACTH is secreted into the blood and triggers the release of cortisol, from the adrenal glands. Cortisol acts in a negative feedback manner in the hypothalamus and in the pituitary to inhibit the production of CRF, ACTH and β-endorphin. Endogenous opioids may be also involved in the regulation of this axis. Reproduced, with permission from Nature Reviews Drug Discovery (Kreek et al., 2002) ©2002 Macmillan Magazines Ltd.

The concentrations of glucocorticoids regulate the level of dopamine release in the NAc (Piazza and Le Moal, 1998). In basal conditions both glucocorticoid secretion and dopamine release are low. An acute stress leads to the increase in glucocorticoid secretion, which enhances dopamine release in the NAc and thereby increases the sensitivity to the reinforcing effects of the drugs of abuse, which can result in increased self-administration. However, since glucocorticoids activate a negative feedback that controls their own release, the system returns to basal levels within 2 hours. Upon repeated stress, the negative feedback loop becomes impaired, resulting in a long-lasting increase in glucocorticoid secretion and, consequently, an increase in dopamine release in the NAc. These changes lead to a long-lasting increase in the sensitivity to the reinforcing effects of the drugs (Piazza and Le Moal, 1998).

During withdrawal, drugs of abuse increase the levels of CRF which may be responsible for stress-induced relapse (reviewed by Sarnyai et al., 2001; Kreek and Koob, 1998).

As described above, drug addiction involves different cellular and molecular adaptations that are specific for each stage of the life cycle of addiction, which are summarized in Figure 1.22.

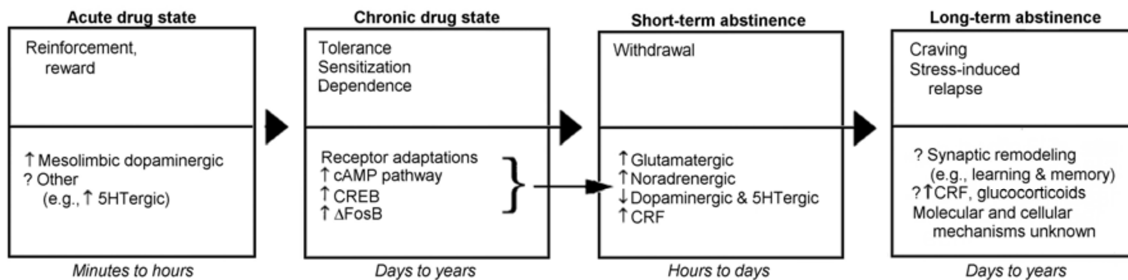


Figure 1.22: Life cycle of addiction. Upper boxes show the processes associated with each stage of drug addiction. Lower boxes show the underlying cellular and molecular mechanisms involved. 5-HT- serotonin. (From Nestler and Aghajanian, 1997. Reprinted with permission from AAAS).

1.6 Drugs of abuse and neurotoxicity

Many drugs of abuse have been shown to induce toxicity in several tissues of the human body. Cytotoxicity was demonstrated for ecstasy (Capela et al., 2006), d-amphetamine, methamphetamine (Cadet et al., 2003), cocaine (Zhang et al., 1999) and morphine (Bhat et al., 2004).

Since addiction may be considered a brain disease (Leshner, 1997), neurotoxicity may underlie some of the effects of the drugs. The brain is one of the most metabolically active tissues. A continuous supply of glucose is essential for the energy-consuming neuronal functions such as axonal transport or synaptic transmission. Although new neurons may be formed by neurogenesis in the adult brain (see section 1.6.2.4) (Gage, 2002), the existing neurons do not divide and thus dysfunction or death of these cells may result in irreversible damage. Furthermore, the brain is highly sensitive to oxidative stress due to its high content in poly-unsaturated

fatty acids, low levels of antioxidants, the presence of transition metals and high levels of oxygen consumption.

Moreover, there are evidences that chronic abuse of heroin (Fishbein et al., 2007), cocaine (Bolla et al., 2000) and amphetamines (Barr et al., 2006) induces impairment of neurocognitive functions.

1.6.1 Glutamate mediated effects of opiate drugs

Many evidences suggest the involvement of glutamatergic neurotransmission in the mechanisms of drug dependence involving the dopaminergic reward circuit in the brain (Tzschentke and Schmidt, 2003).

N-methyl-D-aspartate (NMDA) receptors are a subtype of ionotropic glutamate receptors that plays a key role in excitatory synaptic transmission. These receptors have been implicated in synaptic plasticity associated with learning and memory, and hypofunction of NMDA receptors produces memory dysfunction (Newcomer and Krystal, 2001). In contrast, hyperfunction of these receptors has been associated with acute CNS injury syndromes such as hypoxia, ischaemia, trauma and status epilepticus (Newcomer and Krystal, 2001).

The biophysical and pharmacological properties of these receptors depend on their subunit composition. There are three different subtypes of NMDA receptor subunits, NR1, NR2 (A-D) and NR3 (A-B). NMDA receptors are heteromeric complexes composed of at least one NR1 subunit and one NR2 subunit. Heterotrimeric complexes composed of NR1/NR2 and NR3 have been also described (Cull-Candy et al., 2001).

Glutamate binds to the NR2 subunits and the co-agonist glycine binds to the NR1 subunit. Several agonists and antagonists regulate the activity of NMDA receptors by interacting with different sites of the receptors (Figure 1.23).

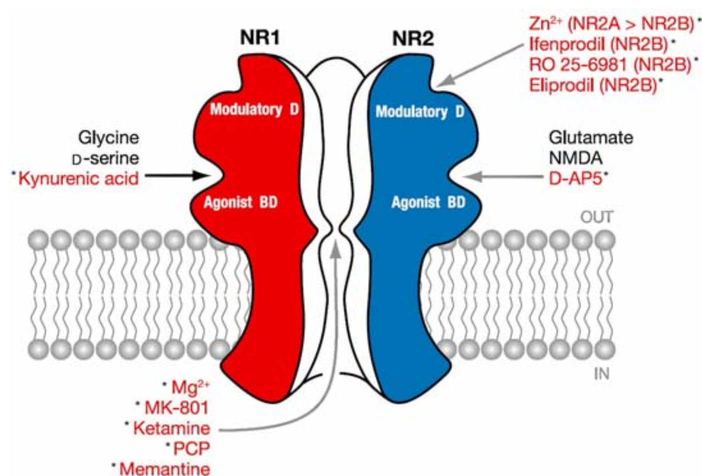


Figure 1.23: Structure of NMDA receptor showing binding sites for agonists and antagonists (*). The extracellular portions of the subunits consist of two domains, the modulatory domain (Modulatory D) and the agonist binding domain (Agonist BD). The NR1 subunit contains the glycine-binding site and the NR2 subunit contains the glutamate-binding site. Reprinted, with permission, from the Annual Review of Pharmacology and Toxicology, Volume 47 ©2007 by Annual Reviews www.annualreviews.org" (Hara and Snyder, 2007).

Subunit composition of NMDA receptors in the brain varies during development. Before birth, NR2B subunits are found in most brain regions and NR2D subunits are found in the diencephalon and brain stem. After birth the levels of NR2A subunits increase in most brain regions and NR2C subunits appear in the cerebellum. Hence, NR2B subunit expression decreases during development, whereas NR2A subunit expression increases (Cull-Candy et al., 2001). However, subunit composition at the same age also differs among different brain regions. The levels of mRNA of the different NR2 subunits in specific brain areas in adult rats (Goebel and Poosch, 1999) are in the following order:

Cortex: NR2B >> NR2A > NR2C >> NR3A > NR2D

Hippocampus: NR2A >> NR2B >> NR2C > NR2D ≈ NR3A

Striatum: NR2B >> NR2A >> NR2C > NR3A ≈ NR2D.

It was previously suggested that glutamatergic neurotransmission involving the NMDA receptor contributes to opiate dependence in humans (Bisaga et al., 2001). NMDA receptor antagonists inhibit the development of physical dependence and tolerance (Trujillo, 2000). Memantine, an antagonist of the NMDA receptor, is capable

of preventing the acquisition of morphine-induced conditioned place-preference, suggesting that the glutamatergic system can modulate opiate reward (Ribeiro Do Couto et al., 2004). Moreover, the NMDA receptor antagonist MK-801 was shown to specifically block morphine-induced tolerance and neuronal apoptosis in the spinal cord (Mao et al., 2002).

Morphine dependence reduces the affinity of glycine for NMDA receptors in the NAc (Siggins et al., 2003; Martin et al., 2004). Moreover, morphine-dependent rats show decreased NR1 and NR2A subunit expression in the frontal cortex and hippocampus and increased levels of NR1 and NR2A in the NAc, whereas NR2B is not affected (Murray et al., 2007). These data suggest that NR2A-containing NMDA receptors in the NAc probably contribute to the development of opiate dependence. Another study also suggested that chronic morphine induces an increase in NR2A subunit function in the NAc, and a decrease in the function of the NMDA receptor subunits NR2B and 2C, which could result in altered excitability and integrative properties (Martin et al., 2004).

However, other authors showed that chronic morphine significantly increased the protein levels of NR1 and NR2B subunits in the NAc (Bajo et al., 2006). It was recently suggested that NR2B subunit-containing NMDA receptors may be involved in the rewarding effect of morphine (Kato et al., 2007; Ma et al., 2007). NR2B-containing NMDA receptors in the NAc and the dorsal hippocampus were proposed to play a significant role in mediating the reinstatement of rewarding responses to morphine (Ma et al., 2007). This effect seems to be specific for morphine because NR2B containing NMDA receptors are more involved in morphine reward rather than in natural rewards (Ma et al., 2006). Nevertheless, the acute exposure to opiates may have different effects on NMDA receptors when compared to the chronic exposure.

NMDA receptors are highly permeable to calcium and, thus, hyperactivation of these receptors leads to excitotoxicity and cell death due to the activation of Ca^{2+}-dependent proteases.

These and other mechanisms of toxicity mediated by the drugs of abuse may involve the mitochondria, organelles with important functions in the life and death of cells.

1.6.2 Mitochondria in cell life and death

Mitochondria are the powerhouses in the cells, producing energy to supply the various energy-consuming cellular activities. Classically, mitochondria are composed of two compartments, separated by two different lipid membranes with different protein compositions (Figure 1.24). The outer mitochondrial membrane (OMM) separates the organelle from the cytoplasm, whereas the inner mitochondrial membrane (IMM) separates the matrix compartment - inside the mitochondria - from the intermembrane space (IMS). Recently, another compartment was shown to exist in the space between cristae, the intracristal space (ICS) (Mannella, 2006), surrounded by inner mitochondrial membrane. The ICS communicates with the IMS through bottleneck-like junctions that create a diffusion barrier.

In these organelles, several important bioenergetic reactions take place. The goal of these reactions is to produce ATP. A series of oxidative reactions mediated by the electron transport chain allows the production of several ATP molecules for each molecule of energetic substrate consumed.

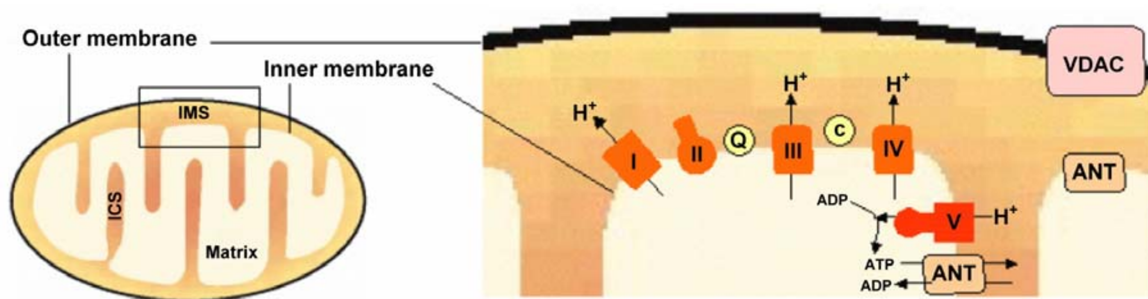


Figure 1.24: Mitochondria and the electron transport chain. ICS: intracristal space, IMS: intermembrane space, VDAC: voltage dependent anion channel, ANT: adenine nucleotide translocator.

The electron transport chain is composed by five protein complexes present in the IMM. NADH/H^+ produced by several bioenergetic reactions (namely in the Krebs

cycle) serves as an electron donor, reducing a flavin mononucleotide (FMN) center in the mitochondrial NADH-ubiquinone reductase- the **complex I** of the mitochondrial electron transport chain. The reduction of FMN initiates a cascade of redox reactions in complex I that result in the reduction of a lipid-diffusible molecule of ubiquinone (or coenzyme Q). These redox reactions also result in the pumping of four protons from the matrix to the IMS.

Complex II consists primarily of the Krebs cycle enzyme that converts succinate in fumarate (succinate dehydrogenase) and two iron-sulfur proteins. This redox reaction results in the reduction of a FAD center in the protein, ultimately resulting in the reduction of a lipid-soluble molecule of ubiquinone.

The reduced lipid-soluble ubiquinone transfers the electrons to another protein complex in the IMM, the cytochrome c reductase or **complex III**. Ubiquinone reduces an iron-sulfur center in complex III, initiating a complex cascade of redox reactions that results in the reduction of two cytochrome c molecules and in the pumping of four protons from the matrix to the IMS.

Reduced cytochrome c transfers one electron to a cupper center in **complex IV** (cytochrome oxidase). Complex IV uses 4 electrons transported by cytochrome c to reduce one oxygen molecule (O_2) into two water molecules, pumping 4 protons to the IMS. The reduction of one O_2 molecule requires the transfer of electrons from two electron carrier molecules. A total of ten protons are pumped across the IMM per electron pair donated by NADH.

Pumping of protons by the mitochondrial electron transport chain generates a proton gradient across the IMM and also an electric potential ($\Delta\Psi_m$). This gradient is used by **complex V** (the ATP synthase) as energy to produce ATP in the mitochondrial matrix.

ADP is transported into the matrix by the adenine nucleotide translocase (ANT) in exchange with ATP, transported into the IMS. Inorganic phosphate (Pi) is transported into the matrix by the Pi translocator, which also transports a proton into the matrix. For each ADP molecule phosphorylated by the ATP synthase in order to form ATP, 3 more protons are transported back to the matrix. Thus, the activity of the

electron transport chain is essential to maintain the proton gradient and also the electric potential.

The oxidative reactions in the mitochondria are also generators of reactive oxygen species (ROS), making the mitochondria a site and a target of oxidative stress.

1.6.2.1 Drug-addiction and oxidative stress

Oxidative stress is defined as the imbalance in the production ROS (oxidants) versus the activity of the detoxifying systems (antioxidants) in the cells, towards an increase in the levels of oxidants.

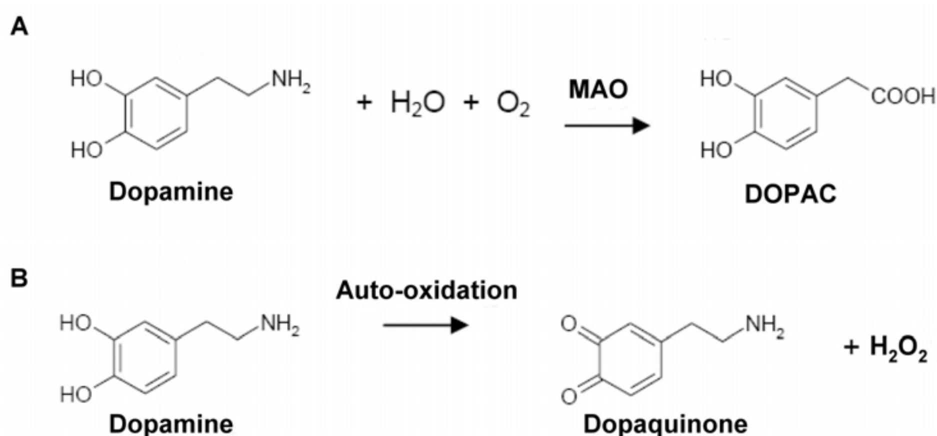
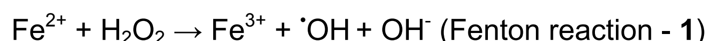


Figure 1.25: Oxidative metabolism of dopamine. A) Enzymatic deamination by MAO, B) Auto-oxidation. Both processes result in the production of H_2O_2.

As described above, all the drugs of abuse induce an increase in the extracellular levels of the neurotransmitter dopamine in specific brain areas. Dopamine is easily oxidized, by enzymatic and non-enzymatic mechanisms (Figure 1.25) and may induce oxidative stress in dopaminergic and neighboring cells. This may contribute to the neurotoxicity of the drugs of abuse. Synaptic activity of dopamine is mainly regulated by two mechanisms: reuptake, responsible for 70-80% of dopamine recycling, and/or inactivation (metabolism). Dopamine may be metabolized intracellularly by MAO (see section 1.5.2.1.1), a mitochondrial enzyme, present in the cytoplasmic side of the outer membrane. MAO catalyses the deamination of dopamine

producing 3,4-dihydroxyphenylacetic acid (DOPAC) and hydrogen peroxide (H_2O_2). Dopamine auto-oxidation also produces H_2O_2.

H_2O_2 may react with transition metal ions, via the Fenton/Haber-Weiss reactions (**1**, **2**), originating the highly toxic hydroxyl radical ($^{\bullet}OH$).



$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$ (Fenton reaction - **1**)

$O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$ (Haber-Weiss reaction - **2**)

Thus, dopamine is both a neurotransmitter and a neurotoxin, and changes in dopamine metabolism may induce oxidative stress and cell death in dopaminergic or surrounding cells (Jones et al., 2000), if the antioxidant systems are not able to deal with the increase in the levels of ROS. The increase in ROS levels may lead to cell death due to the oxidation of important cellular macromolecules such as aminoacids, phospholipids and nucleic acids (Cadet and Brannock, 1998).

1.6.2.1.1 Detoxification of H_2O_2

Cells have specific antioxidant systems (enzymatic and non-enzymatic) that respond to increases in H_2O_2.

Glutathione (γ-L-Glutamyl-L-cysteinyl-glycine) is a tripeptide that can exist in the reduced (GSH) and oxidized (GSSG) forms. Glutathione is an antioxidant that reduces disulfide bonds in cytoplasmic proteins, by acting as an electron donor. When this happens, glutathione is oxidized and a disulfide bond is formed between two cysteines of two GSH molecules. The levels of GSH/GSSG are maintained by two enzymes: **glutathione reductase** (GRed) and **glutathione peroxidase** (GPx). The latter converts two molecules of GSH in GSSG and simultaneously detoxifies H_2O_2 forming two water molecules. GRed reduces GSSG into 2 molecules of GSH by using NADPH/H^+, which is converted into $NADP^+$. NADPH/H+ is produced by glucose-6-phosphate dehydrogenase in the pentose-phosphate pathway. This group of reactions is also known as the glutathione redox cycle (Figure 1.26).

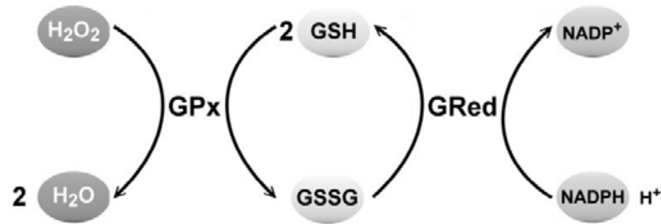
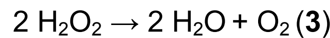


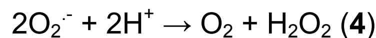
Figure 1.26: Glutathione redox cycle. H_2O_2 is detoxified by glutathione peroxidase (GPx), producing water, using 2 molecules of GSH that are converted into GSSG. Glutathione reductase (GRed) converts GSSG back to GSH, using NADPH/H^+, which is converted into $NADP^+$.

Another enzyme that detoxifies H_2O_2 is **catalase**, present in the peroxisomes, which catalyzes the reaction (**3**):



$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2\ (\mathbf{3})$$

Superoxide dismutase (SOD), another antioxidant enzyme, contributes to increase H_2O_2 levels by detoxification of the superoxide anion ($O_2^{.-}$) (**4**). The H_2O_2 produced by SOD has to be detoxified by the systems described above.



$$2O_2^{.-} + 2H^+ \rightarrow O_2 + H_2O_2\ (\mathbf{4})$$

The levels and the activity of these enzymes are regulated by the cells and are important to maintain cellular homeostasis.

When continuously exposed to oxidants, cells may increase the levels of antioxidant systems in order to maintain homeostasis. Thus, the effects of chronic exposure to oxidants contrast with the effects of acute exposure. For example, acute exposure to H_2O_2 can induce apoptotic cell death in PC12 cells (Benedi et al., 2004; Jang and Surh, 2004), whereas cells chronically exposed to low concentrations of H_2O_2 become resistant to the acute toxicity of this compound (Wiese et al., 1995; Davies, 1999). Acute exposure to H_2O_2 may then be used as a model of cytotoxicity whereas chronic treatment of PC12 cells with a low concentration of H_2O_2 may be a model of cell adaptation (Jackson et al., 1994).

1.6.2.1.2 Amphetamines and oxidative stress

Amphetamines were shown to induce neurotoxicity, both upon acute and chronic exposure. It is believed that the mechanisms involved in amphetamine toxicity are mediated by oxidative stress, due to an increase in the levels of H_2O_2 resulting from enzymatic and non-enzymatic oxidation of dopamine. Table 1.6 summarizes some of the recent studies investigating the role of oxidative stress in the effects of amphetamines in neurons.

Table 1.6: Evidences for amphetamine-induced oxidative stress in the brain or in neuronal cells

Drug	Biological model	Mechanisms	Dose/time of exposure	Reference
Meth	Human SH-SY5Y cell line	↓$\Delta\Psi_m$ ↑ROS Protected by vitamin E	Acute: 0.17-1.68 mM, 24-72h	(Wu et al., 2007)
Meth	Human caudate and frontal cortex	↑4-HNE ↑MDA	--	(Fitzmaurice et al., 2006)
d-Amph	*In vivo*- Wistar rats Prefrontal cortex, striatum, hippocampus	Acute: ↑SOD prefrontal cortex for 1 mg/kg Chronic: ↑SOD hippocampus; ↓SOD striatum for 1 and 4 mg/kg ↑Catalase prefrontal cortex and hippocampus for 4 mg/kg ↓Catalase hippocampus and striatum for 2mg/kg	1 mg/kg, 2 mg/kg, or 4 mg/kg Acute: 1 day Chronic: 7 days	(Frey et al., 2006b)
		Chronic exposure: ↑TBARS and ↑Superoxide in prefrontal cortex and hippocampus		(Frey et al., 2006a)
Meth	Striatal synaptosomes (Wistar rat)	↑ROS Protected by antioxidants	Acute 2h, 2 mM	(Pubill et al., 2005)
Meth	Human caudate	↑SOD ↑GSSG	--	(Mirecki et al., 2004)
d-Amph	Rat brain	↑GST – hypothalamus ↑GPx – striatum, NAc and medial prefrontal cortex ↑GRed - hypothalamus ↑Catalase - medial prefrontal cortex ↓GRed – medial prefrontal cortex	Chronic (14 days) 20 mg/kg/day	(Carvalho et al., 2001)
Meth	*In vivo*- Wistar rats Prefrontal cortex Striatum	↑ SOD ↑ TBARS	Acute 10-15 mg/kg	(Acikgoz et al., 2000)

Abbreviations: Meth- methamphetamine, d-amph- d-amphetamine, $\Delta\Psi_m$- mitochondrial potential; ROS- reactive oxygen species; HNE- hydroxynonenal; MDA- malondialdehyde; SOD- superoxide dismutase; TBARS- thio-barbituric acid reactive substances; GST- glutathione-S-transferase; GPx- glutathione peroxidase; GRed- glutathione reductase

Acute exposure to amphetamine induces an increase in ROS production and interferes with mitochondrial function, whereas chronic exposure induces changes in the activity or expression of antioxidant enzymes. The changes observed seem to be specific for each brain region studied.

Amphetamines interfere with the mitochondrial function in several ways. Methamphetamine was shown to inhibit the mitochondrial respiratory chain (Burrows et al., 2000), and to inhibit ATP synthesis. Moreover, amphetamines may also increase ATP utilization, due to hyperthermia and hyperactivity (reviewed by Brown and Yamamoto, 2003).

Since amphetamines are lipophilic weak bases, they may diffuse into the mitochondria and contribute to the alkalinization of the matrix (Sulzer and Rayport, 1990), disrupting the mitochondrial membrane potential (Cunha-Oliveira et al., 2006a).

1.6.2.1.3 Cocaine and oxidative stress

Cocaine has been shown to induce oxidative stress in the brain. Some recent studies investigating the role of oxidative stress in cocaine neurotoxicity are summarized in Table 1.7. Both acute and chronic cocaine administrations affect the oxidative status of neurons, especially in the striatum, frontal cortex and hippocampus.

Table 1.7: Evidences for cocaine-induced oxidative stress in the brain or neuronal cell cultures

Biological model	Mechanisms	Dose/time of exposure	Reference
In vivo: prenatal exposure- rat hippocampus and cortex	Nitric Oxide TBARS	Repeated: 20 mg/kg/day 4 days	(Bashkatova et al., 2006)
Human neuronal progenitor cells Frontal cortex and striatum	Protein carbonyl Protein HNE ↓GSH	Acute: 1 μM; 30 min; analysed after 6-96 h	(Poon et al., 2007)
In vivo: Swiss mice, striatum and frontal cortex	Low dose: ↓catalase in striatum High dose: ↓catalase activity in cortex and striatum	Low dose: 10-30 mg/kg High dose: 90 mg/kg	(Macedo et al., 2005)
In vivo: rat – frontal cortex and striatum	↑H_2O_2 Lipoperoxidation ↓Complex I activity ↑SOD, ↑GPx	20 mg/kg/day Acute – 1 day Chronic- 10 days	(Dietrich et al., 2005)
In vivo: prenatal exposure – rat brain	↓GSH ↓reduced VitE ↑oxidized Vit E	Single	(Lipton et al., 2003)

Abbreviations: Vit E- vitamin E. For other abbreviations see legend of Table 1.6.

Cocaine was also shown to impair mitochondrial respiration (Devi and Chan, 1997) and to disrupt mitochondrial potential (Cunha-Oliveira et al., 2006a; Yuan and Acosta, Jr., 1996).

1.6.2.1.4 Opiates and oxidative stress

Acute exposure to opiates was also reported to induce oxidative stress in neuronal tissues. Some recent studies showing changes in the levels of oxidants and antioxidants in neurons are summarized in Table 1.8. Acute morphine and heroin exposures affect the levels of antioxidants and increase damage to proteins, lipids and nucleic acids, in the brain.

Table 1.8: Evidences of opiate-induced oxidative stress in neurons

Drug	Biological model	Mechanisms	Dose/time of exposure	Reference
Morphine	Rabbit; brain and spinal tissues	↑Lipid peroxidation ↓GSH ↓Unsaturated fatty acids	6 mg/kg intraspinal	(Ozmen et al., 2007)
Heroin	Mouse brain	↓SOD ↓CAT ↓GPx ↑Oxidative damage to DNA, lipids and proteins	i.p.	(Xu et al., 2006)
Morphine	Rat brain	↓GSH	Single i.p. 3,6 or 12 mg/kg	(Guzman et al., 2006)
Heroin	Mouse brain	↓GSH/GSSG ↓SOD ↓Catalase ↓GPx ↑8-OHdG ↑Protein carbonyls ↑MDA	i.p.	(Qiusheng et al., 2005)

Abbreviations: GSH- reduced glutathione; SOD- superoxide dismutase; CAT- catalase; GPx- glutathione peroxidase; GSSG- oxidized glutathione; 8-OHdG- 8-hydroxy-2-deoxyguanosine; MDA- malondialdehyde, i.p.-intraperitoneal

1.6.2.2 Mitochondria in cell death

Besides being essential for cell survival, mitochondria have been also shown to play a crucial role in cell death. The permeabilization of mitochondrial outer membrane, and disruption of mitochondrial function is a fundamental step in several pathways of cell death (Kroemer et al., 2007). The disruption of the OMM allows the release of soluble proteins that usually are only present in the IMS.

Programmed cell death can occur through several tightly regulated pathways. One of the best characterized types of cell death is apoptosis. Apoptosis was firstly described in 1972 (Kerr et al., 1972) as a form of cell death morphologically distinct from necrosis. Morphological hallmarks of apoptotic cells are cell shrinkage, fragmentation into membrane-bound apoptotic bodies, chromatin condensation and fragmentation.

Biochemically, apoptotic cells maintain membrane integrity and ATP levels, at least in the first steps. Apoptosis is characterized by three phases: initiation, integration/decision and execution/degradation. The signals that trigger apoptosis may come from out of the cell (extrinsic) of from inside (intrinsic). Two main molecular pathways of apoptosis have been characterized, one being activated by extrinsic signals, involving the activation of death-receptors in the cell membrane, and another activated by intrinsic stimuli and involving mitochondrial membrane permeabilization. Both these pathways involve the activation of cysteine aspartic proteases, named caspases, which are activated by proteolysis.

The initiation phase in the **extrinsic** pathway involves the activation of death-receptors, such as tumor necrosis factor (TNF) receptor 1, CD95/Fas or the TNF-related apoptosis inducing ligand receptors 1 and 2 (TRAIL), and the consequent activation of the initiator caspases -8 or -10. The initiation phase in the **intrinsic** apoptotic pathway involves the permeabilization of the mitochondrial membrane, releasing apoptotic factors to the cytosol, such as cytochrome c, second mitochondria-derived activator of caspases (Smac/DIABLO), Omi serine protease (Omi/HtrA2), apoptosis inducing factor (AIF) or endonuclease G (EndoG). Cytosolic cytochrome c reacts with deoxy ATP (dATP) and apoptotic protease activating factor 1 (APAF-1) in

the cytosol, inducing a change in APAF-1 conformation that enables the formation of the apoptosome, a complex composed by seven subunits of APAF-1/cytochrome c/dATP, which activates the initiator caspase-9.

The initiator caspases of both pathways activate the effector caspase -3, initiating the degradation phase, which has common features for the two apoptotic pathways. Caspase-3 cleaves several substrates in the cell, including the effector caspase-6, poly-ADP-ribose polymerase (PARP) – a protein involved in DNA repair, and caspase-activated DNAse (DFF/CAD) – a nuclease that contributes to apoptotic DNA fragmentation.

These mechanisms are involved in the appearance of the hallmarks of apoptosis, namely phosphatydylserine exposure, membrane blebbing, DNA fragmentation and condensation and the formation of apoptotic bodies.

Caspase-independent apoptosis has also been described (Kroemer and Martin, 2005). The mitochondrial factors AIF and EndoG, which are also released upon OMM permeabilization, induce apoptotic DNA fragmentation independently of caspase activity.

1.6.2.2.1 Regulation of mitochondrial membrane permeabilization

The integrity of mitochondrial membranes is essential to maintain different molecular environments. The IMM must be impermeable to protons, in order to allow the establishment of the proton gradient essential to the synthesis of ATP. The permeability of the OMM is also regulated, although it has been assumed that the presence of the voltage dependent anion channel (VDAC) makes this membrane freely permeable to molecules up to 5 kDa. However, VDAC has been shown to control and limit the diffusion of Ca^{2+} (Rizzuto and Pozzan, 2006). OMM permeabilization mainly occurs through a Bax/Bak-mediated mechanism. IMM permeabilization is still a controversial issue, although some authors defend that a mechanism involving the

permeability transition pore (PTP) could contribute to mitochondrial-mediated apoptosis.

1.6.2.2.1.1 Bax/Bak mediated OMM permeabilization

Proteins of the Bcl-2 family regulate the integrity of the OMM. This family of proteins can be divided into sub-families, according to the presence of different Bcl-2 homology (BH) domains in their structure, which confer different functions (Figure 1.27). Multi-domain antiapoptotic proteins with four different BH domains, referred as BH1234, include Bcl-2 and Bcl-X_L. Proapoptotic proteins include the multi-domain proteins (BH123) such as Bax and Bak, and the BH3-only proteins, including Bid and Bad. In all sub-families there are members with a transmembrane domain that allows their insertion into lipid membranes.

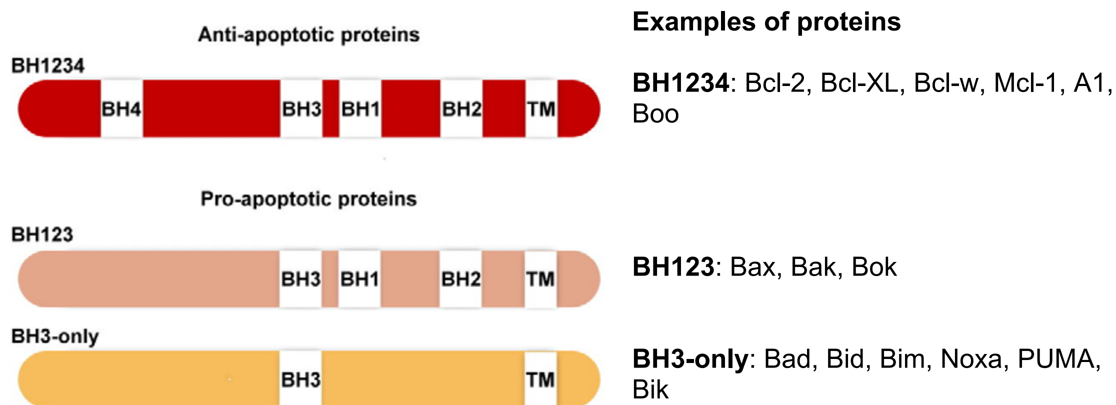


Figure 1.27: Structure of typical members of the subclasses of the Bcl-2 family.

BH1234 normally reside in the OMM, protecting the mitochondria against permeabilization, namely by binding and neutralizing the proapoptotic members of the Bcl-2 family, which induce OMM permeabilization. BH1234 proteins may also be present in the membrane of the endoplasmic reticulum (ER).

Members of the **BH123** proapoptotic proteins have different sub-cellular locations. Bak is normally associated with the OMM, whereas Bax resides in the cytosol, under normal conditions. Bax-mediated OMM permeabilization is initiated by

the translocation of Bax from the cytosol and insertion in the OMM, where it forms openings in the OMM, alone or in association with other pro-apoptotic proteins. These openings can result from the assembly of Bax homo-oligomeric pores or from the destabilization of the lipid bilayer.

BH3-only proteins can exert their pro-apoptotic function either by facilitating or activating BH123 proteins, which initiate OMM permeabilization. *Facilitators*, such as Bad, interact with BH1234 proteins, dissociating them from other pro-apoptotic proteins, which become free to promote OMM permeabilization. The *activators*, such as tBid (which results from the cleavage of Bid by caspase-8), directly activate BH123 proteins, either by stimulating the translocation of Bax to the OMM or by interacting with Bak.

The expression of Bax or Bak has been shown to be required for OMM permeabilization in several models of apoptosis. Models of OMM permeabilization that do not require Bax or Bak can exhibit permeabilization through a VDAC mediated mechanism (reviewed by Kroemer et al., 2007).

1.6.2.2.1.2 IMM permeabilization – the permeability transition pore

The permeability transition pore (PTP) is a voltage-dependent, high-condutance channel located in the OMM, permeable to solutes with a molecular mass up to 1.5 kDa. The molecular composition of the PTP is still controversial, but some consensus in found for the contribution of dynamic interactions between VDAC, ANT and cyclophylin D (CypD). Other proteins thought to be involved in the formation of the PTP are hexokinase, peripheral benzodiazepine receptor (PBR) and Bcl-2 family members, in the OM, creatine kinase, in the IMS, the ANT and CypD, in the IMM. Consequences of the opening of the PTP are mitochondrial swelling and loss of mitochondrial potential. These pores are inhibited by cyclosporin A, a CypD ligand and are activated by Ca^{2+} (Zamzami and Kroemer, 2001).

1.6.2.2.1.3 Reorganization of cristae

The observation that most of the mitochondrial cytochrome c (~85%) is present in the recently described mitochondrial compartment, the ICS (Scorrano et al., 2002), implicates that an additional mechanism is needed to enable complete cytochrome c release upon OMM permeabilization. This mechanism involves remodelling of the cristae, resulting in the removal of the diffusion barrier and mobilization of the cytochrome c from the ICS to the IMS. Reorganization of cristae is mediated by proteins involved in mitochondrial dynamics, in the processes of mitochondrial fusion and fission. The junctions between the ICS and IMS were recently shown to be maintained by Opa1 (optic atrophia 1) (Frezza et al., 2006), an integral protein of the IMM involved in mitochondrial dynamics. The proteolytic activation of Opa1 by the presenilin-associated rhomboid like (PARL), an integral protease present in the IMM, releases truncated Opa1 to the IMS (Cipolat et al., 2006). The soluble and the truncated forms of Opa1 interact to maintain the diffusion barrier between the ICS and the IMS (Frezza et al., 2006). Cleavage of Opa1 by PARL is therefore important to maintain cytochrome c in the ICS and PARL dysfunction may represent a mechanism involved in the triggering of mitochondria-dependent apoptosis.

Changes in mitochondrial dynamics towards an increase in mitochondrial fission have been shown to be associated with mitochondrial-dependent apoptosis (Youle and Karbowski, 2005).

1.6.2.3 Drugs of abuse and apoptosis in the Central Nervous System

Induction of neuronal cell death by apoptosis by some drugs of abuse has been documented. Recent studies about amphetamine-induced neuronal apoptosis are presented in Table 1.9.

Amphetamines induce apoptosis upon acute and repeated exposures. Apoptotic pathways induced by amphetamines in neurons seem to be mainly mediated by the mitochondrial apoptotic pathway, associated with a decrease in Bcl-2 levels and direct interference with mitochondrial potential.

Table 1.9: Evidences of apoptosis induced by amphetamine or its derivatives in the brain or neuronal cells.

Drug	Biological model	Mechanisms	Dose/time of exposure	Reference
MDMA	Rat cortical neurons	Stimulation of serotonin 2A-receptor	200 - 1600 μM	(Capela et al., 2006)
Meth	*In vivo*- mouse striatum	TUNEL positive cells	30 mg/kg, i.p. Tested 24h later	(Zhu et al., 2006)
d-Amph	Rat cortical neurons	Caspases -2, -9 and -3 ↓$\Delta\Psi_m$	Acute (24 h) 500 μM	(Cunha-Oliveira et al., 2006a)
d-Amph	*In vivo* –mouse medium spiny striatal neurons	Mitochondrial pathway Caspase-3, ↑p53, ↑Bax, ↓Bcl-2 Bax-KO mice are resistant	Repeated: 10mg/kg, 4 times, every 2h	(Krasnova et al., 2005)
Meth, MDMA	Rat cerebellar granule cells	Caspase-3; ROS, cytochrome c	Acute (48 h, 1-5 mM)	(Jimenez et al., 2004)
d-Amph	PC12	Caspase-3 Cytochrome c	Acute (5h, 300 μM)	(Oliveira et al., 2003)
d-Amph	PC12	↓ATP/ADP	Acute (96h, 1mM)	(Oliveira et al., 2002)
Meth	*In vivo*- mouse striatum	↑p53; ↓Bcl-2	Repeated: 10mg/kg, 4 times, every 2h	(Imam et al., 2001)
d-Amph Meth MDMA	Neocortical neurons	Bcl-xL/s changes c-Jun	Acute (1-96 h, 125μM-1mM) Apoptosis evaluated at 96h, 500μM	(Stumm et al., 1999)
Meth	Immortalized neural cells from rat mesencephalon	Apoptosis Protection by Bcl-2 overexpression ROS?	1-3 mM (24 h)	(Cadet et al., 1997)

Abbreviations: Meth- methamphetamine, d-Amph- d-amphetamine, MDMA- ecstasy, MDA- malondialdehyde

Cocaine was also described to activate the mitochondrial apoptotic pathway. However, cocaine seems to be less toxic than amphetamine, and in some studies apoptotic neurons were not observed upon exposure to cocaine (Cunha-Oliveira et al., 2006a; Dietrich et al., 2005). Recent studies on cocaine-induced neuronal apoptosis are presented in Table 1.10.

Table 1.10: Evidences of cocaine-induced apoptosis in the brain or neuronal cells.

Biological model	Mechanisms	Dose/time of exposure	Reference
Fetal locus coeruleus neurons	Bax/Bcl-2 Caspase-3	Acute (30 min -24 h) 500 ng/ml	(Dey et al., 2007)
Human neuronal progenitor cells	Oxidative stress (48h) Cell death (72 h)	Acute (30 min)	(Poon et al., 2007)
Rat cortical neurons	Caspases -2,-9 and -3 ↓$\Delta\Psi_m$ Mitochondrial apoptotic pathway No apoptotic morphology	Acute (24 h) 1 mM	(Cunha-Oliveira et al., 2006a)
PC12 cells -rat	Immediate early genes Transcription factors Caspases	Acute (24h) 50-2500 μM	(Imam et al., 2005)
In vivo – rat – dopaminergic brain structures	No apoptosis	20 mg/kg/day Acute – 1 day Chronic- 10 days	(Dietrich et al., 2005)
PC12 - rat	Caspase-3 Cytochrome c	Acute (5 h) 300 μM	(Oliveira et al., 2003)
Prenatal exposure – rat brains	c-Fos Caspases	Acute (binge) 3 x 15 mg/kg – 1h interval in the day before birth Evaluated 24 h after birth	(Mitchell and Snyder-Keller, 2003)
Cerebral vascular muscle cells - dog	TUNEL positive cells	Acute (12-24 h) 1 μM-1mM	(Su et al., 2003)

Abbreviations: TUNEL- terminal dUTP nick-end labelling, $\Delta\Psi_m$ – mitochondrial potential.

Although there are some studies reporting heroin (Fecho and Lysle, 2000) and morphine (Bhat et al., 2004) cytotoxicity, the molecular mechanisms of neurotoxicity induced by these opiate drugs is scarcely documented. However, a few studies have shown the involvement of apoptosis in neuronal dysfunction induced by opiates (Table 1.11).

Table 1.11: Evidences of apoptosis induced by opiates in neuronal cells.

Drug	Biological model	Mechanisms	Dose/time of exposure	Reference
Morphine	Rat spinal cord	Caspase-3, MAPK, PKA	10μg, twice daily, 7 days	(Lim et al., 2005)
Heroin	PC12 cells	Cytochrome c Caspase-3	30 μM (without serum) – 5 h	(Oliveira et al., 2003)
Heroin	PC12 cells	↑Dopamine metabolism	300 μM – 4 days	(Oliveira et al., 2002)
Morphine	Rat spinal cord	↑Caspase-3 ↑Bax ↓Bcl-2 NMDA receptors	20 μg, twice daily for 7 days	(Mao et al., 2002)
Morphine	Fetal human neurons	Caspase-3	1 μM (without serum) – 5 days	(Hu et al., 2002)

Abbreviations: MAPK- mitogen activated protein kinase, PKA- protein kinase A, NMDA- N-methyl-D-aspartate

1.6.2.4 Drugs of abuse and neurogenesis

Besides being toxic to neurons, drugs of abuse have also been shown to induce a decrease in hippocampal neurogenesis (reviewed by Eisch and Harburg, 2006), compromising the capacity of the brain to generate new neurons.

In the adult mammalian brain there are two main neurogenic regions (Figure 1.28), the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus.

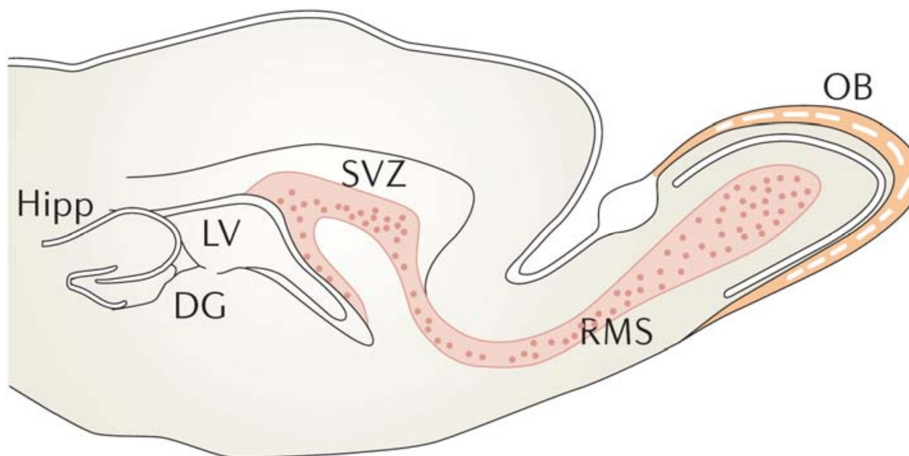


Figure 1.28 Schematic diagram showing the two constitutively neurogenic regions of the adult mammalian CNS (SVZ/olfactory bulb and hippocampal dentate gyrus-DG). Reproduced with permission from Nature Reviews Neuroscience (Lledo et al., 2006) ©2006 Macmillan Magazines Ltd.

The first study showing that morphine and heroin induced a decrease in SGZ neurogenesis was published in 2000 (Eisch et al., 2000). Since then, cocaine (Yamaguchi et al., 2004; Mackowiak et al., 2005) and amphetamines (Teuchert-Noodt et al., 2000) were also reported to decrease hippocampal neurogenesis.

Repeated or chronic exposure seems to be required to observe the inhibition in adult hippocampal neurogenesis induced by opiates or psychostimulants (Eisch and Harburg, 2006)

Although the mechanisms involved in the decrease in neurogenesis induced by drugs of abuse are just starting to be investigated, they may be related with alteration of the proliferative environment, direct action of the drugs of abuse in the progenitor cells or alteration of the cell cycle of SGZ (Eisch and Harburg, 2006).

The effect of drugs of abuse on neurogenesis seems to be selective for the SGZ, and does not seem to occur in the SVZ (Nixon and Crews, 2004). A decrease in hippocampal neurogenesis may result in long-lasting effects on learning, memory and cognition.

1.7 Pharmacotherapies for drug addiction

Some individuals that experiment drugs become addicted to them. The liability to addiction varies from drug to drug, being of 5-10% for cocaine and of 25-33% for opiates, depending not only on the effects of the drugs but also on genetic and environmental factors that lead to repeated drug use (reviewed by Kreek et al., 2002).

Treatment for drug addiction is generally directed to alleviating the withdrawal symptoms, normalizing any physiological functions that were disrupted by drug use, and preventing craving and relapse.

There are three main time points for the pharmacological intervention in drug addiction (Figure 1.29):

1) during active use of the drug of abuse
2) during withdrawal (detoxification)
3) during abstinence (or chronic maintenance or replacement treatment) - relapse prevention

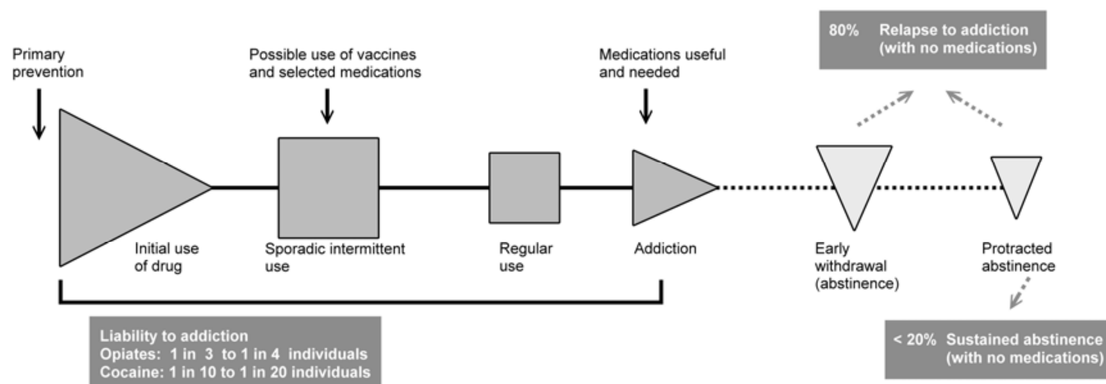


Figure 1.29: Stages of drug addiction and importance of medications. Primary prevention may be useful to stop the whole process of addiction, preventing the initial use of a drug. Vaccines and some medications may be useful to prevent sporadic intermittent use of drugs. Once addiction is established medications are useful and needed, because less than 20% of the addicted individuals sustain abstinence without medications Adapted, with permission from Nature Reviews Drug Discovery (Kreek et al., 2002) ©2002 Macmillan Magazines Ltd.

Opiates are the drugs of abuse that induce a higher number of individuals to search treatment because they induce very severe symptoms. Substitution therapies are needed because more than 80% of the addicted individuals that do not receive medications are not able to sustain abstinence and eventually relapse to drug use. There are three effective **substitution therapies** for the long-term treatment of opiate addiction: methadone, levo-alpha-acetyl-methadol (LAAM) - two opioid receptor agonists, and buprenorphine - a partial opioid receptor agonist - alone or combined with naloxone, an antagonist of opioid receptors. The molecular mechanisms and the main characteristics of these treatments are described in Table 1.12.

Table 1.12: Substances currently used to treat opiate addiction (based on: Pouletty, 2002; Kreek et al., 2002).

Substance	Characteristics
Methadone	μ-opioid receptor agonist
	Prevents withdrawal symptoms without showing opiate-like effects
	Reduces or eliminates drug craving
	Behavioural treatment needed
LAAM	μ-opioid receptor agonist
	Analogue of methadone
Buprenorphine	μ-opioid receptor partial agonist
	Slow onset and long duration of action
	It's more difficult to overdose unintentionally (antagonistic effect prevails in overdosage)
	Partial agonism may limit maximum effectiveness
Naloxone	μ-opioid receptor antagonist
	Combined with buprenorphine to prevent its abuse liability (naloxone is only bioavailable when injected, blocking the buprenorphine agonist effect)
Naltrexone	μ-opioid receptor antagonist
	Little effectiveness in the treatment of opiate addiction
	Prevents endogenous opioid activity

Treatment with methadone consists in the substitution of one opioid for another, but it can improve the physical and psychological health and social functioning. It also reduces illicit drug use, criminality and the risk for contracting infectious diseases (Kaye et al., 2003).

However, susbstitution therapy with methadone has a high initial dropout rate (30-90%) and an early relapse rate (Kaye et al., 2003). Combination of methadone with clonidine, an α2-receptor agonist, may improve treatment success. α2-Receptors are G-protein coupled adrenergic receptors that inhibit adenylyl cyclase. α2-Receptor agonists are thus helpful to suppress noradrenergic hyperactivity observed during withdrawal, which results in symptoms such as nausea, vomiting, cramps, sweating, tachycardia and hypertension.

Another possible treatment is the **ultrarapid detoxification**, which involves the treatment with opioid receptor antagonists, to eliminate the withdrawal symptoms (Kaye et al., 2003). The withdrawal syndrome is precipitated by the administration of opioid

receptor antagonists (naloxone and/or naltrexone – see Table 1.12), under general anaesthesia. This treatment has the advantage of being a very short detoxification process, lasting 4 to 6 hours. However, this treatment only prevents further withdrawal symptoms and does is not effective in preventing relapse.

Other pharmacotherapies for drug addiction are being developed (reviewed by Kreek et al., 2002). These are mainly directed against μ-opioid receptor and their endogenous ligands (β-endorphin and enkephalin peptides), the stress-responsive axis, components of the dopaminergic system (dopamine receptor antagonists, partial agonists or high efficacy agonists and monoamine reuptake inhibitors) and k-opioid receptor and dynorphin peptides (k-opioid receptor antagonists, partial agonists and high efficacy, selective agonists).

Vaccines against the drugs of abuse could also be useful to decrease the kinetics of entry of the drugs into the brain and reduce their acute effects.

Other therapeutic targets could be explored in the future, namely 1) preventing the toxicity and destruction of synapses, 2) enhancing cognition and improving decision-making capacities and 3) reducing the development of drug-associated memories coupled to conditional cues, which lead to relapse.

OBJECTIVES

Substance abuse and addiction are the most costly of all neuropsychiatric disorders (Madras, 2006).

In the last decades, much progress has been achieved in the understanding the effects of the drugs of abuse on the brain. However, efficient treatments that prevent relapse have not been developed.

Understanding the mechanisms that underlie brain dysfunction observed in these individuals may contribute to improve the treatment of drug addiction, which may have social and economic consequences.

The main objective of the work presented in Part III, is to study the neuronal dysfunction and neurotoxicity induced by the drugs of abuse cocaine, amphetamine (Chapter 1) and heroin (Chapter 2).

The specific objectives were:

1) To study the dysfunction of dopaminergic cells upon chronic exposure to cocaine, amphetamine – Manuscripts 1 (Chapter 1) - and *street* heroin- Manuscript 2 (Chapter 2), compared to H_2O_2

2) To analyse the contribution of NMDA receptor subunits (NR1/NR2A or NR1/NR2B) to *street* heroin toxicity – Manuscript 3 (Chapter 2)

3) To investigate the mechanisms of neurotoxicity of *street* heroin in cortical neurons – Manuscript 4 (Chapter 2)

PART II - MATERIAL AND METHODS

2.1 Materials

Optimem medium, Neurobasal medium and B27 supplement were supplied by *Gibco* (Paisley, UK). RPMI, DMEM, Chymostatin, leupeptin, antipain, pepstatin A, N-Acetyl-Ile-Glu-Pro-Asp-p-nitroaniline (Ac-IEPD-pNA), N-Acetyl-Val-Asp-Val-Ala-Asp-p-nitroanilide (Ac-VDVAD-pNA), N-Acetyl-Val-Glu-Ile-Asp-p-nitroanilide (Ac-VEID-pNA) and N-Acetyl-Leu-Glu-His-Asp-p-nitroanilide (Ac-LEHD-pNA) trypsin, Soybean Trypsin Inhibitor (SBTI), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), anti-microtubule associated protein 2 (MAP-2), ifenprodil and glutathione ethyl ester (GSH-EE) were supplied by *Sigma Chemical Co* (St Louis, MO, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), trolox and N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) were supplied by *Calbiochem* (Darmstadt, Germany). PC12 cells and HEK293 cells were obtained from *ATCC* (Manassas, VA, USA). NT-2 cells were a kind gift from Dr. Russell Swerdlow (University of Virginia Health System, VA, USA). The superfect transfection reagent was obtained from Qiagen (Hilden, Germany). The rat NR1 (NR1a), NR2A and NR2B cDNA clones inserted respectively in pEGFP-N3, pcDNAI, or pDP3 were a generous gift from Dr. John Woodward (Medical University of South Carolina, Charleston, SC, USA). The antibodies anti-caspase 3, anti-caspase 9, anti-Bax and anti-cleaved poly(ADP-ribose) polymerase (PARP) were supplied by *Cell Signaling* (Beverly, MA, USA); ECF and anti-rabbit IgG were obtained from *Amersham Biosciences* (Piscataway, NJ, USA); MitoTracker Green, Hoechst 33342, Alexa anti-rabbit IgG 488, Alexa anti-mouse IgG 594 and rhodamine 123 were supplied by *Molecular Probes* (Eugene, OR, USA). Antibodies against the native and denatured forms of cytochrome c were obtained from *PharMingen* (San Diego, CA, USA). MK-801 was a kind gift from *Merck Sharp & Dohme Research Laboratories* (Merck & Co. Inc., Whitehouse Station, NJ, USA). Idebenone was a kind gift from *Seber* (Odivelas, Portugal). Clocinnamox, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide (CTOP), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydro-benzoquinoxaline-7-sulfonamide (NBQX), d-2-amino-5-phosphono-pentanoic acid (d-AP-5) and naltrindole were purchased from *Tocris* (Bristol, UK). Anti-Bcl-2 was

obtained from *Santa Cruz Biotechnology Inc.* (Santa Cruz, CA, USA). Amphetamine, May-Grünwald and Giemsa solutions were obtained from *Merck* (Darmstadt, Germany). Cocaine and a sample of seizure *street* heroin was provided by the *Instituto da Droga e da Toxicodependência* (IDT, Lisbon, Portugal). Morphine and codeine hydrochlorides were obtained from Uquipa (Lisbon, Portugal).

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Culture and incubation of PC12 cells

PC12 cells were cultured in 75 cm^2 flasks, in RPMI 1640 medium supplemented with 10% (v/v) horse serum, 5% (v/v) bovine serum, 50 U/mL penicillin, and 50 mg/mL streptomycin. Cultures were maintained at 37ºC in a humidified incubator containing 95% air and 5% CO_2. In one study, the cells were plated on poly-L-lysine coated multiwells at a density of 50,000 cells/cm^2 for MTT studies and 160,000 cells/cm^2 for HPLC analysis.

For chronic treatment, PC12 cells were cultured for 7-12 months in RPMI medium supplemented with non-toxic concentrations of cocaine (30 μM), amphetamine (30 μM), *street* heroin (10 μM) or H_2O_2 (10 μM). Toxicity studies revealed similar responses in cells chronically exposed to the drugs or H_2O_2 from 7 to 12 months (data not shown). For acute treatment, the cells were exposed to toxic concentrations of cocaine (3 mM), amphetamine (1 mM), *street* heroin (300 μM) or H_2O_2 (50-75 μM), for 24-96 h. Concentrations of the stimulant drugs (Oliveira et al., 2002) or H_2O_2 (data not shown) for chronic or acute experiments were previously established in the PC12 cells. *Street* heroin was dissolved in DMSO and the maximal concentration of DMSO (0.2 %) used in the experiments was not toxic *per se*.

For the study of the activity of antioxidant enzymes, the cells were grown in suspension, and passed twice a week, in culture medium (control) or in culture medium supplemented with non-toxic concentrations of cocaine (30 μM), amphetamine (30 μM) or H_2O_2 (10 μM), for 1 to 4 weeks.

2.2.1.2 Culture and transfection of HEK293 cells

HEK 293 cells were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.) and were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (Biochrom AG, Berlin, Germany), penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were grown to 50-75% confluence in poly-L-lisine coated multiwells and transfected using the superfect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In co-transfections the NR1:NR2 plasmid ratio was 1:4. Cells were transfected with the DNA-superfect mixture for 4 h and further incubated for 24 h with 10- 1000 µM heroin (provided by the *Instituto da Droga e da Toxicodependência*, Lisbon, Portugal) and/or 10 µM MK-801, in fresh culture media. The rat NR1 (NR1a), NR2A, and NR2B cDNA clones inserted respectively in pEGFP-N3, pcDNAI or pDP3 were a generous gift from Dr. John Woodward (Medical University of South Carolina, Charleston, U.S.A.). NR1 is expressed as a fusion protein with EGFP and is referred in the manuscript as NR1-GFP.

2.2.1.3 Culture of cortical neurons

The frontal cortices of rat embryos (16-17 days) were dissected and the cells washed with isolation medium (in mM: 120.9 NaCl, 4.83 KCl, 1.22 KH_2PO_4, 25.5 $NaHCO_3$, 13.0 glucose, 10.0 HEPES) containing 0.3% BSA. The cells were sedimented at 1000 rpm and dissociated with isolation medium, supplemented with 0.02% trypsin and 0.04 mg/mL DNAse for 10 min at 37ºC. Trypsin was inactivated by adding 0.075% SBTI (trypsin inhibitor) and the cells were centrifuged at 1000 rpm for 5 min. The cells were then mechanically dissociated in isolation medium containing

0.012% SBTI and centrifuged at 1000 rpm for 5 min. The resulting pellet was ressuspended in Neurobasal medium with B27 supplement, 0.2 mM glutamine, 0.1 mg/mL streptomycin and 100 U/mL penicillin (in 5% CO_2/ 95% air), and plated on poly-L-lysine (1 mg/mL) coated multiwells or coverslips. Cortical cultures contained a small percentage of glial cells (less than 10%) as assessed by immunofluorescence using anti- MAP2 and anti-glial fibrillary acidic protein (GFAP) (not shown). After 6 days in culture, the cells were incubated with *street* heroin (4.3- 1280 µg/mL), pure heroin (215 or 840 µM), morphine (4.5 or 17.6 µM) or 6-MAM (47 or 183 µM), for 24 h, unless otherwise specified. *Street* heroin was dissolved in DMSO and the maximal concentration of DMSO (0.2 %) used in the experiments was not toxic *per se* (not shown). Where specified, 1 µM z-VAD-fmk, 1 µM naloxone, 1 µM clocinnamox, 1 µM CTOP, 1 µM naltrindole, 2 µM MK-801, 3 µM ifenprodil, 100 µM d-AP-5, 10 µM NBQX, 3 µM idebenone, 100 µM trolox or 100 µM GSH-EE were preincubated for 30-60 min and were present in the culture media throughout all the experiments. The concentrations of the compounds tested were chosen based on previous studies (Gil et al., 2003; Araujo et al., 2003; Williams et al., 2001).

2.2.1.4 Culture of NT-2 cells

The production of the NT2 rho^0 cell line used in these experiments was previously described (Swerdlow et al., 1997). NT-2 rho^+ and rho^0 cells were grown routinely in 75 cm^2 tissue culture flasks in Optimem Medium, supplemented with 10% heat inactivated fetal calf serum, penicillin (50 U/mL), and streptomycin (50 µg/mL). Uridine (50 µg/mL) and pyruvate (200 µg/mL) were also added to rho^0 cell growth medium. The cells were grown and maintained at 37°C in a humidified incubator containing 95% air and 5% CO_2. The cells were plated at 0.1×10^6 cm^{-2} for cell viability assay and incubated with *street* heroin (128 µg/mL) for 24 h.

2.2.2 Assessment of cell viability

2.2.2.1 MTT assay

Metabolic cell viability was measured using the MTT reduction assay, at 570 nm (Mosmann, 1983). The cells were incubated with 0.5 mg/mL MTT in Na^+ medium (in mM: 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 NaH_2PO_4, 1.5 $CaCl_2$, 5.6 glucose, 20 HEPES, pH 7.4) for 2 h and the precipitated salt was dissolved with 0.04 M HCl in isopropanol. The capacity of treated cells of reducing the tetrazolium salt was expressed as a percentage of absorbance (at 570 nm) in control cells.

2.2.2.2 LDH assay

The integrity of the plasma membrane of cortical neurons was determined by monitoring the leakage of lactate dehydrogenase (LDH), by following the rate of conversion of NADH to NAD^+ at 340 nm, according to Bergmeyer and Bernt (1974). Alterations in membrane integrity were expressed as a percentage of LDH release, over the total LDH, compared to control cells.

2.2.3 Measurement of dopamine and DOPAC levels

The intracellular levels of dopamine and dihydroxyphenylacetic acid (DOPAC) were determined after cell extraction with 0.1 M perchloric acid (0-4ºC). The cells were centrifuged at 15,800 x*g* for 10 min, and the pellet was solubilized with 1 M NaOH for total protein analysis using the Sedmak method (Sedmak and Grossberg, 1977). The resulting supernatants, stored at –80ºC, were extracted with alumina, using dihydroxybenzylamine (DHBA) as an internal standard, and assayed for dopamine and DOPAC analysis by HPLC with electrochemical detection, as described previously

(Warnhoff, 1984). Dopamine accumulated in the cultured media upon acute exposure to cocaine or amphetamine was determined using the same procedure.

2.2.4 Morphological analysis of PC12 cells

Cell morphology was evaluated by staining cell smears with May-Grünwald-Giemsa staining procedure. Naïve cells and cells chronically exposed to the stimulant drugs or to H_2O_2 ($1x10^6$ cells), before or after an acute exposure to 50 μM H_2O_2, were centrifuged at 200 x*g* for 5 min, ressuspended in 30 μl of FBS and placed on a slide for analysis at the microscope. Cell smears were stained with May-Grünwald and Giemsa solutions. Cell morphology was analysed by optic microscopy using a Leitz Dialux 20 microscope associated with a digital camera.

2.2.5 Measurement of intracellular ATP levels

Intracellular ATP, ADP and AMP levels were determined after cell extraction with 0.3 M perchloric acid (0-4ºC). The cells were centrifuged at 15,800 x*g* for 10 min, and the pellet was solubilized with 1 M NaOH for total protein analysis using the Biorad protein assay (Biorad). The supernatants were neutralized with 3 M KOH in 1.5 M Tris, and centrifuged at 15,800 x*g* for 10 min. The resulting supernatants, stored at –80ºC, were assayed for adenine nucleotide determination, by separation in a reverse-phase HPLC, as described previously (Rego et al., 1997).

2.2.6 Evaluation of the activity of antioxidant enzymes

PC12 cells were centrifuged at 1000 x g for 10 minutes and then ressuspended in 200 µl of a lysis buffer containing 10 mM HEPES, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 0.1% Triton X-100, 2 mM DTT, 1:1000 of a protease inhibitor cocktail (chymostatin, leupeptin, antipain- serine and cysteine protease inhibitors and pepstatin

A - potent inhibitor of acid proteases; 1mg/mL each); 0.1 mM PMSF, 50 mM NaF, pH 7.5. The cells were allowed to lyse on ice, for 40 minutes, and then the samples were centrifuged at 2300 x g for 12 min, 4ºC. The supernatants were collected and stored at -20ºC.

2.2.6.1 Glutathione peroxidase (GPx) activity assay

A reaction mixture containing 5 mM K_2HPO_4, 5 mM KH_2PO_4, 0.1 mM EDTA, 1mM GSH, 0.24 U/mL glutathione reductase and 0.25 mM NADPH was freshly prepared from stock solutions. Five µl of sample were added to 175 µl of the reaction mixture and tested for GPx activity by initiation with 1.1 mM *tert*-butyl hydroperoxide (20 µl). The oxidation of NADPH was monitored at 340 nm for 5 minutes, against blanks prepared without NADPH, at 25ºC in a Spectramax Plus 384 microplate reader (Molecular Devices). The acitivity of GPx was calculated and normalized in percentage of the control.

2.2.6.2 Glutathione reductase (GRed) activity assay

A reaction mixture containing 100 mM K_2HPO_4, 1 mM EDTA and 0.1 mM NADPH was freshly prepared from stock solutions. Twenty µl of sample were added to 170 µl of the reaction mixture and tested for GRed activity by initiation with 1 mM GSSG (10 µl). The oxidation of NADPH was monitored at 340 nm for 5 minutes, at 30 ºC, against blanks prepared without GSSG, in a Spectramax Plus 384 microplate reader (Molecular Devices). The acitivity of GRed was calculated and normalized in percentage of the control.

2.2.6.3 Superoxide dismutase (SOD) activity assay

A reaction mixture containing 43.75 mM KH_2PO_4, 0.088 mM EDTA and 0.025 mM hypoxanthine, 0.025% triton X-100 and 0.1 mM NBT was freshly prepared from stock solutions. 10 µl of sample were added to 187 µl of the reaction mixture and tested for SOD activity by initiation with 0.025 U/mL Xanthine Oxidase (3 µl). The reduction of NBT was monitored at 560 nm for 5 minutes at 25ºC, against blanks prepared without hypoxanthine, in a Spectramax Plus 384 microplate reader (Molecular Devices). The maximum rate of NBT reduction mediated by the superoxide radical was determined in wells prepared without any test sample. The activity of SOD in the test samples was proportional to the percentage of inhibition of the maximum NBT reduction. The acitivity of superoxide dismutase was calculated and normalized in percentage of the control.

2.2.7 Chemical analysis

A street heroin sample was analysed in order to quantify diacetylmorphine, morphine, 6-MAM, codeine and acetylcodeine content. HPLC analyses were conducted on an HPLC system (Merck/Hitachi–LaChrom) equipped with a diode-array detector (DAD). The analytical column was a commercially prepacked reverse phase (RP-18) column (250 × 4.0 mm i.d., 5 µm) with precolumn (Waters, Watford, UK). An isocratic elution was performed at a flow rate of 1.5 mL min^{-1} and the absorbance was measured at 216 nm. The analysis was carried out, at room temperature, using a solution of 60% 10 mM aqueous ammonium acetate (pH 3) plus 40% acetonitrile as mobile phase. Prior to use, the solutions were filtered and subsequently sonicated for a minimum of 15 min. The volume of sample injected was 20 µL. A calibration curve of six standards was prepared for each of the compounds (heroin, morphine, 6-MAM, codeine and acetylcodeine). Compounds on seizure *street* heroin samples were

identified by comparison of their retention times with those of known standards. For this study heroin and analogs were synthesized due to the nonavailability of the compounds from commercial sources. Heroin and acetylcodeine were synthesized by the classic method of acetylation, using acetic anhydride and pyridine (Garrido et al., 2004b; Garrido et al., 2004a) at room temperature. 6-Monoacetylmorphine was obtained by deacetylation of the phenolic group of heroin under mild conditions (Garrido et al., 2004b). Qualitative and quantitative analysis of the seizure *street* heroin was accomplished using HPLC with UV detection. *Street* heroin maintained the same composition throughout the experiments. Qualitative thin-layer chromatography was used to detect the presence of caffeine and sugars (Moffat, 1986; Chiarotti et al., 1991; Sharma et al., 2005).

2.2.8 Immunocytofluorescence

2.2.8.1 Cytochrome c

The cells were incubated with MitoTracker Green (1 μM) in Na^+ medium for 1 h. After fixation in 4% paraformaldehyde containing 4% sucrose in saline buffer (PBS, in mM: NaCl 137, KCl 2.7, K_2HPO_4 1.4, KH_2PO_4 4.3 at pH 7.4), the cells were incubated with 20 mM glycine. After cell permeabilization in the presence of saponin (0.1% in 20 mM glycine), the cells were incubated with a specific antibody against the native form of cytochrome c (PharMingen, 1:100 in 0.1% saponin), which was detected by using a secondary antibody Alexa Fluor 594 anti-mouse IgG (1:200 in 0.1% saponin). The cells were visualized in a confocal microscope (Bio-Rad MRC 600).

2.2.8.2 Cleaved PARP

The cells were fixed in 4% paraformaldeyde, permeabilized in 0.2% Triton X-100, for 2 min, and blocked in 3% BSA for 30 min. The fragment resulting from the cleavage of PARP by caspase-3 was detected by using a specific primary antibody

(1:100 in 3% BSA) and a secondary Alexa anti-rabbit 488 antibody (1:200 in 3% BSA). The nuclei were stained by using Hoechst 33342 (1 μg/mL) and the cells were visualized in an epifluorescence microscope (Zeiss Axioscope).

2.2.9 Fluorimetric evaluation of rhodamine-123 cellular retention

The cells were loaded with 1 μM rhodamine-123 for 10 min, in the dark, at 37ºC. The fluorescence (λex 505nm and λem 525nm) was recorded for 10 min, before and after permeabilization with 0.5% Triton X-100, in a SPEX Fluorolog spectrometer equipped with a thermostatic water bath. Rhodamine-123 retention was determined by the difference between total fluorescence (after permeabilization) and the initial value of fluorescence. Because positively charged rhodamine-123 is retained by the mitochondria under normal conditions, corresponding to a high mitochondrial membrane potential ($\Delta\Psi_m$), a decrease in cellular retention of rhodamine-123 was associated with a decrease in $\Delta\Psi_m$ (Palmeira et al., 1996).

2.2.10 Colorimetric evaluation of caspases-like activity

The cells were dissociated in lysis buffer [in mM: 25 HEPES, 2 $MgCl_2$, 1 EDTA, 1 EGTA, 2 DTT, 0.1 PMSF and 1 μg/mL of protease inhibitor cocktail. The resulting extracts were frozen and thawed three times, centrifuged at 15,000 g for 10 min (4ºC), and the protein (supernatant fraction) was quantified by the Bio-Rad protein assay. The supernatant was tested for the activity of caspases 2, 3, 6, 8 and 9, at 405 nm, after reaction with the respective substrates Ac-VDVAD-pNA, Ac-DEVD-pNA, Ac-VEID-pNA, Ac-IEPD-pNA and Ac-LEHD-pNA (100 μM, for 2 h at 37ºC) as described by Gurtu et al (1997). The results were normalized over the control absorbance value, using the same amount of protein (25-40 μg/assay).

2.2.11 Western blotting

2.2.11.1 Sample preparation

For the analysis of caspases activation by western blotting, the cells were treated as for the caspases activity assay. For the analysis of Bcl-2 and Bax levels, total extracts were obtained using total extraction buffer (in mM: 20 Tris (pH 7); 100 NaCl; 2 EDTA; 2 EGTA, 50 NaF; 1 sodium orthovanadate; 0.1 PMSF) supplemented with 100 nM okadaic acid, 1 µg/mL of protease inhibitor cocktail, 0.5% SDS and 0.5% Triton X-100. For the analysis of cytochrome c release, cell lysates were homogenized in sucrose buffer (in mM: 250 sucrose; 20 HEPES, 10 KCl; 1.5 $MgCl_2$; 1 EDTA; 1 DTT; 0.1 PMSF) supplemented with 1 µg/mL of protease inhibitor cocktail. The mitochondrial fraction (P2) was separated by centrifugation at 500 x *g* for 12 min (4ºC) followed by centrifugation of the resulting supernatant at 12,000 x *g* for 20 min (4ºC). Cytosolic fraction (P3) was obtained upon protein precipitation of the resulting supernatant with 5% trichloroacetic acid, followed by centrifugation at 15,800 x *g* for 10 min and pH neutralization with KOH.

2.2.11.2 Immunoblotting procedures

Proteins (30-50 µg/sample) were denatured and separated by SDS-PAGE (12%), and then transferred to a PVDF membrane, which was then incubated with rabbit anti-caspase-3 (1:1000), rabbit anti-caspase-9 (1:1000), mouse anti-Bcl-2 (1:500), rabbit anti-Bax (1:1000), mouse anti-cytochrome c (1:500) and mouse anti-GAPDH (1:2500) antibodies. The secondary detection was performed using anti-mouse IgG or anti-rabbit IgG alkaline-phosphatase-bound antibodies (1:20000). The bands were developed with ECF, visualized in a VersaDoc imaging system (Bio-Rad) and quantified using the Quantity One software (Bio-Rad).

2.2.12 Statistical analysis

Data are the mean ± SEM from at least three independent experiments, performed in duplicate or triplicate. Statistical analysis was performed by one-way ANOVA with Bonferroni post *hoc* test or by the Student's t-test, when comparing two Gaussian populations (a $P<0.05$ was considered significant).

PART III: RESULTS AND DISCUSSION

3.1 CHAPTER 1: Neuroadaptation induced by psychostimulants

3.1.1 MANUSCRIPT 1- Differential cytotoxic responses of PC12 cells chronically exposed to psychostimulants or to hydrogen peroxide

Based on: Teresa Cunha-Oliveira, A. Cristina Rego, M. Teresa Morgadinho, Tice Macedo and Catarina R. Oliveira, Differential cytotoxic responses of PC12 cells chronically exposed to psychostimulants or to hydrogen peroxide, Toxicology (2006) 217: 54-62 and unpublished results.

3.1.1.1 Abstract

Repeated abuse of the stimulant drugs cocaine and amphetamine, is associated with extraneuronal dopamine accumulation in specific brain areas. Dopamine may be cytotoxic through the generation of ROS, namely H_2O_2, resulting from dopamine oxidative metabolism. In this work we studied the cytotoxicity in PC12 cells (a dopaminergic neuronal model) chronically and/or acutely exposed to cocaine or amphetamine, as compared to H_2O_2 exposure. Chronic cocaine treatment induced sensitization to acute cocaine insult and increased cocaine-evoked accumulation of extracellular dopamine, although no changes in DOPAC levels were observed. Moreover, dopamine was depleted in cells chronically exposed to amphetamine and acute amphetamine toxicity persisted in these cells, indicating that dopamine was not involved in amphetamine cytotoxicity. PC12 cells chronically treated with H_2O_2 were totally resistant to acute H_2O_2, but not to acute cocaine or amphetamine exposure, suggesting that the toxicity induced by these stimulant drugs is unrelated to adaptation to oxidative stress. Interestingly, chronic cocaine treatment largely, but not completely, protected the cells against a H_2O_2 challenge, whilst a decrement in intracellular ATP was observed. H_2O_2 resistance of cells chronically exposed to H_2O_2 appears to involve changes in the activity of GPx, GRed and SOD, whereas chronic cocaine increased GPx activity only, possibly explaining the incomplete resistance to acute H_2O_2. PC12 cells chronically exposed to amphetamine initially exhibited changes in GPx, GRed and SOD activities that returned to control levels after 4 weeks of exposure. This biphasic effect may be explained by dopamine depletion evoked by amphetamine, and may explain the lower level of resistance to acute H_2O_2 of cells chronically exposed to amphetamine, in comparison with cells chronically exposed to cocaine. Together, these results indicate that cellular adaptations of PC12 cells to cocaine and amphetamine are associated with changes in dopamine levels and in the activity of antioxidant enzymes, suggesting the involvement of oxidative stress in the chronic effects of these drugs of abuse.

Keywords: Amphetamine, cocaine, drug abuse, neurotoxicity, oxidative stress

3.1.1.2 Introduction

The continuous abuse of drugs is sustained by the activation of the reward circuits in the brain, which is mainly associated with changes in dopaminergic activity (Di Chiara and Imperato, 1988). The stimulant drugs amphetamine and cocaine are known to increase extraneuronal dopamine levels through different mechanisms. Amphetamine can redistribute the dopamine stored in vesicles to the cytoplasm (Sulzer et al., 1995), inducing non-vesicular release of dopamine, which is mediated by the plasma membrane DAT (Kahlig et al., 2005). Amphetamine induces an increase in cytosolic dopamine that may contribute to its increased intracellular metabolism by the mitochondrial enzyme MAO. On the other hand, cocaine inhibits DAT (Brown et al., 2001), preventing the reuptake of dopamine to the nerve terminal, thereby increasing the levels of dopamine in the synaptic cleft.

Dopaminergic cells are particularly sensitive to oxidative stress because dopamine is easily oxidized by MAO or by auto-oxidation, resulting in the production of H_2O_2. Although amphetamine may inhibit MAO (Ramsay and Hunter, 2002), it was previously demonstrated that amphetamine-induced H_2O_2 production is MAO-dependent (Duarte et al., 2004). Another source of intracellular H_2O_2 is the enzyme SOD, which detoxifies the radical $O_2^{\cdot-}$, with consequent formation of H_2O_2. H_2O_2 is detoxified by the antioxidant enzymes GPx and catalase. GPx reduces H_2O_2 into water, and simultaneously oxidizes two molecules of GSH, forming GSSG. GSSG is converted back to GSH by GRed. Catalase also converts H_2O_2 into water. However, while GPx is present in the brain mainly in the cytosol and mitochondria, catalase is restricted to the peroxisomes and has a modest activity in the brain (Mavelli et al., 1982).

ROS have been frequently associated with neuronal cell death due to the oxidation of amino acids, phospholipids and nucleic acids (reviewed by Cadet and Brannock, 1998). Accordingly, acute exposure to H_2O_2 has been reported to induce apoptotic cell death in PC12 cells (Benedi et al., 2004; Jang and Surh, 2004). In contrast, cells chronically exposed to low concentrations of H_2O_2 become resistant to H_2O_2-induced toxicity (Wiese et al., 1995; Davies, 1999). Thus, acute H_2O_2 can be

used as a model of cytotoxicity, whereas chronic treatment of PC12 cells with H_2O_2 is a model of cellular adaptation (Jackson et al., 1994).

Chronic exposure to the drugs of abuse in humans induces long-lasting changes in gene expression, and some of these changes may be correlated with the development of the compulsive behaviour associated with drug addiction (Rhodes and Crabbe, 2005, for review). Changes in gene expression induced by chronic drug exposure may underlie changes in the cellular responses to stress. These alterations are also in the basis for the development of sensitization or tolerance, in human drug abusers, in which the response to the same dose of a drug is increased or decreased, respectively. These processes contribute to the withdrawal symptoms and to increased motivation to drug abuse (Nestler, 2004b). Thus, we hypothesize that chronic drug exposure may also be involved in altered responses to cytotoxic insults, such as an acute exposure to the same drug or to other cytotoxic stimuli, such as oxidative stress.

Psychostimulant drugs of abuse have been suggested to induce oxidative stress in the brain, upon chronic exposure. Moreover, there are evidences of adaptations to oxidative stress upon psychostimulant exposure *in vivo* (Frey et al., 2006b; Carvalho et al., 2001; Acikgoz et al., 2000). Chronic d-amphetamine exposure has been shown to induce changes in the activity of GPx, GRed (Carvalho et al., 2001), SOD and catalase (Frey et al., 2006b) in the rat brain. Changes in the activity of SOD, GPx and in the levels of GSH and hydroperoxides in the rat brain were also observed upon chronic exposure to cocaine (Dietrich et al., 2005). However, it has not been previously demonstrated if adaptation to oxidative stress occurs upon direct exposure of dopaminergic cells to amphetamine and cocaine.

Taking into account our previous studies with PC12 cells exposed to the stimulant drugs (Oliveira et al., 2002), we analyzed the toxicity of cocaine, amphetamine or H_2O_2 (acute exposure) in PC12 cells chronically exposed to non-toxic concentrations of cocaine, amphetamine or H_2O_2 for 7-12 months, as compared to PC12 cells non-exposed to the drugs of abuse (naïve cells). We also evaluated the intracellular ATP/ADP levels and the activity of the antioxidant enzymes GPx, GRed and SOD upon continuous exposure of PC12 cells to cocaine, amphetamine and H_2O_2 in the initial phases of cell adaptation, at 1 to 4 weeks.

Our data suggest that the cytotoxic responses of PC12 cells are modified depending on the chronic exposure to cocaine, amphetamine or H_2O_2. In particular, increased toxicity of cocaine in cells chronically exposed to this stimulant drug may contribute to the behavioural sensitization of drug addicts after acute cocaine exposure. Furthermore, changes in energy metabolism and in the activity of antioxidant enzymes are involved in the chronic effects of cocaine, amphetamine and H_2O_2.

3.1.1.3 Results

3.1.1.3.1 Chronic cocaine treatment sensitizes PC12 cells to acute cocaine

The toxicity of acute exposure to stimulant drugs was analyzed in naïve PC12 cells and cells chronically exposed to cocaine, amphetamine or H_2O_2, by following the MTT reduction assay (Figure 3.1).

Figure 3.1: Acute effect of cocaine, amphetamine or H_2O_2 on the viability of PC12 cells chronically exposed to the stimulant drugs of abuse or H_2O_2. Naïve PC12 cells or PC12 cells chronically exposed to cocaine, amphetamine or H_2O_2 for 7-12 months were incubated with 1 mM amphetamine (amph) or 3 mM cocaine for 4 days or 75 μM H_2O_2 for 24 h. Cell viability was determined by the MTT reduction assay. Data were normalized in percentage of the respective controls, typically with absorbance values (at 570 nm) of 0.42 (naïve), 0.84 (cocaine), 0.83 (amphetamine) and 0.90 (H_2O_2). Data are the means ± SEM of at least 3 experiments performed in triplicate. Statistical significance: ***P<0.001 as compared with the respective control; ###P<0.001 compared to naïve cells.

The effect of long-term exposure to cocaine, amphetamine or H_2O_2 in cell viability was not possible to assess using the MTT assay because cells chronically exposed to these agents revealed an increased capacity to reduce MTT (about 2 fold), which can result from an increase in cell proliferation and/or an increase in cell viability. Exposure of dividing mammalian fibroblasts to low concentrations of oxidants (3-15 µM H_2O_2) was previously reported to stimulate cell growth and proliferation (Davies, 1999). Thus, it is possible that the increase in MTT reduction observed in cells chronically exposed to H_2O_2 or to the stimulants is due to an increase in cell proliferation rather than a direct effect in cellular reduction systems or in cell viability. Therefore, MTT reduction data were normalized as a percentage of control for each sub-cell line to evaluate the susceptibility induced by acute exposure to the agents, after different long-term pre-treatments. Acute cocaine exposure (3 mM, for 96 h) decreased the viability of naïve cells by about 40%, whereas in cells previously exposed to cocaine the decrement in cell viability was about 60%. Thus, chronic cocaine sensitized PC12 cells to acute cocaine toxicity by about 20%. In contrast, acute incubation with amphetamine (1 mM, for 96 h) induced the same decrease in cell viability (about 40%) in naïve cells or in cells previously exposed to amphetamine (Figure 3.1).

Since cocaine and amphetamine are known to interfere with the dopaminergic system, we evaluated the effects of chronic and acute exposure to these psychostimulants in the accumulation of extracellular dopamine (Figure 3.2 A,B), intracellular dopamine levels (Figure 3.2 C,D) and the endogenous levels of its metabolite, DOPAC (Figure 3.2.E,F).

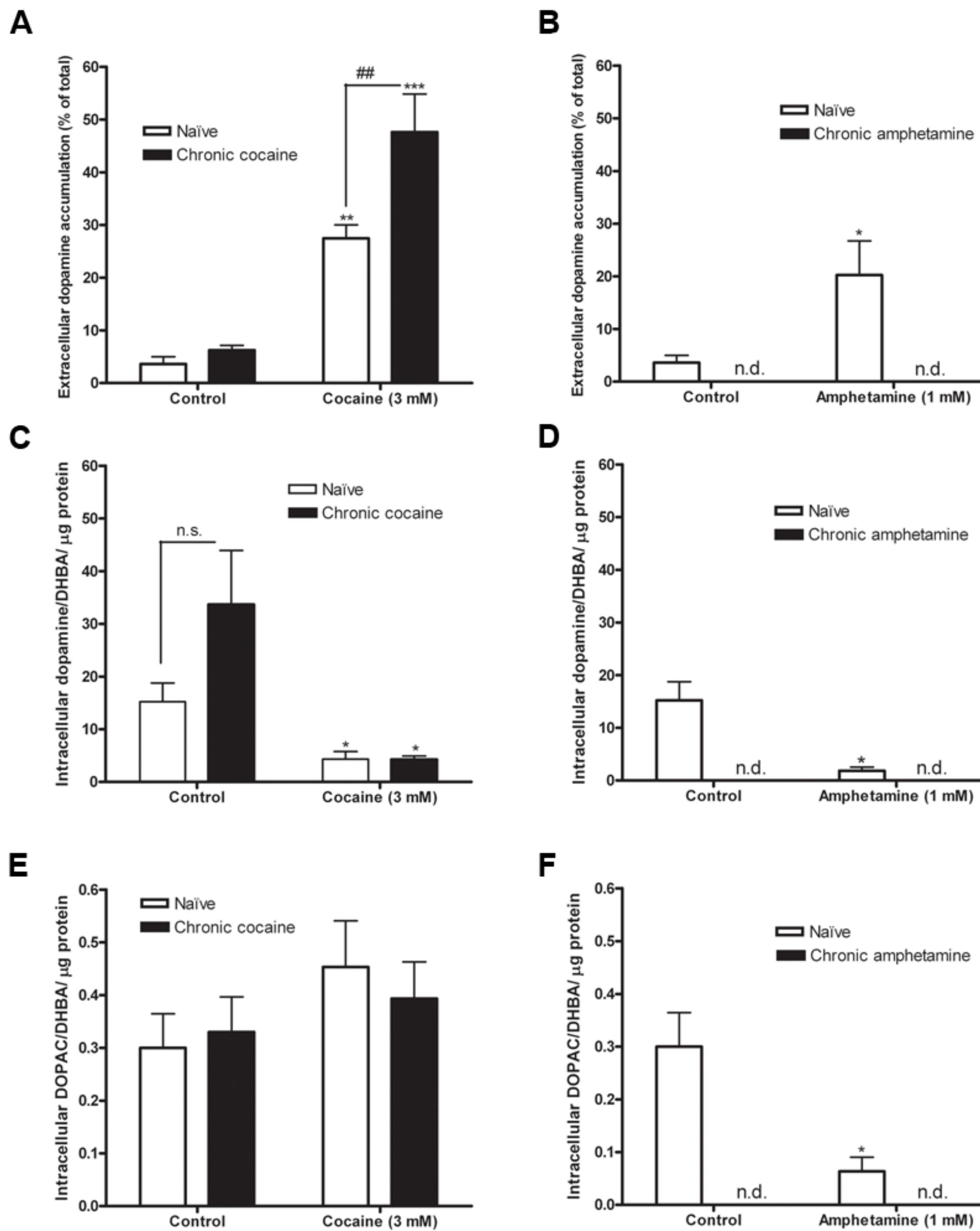


Figure 3.2: Dopamine and DOPAC levels upon an acute exposure to cocaine or amphetamine, of naïve cells or cells chronically exposed to cocaine (**A,C,E**) or amphetamine (**B,D,F**), for 7-12 months. The cells were acutely exposed to cocaine (3 mM) or amphetamine (1 mM), for 24 h. Intra and extracellular dopamine and DOPAC levels were determined by HPLC with electrochemical detection. Data are the means ± SEM of at least 3 experiments performed in triplicate. Statistical analysis: *P<0.05,**P<0.01 and ***P<0.001, compared with the respective control; ##P<0.01 compared to naïve cells. n.d. - non-detectable levels.

Acute cocaine (3 mM) or amphetamine (1 mM) exposure for 24 h in naïve PC12 cells, increased extracellular dopamine accumulation, by about 7.6-fold and 5.6-fold, respectively (Figure 3.2.A,B). Concordantly, under these conditions, a decrease in intracellular dopamine levels was observed in the presence of cocaine (decreased by 3.5 fold) or amphetamine (decreased by 8.3-fold) (Figure 3.2.C,D). Moreover, acute amphetamine treatment largely decreased DOPAC levels, compared to the control, by 4.7-fold (Figure 3.2.F), whereas no significant changes were observed in cells exposed to cocaine (Figure 3.2.E). The decrement of DOPAC levels in cells exposed to amphetamine is consistent with the ability of amphetamine to inhibit MAO (Ramsay and Hunter, 2002).

In cells chronically treated with cocaine, further exposure to cocaine evoked a significant increase in extracellular dopamine by about 18.5% (Figure 3.2.A), when compared to naïve cells. This increase can be related with a non-statistical increase (by 2.2-fold) in intracellular dopamine levels in cells chronically exposed to cocaine, when compared to naïve cells (Figure 3.2.C). DOPAC levels were not significantly changed upon incubation with cocaine in cells chronically treated with the same drug, indicating that the extra dopamine was not metabolized by MAO (Figure 3.2.E). These data suggested that cocaine sensitization in cells chronically exposed to cocaine (Figure 3.1) was related with an increase in cocaine-evoked extracellular dopamine accumulation.

Interestingly, no detectable levels of extracellular or intracellular dopamine (Figure 3.2.B,D) or intracellular DOPAC (Figure 3.2.F) were observed in cells chronically exposed to amphetamine. Thus, amphetamine toxicity in cells chronically exposed to amphetamine (Figure 3.1) can not be attributed to changes in extracellular or intracellular dopamine. Amphetamine-induced depletion of endogenous dopamine can be explained by long-term inhibition of tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of dopamine (Bowyer et al., 1998). On the other hand, amphetamine is also known to decrease DAT activity (Kahlig et al., 2004), decreasing the reuptake of released dopamine.

3.1.1.3.2 Cells chronically exposed to cocaine are partially resistant to H_2O_2

Repeated exposure to H_2O_2 is known to induce cellular adaptation to acute H_2O_2 in PC12 cells (Jackson et al., 1994) and in other cell types (Wiese et al., 1995; Seong et al., 2002). In this study, we compared the toxicity of H_2O_2 in cells chronically exposed to the drugs of abuse or to H_2O_2. In naïve cells, acute exposure to H_2O_2 (75 μM, for 24 h) induced a decrease in cell viability by about 80% (Figure 3.1). In contrast, in cells chronically exposed to H_2O_2 the same acute exposure did not significantly change cell viability, indicating that these cells are completely resistant to H_2O_2-induced toxicity and thus adapted to oxidative stress injury. Moreover, upon chronic exposure to H_2O_2, the toxicity of cocaine or amphetamine was similar to the toxicity observed in naïve cells (Figure 3.1), suggesting that acute toxic effects mediated by the stimulant drugs were independent of adaptive changes induced by oxidative stress. Interestingly, cells chronically exposed to cocaine were more resistant to acute H_2O_2 than naïve cells, showing a decrease in cell viability by about 25% only (Figure 3.1). In contrast, cells chronically exposed to amphetamine were only slightly more resistant to acute exposure to H_2O_2, (cell viability decreased by about 70%), in comparison with naïve cells. No significant changes in Bcl-2/Bax were observed in PC12 cells chronically exposed to cocaine, amphetamine or H_2O_2 (data not shown), thus not explaining the different susceptibility of these cells.

Since chronic cocaine appeared to mediate some resistance to H_2O_2 toxicity (Figure 3.1), we also analyzed the morphology of PC12 cells and the intracellular levels of ATP upon an acute exposure to H_2O_2 in cells chronically treated with cocaine, which were compared to cells adapted to H_2O_2. Exposure of naïve cells to H_2O_2 (50 μM, for 24 h) induced a decrease in cell viability by about 70% (data not shown), similar to 75 μM H_2O_2 (Figure 3.1). Under these conditions, the cells showed membrane blebbing and cell shrinkage (Figure 3.3.A), indicating some degree of lesion, compatible with the viability assay. In contrast, cells chronically exposed to H_2O_2 or to cocaine, showed a

normal morphology after an acute exposure to H_2O_2 (Figure 3.3.A). These data were in accordance with data shown in Figure 3.1.

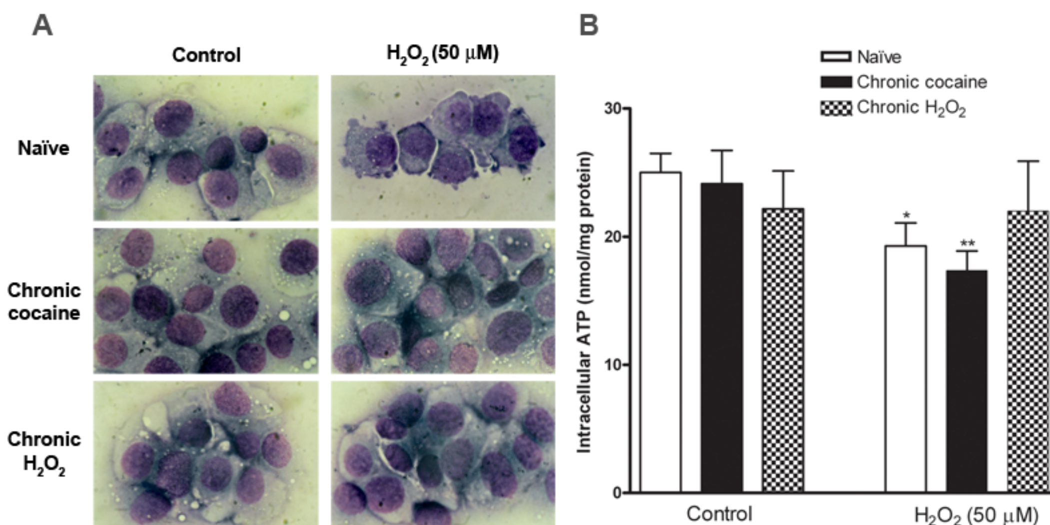


Figure 3.3: Effect of an acute exposure to H_2O_2 in cells chronically exposed to cocaine or H_2O_2. (**A**) Cell morphology: cell smears were stained following May-Grunwald staining procedure and photographed under 100 x magnification in a Leitz Dialux 20 light microscope associated with a camera. Note the formation of blebs and cell shrinkage upon exposure of naïve cells to 50 μM H_2O_2 for 24 h. Images are representative of 3 experiments performed in duplicate. (**B**) Intracellular ATP levels were detected by HPLC with UV detection, before or after incubation with 50 μM H_2O_2 for 24 h. Data are the means ± SEM of 4 experiments performed in triplicate. Statistical significance: *P <0.05 and **P <0.01 compared to the control.

Acute exposure of naïve cells to H_2O_2 induced a decrease in intracellular ATP levels, by about 23%, whereas in cells chronically exposed to H_2O_2, ATP levels were maintained after an acute exposure to H_2O_2 (Figure 3.3.B), indicating a complete adaptation to oxidative stress-mediated changes in energy metabolism. However, in cells chronically exposed to cocaine, ATP levels were decreased by about 28% after exposure to H_2O_2. Thus, although cells chronically exposed to cocaine were more resistant to H_2O_2, intracellular ATP levels were still affected, suggesting that this may account for the increased susceptibility of cells chronically treated with cocaine, compared to cells treated with H_2O_2, when submitted to an acute H_2O_2 challenge (Figure 3.1). Endogenous levels of ADP were not significantly affected by acute exposure to H_2O_2 in naïve cells or in cells chronically exposed to cocaine or H_2O_2 (data not shown), suggesting a decrease in ATP synthesis. The apparent lack of correlation

between the high toxicity of acute H_2O_2 (Figure 3.1) and the moderate decrease in ATP levels (Figure 3.3), under similar experimental conditions, can be explained by the normalization of ATP levels over the protein content, which is not taken into consideration in the MTT reduction assay.

3.1.1.3.3 Chronic exposure to cocaine or amphetamine induces changes in the activity of antioxidant enzymes

The involvement of oxidative stress in the chronic effects of cocaine and amphetamine was further evaluated in PC12 cells exposed to cocaine (30 μM), amphetamine (30 μM) and H_2O_2 (10 μM) in the initial phases of cell adaptation, for 1 - 4 weeks, by analyzing the activity of the antioxidant enzymes GPx (Figure 3.4.A), GRed (Figure 3.4.B) and SOD (Figure 3.4.C). Results in this section are presented as unpublished data, which are complementary to the results previously described.

GPx detoxifies H_2O_2 producing water, by using GSH which is converted into GSSG. Chronic exposure to cocaine induced an increase in the activity of GPx (136.6 %) in PC12 cells, upon 4 weeks exposure (Figure 3.4.A). In the case of chronic exposure to amphetamine, the activity of GPx was increased in PC12 cells upon 3 weeks exposure (159.3 %), and returned to control levels upon 4 weeks exposure to amphetamine. Chronic exposure to H_2O_2 induced an increase in GPx activity in PC12 cells, upon 3 (172.3 %) and 4 (157.8 %) weeks of exposure, which compensates for the increased need of the cells to detoxify H_2O_2.

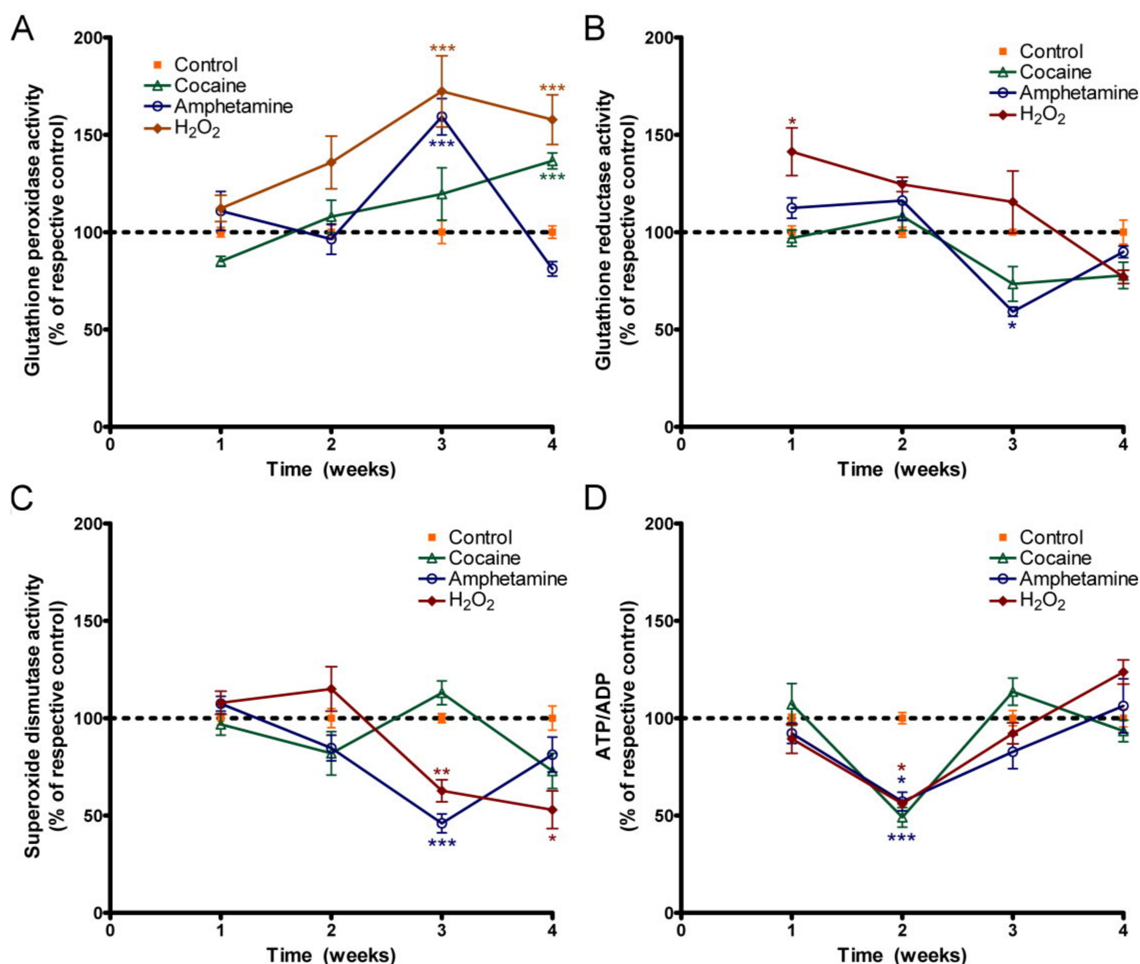


Figure 3.4: Time course of glutathione peroxidase (**A**), glutathione reductase (**B**) and (**C**) superoxide dismutase activities and ATP/ADP levels (**D**) upon continuous exposure to cocaine, amphetamine or H_2O_2 for 1-4 weeks. PC12 cells were incubated with cocaine (30 µM), amphetamine (30 µM) or H_2O_2 (10 µM). The activity of antioxidant enzymes and the ATP/ADP ratio were normalized over the values of the respective controls. Data are the mean ± SEM of 5 experiments performed in duplicate. *P< 0.05 **P< 0.01 ***P< 0.001 when compared to control PC12 cells.

GRed reduces GSSG, regenerating GSH. Chronic exposure to cocaine did not significantly affect GRed activity whereas chronic exposure to amphetamine induced a decrease in GRed activity (59.2 %) after 3 weeks of exposure, returning to control levels upon 4 weeks of exposure to amphetamine (Figure 3.4.B). H_2O_2 initially induced an increase in GRed activity (141.3 %) upon 1 week exposure, returning to control levels upon 2 weeks of exposure of PC12 cells to H_2O_2.

SOD converts the superoxide anion into H_2O_2, a less toxic molecule. SOD activity was not significantly affected by chronic exposure to cocaine (Figure 3.4.C). In contrast, chronic exposure to amphetamine induced a decrease in SOD activity (46.0 %) upon 3 weeks of exposure, returning to control levels upon 4 weeks exposure to amphetamine. H_2O_2 induced a decrease in SOD activity in PC12 cells, upon 3 (62.7 %) and 4 (52.9 %) weeks exposure.

Oxidative stress induces changes in cell metabolism. We have previously shown that acute exposure to H_2O_2 induces a decrease in ATP levels in PC12 cells, whereas chronic (7-12 months) exposure showed no significant changes in cellular ATP (Figure 3.3.B). Therefore, earlier stages of exposure to H_2O_2 may involve changes in energy metabolism. In this context, we analyzed the intracellular levels of ATP/ADP in PC12 cells exposed to cocaine, amphetamine and H_2O_2 for 1-4 weeks (Figure 3.4.D). We observed a decrease in ATP/ADP levels in comparison with the controls, upon 2 weeks of exposure to cocaine (49.1 %), amphetamine (57.2 %) or H_2O_2 (56.1 %). Interestingly, the levels of ATP/ADP returned to control levels upon 3 weeks of exposure to the drugs of abuse or to H_2O_2.

3.1.1.4 Discussion

3.1.1.4.1 Cocaine sensitization and dopamine toxicity

In this study we showed that chronic cocaine increases the susceptibility of PC12 cells to acute cocaine toxicity, occurring concomitantly with enhanced cocaine-evoked dopamine release. PC12 cells chronically exposed to cocaine showed a non-statistical increase (by 2.2-fold) in intracellular dopamine levels, which could help to explain the increase in cocaine-evoked extracellular dopamine accumulation observed in Figure 3.2.A, since dopamine release induced by cocaine is dependent on the vesicular pool of the neurotransmitter (Pifl et al., 1995). Repeated cocaine administration has been described to increase tyrosine hydroxylase activity, the rate-limiting enzyme in the biosynthesis of dopamine, in brain areas affected by cocaine, such as the ventral tegmental area (Sorg et al., 1993) or the substantia nigra (Vrana et al., 1993). Since reuptake of dopamine is inhibited by cocaine (Brown et al., 2001), increased intracellular levels of dopamine could be due to increased dopamine biosynthesis. Furthermore, under control conditions, extracellular dopamine levels in cells chronically exposed to cocaine were not statistically different from those in naïve cells (Figure 3.2A). This observation could be due to an increase in expression of plasma membrane DAT, as reported previously in cells subjected to low concentrations of cocaine (Kahlig and Galli, 2003, for review). Thus dopamine uptake during chronic treatment with cocaine could be preserved. Dopamine has been described to be cytotoxic to PC12 cells, inducing oxidative stress and apoptosis (Wang et al., 2005), in a process occurring through activation of NF-kB (Panet et al., 2001) or the SAPK/JNK pathway (Luo et al., 1998). Nevertheless, no evidences of changes in dopamine metabolization by MAO were found, as examined by the maintenance of DOPAC levels in cells exposed to cocaine (Figure 3.2.E). Importantly, the observed increase in cocaine-evoked dopamine release may be related to the behavioral sensitization induced by this drug (Williams and Steketee, 2005), which is defined by increased motor activity after repeated administration.

3.1.1.4.2 Amphetamine-induced dopamine depletion and amphetamine toxicity

Chronic exposure to amphetamine induced dopamine depletion (Figure 3.2.B,D). Nevertheless, acute amphetamine toxicity persisted in cells lacking dopamine (Figure 3.1), indicating that dopamine is not involved in amphetamine toxicity. *In vivo* studies demonstrated that amphetamine toxicity is associated with a decrease in striatal dopamine levels (Wagner et al., 1980), decreased tyrosine hydroxylase activity (Ellison et al., 1978) and loss of dopamine transporter activity (Saunders et al., 2000). Although toxic mechanisms caused by amphetamine have been largely associated with the dopaminergic system, a more general mechanism of toxicity for amphetamine was also described, which involves disruption of the mitochondrial potential due to the net positive charge of amphetamine (Davidson et al., 2001). In accordance, we have previously observed that acute exposure of PC12 cells to amphetamine (1 mM, for 96 h) resulted in a decrease in intracellular ATP/ADP ratio (Oliveira et al., 2002). Moreover, increasing evidence suggest that amphetamine, or amphetamine derivatives, can have toxic effects in non-dopaminergic cells, such as cerebellar granule cells exposed *in vitro* to 4 mM methamphetamine or methylenedioxy-methamphetamine (Jimenez et al., 2004) and rat medium spiny projection neurons in the striatum, upon *in vivo* exposure to 4 times 10 mg/kg d-amphetamine (Krasnova et al., 2005).

3.1.1.4.3 Cocaine and amphetamine-induced partial resistance to H_2O_2

Chronic cocaine treatment was shown to partially protect PC12 cells against H_2O_2-induced toxicity, despite the decrease in ATP (by ~28%), which reflects metabolic dysfunction. In contrast, chronic treatment with H_2O_2 completely prevented H_2O_2 acute toxicity, as evaluated by MTT reduction (Figure 3.1), cell morphology (Figure 3.3.A)

and ATP levels (Figure 3.3.B). Several mechanisms have been proposed to explain cellular adaptation to H_2O_2. Moderate concentrations of H_2O_2 (0.25 mM, for 24 h) increase the capacity of oxidant degradation by antioxidant enzymes, whereas lower concentrations (0.05 mM, for 24 h) block the signalling pathways triggered by toxic concentrations of H_2O_2 (1 mM) (Lee and Um, 1999).

3.1.1.4.3.1 Involvement of antioxidant enzymes

Our results showed that changes in energy metabolism and in the activity of antioxidant enzymes are involved in the chronic effects of cocaine and amphetamine and also H_2O_2.

Chronic exposure to H_2O_2 induced an increase in GPx activity, statistically significant upon 3 and 4 weeks of exposure (Figure 3.4.A). This was expected because of the increased need to detoxify H_2O_2. Upon 1 week exposure to H_2O_2, GRed activity was also increased (Figure 3.4.B), decreasing to normal levels upon 2 weeks of exposure. This may be explained by an initial need to convert GSSG into GSH. The restoration of GRed activity suggests the maintenance of GSH/GSSG by other mechanisms. One possibility is the increase in GSH synthesis, which could account for the GSH needed to supply the increased activity of GPx observed at a later time point.

Repeated exposure to H_2O_2 also caused a decrease in SOD activity (Figure 3.4.C), upon 3 and 4 weeks of exposure, in agreement with what was described by Ramasarma (1990). This decrease paralleled the increase in GPx, suggesting that increased levels of H_2O_2 are then reduced to water.

Energy levels, measured by the ratio ATP/ADP, were decreased upon 2 weeks of exposure (Figure 3.4.D), returning to normal levels upon 3 weeks. This may be explained by an increased need for ATP upon 2 weeks of exposure to support the changes in cellular activity that are mainly manifested upon 3 weeks of exposure to H_2O_2. After this initial energy-requiring phase, the cells became adapted to the presence of H_2O_2, which may account for the restoration of the energy levels.

Repeated exposure to cocaine induced an increase in GPx activity in PC12 cells (Figure 3.4.A), statistically significant upon 4 weeks of exposure, one week later, compared with cells exposed to H_2O_2. GRed activity showed a tendency for a decrease upon 3 and 4 weeks of exposure to cocaine (Figure 3.4.B), in contrast with cells exposed to H_2O_2. Stimulation of GPx activity without a concomitant increase in GRed activity suggests an impairment of GSH regeneration by GRed. In this case, if GSH synthesis does not increase, stimulation of GPx activity may lead to GSH depletion. In accordance, total GSH was shown to decrease in the prefrontal cortex and in the striatum upon *in vivo* exposure to cocaine (Dietrich et al., 2005; Poon et al., 2007). Furthermore, the activity of SOD was not affected by cocaine (Figure 3.4.C). Together, these findings suggest that repeated exposure to cocaine is accompanied by the generation of H_2O_2, which activates GPx, thus helping to decrease endogenous levels of intracellular H_2O_2. Because SOD activity is not affected, intracellular H_2O_2 levels should be lower than the levels achieved upon direct exposure of cells to 10 μM H_2O_2. The differential effect of chronic exposures to cocaine or H_2O_2 in the activity of antioxidant enzymes may explain the partial resistance of cells chronically exposed to cocaine, when acutely exposed to H_2O_2.

It was previously reported that *in vivo* cocaine exposure induced an increase in GPx activity in the prefrontal cortex and in the striatum (Dietrich et al., 2005). However, these authors also observed an increase in SOD activity in the same brain regions. These observations may be explained by the fact that the structures analysed are composed by several types of neurons, which may respond to the drugs in different ways. PC12 cells synthesize only dopamine, noradrenaline and acetylcholine (Shafer and Atchison, 1991) and, thus, the effects of the drugs of abuse in PC12 cells may only be mediated by these neurotransmitters.

Repeated exposure to amphetamine induced an increase in GPx activity (Figure 3.4.A) and a decrease in GRed (Figure 3.4.B) and SOD (Figure 3.4.C) activities, after 3 weeks of exposure. However, upon 4 weeks of exposure to amphetamine, the activities of these enzymes returned to control levels. Similarly to the effects described for cocaine, these results may represent an initial increase in H_2O_2

production from dopamine metabolism, via MAO or auto-oxidation, which may explain increased activity of GPx. An increase in GPx activity and a concomitant decrease in GRed activity suggest GSH depletion and a decrease in GSH/GSSG. In accordance, an increase in GSSG was observed in the dopamine-rich caudate of human methamphetamine abusers (Mirecki et al., 2004).

The fact that, upon 4 weeks of exposure to amphetamine the activities of GPx, GRed and SOD returned to control levels, suggests a decrease in H_2O_2 production upon 4 weeks of exposure to amphetamine. This may be explained by the inhibition of TH by amphetamine, decreasing the intracellular levels of dopamine (as observed in PC12 cells exposed to amphetamine for 7-12 months - Figure 3.2) and thus also decreasing H_2O_2 produced by dopamine oxidation. Amphetamine inhibition of MAO (Ramsay and Hunter, 2002) may also be more evident upon 4 weeks of exposure to amphetamine, further decreasing the H_2O_2 produced by MAO. The biphasic effect of amphetamine may explain the lower level of resistance of cells chronically exposed to amphetamine to acute H_2O_2 toxicity upon 7-12 months of exposure (Figure 3.1), in comparison with cells chronically exposed to cocaine.

It was previously reported that exposure to amphetamine *in vivo* induced an increase in GPx in the striatum, NAc and prefrontal cortex (Carvalho et al., 2001). The same authors reported an increase in catalase and a decrease in GRed in the prefrontal cortex. Other authors reported that amphetamine exposure induced a decrease in SOD in the striatum and an increase in catalase in the prefrontal cortex and hippocampus (Frey et al., 2006b). The results of these studies are in accordance with the results of the present study. However, in the same studies, the reverse changes were observed in other brain regions such as the hypothalamus (Carvalho et al., 2001) and hippocampus (Frey et al., 2006b). Thus, drugs of abuse may induce changes in antioxidant enzymes through different mechanisms in distinct brain regions.

Interestingly, the decrease in ATP/ADP observed upon 2 weeks of exposure to cocaine or amphetamine (Figure 3.4.D), was restored to control levels upon 3 weeks of exposure to the drugs of abuse or H_2O_2. This may be explained by an increase in energy demanding cellular activities upon 2 weeks of exposure, which may underlie

cellular adaptation to these stress conditions. Moreover, changes in the activity of antioxidant enzymes may help to recover the energy levels.

3.1.1.4.3.2 Involvement of intracellular signalling pathways and energy metabolism

H_2O_2 adaptation may involve increased translocation of NF-kB to the nucleus, which contributes to increased cell survival (Kim et al., 2001a), not involving an increase in Bcl-2 or Bcl-XL (Lee and Um, 1999). Another signalling pathway regulated by H_2O_2 is the SAPK/JNK pathway, which is activated by high concentrations of H_2O_2 and suppressed by low adaptive concentrations of H_2O_2 (Kim et al., 2001b). The interference with these signalling pathways may confer increased cell resistance to H_2O_2, independently of an increase in the capacity of degrading H_2O_2 (Lee and Um, 1999). Moreover, H_2O_2 adaptation induces cross-resistance to serum withdrawal and C2-ceramide by blocking their ability to activate the SAPK/JNK pathway (Kim et al., 2001b). Acute H_2O_2 toxicity has been reported to involve the inhibition of ADP phosphorylation, namely through the inhibition of glyceraldehyde-3-phosphate dehydrogenase, in glycolysis, and the ATP-synthase complex, at the mitochondrial respiratory chain (Hyslop et al., 1988). This could account for the decrease in intracellular ATP levels observed in naïve PC12 cells and in cells chronically exposed to cocaine, following acute exposure to H_2O_2 (Figure 3.3.B).

Furthermore, proteomic analysis showed that adaptation to low concentrations of H_2O_2 may induce upregulation of proteins involved in energy metabolism, such as ATP synthase and glyceraldehyde-3-phosphate dehydrogenase (Seong et al., 2002), which may explain the maintenance of intracellular levels of ATP (Figure 3.3.B). In addition, proteomic analysis also suggests that upregulation of translation and RNA processing, chaperoning and redox regulation may also be in the basis for adaptation to H_2O_2 (Seong et al., 2002).

In cells chronically exposed to cocaine, H_2O_2 toxicity seemed to affect cell metabolism (Figure 3.3.B). Thus, the mechanism involved in the partial resistance of

these cells to acute H_2O_2 is certainly not related to stimulation of energy metabolism. Similarly to H_2O_2, chronic cocaine was also reported to induce the activation of NF-kB (Ang et al., 2001) and AP-1 (Lee et al., 2001), two transcription factors described to be activated under oxidative stress conditions. Thus the mechanism of partial resistance to H_2O_2 toxicity in cells chronically exposed to cocaine may possibly be explained by the activation of intracellular signalling pathways involved in the adaptation to H_2O_2, by low concentrations of cocaine. Nevertheless, high concentrations of cocaine induced toxicity in H_2O_2-treated cells, suggesting that toxic effects mediated by high concentrations of cocaine are unrelated to adaptive changes induced by oxidative stress.

In conclusion, this study provides further data confirming functional alterations in dopaminergic cells chronically exposed to amphetamine or cocaine. We showed that chronic treatment with amphetamine induced dopamine depletion, which did not affect amphetamine or H_2O_2 cytotoxicity in these cells. Furthermore, exposure to cocaine increased the sensitivity to an acute cocaine exposure, along with cocaine-evoked extracellular dopamine accumulation, which may be related with increased motor activity after repeated administration of this drug of abuse (Williams and Steketee, 2005). Moreover, in contrast to prolonged exposure to H_2O_2, chronic cocaine induced a partial resistance to H_2O_2-mediated cytotoxicity, which was not correlated with the maintenance of energy levels. These data suggest that distinct cellular mechanisms operate during prolonged exposure to cocaine or H_2O_2, implicating major changes in cell susceptibility. Moreover, oxidative stress, probably mediated by H_2O_2, is involved in the effects of exposure to sub-toxic concentrations of cocaine and amphetamine in a catecholaminergic cell line. Amphetamine seems to have a biphasic effect on oxidative stress, which may be due to the downregulation of TH and MAO activities. Furthermore, H_2O_2 resistance of PC12 cells chonically exposed to H_2O_2 involves adaptation to oxidative stress.

3.2 CHAPTER 2: Neuroadaptation and neurotoxicity induced by *street* heroin

3.2.1 MANUSCRIPT 2: Chronic effects of heroin and hydrogen peroxide in PC12 cells

Based on: Teresa Cunha-Oliveira, A. Cristina Rego, M. Teresa Morgadinho, Tice Macedo and Catarina R. Oliveira, *Efeitos crónicos da heroína e do peróxido de hidrogénio em células PC12*, *Coimbra Médica* V série (2006) Vol.2, pp 21-29

3.2.1.1 Abstract

The repeated use of opiates and other drugs of abuse leads to drug dependence. The most studied mechanism involved in this process is the activation of the dopaminergic neurotransmission in the mesolimbic/ mesocortical pathway. Dopamine is potentially neurotoxic, due to the generation of ROS, namely H_2O_2, following its oxidative metabolism. In this work we evaluated the effect of chronic exposure of PC12 cells to *street* heroin or H_2O_2 upon an acute exposure to these compounds, in comparison with naïve PC12 cells. Cells chronically exposed to heroin or H_2O_2 were partially or totally resistant to an acute exposure to H_2O_2, respectively. This effect was related to the maintenance of ATP levels upon acute exposure to H_2O_2. Moreover, the viability of cells chronically exposed to heroin and H_2O_2 was highly affected by an acute heroin exposure. This sensitization may be related to the increase in extracellular dopamine accumulation induced by these compounds. On the other hand, acute exposure to heroin in cells chronically exposed to this drug was more cytotoxic than in cells chronically exposed to H_2O_2. This observation may be related to the decrease in intracellular levels of ATP and ADP induced by chronic exposure to *street* heroin. In summary, chronic exposure to *street* heroin induces changes in cellular energy levels, in extracellular dopamine levels and in the response to cytotoxic stimuli. Together, these observations reflect the harmful effects of repeated heroin abuse.

Keywords: adenine nucleotides, cytotoxicity, dopamine, heroin, oxidative stress

3.2.1.2 Introduction

The repeated abuse of drugs of abuse is responsible for the development of drug dependence. The most studied brain circuit involved in drug dependence, which is shared by the majority of the drugs of abuse, is the reward pathway. In this pathway dopamine neurons of the mesolimbic/mesocortical structures play a major role in mediating the effects of the drugs of abuse. The drugs interact directly or indirectly with these dopaminergic neurons, increasing dopaminergic neurotransmission (Di Chiara and Imperato, 1988). Dopamine is both a neurotransmitter and a neurotoxin. Oxidative metabolism of dopamine generates ROS, namely H_2O_2, which can originate the highly reactive $^{\bullet}OH$ in the presence of transition metal ions. Thus, dopamine metabolism causes oxidative stress and cell death in dopaminergic and surrounding cells (Jones et al., 2000). In this context, acute exposure to H_2O_2 induces apoptotic cell death in PC12 cells (Benedi et al., 2004; Jang and Surh, 2004), whereas cells chronically exposed to low concentrations of H_2O_2 become resistant to the acute toxicity of this compound (Wiese et al., 1995; Davies, 1999). Thus, H_2O_2 chronic treatment of PC12 cells with H_2O_2 has been considered to be a model of cell adaptation (Jackson et al., 1994).

In this work we investigated the effect of chronic exposure of PC12 cells to sub-toxic concentrations of *street* heroin or H_2O_2, in comparison with non-chronically exposed PC12 cells (naïve cells). The cells were subsequently treated with toxic concentrations of *street* heroin or H_2O_2 (acute exposure), in accordance with our previous work (Oliveira et al., 2002; Cunha-Oliveira et al., 2006b). The results suggest that chronic exposure to *street* heroin changes the capacity of PC12 cells to respond to cytotoxic stimuli, namely due to a compromise of the energy metabolism, induced by this drug.

3.2.1.3 Results

In order to compare the effects of *street* heroin and H_2O_2 in the metabolic viability of naïve and chronically exposed PC12 cells we evaluated the capacity to reduce MTT. We observed that incubation with *street* heroin (300 µM) induced a decrease in cell viability, by about 30% in naïve cells, as previously observed (Oliveira et al., 2002). In cells previously exposed to *street* heroin, the same acute concentration of *street* heroin induced a decrease in cell viability by about 70% (Figure 3.5.A), suggesting a higher susceptibility of cells chronically exposed to *street* heroin, when exposed to a toxic concentration of the same drug. In cells previously exposed to H_2O_2, *street* heroin induced a decrease in cell viability by about 50% (Figure 3.5.A), reflecting a lower degree of sensitization to the toxic effects of *street* heroin, in comparison with cells chronically exposed to *street* heroin. As described in Chapter 3.1 in cells exposed to cocaine in these conditions, we could not compare the controls of chronically exposed cells by the MTT assay, since there were differences in cell proliferation along the time of cell adaptation to H_2O_2 or *street* heroin. Exposure to H_2O_2 (50 µM, for 24 h) induced a decrease in cell viability by about 70% in naïve cells, whereas in cells chronically exposed to *street* heroin the decrease in cell viability was of just about 40% (Figure 3.5.A). However, the increase in H_2O_2 concentration to 75 µM induced a decrease in metabolic activity of naïve cells by about 80%, in comparison with the control, whereas in cells chronically exposed to *street* heroin the decrease was of about 60% (Figure 3.5.A). The fact that in cells chronically exposed to H_2O_2 the acute exposure to the same compound did not induce a significant change in metabolic cell viability appears to represent a cellular adaptation to H_2O_2 in cells chronically exposed to a sub-toxic concentration of this compound. Interestingly, cells chronically exposed to *street* heroin are partially resistant to acute H_2O_2 (Figure 3.5.A).

The acute exposure to H_2O_2 induced changes in cell morphology, as evaluated by May-Grunwald-Giemsa staining (Figure 3.5.B), leading to loss of membrane integrity and to the appearance of membrane blebs. In cells chronically exposed to H_2O_2, and subsequently exposed to acute H_2O_2, the morphology was similar to the controls, confirming the total resistance of these cells to H_2O_2. In cells chronically exposed to

street heroin, acute exposure to H_2O_2 induced some changes in cell morphology, when compared to the control (Figure 3.5.B). These changes were less evident than the changes observed in naïve cells, which is in accordance with the partial resistance suggested by the MTT assay.

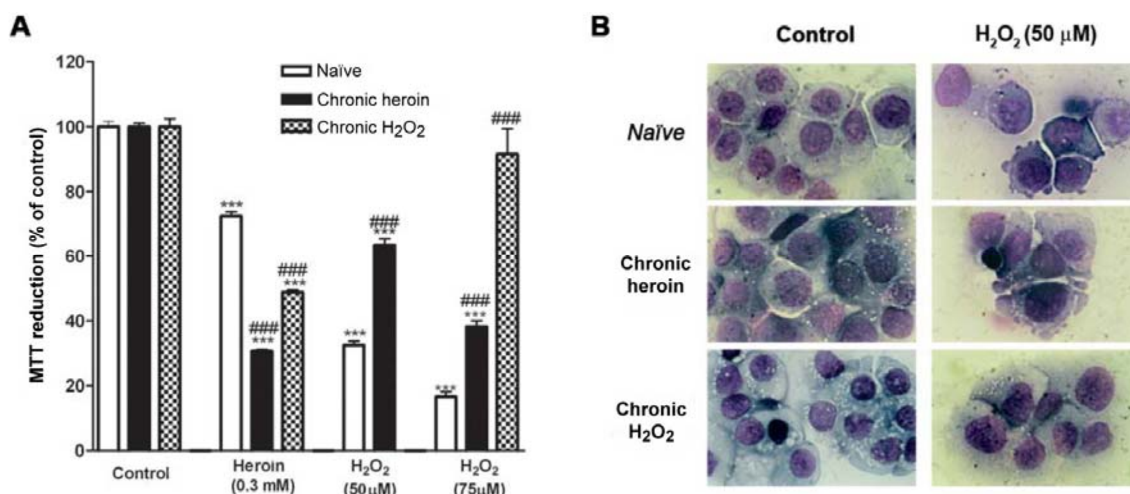


Figure 3.5: Analysis of cell viability of PC12 cells chronically exposed to *street* heroin and H_2O_2, before and after acute exposure to cytotoxic concentrations of these compounds. Naïve PC12 cells and PC12 cells chronically exposed to *street* heroin or H_2O_2 (for 7-12 months) were incubated with *street* heroin (0.3 mM, for 96 h) or H_2O_2 (50 or 75 μM, for 24 h). A) Cell viability was evaluated by the MTT reduction assay. Data were normalized in percentage of the respective controls. Data are mean ± SEM of at least 3 experiments performed in triplicate. ***P<0.001, compared with the respective control, ###P<0.001, compared with the same exposure in naïve cells. B) Cell morphology was analysed by staining cell smears using the May-Grünwald-Giemsa technique. Note the formation of membrane blebbings and cell shrinkage in naïve cells and in cells chronically exposed to *street* heroin, upon acute exposure to H_2O_2 (50 μM, for 24 h). Images are representative of 3 experiments performed in duplicate.

3.2.1.3.1 Street heroin induced changes in energy metabolism

Since H_2O_2 interferes with energy metabolism, we investigated if the partial resistance to H_2O_2 in cells chronically exposed to *street* heroin or the total resistance in cells chronically exposed to H_2O_2, would be related to changes in intracellular levels of the adenine nucleotides ATP (Figure 3.6.A) and ADP (Figure 3.6.B). We observed that acute exposure of naïve cells to H_2O_2 (50 μM) induced a decrease in intracellular ATP

levels (Figure 3.6.A). However, this decrease was not compensated by an increase in intracellular ADP (Figure 3.6.B) or AMP (not shown). Chronic exposure to *street* heroin induced a decrease in intracellular ATP (Figure 3.6.A) and ADP (Figure 3.6.B) levels, suggesting an energetic compromise induced by this drug of abuse. In these cells, acute exposure to H_2O_2 did not induce significant alterations in ATP levels, as observed in naïve cells, possibly explaining the partial resistance of these cells to H_2O_2, observed in Figure 3.5.A. In addition, and in accordance with the total resistance to H_2O_2, we did not observe significant alterations in intracellular ATP or ADP levels in cells cronically exposed to H_2O_2, before or after incubation with 50 μM H_2O_2 (Figure 3.6).

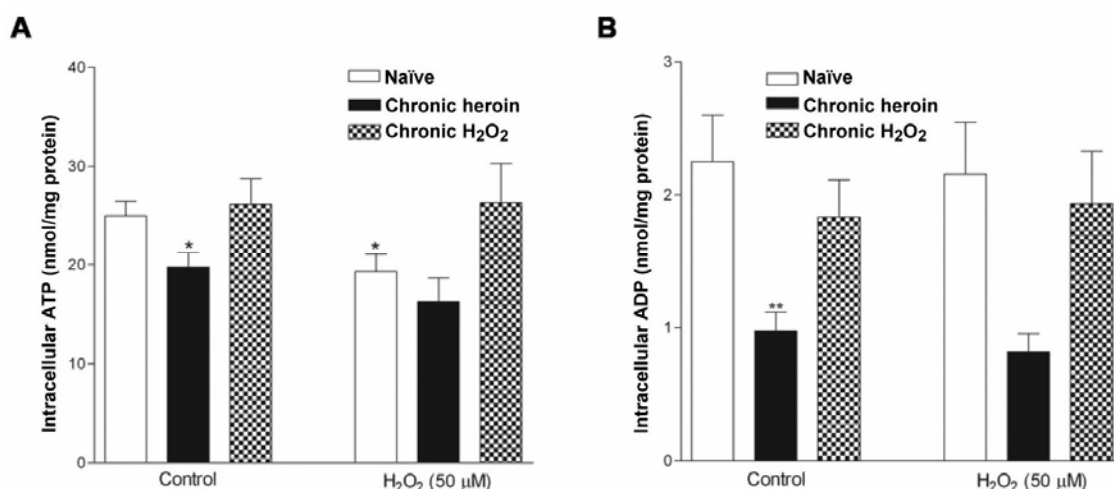


Figure 3.6: Analysis of intracellular ATP (A) and ADP (B) levels, before and after acute exposure to H_2O_2. Naïve cells and cells chronically exposed to *street* heroin or H_2O_2 were incubated with H_2O_2 50 μM, for 24 h. The levels of adenine nucleotides were determined by HPLC with UV detection. Data correspond to mean ± SEM of 4 independent experiments, performed in triplicate. *P<0.05 and **P<0.01, compared to control naïve cells.

3.2.1.3.2 Role of dopamine in heroin sensitization

Because PC12 cells are a catecholaminergic cell line and dopamine is potentially neurotoxic, we investigated whether *street* heroin sensitization observed in cells chronically exposed to *street* heroin would be related with changes in intra- or extracellular dopamine levels (Figure 3.7). We observed that acute exposure to *street* heroin induced a decrease in intracellular dopamine levels (Figure 3.7.A) and in

parallel, an increase in extracellular accumulation of this neurotransmitter (Figure 3.7.B), in all sub-cell lines tested. Moreover, chronic exposure to *street* heroin and H_2O_2 induced an increase in extracellular dopamine accumulation (Figure 3.7.B). Upon acute exposure to *street* heroin, extracellular dopamine accumulation in cells chronically exposed to H_2O_2 was significantly increased, when compared to naïve cells (Figure 3.7.B). This increase can be related to the increase (although non-statistically significant) of intracellular dopamine in these cells (Figure 3.7.A). No significant alterations in intracellular DOPAC levels were observed in any of the experimental conditions tested (data not shown).

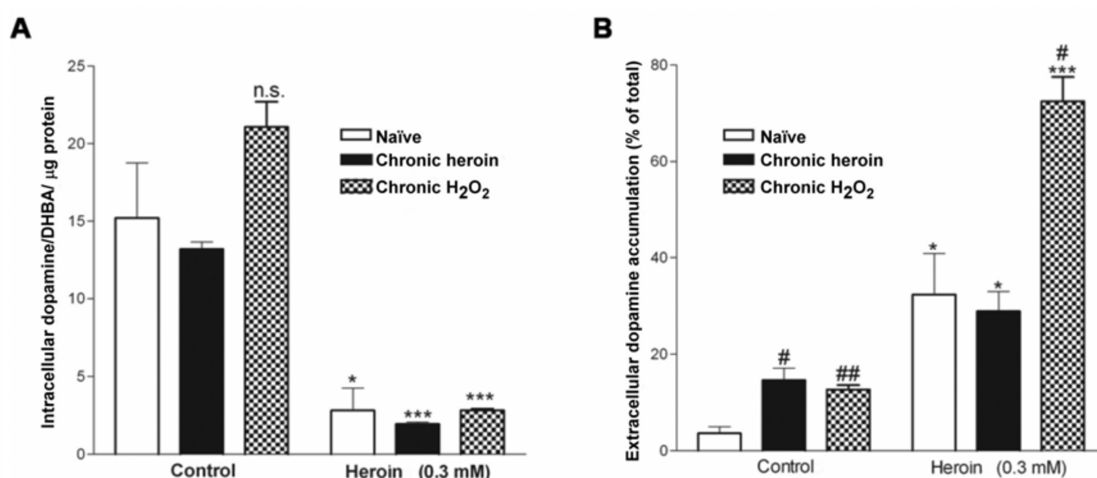


Figure 3.7: Analysis of intracellular (A) and extracellular (B) dopamine levels, before and after acute exposure to *street* heroin. Naïve cells and cells chronically exposed to *street* heroin and to H_2O_2 were incubated with *street* heroin (0.3 mM), for 96 h. Intra- and extracellular dopamine levels were analysed by HPLC with electrochemical detection. Data are the mean ± SEM of 3 experiments performed in triplicate. *P<0.05 **P<0.01 and ***P<0.001 compared to the respective control. #P<0.05 and ###P<0.001 compared to naïve cells. n.s.= not significant.

3.2.1.4 Discussion

In this work we showed that chronic treatment of PC12 cells with *street* heroin or H_2O_2 induced, respectively, a partial or total resistance to the acute H_2O_2 cytotoxicity, accompanied by maintenance of intracellular ATP levels, upon exposure to H_2O_2. Cells chronically exposed to *street* heroin showed higher susceptibility to an acute exposure to the same drug, in comparison with cells chronically exposed to H_2O_2. This fact can be related to the changes in energy metabolism in cells chronically exposed to *street* heroin, as evidenced by the decrease in intracellular ATP and ADP levels. In addition, extracellular dopamine accumulation seems to contribute to the susceptibility of cells chronically exposed to *street* heroin or H_2O_2, upon acute *street* heroin exposure, suggesting a cytotoxic role for dopamine.

Cellular adaptation to H_2O_2 can be explained by two mechanisms. When present in low concentrations (0.25 mM for 24 h), H_2O_2 induces an increase in cellular antioxidant capacity, namely an increase in glutathione peroxidase expression (Lee and Um, 1999). Moreover, when present in low concentrations (0.05 mM for 24h), H_2O_2 induces an adaptive process that may not to be associated with an increase in cellular antioxidant capacity. In these conditions, a blockade of cell signalling pathways involving nuclear factor kB (Kim et al., 2001a) or the stress activated protein kinase (SAPK/JNK) pathway (Kim et al., 2001b) can occur, which are usually activated by toxic concentrations of H_2O_2 (1 mM) (Lee and Um, 1999). Cellular adaptation induced by low concentrations of H_2O_2 may also be related with an increase in the expression of proteins involved in energy metabolism, such as ATP synthase (at the mitochondrial respiratory chain) and glyceraldehyde-3-phosphate (GAPDH, at the glycolytic pathway), as demonstrated by proteomic analysis (Seong et al., 2002). The increase in the expression of these enzymes may explain the maintenance of intracellular ATP levels and the consequent cellular resistance upon acute exposure to H_2O_2, in cells chronically exposed to H_2O_2 (Figure 3.6.A), but not in cells chronically exposed to *street* heroin, since these cells showed a decrease in the intracellular levels of adenine nucleotides (Figure 3.6). Proteomic analysis also suggests that H_2O_2 adaptation can

induce an increase in the expression of proteins associated with RNA processing, chaperones and proteins involved in redox regulation (Seong et al., 2002). The acute toxicity of H_2O_2 can be related to the inhibition of ADP phosphorylation, namely by inhibition of ATP synthase or GAPDH (Hyslop et al., 1988). These data could explain the decrease in intracellular ATP levels observed in naïve cells upon acute exposure to H_2O_2 (Figure 3.6.A), similar to what was previously observed by other authors (Milusheva et al., 2003). In addition, the decrease in intracellular energy levels induced by *street* heroin may be related to the inhibition of oxidative phosphorylation, since previous studies reported that the heroin metabolite morphine inhibits ATP synthase and the ATPase from the inner mitochondrial membrane (Gegenava and Chistyakov, 1975).

Previous studies demonstrated that H_2O_2 exposure induces dopamine release in rat striatal slices (Milusheva et al., 2005). These authors suggested that H_2O_2 may interfere with dopamine reuptake and/or storage of dopamine in presynaptic vesicles. It was also demonstrated that H_2O_2 induces a reversible inhibition of the activity of the DAT present in the plasma membrane of dopaminergic cells, via a calcium-dependent redox regulation (Huang et al., 2003). Taking this into account, in our conditions the increase in extracellular dopamine accumulation upon chronic exposure to H_2O_2, may be explained by H_2O_2-induced inhibition of DAT. We have previously observed that chronic exposure to cocaine (a classic DAT inhibitor) in PC12 cells induced similar effects in extracellular and intracellular dopamine levels, when compared to chronic exposure to H_2O_2 (Figure 3.2).

Interestingly, chronic exposure to *street* heroin also induced an increase in the extracellular levels of dopamine. Moreover, acute exposure to this drug reinforced the accumulation of extracellular dopamine in cells chronically exposed to H_2O_2, in comparison with cells chronically exposed to *street* heroin (Figure 3.7.B). Heroin exposure has been reported to cause oxidative stress when intraperitoneally injected in mice (Pan et al., 2005; Qiusheng et al., 2005). In previous studies, we observed that acute treatment of PC12 cells with *street* heroin induced a non-statistical increase in intracellular hydroperoxides that was correlated with an increase in intracellular DOPAC levels (Oliveira et al., 2002). These data suggested that the cytotoxic effects of

street heroin could involve an unbalance between the cellular levels of oxidants and antioxidants. Thus, the partial resistance to H_2O_2 observed upon chronic exposure to *street* heroin, as well as the increase in extracellular dopamine, may be due to some degree of adaptation to oxidative stress.

Together, the present results show that prolonged exposure to catecholaminergic cells to *street* heroin induces an energetic compromise in these cells and an increase in the sensitivity to high concentrations of the same drug. However, these cells are more resistant to H_2O_2, suggesting adaptation to oxidative stress. These data are important may contribute to increase our knowledge on the molecular and cellular mechanisms involved in drug addiction.

3.2.2 MANUSCRIPT 3: Expression of NR1/NR2B *N*-methyl-D-aspartate receptors enhance heroin toxicity in HEK293 cells

Based on: António Domingues and Teresa Cunha-Oliveira, Tice Macedo, Catarina R. Oliveira and A. Cristina Rego, *Expression of NR1/NR2B N*-methyl-D-aspartate receptors enhances heroin toxicity in HEK293 cells, *Annals of the New York Academy of Sciences* 1074: 458-465

3.2.2.1 Abstract

Repeated use of drugs of abuse, namely opiates, has been shown to affect glutamate-releasing neurons. Moreover, blockade of NMDA receptors prevents cell death by apoptosis induced by morphine, a heroin metabolite. Thus, in this study we investigated the involvement of different NMDA receptor subunits in *street* heroin cytotoxicity. HEK293 cells, which do not express native NMDA receptors, were transfected with NR1/NR2A or NR1/NR2B subunits. As a control, cells were transfected with NR1 alone, which does not form functional channels. Incubation with *street* heroin for 24 h induced a dose-dependent decrease in cell viability both in NR1-transfected and non-transfected cells. The loss of membrane integrity induced by *street* heroin was more evident in cells transfected with NR1/NR2B than in cells transfected with NR1 alone or NR1/NR2A. This decrease in cell viability was blocked by MK-801, a selective and non-competitive antagonist of NMDA receptors. Nevertheless, no significant changes in intracellular ATP were observed in cells treated with *street* heroin. These data implicate NR2B-composed NMDA receptors as important mediators of *street* heroin neurotoxicity.

Keywords: cytotoxicity, drugs of abuse, HEK293 cells, heroin, NMDA receptors

3.2.2.2 Introduction

The abuse of opiates and other drugs of abuse is associated with severe physical and mental health problems. These drugs interact with the CNS, inducing changes in the release of neurotransmitters, namely in the dopaminergic system. Many evidences also suggest the involvement of glutamatergic neurotransmission in the mechanisms of drug dependence involving the dopaminergic reward circuit in the brain (Tzschentke and Schmidt, 2003). On the other hand, some drugs of abuse, such as methamphetamine, are responsible for neurodegenerative mechanisms that lead to the irreversible loss of neurons, with the involvement of dopaminergic and/or glutamatergic systems (Ohmori et al., 1996). Under this perspective, *street* heroin is able to induce cell death in PC12 cells (Oliveira et al., 2002; Oliveira et al., 2003) and morphine, a heroin metabolite, induces cell death by apoptosis in the spinal cord (Mao et al., 2002) and in human cerebrocortical neurons (Hu et al., 2002).

Among the processes leading to cell death, the excitotoxic mechanism, which involves the hyperactivation of ionotropic glutamate receptors, has been largely studied due to its involvement in ischemia and several neurodegenerative diseases (Rego and Oliveira, 2003). NMDA receptors have an outstanding role in excitotoxic processes, due to their high permeability to calcium, which is responsible for the activation of several intracellular enzymes, leading to mitochondrial dysfunction and cell death. NMDA receptors are composed by NR1 subunits which can interact with NR2 (A-D) subunits, and less frequently with NR3 (A,B). NR1 subunits contain the glycine-binding site, whereas the NR2 subunits contain the glutamate-binding site, conferring heterogeneity to these receptors. Previous studies demonstrated that NR2B subunits, which are mainly localized in extra-synaptic sites, are associated to increased toxicity in comparison with NR2A synaptic subunits (Hardingham et al., 2002).

It was previously suggested that glutamatergic neurotransmission involving the NMDA receptor contributes to opiate dependence in humans (Bisaga et al., 2001). Moreover, the NMDA receptor antagonist MK-801 was shown to specifically block morphine tolerance and neuronal apoptosis in the spinal cord (Mao et al., 2002). In this

context, in this work we analysed the involvement of NR1/NR2A and NR1/NR2B subunits in *street* heroin cytotoxicity.

3.2.2.3 Results and Discussion

Previously, we showed that *street* heroin induces a dose-dependent decrease in cell viability in PC12 cells (Oliveira et al., 2002) and in primary cortical neurons (unpublished results). In the present study we observed a dose-dependent decrease in metabolic viability after 24 h exposure to *street* heroin in both untransfected and NR1-GFP transfected HEK293 cells (Figure 3.8).

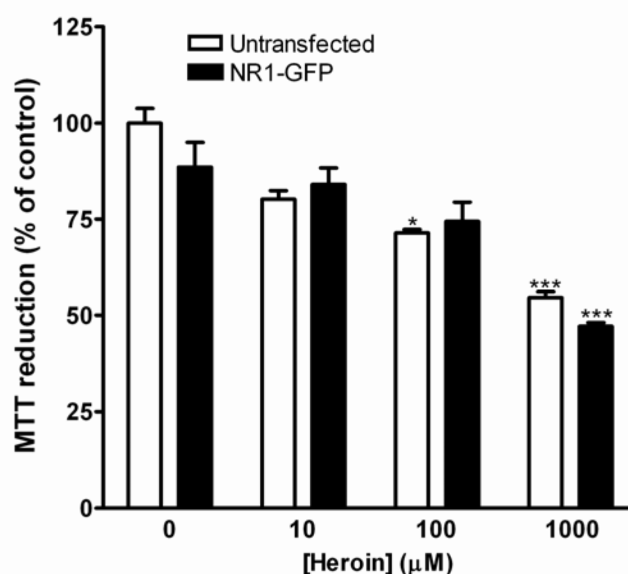


Figure 3.8: Analysis of cell viability in HEK293 cells exposed to *street* heroin. The viability of untransfected HEK293 cells and cells transfected with NR1-GFP was evaluated upon exposure to increasing concentrations of *street* heroin (10-1000 μM) for 24 h, by using the MTT reduction assay. Data presented as mean ± SEM of at least three experiments, performed in triplicate, were normalized in percentage of non-treated untransfected cells (control). Statistical analysis: *P<0.05 and ***P<0.001 compared to the respective untreated cells.

The decrease in metabolic viability was statistically significant for both populations at a high *street* heroin concentration (1 mM). A decrease in cell viability was also observed in untransfected (p<0.05) and NR1-GFP transfected (non-statistically significant) HEK293 cells exposed to 100 μM *street* heroin. This concentration of *street* heroin was used in the following experiments, because a

moderate decrease in cell viability was observed under these conditions. These results demonstrated that *street* heroin is toxic to HEK293 cells. Furthermore, expression of non-functional NMDA receptors (NR1) did not affect *street* heroin toxicity.

Next we evaluated the effect of functional NMDA receptors, composed of NR1/NR2A or NR1/NR2B, on *street* heroin-induced toxicity. NMDA receptor expression in the non-neuronal cell line *per se* led to a non-statistical increase (approximately 5-10%) in LDH release, indicative of some loss of membrane integrity (Figure 3.9).

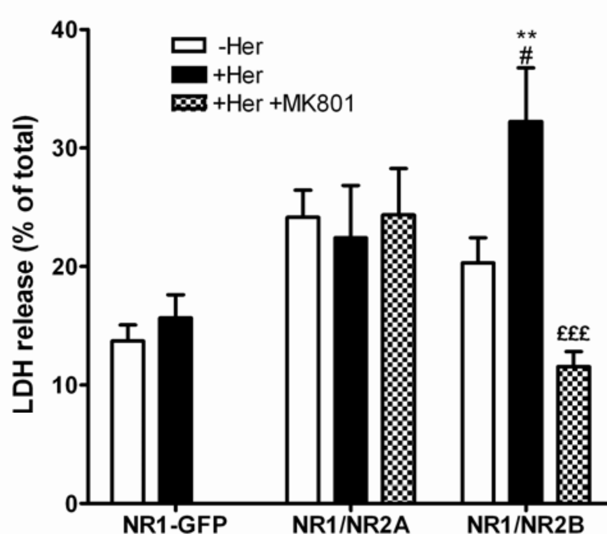


Figure 3.9: Analysis of plasma membrane integrity in HEK293 cells transfected with different subunits of the NMDA receptor upon acute exposure to *street* heroin. The cells were transfected with NR1/NR2A or NR1/NR2B subunits of the NMDA receptor or with NR1-GFP (transfection control) and exposed to 100 μM *street* heroin and/or 10 μM MK-801 for 24 h. The integrity of the plasma membrane was evaluated by the LDH leakage assay. Data were expressed as a percentage of total LDH and presented as mean±SEM of at least 3 experiments, performed in triplicate. Statistical significance: **P<0.01 as compared to NR1-GFP, #P<0.05 compared to the respective untreated cells and £££P<0.001 compared to *street* heroin-treated cells expressing NR1/NR2B.

Exposure to *street* heroin (100 μM, for 24 h) did not significantly change membrane integrity of NR1-GFP or NR1/NR2A-expressing cells (Figure 3.9). However, NR1/NR2B transfected cells showed a significant increase in membrane permeability in

the presence of the drug ($p<0.05$), and this effect was completely prevented by the NMDA receptor antagonist MK-801 (Figure 3.9). The protective effect of MK-801 suggests that activation of NR1/NR2B receptor is necessary for *street* heroin-induced increase in membrane permeability, because MK-801 is an open-channel blocker of NMDA receptors. Interestingly, NR1/NR2B subtype was previously linked to cellular demise that occurs both in an excitotoxic paradigm (Hardingham et al., 2002) and in Huntington´s disease (Zeron et al., 2002).

Cellular energy deficits are often associated with NMDA receptor-mediated excitotoxicity. Overactivation of NMDA receptor leads to an excessive increase in intracellular calcium concentration, which is exacerbated by a dysfunction of calcium extrusion mechanisms. This has been suggested to occur as a consequence of mitochondrial dysfunction and resulting decrement in ATP production (Rego and Oliveira, 2003). Nevertheless, neither the presence of *street* heroin nor the expression of functional NMDA receptor subtypes, NR1/NR2A or NR1/NR2B, affected ATP intracellular levels significantly (Figure 3.10).

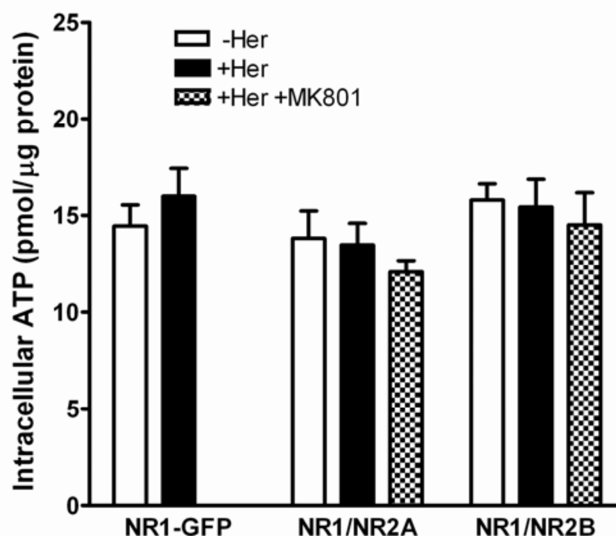


Figure 3.10: Measurement of intracellular ATP levels in HEK293 cells transfected with different subunits of the NMDA receptor upon acute exposure to *street* heroin. Cells transfected with NR1-GFP (transfection control) or with the different subunits of the NMDA receptor (NR1/NR2A or NR1/NR2B) were treated as described in Figure 3.9. Intracellular ATP levels were analysed by HPLC with UV detection. Data were expressed as pmol/µg protein and presented as mean ± SEM of at least 3 experiments, performed in triplicate.

Because intact cellular energy levels are needed for the prosecution of apoptotic cell death (Nicotera and Melino, 2004), we hypothesize that NR1/NR2B-mediated increase in *street* heroin cytotoxicity is initially mediated through apoptosis, rather than necrosis. Later stages of apoptosis also involve an increase in membrane permeability (Schwab et al., 2002), explaining the loss of membrane integrity observed in Figure 3.9. However, the occurrence of necrotic cell death cannot be excluded because, as ATP levels are normalized over the protein content in each sample, intracellular ATP can be over-estimated due to the detachment of necrotic cells from the multiwells. Therefore, it is possible that a decrease in intracellular ATP occurs in the total population of cells, but not in the cells that remain attached to the multiwell and contribute to the determination of intracellular ATP levels.

Taken together our results indicate that the NR1/NR2B subtype of NMDA receptors is a mediator of *street* heroin-induced cytotoxicity. Previously, chronic exposure to morphine, a heroin metabolite that can coexist in heroin solutions (Hutchinson and Somogyi, 2002), was reported to alter NMDA receptor subunit composition in rat NAc neurons. Neuroadaptation to chronic morphine exposure was shown to increase NR2A activity and decrease NR2B activity (Martin et al., 2004). In the present work we hypothesize that overactivation of NR2B-composed receptors is responsible for the deleterious cellular effects induced by *street* heroin. It is possible that the decrease in the activity of NR2B-composed NMDA receptors observed by Martin *et al.* (2004) is due to selective cell death of neurons exhibiting higher expression levels of this NMDA receptors subunit. In this case, these neurons may be more susceptible to toxic insults, similarly to what was reported in Huntington´s disease (Zeron et al., 2002).

3.2.3 MANUSCRIPT 4: *Street* heroin induces mitochondrial dysfunction and apoptosis in rat cortical neurons

Based on: Teresa Cunha-Oliveira, A. Cristina Rego, Jorge Garrido, Fernanda Borges, Tice Macedo and Catarina R. Oliveira, *Street* heroin induces mitochondrial dysfunction and apoptosis in rat cortical neurons, *J. Neurochem.* 101: 543-554

3.2.3.1 Abstract

Cortical function has been suggested to be highly compromised by repeated heroin self-administration. We have previously showed that *street* heroin induces apoptosis in neuronal-like PC12 cells. Thus, we analyzed the apoptotic pathways involved in *street* heroin neurotoxicity using primary cultures of rat cortical neurons. Our *street* heroin sample was shown to be mainly composed by heroin, 6-monoacetylmorphine and morphine. Exposure of cortical neurons to *street* heroin induced a slight decrease in metabolic viability, without loss of neuronal integrity. Early activation of caspases involved in the mitochondrial apoptotic pathway was observed, culminating in caspase-3 activation, PARP cleavage and DNA fragmentation. Apoptotic morphology was completely prevented by the non-selective caspase inhibitor z-VAD-fmk, indicating an important role for caspases in neurodegeneration induced by *street* heroin. Ionotropic glutamate receptors, opioid receptors and oxidative stress were not involved in caspase-3 activation. Interestingly, *street* heroin cytotoxicity was shown to be independent of a functional mitochondrial respiratory chain, as determined using NT-2 rho^0 cells. Nonetheless, in *street* heroin treated cortical neurons, cytochrome c was released, accompanied by a decrease in mitochondrial potential and Bcl-2/Bax. Pure heroin hydrochloride similarly decreased metabolic viability but only slightly activated caspase-3. Altogether, our data suggest an important role for mitochondria in mediating *street* heroin neurotoxic effects.

Keywords: Apoptosis, cell death, heroin, mitochondrial dysfunction

3.2.3.2 Introduction

Heroin abuse has been associated with loss of impulse control (Pau et al., 2002), a function of the frontal cortex associated with drug dependence. Furthermore, downregulation of μ-opioid receptors was reported to occur in the prefrontal cortex in human heroin addicts, as detected by post-mortem analysis (Ferrer-Alcon et al., 2004).

Street heroin may contain different quantities of heroin and other components depending on its origin and on the method of illicit synthesis. Heroin is a semi-synthetic drug, obtained from acetylation of morphine. Heroin possesses little or no opioid activity (White and Irvine, 1999) but its metabolism, which may occur *in vivo* and *in vitro* (Hutchinson and Somogyi, 2002), generates 6-MAM and morphine, two μ-opioid receptor agonists (White and Irvine, 1999). *Street* heroin is illegally synthesized from morphine purified from opium extracts. Opium contains about 40 alkaloids that make up 10-20% of total opium substances. The most abundant opium alkaloids are morphine (8-17%), codeine (0.7-5%), thebaine (0.1-1.5%), papaverine (0.5-1.5%) and noscapine (narcotine, 1-10%) (Schiff, 2002). Upon illicit purification, morphine is often contaminated with other alkaloids, which may also suffer synthetic acetylation during heroin manufacture. Depending on the purification procedure, *street* heroin may contain some impurities (Moore et al., 1984), namely morphine and 6-MAM (heroin metabolites), codeine and acetylcodeine (Soine, 1986, for review). Heroin seized samples may also contain various inert diluents (starch, lactose, fructose, sucrose, mannitol, powdered milk) and active adulterants (caffeine, paracetamol, strychnine, acetylsalicylic acid, barbiturates, quinine and amphetamines) (Chiarotti et al., 1991; Sharma et al., 2005).

Apoptotic cell death appears to be involved in the loss of neuronal function induced by opiates. We have previously shown that *street* heroin induces apoptosis in neuronal-like PC12 cells (Oliveira et al., 2002; Oliveira et al., 2003). Furthermore, morphine was described to induce apoptosis in neurons (Mao et al., 2002) and microglia (Hu et al., 2002).

Apoptosis may be initiated by the activation of the extrinsic and/or the intrinsic pathways. The extrinsic pathway is activated by death receptors in the cell membrane that further activate the initiator caspases 8 or 10 (Chen and Wang, 2002) and possibly caspase 2 (Zhivotovsky and Orrenius, 2005). The intrinsic pathway is initiated by an increase in mitochondrial membrane permeability, regulated by pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family, and associated with the loss of mitochondrial potential ($\Delta\Psi_m$) and the release of mitochondrial proteins to the cytosol (Scorrano and Korsmeyer, 2003). Among these proteins, the most studied is cytochrome c, which induces the formation of the apoptosome and the consequent activation of caspase-9. Caspase-2 may also trigger the intrinsic pathway (Enoksson et al., 2004). Initiator caspases activate effector caspases (such as caspases 3 or 6) that further induce degradation of cellular components (Fischer et al., 2003, for review). Caspase-3 cleaves PARP, a protein involved in DNA repair, and activates DFF/CAD leading to apoptotic DNA fragmentation. However, apoptotic cell death may occur in a caspase-independent manner (Kroemer and Martin, 2005).

In this study we evaluate the neurotoxicity of *street* heroin in primary cultures of rat cortical neurons. As many drugs of abuse, such as amphetamines (Cunha-Oliveira et al., 2006a; Davidson et al., 2001, for review), cocaine (Cunha-Oliveira et al., 2006a; Nassogne et al., 1998, for review) or morphine (Mao et al., 2002; Hu et al., 2002), have been reported to induce neuronal apoptosis, we characterize the involvement of apoptotic pathways in the neurotoxicity of *street* heroin. Briefly, we show that *street* heroin, in a concentration that slightly decreases metabolic viability, promotes caspase-dependent mitochondrial apoptosis, characterized by a downregulation of Bcl-2 and a loss of $\Delta\Psi_m$. The decrease in metabolic viability may be attributed to heroin itself, but caspase-3 activation may be due to drug-drug interaction in the cocktail of compounds that were identified in *street* heroin.

3.2.3.3 Results

3.2.3.3.1 *Street* heroin dose-dependently affects cell viability

Street heroin was shown to be composed by 62% heroin, 12% 6-MAM, 1% morphine, with trace quantities of codeine, acetylcodeine (less than 0.2% each), starch and caffeine, and other unidentified diluents and adulterants (about 25%). The results are in accordance with the data published by the U.S. Drug Enforcement Administration (DEA) which show that the purity of the majority of *street* heroin samples ranges from 10% to 70%.

In order to define experimental conditions of moderate neurotoxicity, we performed a dose-response analysis of cell viability, following the MTT reduction assay (Figure 3.11.A) and the LDH release assay (Figure 3.11.B), after incubation with *street* heroin, for 24 h. *Street* heroin induced a dose-dependent decrease in metabolic viability, showing an IC_{10} value of 128 μg/mL, corresponding to 215 μM heroin, 47 μM 6-MAM and 4.5 μM morphine. Under these conditions, plasma membrane integrity, determined by the LDH leakage assay, was maintained (Figure 3.11.B). The IC_{50} was calculated to be 500 μg/mL, corresponding to 840 μM heroin, 183 μM 6-MAM and 17.6 μM morphine. Membrane integrity was only affected by high concentrations of *street* heroin (Figure 3.11.B), suggesting that, for concentrations up to 427 μg/mL, the neurotoxicity induced by *street* heroin is independent of necrosis. As the *street* heroin sample was not pure, we tested the effect of the identified components (pure heroin, 6-MAM and morphine) on metabolic viability (Figure 3.11.C). While no toxic effects were induced by either 6-MAM or morphine, pure heroin induced a similar decrease in MTT reduction, compared to treatment with *street* heroin. Despite acetylcodeine and codeine were present in very low quantities in *street* heroin, we tested the effect of these compounds on neuronal viability, as acetylcodeine has been reported to be more toxic than heroin (Soine, 1986). Acetylcodeine (1-100 μM) did not significantly affect

cortical neuron metabolic viability, whereas codeine (10-30 μM) induced a slight decrease (by about 10%) in MTT reduction (not shown).

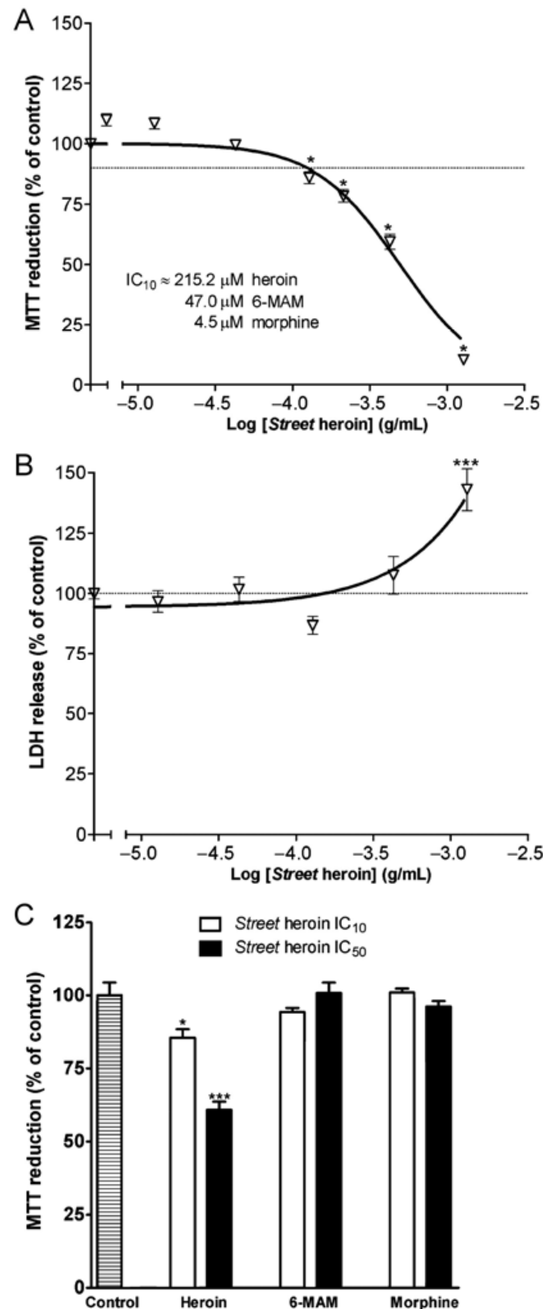


Figure 3.11: Analysis of cell viability upon exposure to *street* heroin. Cortical neurons were incubated with increasing concentrations of *street* heroin (4,3 – 1280 μg/mL), for 24 h, and the dose-dependent changes in cell viability were evaluated by A) the MTT reduction assay, which evaluates changes in metabolic viability, and by B) the LDH leakage assay, in order to analyze changes in membrane integrity. Data are the mean ± SEM of 3-5 experiments performed in duplicate or triplicate. The dotted line in A) represents the IC10 interpolation, corresponding to 128 μg/mL (215.2 μM heroin, 47.0 μM 6-monoacetylmorphine, 4.5 μM morphine). Statistical significance: *P< 0.001 as compared to the control. C) Analysis of cell viability upon exposure to pure heroin (215 and 840 μM), 6-MAM (47 and 183 μM) and morphine (4.5 and 17.6 μM) using the concentrations found in *street* heroin, corresponding to IC10 and IC50. Statistical significance: *P< 0.05, ***P< 0.001 as compared to the control.

3.2.3.3.2 Caspases of the mitochondrial apoptotic pathway are preferentially activated by *street* heroin

We investigated the involvement of apoptotic signalling, namely caspases activation, induced by the concentration of *street* heroin corresponding to the IC_{10} values determined in Figure 3.11.A. Initiator caspases of the mitochondrial pathway, caspases 2 and 9, were significantly activated after 12 h of *street* heroin exposure (Figure 3.12.A), whereas caspase-8 was only slightly activated at a later time point (24 h), indicating that the extrinsic pathway was not involved in the initiation of the apoptotic cascade. Caspase-3 activity in cells treated with *street* heroin was significantly increased after 12 h exposure, when compared with the control (Figure 3.12.A), and remained elevated up to 24 h. The activity of the effector caspase-6 only slightly increased in 24 h *street* heroin-treated cells (not shown). Proteolytic processing of caspases -9 and -3 was confirmed by western blotting (Figure 3.12.B,C). Forty-eight hours exposure to *street* heroin resulted in a massive cell death, as indicated by a large decrease in MTT reduction and a major decrease in caspase-3 activity (data not shown). *Street* heroin-induced increase in caspase-3 activity at 24 h was completely prevented in the presence of 1 μM z-VAD-fmk, a non-selective caspase inhibitor (Figure 3.12.D). Pure heroin (215 μM) only slightly activated caspase-3 (1.4 fold above the control, Figure 3.12.E), suggesting that caspase-3 may be further activated by other substances present in *street* heroin and/or that combination of *street* heroin components act synergistically to cause apoptotic cell death.

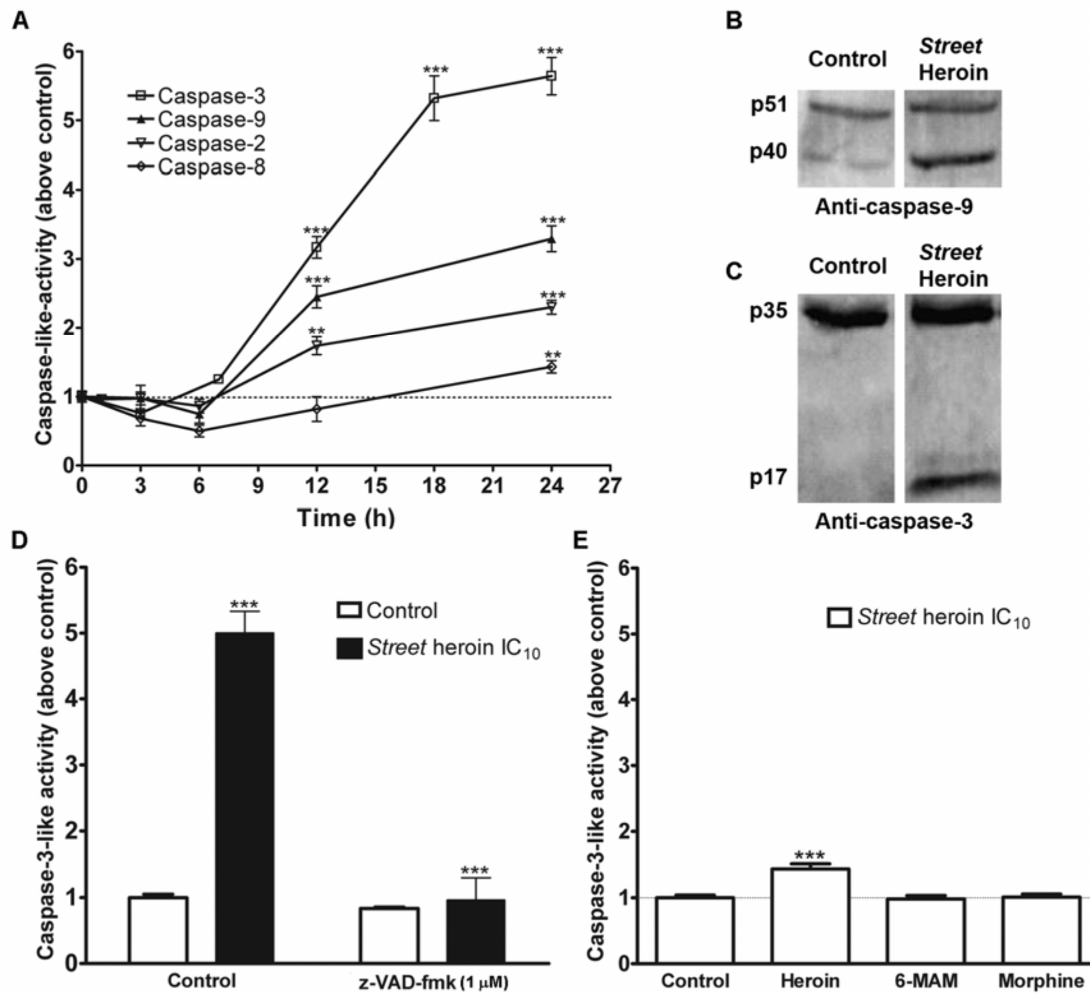


Figure 3.12: Caspases activation in cells incubated with *street* heroin. A) Time course analysis of caspase activation. Cellular extracts were tested for the activity of caspases 3, 9, 2 and 8, by determining the extent of cleavage of caspases substrates: Ac-DEVD-pNA, Ac-LEHD-pNA, Ac-VDVAD-pNA and Ac-IEPD-pNA, respectively. The cells were incubated with *street* heroin (IC10), for 1-24 h. Pro-caspases 3 (B) and 9 (C) processing, after 24 h of exposure to *street* heroin, was also examined by western blotting D) Complete inhibition of *street* heroin-induced caspase-3 activation in the presence of 1μM z-VAD-fmk. E) Caspase-3 activity upon 24 h exposure to 215 μM pure heroin, 47 μM 6-MAM or 4.5 μM morphine, corresponding to *street* heroin IC10. Data are the mean ± SEM of the fold increase above control absorbance values of 3 experiments performed in duplicate. Statistical significance: **P< 0.01, ***P<0.001 compared to the control.

3.2.3.3.3 DNA fragmentation induced by *street* heroin is completely dependent on caspase activity

According to data on Figure 3.12, caspase-dependent apoptotic cell death seems to be involved in the neurotoxic effects of *street* heroin. This was further evaluated by analyzing the occurrence of nuclear apoptotic morphology and PARP cleavage in cells treated with the drug of abuse (Figure 3.13). *Street* heroin induced an increase (by about 17%) in the number of cells showing fragmented or condensed DNA, as evaluated by nuclear staining with Hoechst 33342 (Figure 3.13). The number of cells showing immunoreactivity to PARP, cleaved on the caspase cleavage site, also increased (Figure 3.13.A), reflecting the activation of caspase-3. DNA fragmentation induced by *street* heroin was completely prevented by 1 µM z-VAD-fmk (Figure 3.13.B), a concentration previously shown to prevent the increase in caspase-3 activity induced by *street* heroin (Figure 3.12.D). These data support an important role for caspases in the neurotoxic effects of *street* heroin.

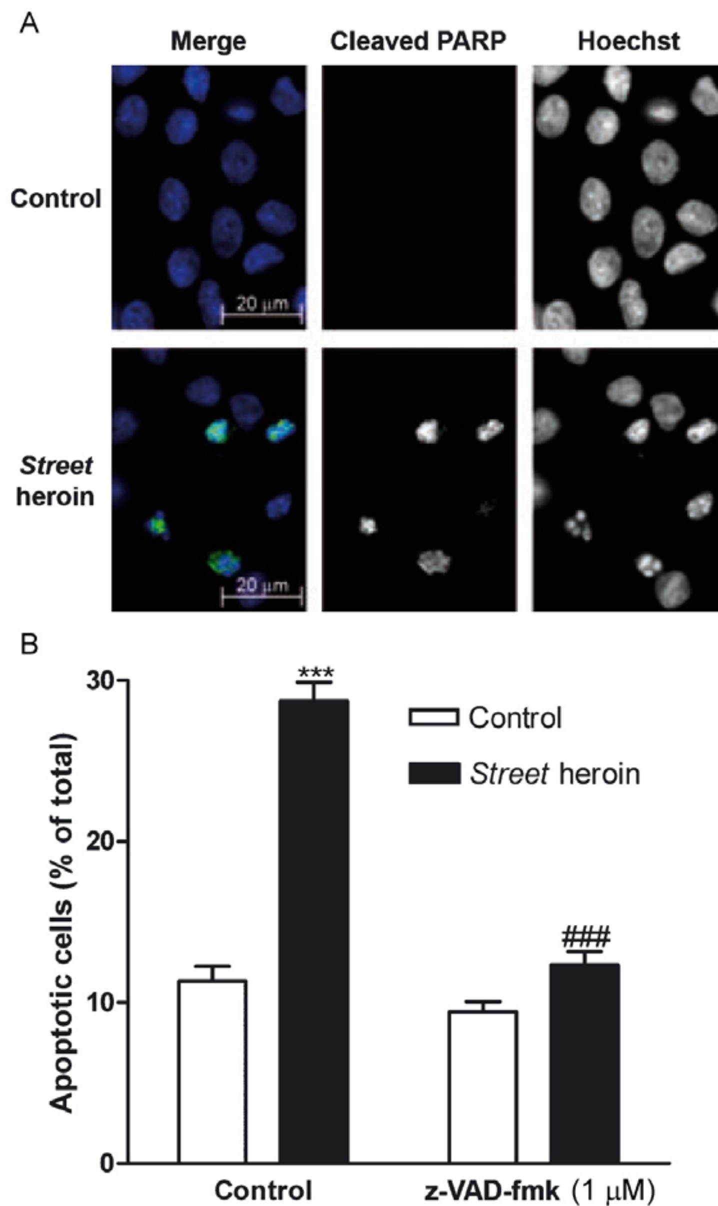


Figure 3.13: Analysis of nuclear apoptotic morphology in cortical neurons exposed to *street* heroin. A) Immunocytochemical analysis of apoptotic morphology. Apoptotic cell death upon exposure to *street* heroin (IC10, for 24 h) was evaluated by labeling cleaved PARP, using a specific antibody (in green), and by nuclear morphology following Hoechst 33342 staining (in blue). B) Complete prevention of DNA fragmentation induced by *street* heroin, in the presence of 1 µM z-VAD-fmk. About 500-700 cells were counted per coverslip. Data are the mean ± SEM of 3-4 experiments performed in duplicates. Statistical significance: ***P< 0.001 compared to the control, ###P< 0.001 compared to *street* heroin alone.

3.2.3.3.4 Caspase activation is not mediated by opioid or ionotropic glutamate receptors and appears to be independent of oxidative stress

We further investigated the mechanisms leading to caspase-3 activation induced by *street* heroin by analyzing the involvement of opioid and ionotropic glutamate receptors and the contribution of ROS. The involvement of opioid receptors was determined by using a non-selective opioid receptor antagonist, naloxone, the μ-opioid receptor antagonists, clocinnamox and CTOP, and the delta-opioid receptor antagonist, naltrindole (Table 3.1.A). Under these conditions, a partial prevention of caspase-3 activation was observed in the presence of naloxone (about 30%). Naloxone also prevented (by about 25%) the appearance of nuclear apoptotic morphology induced by *street* heroin (not shown). However, the selective opioid receptor antagonists did not significantly affect caspase-3 activity induced by *street* heroin. These data suggest that opioid receptors do not mediate *street* heroin neurotoxicity, and that naloxone may prevent caspase-3 activation, and apoptosis, by an opioid receptor-independent mechanism, as previously reported (Liu et al., 2002).

Table 3.1 Analysis of cellular mechanisms involved in caspase-3 activation in cells incubated with *street* heroin (IC10).

		Antagonists	caspase 3-like activity (above control)	caspase 3-like activity (above heroin)
			− *Street* heroin	+ *Street* heroin
A)	Opioid receptors	None	1.00 ± 0.03	1.00 ± 0.02
	Non-selective	Naloxone (1 μmol/L)	0.87 ± 0.12	0.71 ± 0.03**
	Micra	Cloccinamox (1 μmol/L)	0.98 ± 0.05	0.93 ± 0.06
		CTOP (1 μmol/L)	0.92 ± 0.16	0.94 ± 0.05
	Delta	Naltrindole (1 μmol/L)	1.04 ± 0.06	0.97 ± 0.07
B)	Glutamate receptors	None	1.00 ± 0.02	1.00 ± 0.02
	NMDA	MK-801 (2 μmol/L)	1.27 ± 0.10	1.01 ± 0.06
		Ifenprodil (3 μmol/L)	1.24 ± 0.12	1.11 ± 0.11
		AP-5 (100 μmol/L)	1.11 ± 0.19	1.21 ± 0.08
	AMPA	NBQX (10 μmol/L)	1.22 ± 0.12	0.97 ± 0.12
C)	Antioxidants	None	1.00 ± 0.08	1.00 ± 0.04
		Idebenone (3 μmol/L)	1.48 ± 0.16	1.08 ± 0.09
		Trolox (100 μmol/L)	1.02 ± 0.17	1.00 ± 0.03
		GSH (100 μmol/L)	1.30 ± 0.12	1.01 ± 0.09

A) The involvement of opioid receptors was investigated by testing the effect of a non-selective antagonist (1 μM naloxone) and antagonists of mu (1 μM clocinnamox and 1 μM CTOP) and delta (1 μM naltrindole) opioid receptors. B) The involvement of ionotropic glutamate receptors in caspase-3 activation was investigated using antagonists of NMDA (2 μM MK-801, 3 μM ifenprodil or 100 μM d-AP-5) and AMPA (10 μM NBQX) receptors. C) The involvement of reactive oxygen species in caspase-3 activation induced by *street* heroin was investigated using antioxidants idebenone (3 μM), trolox (100 μM) and glutathione ethyl ester (100 μM GSH-EE). Data are the mean ± SEM of 3 experiments performed in duplicate. Statistical significance: **P< 0.01, compared to *street* heroin alone.

Moreover, as apoptotic cell death frequently depends on excitotoxic events mediated by ionotropic glutamate receptors, and these receptors were reported to be present in embryonic cortical neurons (Babb et al., 2005), we also tested the effect of the NMDA receptor antagonists MK-801, ifenprodil and d-AP-5, and the AMPA receptor antagonist NBQX, in the activation of caspase-3 induced by *street* heroin (Table 3.1.B). The results indicate that ionotropic glutamate receptors are not involved in *street* heroin neurotoxicity, as the antagonists of NMDA or AMPA receptors did not prevent *street* heroin-induced caspase-3 activation.

Because apoptotic cell death has been associated with oxidative stress we tested whether the antioxidants idebenone (a benzoquinone derivative of coenzyme

Q10), trolox (an analog of vitamin E) or glutathione ethyl ester (a cell-permeable form of reduced glutathione), protected against *street* heroin-induced apoptosis (Table 3.1.C). None of the antioxidants tested prevented *street* heroin-induced caspase-3 activation, indicating that oxidative stress is not involved in the apoptotic process induced by this drug of abuse.

3.2.3.3.5 *Street* heroin induces cytochrome c release and mitochondrial dysfunction

Initiator caspases (2 and 9) activated by *street* heroin at early time points (Figure 3.12.A) have been largely associated with the mitochondrial apoptotic pathway. Thus we analyzed whether the release of cytochrome c and mitochondrial dysfunction contributed to the neurotoxic effects of *street* heroin.

Analysis of cytochrome c release by immunocytochemistry (Figure 3.14.A) showed a decrease in co-localization between cytochrome c (labeled with a specific antibody) and the mitochondria (labeled with MitoTracker Green) upon treatment with *street* heroin, indicating a decrease in mitochondrial cytochrome c content. *Street* heroin-mediated release of cytochrome c to the cytosol was further evidenced by western blotting, showing a decrease in mitochondrial cytochrome c content (by about 60%) and a consequent increase in the cytosolic fraction (Figure 3.14.B). An increase in mitochondrial permeability, responsible for cytochrome c release, has been associated with a decrease in $\Delta\Psi_m$. Thus, we evaluated the changes in $\Delta\Psi_m$, induced by *street* heroin (Figure 3.14.C), by following the cellular retention of rhodamine 123, described to be higher in cells maintaining $\Delta\Psi_m$ (Palmeira et al., 1996). Our data suggest that exposure to *street* heroin causes a great decrease in $\Delta\Psi_m$ (by about 55%).

Because *street* heroin neurotoxicity involved mitochondrial dysfunction, we investigated the requirement of a functional respiratory chain. Thus, we analyzed *street* heroin neurotoxicity in NT-2 rho^0 cells, which do not possess a functional electron transport chain (Cardoso et al., 2001), in comparison with NT-2 rho^+ cells (Figure 3.14.D). The toxicity of *street* heroin in NT-2 rho^0 cells was not significantly different

compared to NT-2 rho^+ cells, suggesting that *street* heroin neurotoxicity is independent of an interaction with a functional mitochondrial respiratory chain.

Pro-apoptotic proteins, such as Bax, have been shown to mediate the release of cytochrome c, whereas anti-apoptotic proteins, such as Bcl-2, modulate the activity of pro-apoptotic proteins. Thus, we determined the total levels of Bcl-2 and Bax, by western blotting, after exposure to *street* heroin (Figure 3.14.E and F). We found a significant decrease (by about 37%) in Bcl-2 levels (Figure 3.14.E), without major changes in the levels of Bax (Figure 3.14.F) in cortical neurons treated with *street* heroin. These results indicate that *street* heroin induces a decrease in Bcl-2/Bax ratio, favoring the proapoptotic activity of Bax, which could help to explain the mechanism involved in cytochrome c release induced by *street* heroin. The fact that *street* heroin neurotoxicity seems to be independent of a functional mitochondrial respiratory chain, suggests that *street* heroin-induced decrease in $\Delta\Psi_m$ in cortical neurons may be due to the formation of mitochondrial permeability transition or Bax-composed pores. As it was previously described that Bax channel inhibitors can block the decrease in $\Delta\Psi_m$ induced by a pro-apoptotic inducer (Hetz et al., 2005), a decrease in Bcl-2/Bax may lead to mitochondrial permeabilization and consequent loss of $\Delta\Psi_m$.

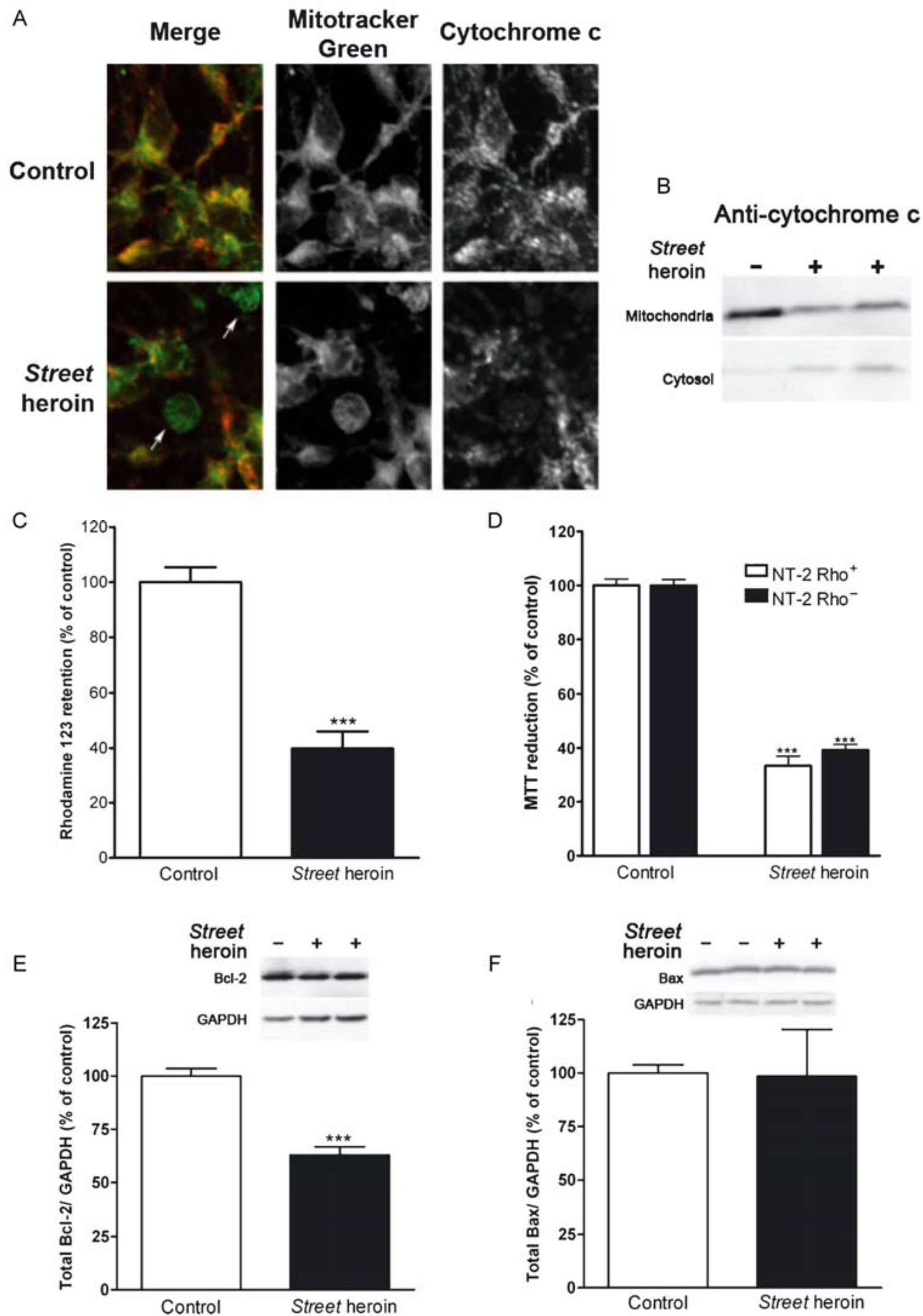


Figure 3.14: Analysis of cytochrome c release and mitochondrial dysfunction after exposure to *street* heroin (IC10, for 24 h). A,B) Analysis of cytochrome c release from the mitochondria, by immunocytofluorescence (A), using MitoTracker Green to label the mitochondria (in green), and an antibody against the native form of cytochrome c (in red), or by western blotting (B)

using subcellular fractions corresponding to the same samples. C) Analysis of rhodamine 123 (Rh123) retention capacity of cortical neurons after exposure to the drug of abuse. Rh123 retention capacity was used to evaluate $\Delta\Psi m$ in cortical neurons incubated with *street* heroin (IC10). The cells were incubated with 1 μM Rh123, for 10 min. The fluorescence of the probe was recorded before (Fi) and after (Ff) cell permeabilization with Triton X-100. Rh123 retention capacity was calculated by the difference: Ff - Fi. D) Analysis of *street* heroin cytotoxicity in NT-2 rho+ and rho0 cells by following the cellular capacity to reduce MTT after exposure to *street* heroin (IC10, 24 h). E,F) Analysis of total Bcl-2 and Bax levels, by western blotting. Images are representative of at least 3 experiments, performed in duplicate. Quantitative data are the mean $\pm$ SEM of at least 3 experiments performed in duplicate or triplicate. Statistical significance: ***P<0.001 compared to the control.

3.2.3.4 Discussion

In this study we show that *street* heroin induces cell death by a mitochondrial-dependent apoptotic pathway, initiated by caspases 2 and 9, and involving cytochrome c release, loss of mitochondrial potential and downregulation of Bcl-2. This process is not dependent on the activation of opioid or ionotropic glutamate receptors, nor is it dependent on oxidative stress. Due to the fact that the *street* heroin sample was a mixture, the effects observed in this study may be either due to heroin, or to the combination of *street* heroin components, which can act synergistically.

Acetylcodeine has been reported to be more toxic than heroin (Soine, 1986). However, in our experimental conditions, acetylcodeine did not significantly affect cortical neurons metabolic viability, whereas codeine induced a slight decrease in MTT reduction, when used in higher concentrations than those achieved in *street* heroin. Noscapine (20 µM), another alkaloid present in opium extracts, has been shown to induce apoptosis in HeLa cells and thymocytes (Ye et al., 1998). In addition to opium substances, *street heroin* often contains some adulterants, namely paracetamol (acetaminophen), caffeine and theophylline (Zhang et al., 2004), which may also exhibit toxic effects. Very toxic adulterants are not usually detected in this type of samples because when present, they are found in very low concentrations (Chiarotti et al., 1991; Sharma et al., 2005).

The mechanisms of cell death induced by heroin are largely unknown. Fecho and Lysle (2000) showed that heroin decreased the number of leukocytes in the rat spleen, which presented several apoptotic features, such as annexin V labeling and DNA fragmentation. In contrast, the cytotoxicity of morphine, a metabolite of heroin that may coexist in heroin solutions (Hutchinson and Somogyi, 2002), has been investigated by several groups. Hu *et al.* (2002) showed that morphine increased the number of apoptotic microglia and neurons. This process of cell death was blocked by naloxone and involved caspase-3 activation and DNA fragmentation. Other authors (Mao et al., 2002) showed that prolonged morphine administration increased rat spinal

neuronal apoptosis, with upregulation of caspase-3 and Bax, and downregulation of Bcl-2 in the spinal dorsal horn. The non-selective caspase inhibitor, z-VAD-fmk and the caspase-3 inhibitor Ac-DEVD-CHO blocked neuronal apoptosis induced by morphine. Moreover, in this study, the apoptotic pathway was reported to be mediated by the NMDA receptors (Mao et al., 2002). In another study, Yin *et al.* (1999) showed that morphine induced the expression of Fas and promoted Fas-L-mediated apoptosis of lymphocytes. This effect was blocked by naloxone, suggesting the involvement of the opioid receptors. Furthermore, Singhal *et al.* (2002) showed that morphine induced apoptosis of T-cells, with activation of caspases -3, -8 and -10 and PARP cleavage. This apoptotic pathway was reported to be mediated by c-Jun N-terminal kinase (JNK) activation. Together, these studies suggest that morphine is able to induce apoptosis in several cell types, including neurons, in a process mediated by opioid receptors. Another study (Jiang et al., 2003) showed that morphine, heroin and cocaine upregulate mouse double minute-2 (MDM2) in several brain regions. MDM2 is known for suppressing the activity of p53. Increased MDM2 may reflect an increase in p53 activity induced by the drugs of abuse (Jiang et al., 2003). The neurotoxicity and apoptotic cell death induced by morphine were also suggested to be mediated by an increase in JNK3 expression (Fan et al., 2003). According to our results only pure heroin slightly contributed for caspase-3 activation and loss of cell viability, whereas morphine did not exhibit toxic effects.

Cortical neurons were previously reported to express both μ and δ opioid receptors (Lee et al., 2002). In the present work, although we observed a reduction of caspase-3 activation (by about 30%) and a reduction in apoptotic nuclear morphology (by about 25%, data not shown) in the presence of naloxone, the involvement of opioid receptors in apoptotic cell death induced by *street* heroin was not corroborated by using specific opioid receptor antagonists (Table 3.1.A). Naloxone was previously shown to mediate neuroprotection independently of the interaction with opioid receptors (Liu et al., 2002). Moreover, the stereoisomer (+)-naloxone, which has no activity as an opioid receptor antagonist, effectively inhibits microglial activation and has been demonstrated to be neuroprotective (Liu et al., 2002). It was previously

suggested that naloxone could also directly interact with the nicotinic receptor (Tome et al., 2001). In our cellular model, *street* heroin's neurotoxicity is largely independent of opioid receptors, and naloxone may prevent apoptotic cell death induced by *street* heroin by a mechanism independent of the interaction with opioid receptors. Morphine was previously reported to induce loss of $\Delta\Psi_m$ in human glioma cells by a mechanism mediated by naloxone-sensitive receptors (Mastronicola et al., 2004). Thus, naloxone-mediated neuroprotection observed in the present work may be related with the inhibition of *street* heroin-induced loss of $\Delta\Psi_m$. In addition to these results, the PKA activator forskolin (1-30 µM) was not able to prevent *street* heroin-induced caspase-3 activation (data not shown), suggesting that the mechanism involved in apoptosis induced by *street* heroin is not dependent on PKA inhibition, which is known to occur upon activation of opioid receptors. Moreover, the fact that opioid receptors are not involved in *street* heroin-induced apoptosis is in agreement with the lack of major apoptotic effects of the opioid components identified in our *street* heroin sample (Figure 3.12.E).

Ionotropic glutamate receptor activation, frequently associated with excitotoxic cell death, was not involved in *street* heroin-induced apoptosis either, because antagonists of the NMDA or AMPA receptors were not able to rescue caspase-3 activation induced by heroin (Table 3.1.B). Moreover, nicotine, an agonist of the nicotinic acetylcholine receptor, previously shown to be neuroprotective in cortical cell cultures through a decrease in caspase activation and inhibition of apoptosis induced by oxygen deprivation (Hejmadi et al., 2003), did not prevent *street* heroin-induced caspase-3 activation, when used at 10-30 µM (data not shown).

Other mediators of cell death involving mitochondrial dysfunction are ROS. Previously, other authors reported that *in vivo* exposure of mice to heroin induced oxidative stress (Pan et al., 2005; Qiusheng et al., 2005). However, in the present study the antioxidants idebenone, trolox and glutathione ethyl ester did not prevent *street* heroin-induced caspase-3 activation (Table 3.1.C), indicating that, in our conditions, an increase in ROS levels is not upstream of caspase-3 activation. In addition, although nitric oxide synthase activation has been reported to mediate

mitochondrial dysfunction and apoptosis in cortical neurons subjected to glutamate (Almeida and Bolanos, 2001), in this work the nitric oxide synthase inhibitors N-omega-nitro-L-arginine methyl ester (100-500 μM L-NAME) or 7-nitroindazole (10-100 μM 7-NI) were also unable to prevent *street* heroin-induced caspase-3 activation (data not shown).

The time course of apoptotic events in this work suggests the activation of caspases 2 and 9 upon 6-12 h of *street* heroin exposure, resulting in the activation of caspase 3. Caspase-3 activity is also suggested by the occurrence of PARP cleavage and DNA fragmentation. In addition, upon 24 h exposure to *street* heroin, metabolic viability was decreased by about 10% but there were no evidences of changes in plasma membrane integrity, suggesting that necrosis was not involved at this time point. Although the mechanism underlying *street* heroin-induced apoptosis does not seem to involve the activation of plasma membrane receptors (opioid or glutamate receptors), it is likely to be explained by intracellular drug-drug interactions of one or more components of *street* heroin. This interaction is likely to be directed to Bcl-2 expression and to the mitochondria, resulting in the downregulation of Bcl-2 and the loss of $\Delta\Psi_m$. These changes may help to explain cytochrome c release. Moreover, *street* heroin-induced loss of $\Delta\Psi_m$ in cortical neurons was similar to that induced by amphetamine in cortical neurons (Cunha-Oliveira et al., 2006a), although activation of all of the caspases studied was more evident upon treatment with *street* heroin. Furthermore, a possible interaction of heroin (or other components of *street* heroin) with the mitochondria does not require a functional mitochondrial respiratory chain, since the drug was as toxic in NT-2 rho^0 cells as in NT-2 rho^+ cells.

Concluding, we demonstrate the importance of the mitochondrial apoptotic pathway involving caspases activation in the neurotoxicity of *street* heroin, which may be caused either by the drug itself or by the cocktail of *street* heroin components that can act synergistically.

Although further studies are required to determine the mitochondrial changes upon *in vivo* heroin administration, the intrinsic apoptotic pathway appears to be an

important target for neuroprotective strategies in heroin addicted individuals, for whom the use of impure heroin represents an increased neurotoxic risk.

PART IV - CONCLUSIONS

In Part III, several functional alterations were observed in PC12 cells upon chronic exposure to amphetamine, cocaine and *street* heroin. PC12 cells chronically exposed to H_2O_2 were used as a model of cellular adaptation to oxidative stress. Some changes observed in cells chronically exposed to the drugs of abuse were comparable to those induced by chronic exposure to H_2O_2, suggesting a role of oxidative stress on the adaptation of catecolaminergic cells to the drugs of abuse.

Chronic exposure to amphetamine caused dopamine depletion upon 7-12 months of exposure. At initial time-points of chronic exposure to amphetamine alterations in the activity of antioxidant enzymes were observed, which returned to control levels after 4 weeks. Moreover, acute amphetamine toxicity was shown to be independent of dopamine and oxidative stress. Briefly, amphetamine exposure induced the following changes in PC12 cells:

i. Dopamine depletion, suggesting long-term tyrosine hydroxylase inhibition;
ii. Toxicity when incubated in dopamine-depleted cells, suggesting that acute amphetamine effects are independent of dopamine, and possibly mediated by mitochondrial dysfunction;
iii. Toxicity in cells chronically exposed to H_2O_2, suggesting that acute amphetamine effects are independent of oxidative stress; and
iv. Biphasic changes in the activity of the antioxidant enzymes GPx, GRed and SOD during initial chronic amphetamine exposure, indicating the involvement of oxidative stress mediated by H_2O_2, which tends to recover at a later time point possibly due to dopamine depletion and/or MAO inhibition.

Chronic cocaine increased PC12 sensitivity to a toxic cocaine insult. Chronic cocaine also increased the resistance against acute H_2O_2 toxicity, although only partially, in contrast to the complete resistance of PC12 cells chronically exposed to H_2O_2, suggesting some degree of adaptation to oxidative stress. The incomplete adaptation to oxidative stress may be explained by differential effects of chronic exposure to cocaine or H_2O_2 on the activity of antioxidant enzymes, observed in early time-points of chronic exposure. However, acute exposure to cocaine was suggested

to be independent of oxidative stress. Briefly, exposure of PC12 cells to cocaine caused the following:

i. Increased sensitivity to acute cocaine toxicity in cells chronically treated with cocaine;
ii. Increased cocaine-evoked extracellular dopamine accumulation, which may explain increased motor activity in response to cocaine, upon repeated cocaine administration *in vivo;*
iii. Toxicity in cells chronically exposed to H_2O_2, suggesting that acute cocaine effects are independent of oxidative stress;
iv. Increased resistance to H_2O_2 acute toxicity, highly suggesting that chronic exposure to cocaine involves adaptation to oxidative stress; and
v. Increased GPx activity, probably occurring as a consequence of enhanced H_2O_2 production.

Chronic *street* heroin caused a decrease in energy levels that may underlie the observed increase in cellular sensitivity to acute *street* heroin treatment. Cells chronically exposed to street heroin exhibited a partial resistance against oxidative stress. The following changes were observed upon exposure of PC12 cells to street heroin:

i. Decreased intracellular levels of ATP and ADP;
ii. Increased toxicity upon acute exposure, probably mediated by an increase in extracellular dopamine accumulation;
iii. Increased toxicity in cells chronically exposed to H_2O_2, suggesting that acute *street* heroin effects are independent of oxidative stress; and
iv. Increased resistance against acute H_2O_2 treatment, suggesting adaptation to oxidative stress upon chronic exposure to *street* heroin.

Furthermore, acute cytotoxic effects of *street* heroin were shown to be mediated by NR1/NR2B composed NMDA receptors in transfected HEK293 cells. In these cells,

the cytotoxic effects of *street* heroin involved loss of plasma membrane integrity, suggesting the occurrence of necrotic cell death.

In rat primary cortical neurons, a concentration of *street* heroin that induced a mild decrease in metabolic cell viability, without loss of membrane integrity, was shown to induce apoptotic cell death, involving early caspase -2, -3 and -9 activation, and a late activation of caspases -6 and -8. *Street* heroin-exposed cortical neurons exhibited chromatin condensation and fragmentation, which were caspase-dependent. Apoptotic cell death induced by *street* heroin was mediated by the mitochondrial apoptotic pathway and involved mitochondrial dysfunction. However, NMDA-receptors were not involved in *street* heroin-induced apoptosis. This result suggests that NMDA receptors mediate more severe forms of *street* heroin cytotoxicity, involving loss of membrane integrity, probably associated with necrotic cell death. Briefly, acute *street* heroin toxicity was shown to involve:

i. Increased toxicity in HEK293 cells expressing NR1/NR2B composed receptors, in comparison with cells expressing NR1/NR2A functional receptors;

ii. Activation of the mitochondrial apoptotic pathway in rat primary cortical neurons showing:

   1. Decreased Bcl-2/Bax, cytochrome c release and increased activity of caspases associated with the mitochondrial apoptotic pathway;
   2. Prevention of apoptosis by the non-selective caspase-inhibitor z-VAD-fmk and by naloxone;
   3. Caspase activation independently of the activation of opioid receptors, ionotropic glutamate receptors or oxidative stress;
   4. Mitochondrial dysfunction, as determined by a decrease in mitochondrial membrane potential;
   5. Loss of metabolic viability similarly caused by pure heroin, but the latter was less efficient in activating caspase-3, highly suggesting that the use of impure heroin represents an increased neurotoxic risk.

In conclusion, drugs of abuse cause the dysfunction of PC12 cells and cortical neurons. Chronic and acute exposures to the drugs of abuse in dopaminergic cells involve neuronal dysfunction and neurotoxicity mediated by changes in dopamine levels. Increased oxidative stress may also underlie the chronic effects of these drugs in dopaminergic cells. Acute, severe, *street* heroin toxicity seems to be mediated by NR1/NR2B composed NMDA receptors, in transfected HEK293 cells. However, apoptosis evoked by a mild insult with *street* heroin in primary cortical neurons does not involve activation of NMDA receptors or oxidative stress. Hence, apoptotic effects of *street* heroin are mediated by mitochondrial dysfunction and are dependent on the activity of caspases.

Moreover, we showed that chronic exposure to cocaine or *street* heroin may sensitize the cells for subsequent drug exposure, increasing drug toxicity, and contributing to further brain dysfunction that may potentiate the development of drug addiction. Chronic exposure to amphetamine caused dopamine depletion, which may contribute to long-term amphetamine effects, since dopamine mediates several important brain functions.

Future therapies for drug addiction disorders should take into account the impact of the neurotoxic effects of these drugs. Oxidative stress and mitochondrial dysfunction are suggested as new targets for the development of future therapeutic strategies against drug addiction.

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