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Vulnerability to cognitive, neurotoxic and neuroinflammatory effects of
toxins that induce Parkinson's disease after administration of
amphetamine-related drugs in mice

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List of abbreviations

α -MeDA α -methyldopamine
5-HIAA 5-hydroxyindoleacetic acid
5-HT 5-hydroxytryptamine
ATP adenosine triphosphate
BBB blood brain barrier
CD11b complement type 3 receptor
CNS central nervous system
COMT catechol-O-methyl transferase
CSF cerebrospinal fluid
CYP cytochrome P450
D density of TH-positive neurons in SNc
DA dopamine
DAT dopamine transporter
DOPAC 3,4-dihydroxyphenylacetic acid
DOPAL 3,4-dihydroxyphenylacetaldehyde
EMCDDA European Monitoring Centre for Drugs and Drug Addiction
fMRI functional magnetic resonance imaging
GFAP glial fibrillary acidic protein
GLU glutamate
GSH glutathione
HHA 3,4-dihydroxyamphetamine
HHMA 3,4-dihydroxymethamphetamine
HMA 4-hydroxy-3-methoxyamphetamine
HMMA 4-hydroxy-3-methoxymethamphetamine
HO• hydroxyl radical
HVA homovanillic acid
i.m. intramuscular administration
i.p. intraperitoneal administration
i.v. intravenous administration
IL-1 β interleukin-1 β
iNOS inducible nitric oxide synthase
INT- γ interferon γ
L-DOPA L-3,4-dihydroxyphenylalanine
MAO monoamine oxidase
MDA 3,4-methylenedioxyamphetamine
MDMA 3,4-methylenedioxymethamphetamine
METH methamphetamine
MPDP⁺ 1-methyl-4-phenyl-2,3-dihydropyridium
mPFC medial prefrontal cortex
MPP⁺ 1-methyl-4-phenylpyridinium
MPPP 1-methyl-4-phenyl-4-propionpiperidine
MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
N total number of TH-positive neurons in SNc
NAc nucleus accumbens
NE noradrenaline
NET noradrenaline transporter
NMDA N-methyl-D-aspartic acid
N-Me- α -MeDA N-methyl- α -methyldopamine
NO nitric oxide

NOR novel object recognition task
ONOO⁻ peroxynitrite
nNOS neuronal nitric oxide synthase
O₂⁻ superoxide
PD Parkinson's disease
PET positron emission tomography
PFC prefrontal cortex
RNS reactive nitrogen species
ROS reactive oxygen species
s.c. subcutaneous administration
SERT 5-hydroxytryptamine transporter
SN substantia nigra
SNc substantia nigra pars compacta
SOD superoxide dismutase
T-4,5-D tryptamine-4,5-dione
TH tyrosine hydroxylase
TNF- α tumour necrosis factor α
TPH tryptophan hydroxylase
VMAT₂ vesicular monoamine transporter

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Abstract

Clinical observations report a higher propensity to develop Parkinson's disease (PD) in amphetamine users. 3,4-Methylenedioxymethamphetamine (MDMA) is an amphetamine-related drug which may have neuroinflammatory and neurotoxic effects. The present study was aimed at evaluating in mice whether administration of MDMA during adolescence might influence neurotoxicity towards dopaminergic neurons and neuroinflammatory effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a toxin known to induce PD in humans, in motor, limbic and cortical areas, and consequently affects cognitive performance.

Mice received MDMA (10 mg/kg, twice a day/a week) for 9 weeks, followed by MPTP (20 mg/kg × 4 administrations), starting 2 weeks after MDMA discontinuation. Activation of astroglia and microglia by GFAP and CD11b immunohistochemistry in motor areas, as substantia nigra compacta (SNc) and striatum, limbic and cortical areas, as hippocampus and medial prefrontal cortex (mPFC), was assessed. Degeneration of dopaminergic neurons by tyrosine hydroxylase (TH) immunohistochemistry in SNc and striatum was also evaluated. Neurochemical evaluations were paired with assessment of cognitive performance by means of the novel object recognition (NOR) and spontaneous alternation in a Y-maze tests.

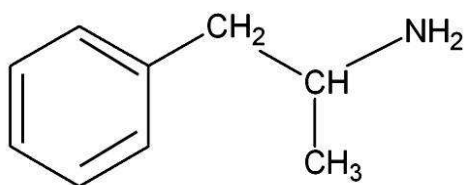
MPTP administration to MDMA-pretreated mice elicited a stronger increase in CD11b and GFAP levels in motor, limbic and cortical areas, and a stronger decrease of TH-positive neurons and fibers in motor areas, compared with either substance administered alone. Furthermore, NOR performance in the same group was lower, compared with mice that received either substance alone.

Results demonstrate that MDMA administration during adolescence influence negatively MPTP effects on motor, limbic and cortical areas and result in cognitive impairment.

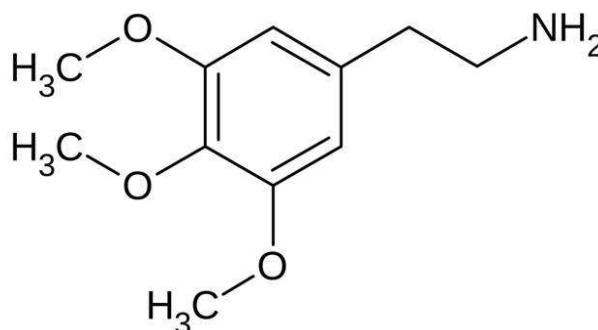
Introduction

1. MDMA

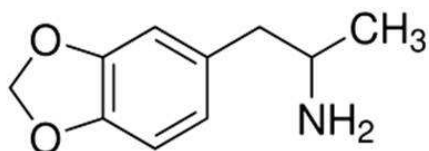
3,4-Methylenedioxyamphetamine (MDMA), otherwise called “Ecstasy” (Figure 1), is a ring-substituted drug structurally correlated to the psychostimulant compounds as amphetamine, mescaline, 3,4-methylenedioxyamphetamine (MDA) and also to monoamine neurotransmitters. MDMA is a member of the larger group of ring-substituted phenylethylamines, and is characterized by a N-methyl group and a methylenedioxy ring on the third and fourth carbon of the phenyl ring, respectively.



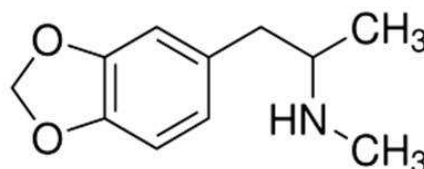
(A) Amphetamine



(B) Mescaline



(C) MDA 3,4-Methylenedioxyamphetamine



(D) MDMA 3,4-Methylenedioxyamphetamine

Figure 1. Chemical structures of (A) amphetamine, (B) mescaline, (C) MDA and (D) MDMA.

MDMA was first synthesized and patented by Köllisch at the German pharmaceutical company Merck in Darmstadt around 1912 and, at that time, it was not tested pharmacologically because it was regarded only as in a new synthetic procedure for haemostatic substances (Freudenmann et al. 2006). Moreover, MDMA was not mentioned in the Merck archive files for the next 15 years. In 1927, a Merck chemist, Oberlin, noted that the structure of MDMA was similar to that of ephedrine and adrenaline, and performed the first proven pharmacological test comparing the effects of MDMA with those of ephedrine. In 1952, another Merck chemist, van Schoor, conducted a simple toxicological experiment documented only by a short note in his personal laboratory book. In 1973, the first study was published in the literature that mentioned MDMA,

based upon toxicological research conducted in five animal species, including the mouse, in the early 1950s by the Army's Chemical Center (Hardman et al. 1973). The next phase of research on MDMA commenced when Alexander Shulgin appeared on the stage. According to Shulgin's autobiography and to interviews, he synthesized and tested the effects of more than two hundred potentially psychoactive substances, including MDMA, often by taking these substances himself, and/or administering them to a close circle of collaborators. In 1990, Shulgin wrote a comprehensive "history of MDMA", where it is reported that MDMA was first introduced into clinical practice on the West Coast of the United States in 1976, and was used by psychotherapists on the East Coast shortly after. Later, he specified that in 1977 he had introduced MDMA to Leo Zeff, a retired psychologist in Oakland, CA, who was so impressed by the effects of MDMA that he abandoned retirement and start to introduce MDMA to many psychotherapists across the United States (Benzenhöfer and Passie 2010). Meanwhile, in 1977, the UK classified MDMA as a class A schedule 1 drug, thus prohibiting to possess, sell, or give away the substance. In the USA, since the early 1980s, MDMA became popular in the streets as a recreational drug, and as a "fun drug" that was "good to dance". In 1985, the American agency DEA classified MDMA as a Schedule 1 drug, due to its high abuse potential, lack of clinical applications, lack of certified safety for use under medical supervision, and suspects that it could be neurotoxic. The fame MDMA acquired in the USA soon spread across the Atlantic. Globally, the Europe remains the main center of MDMA production, although its relative importance appears to be declining, as MDMA manufacture has spread to other parts of the world in recent years, notably to USA and east and southeast Asia (European Monitoring Centre for Drugs and Drug Addiction –EMCDDA-, 2007).

MDMA is almost exclusively sold and consumed orally in the form of tablets (rarely capsules), which frequently contain smart symbols (logos) and are colored, in order to capture the consumers' attention. The amount of MDMA contained in the tables sold as "ecstasy" greatly varies from batch to batch (even among those tablets with the same logo), both between and within countries. Between 2008 and 2010, a change has occurred in the content of illicit drug tablets in Europe, from a situation where most of the tablets analysed contained MDMA (60–70 mg of MDMA) or another ecstasy-like substance (MDA; 3,4-methylenedioxyethylamphetamine; paramethoxyamphetamine; 2,5-dimethoxy-4-bromoamphetamine; 4-methylthioamphetamine) as the only psychoactive substance, to one where the contents are more heterogeneous, and MDMA-like substances are often present in traces, or absent. This shift was most pronounced in 2009, when only three countries reported that MDMA-like substances accounted for a large proportion of the content of the tablets analyzed. However, recent data indicate a rise in the

amount of MDMA contained in tablets. In 2011, average values ranging from 43 mg (Denmark) to 113 mg (Turkey) of MDMA per tablet were reported across Europe; moreover, the number of countries reporting a predominance of tablets containing MDMA-like substances has increased to eleven (EMCDDA, Table PPP-9, Composition of illicit drug tablets, 2011).

1.1 Pharmacokinetics

MDMA, similar to other amphetamine-related drugs, is a weak base ($pK_a = 9.9$) with a low molecular weight, a low protein binding and a high volume of distribution (Capela et al. 2009). Moreover, MDMA is highly lipid soluble: this means that it is able to easily cross the blood brain barrier (BBB) and enter the brain without significant delay.

Numerous studies have indicated that MDMA is well absorbed by the oral route, and reaches its maximum concentration after 1.5-3 hours from its intake, with a plasma half-life around 6-7 hours (de la Torre et al. 2004).

In both humans and rodents, MDMA is metabolized chiefly by the liver (de la Torre et al. 2004). In humans, the major metabolic pathway of MDMA is O-demethylation catalyzed by the hepatic cytochrome P450 (CYP) 2D6 (CYP2D6), which leads to the generation of 3,4-dihydroxymethamphetamine (HHMA). MDMA is also a mechanism-based inhibitor of CYP2D6, a phenomenon that is associated with a decrease in the amount of effective enzyme (O'Mathùna et al. 2008). HHMA is further O-methylated by catechol-O-methyl transferase (COMT), generating 4-hydroxy-3-methoxymethamphetamine (HMMA) (de la Torre et al. 2004). In rats, N-demethylation to MDA predominates (de la Torre et al. 2004). MDA is further metabolized to the catechol intermediate, 3,4-dihydroxyamphetamine (HHA) and finally O-methylated to 4-hydroxy-3-methoxyamphetamine (HMA). HHA and HHMA are the precursors of neurotoxic species.

The metabolism of MDMA in experimental animals is qualitatively similar as for its major metabolic reactions in most animal species, even though quantitative differences exist. In rodents, the conversion to MDA is the main metabolic pathway of MDMA, but sex and strain differences in drug disposition exist, which have been attributed to the polymorphism of CYP isoenzymes (Koenig et al. 2005). In mice acutely administered with high doses of MDMA, the ratio between the parent compound and its metabolites is significantly higher than in rats, in both the plasma and the brain. However, this species difference largely disappeared when low doses of MDMA were tested in mice (Mueller et al. 2013).

With regard to the clearance of MDMA in humans, about 80% of the drug is transformed metabolically through the liver, and the remaining 20% is excreted unaltered in urine in a dose-independent fashion (Capela et al. 2009). It is quite difficult to make direct comparisons between

pharmacokinetics results obtained in humans and the few pharmacokinetics results from animal studies, since large mammals tend to dispose drugs at a lower rate than small mammals. In spite of that, it has been published that the clearance of MDMA and its metabolites was significantly faster in the mouse compared with the rat (Mueller et al. 2013).

1.2 Pharmacology and toxicology

Preclinical data gathered from research performed in laboratory animals, indicate that the systemic administration of MDMA affects the functions of both peripheral and central nervous system (CNS), mainly by acting on monoaminergic systems. Thus, initial studies showed that MDMA and its main metabolite, MDA, stimulate the efflux of 5-hydroxytryptamine (5-HT) (de la Torre et al. 2004) and dopamine (DA) from preloaded synaptosomes (Johnson et al. 1986; Schmidt et al. 1987). Subsequent reports have demonstrated that MDMA binds all the three presynaptic monoamine transporters with differences between species, exhibiting, in rodents, the highest affinity for the 5-hydroxytryptamine transporter (SERT), whilst the affinities for the noradrenaline (NET) and dopamine (DAT) transporters are lower (Rudnick and Wall 1992; Steele et al. 1987). MDMA also enhances the release of acetylcholine both *in vitro* in striatal slices and *in vivo* in prefrontal cortex (PFC) and striatum of rats, an effect that appears to derive from the activation of serotonergic, dopaminergic and/or histaminergic receptors (Gudelsky and Yamamoto 2008). At variance with data obtained in studies concerned with rodent transporters, it has been reported that, in humans, MDMA displays higher affinity for NET and lower, but similar, affinities for SERT and DAT (Verrico et al. 2007).

Once translocated to the cytoplasm, MDMA increases the extracellular levels of 5-HT, DA and noradrenaline (NA) in multiple brain regions (Gudelsky and Yamamoto 2008). MDMA causes the dissipation of the proton gradient between the vesicles and the cytosol that is necessary for the proper functioning of the vesicular monoamine transporter (VMAT₂), also inhibiting VMAT₂-mediated influx and proper storage of 5-HT in the terminal (Rudnick and Wall 1992). This event is boosted by the block of reuptake on presynaptic terminals, and by the inhibition of the monoamine oxidase (MAO) type B (Leonardi and Azmitia 1994). MDMA also blocks the activity of tryptophan hydroxylase (TPH, the rate-limiting enzyme in the synthesis of 5-HT), an effect that occurs as soon as fifteen min after MDMA intake, and persists for up to two weeks (Schmidt and Taylor 1987). Furthermore, MDMA causes a global increase in extracellular 5-HT throughout the brain regions bearing raphe afferents (Rudnick and Wall 1992).

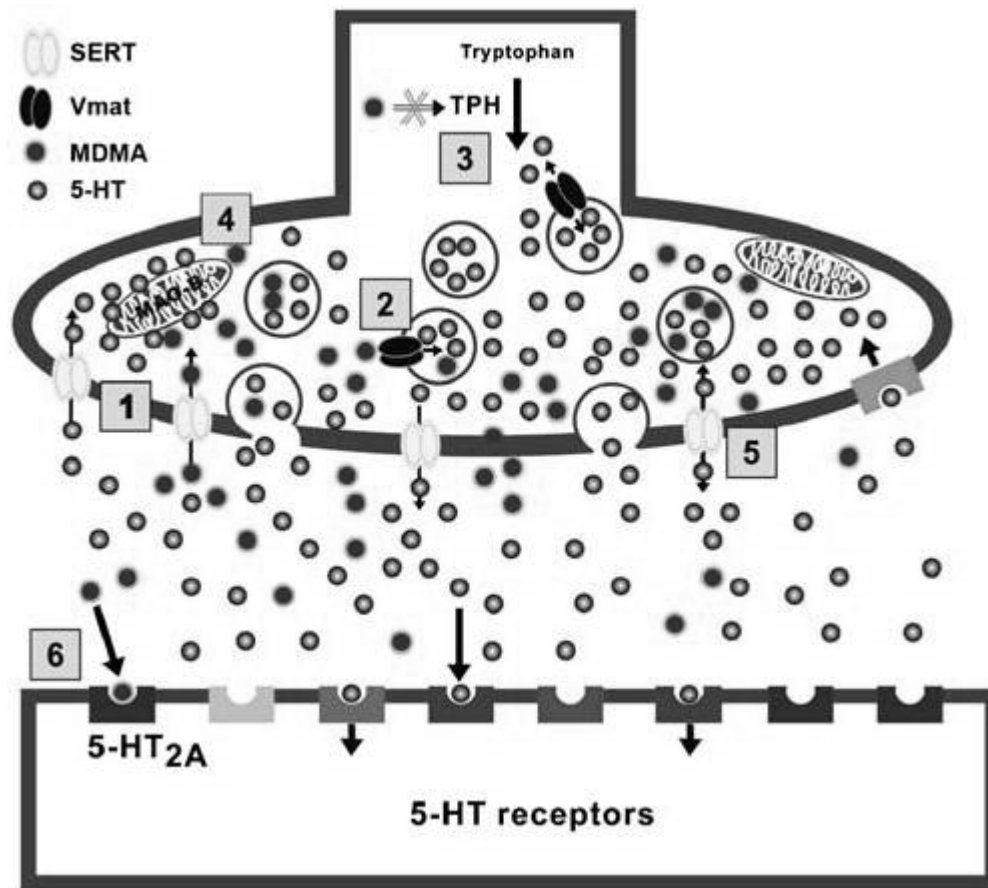


Figure 2. Mechanism of action of MDMA. (1) MDMA is a substrate of SERT by which enters inside the neuronal terminal; (2) once inside, MDMA produces an acute and rapide release of 5-HT from the storage vesicles; (3) MDMA is able to inhibits TPH and (4) MAO type B enzymes, further increasing the levels of free cytoplasmatic 5-HT; (5) MDMA promotes a rapid release of intracellular 5-HT to the neuronal synapse via reversal of the SERT activity; (6) 5-HT acts as agonist on 5-HT_{2A} receptors (Capela et al. 2009).

1.2.1. Toxicology in humans

The appeal of MDMA is due to its special profile of actions, which includes amphetamine-like stimulant effects, coupled with feelings of increased emotional sensitivity and closeness to others. Based on this, Nichols (1986) postulated that MDMA is member of a novel class of compounds, in that it possesses unique effects, which means that MDMA cannot be classified as either a hallucinogen or a psychostimulant. Nichols proposed the term “entactogen” to describe the effects of MDMA and related compounds. MDMA “high” period is characterized mainly by disinhibition in social relations, greater openness of spirit, better acceptance of others (empathy effects), increased esteem and self-confidence, feeling of euphoria, increased vigilance, improvement of mood, and abolition of fatigue.

However, not every MDMA experience is positive, since up to the 25% of users report having had at least one adverse reaction, with prevalence of unpleasant feelings and bodily sensations (Davison and Parrott 1997). However, the possibility exists that the positive effects of MDMA may subside with repeated use, as suggested by Peroutka et al. (1988). Peripheral adverse effects of MDMA in humans include cardiac arrhythmias, hypertension, hyperthermia, hyponatremia, liver complications, seizures, coma, and death (Schifano 2004). Hyperthermia is a very relevant clinical problem in MDMA users, since body temperature elevation produced by the drug may reach up to 43 °C (Capela et al. 2009), and around 85–90% of recreational MDMA users report an increase in body temperature and sweating, accompanied by dehydration (Davison and Parrott 1997). The issue of MDMA-induced hyperthermia is complex, since the biological mechanisms involved in heat production and progression to hyperthermia after exposure to the drug are not clearly understood. It is conceivable that the administration of a large dose of MDMA evokes 5-HT release that activates sufficient 5-HT_{1B} and 5-HT_{2A} receptors within vasculature (Gudelsky et al. 1986) to induce constriction, interfering with the normal thermoregulatory mechanisms of the body (Sprague et al. 2003). In this regard, it is worth to mention the study of Blessing and colleagues (2003) where it is demonstrated that clozapine and olanzapine, atypical antipsychotic agents that act as antagonists respectively at the 5-HT_{1A} and 5-HT₂ receptors, reverse severe and potentially fatal hyperthermia elicited by MDMA acute administration in rats and rabbits. Several reports in humans and in laboratory animals indicate that the rises in body temperature induced by MDMA are strictly influenced by the external environment (Carvalho et al. 2002; Huether et al. 1997), with an important factor for the toxicity of the drug being the social gathering (also called “aggregation toxicity”). Thus, the typical conditions encountered in “raves” and clubs parties, where the music is deafening, room temperatures are high due to crowding, and people usually assume few water and lots of alcohol are crucial to amplify MDMA-induced hyperthermia, and also all the other acute toxic effects of MDMA described above (Green et al. 2003).

Another problem related to the MDMA use is the so-called “serotonin syndrome”, which is caused by drug-induced excess of intersynaptic 5-HT (Gillman 1999; Hall and Henry 2006; Huether et al. 1997). The symptoms include behavioral hyperactivity, mental confusion, agitation, hyperreflexia, hyperpyrexia, tachycardia, shivering, clonus, myoclonus, ocular oscillations, and tremor (Gillman 1999; Hall and Henry 2006; Huether et al. 1997). The “serotonin syndrome” is often conceptualized as an unusual, or atypical, severe adverse MDMA reaction. However, Gillman (1999) argued that this syndrome was neither rare nor idiosyncratic, but represented a continuum of responses from mild to severe. The mild “serotonin syndrome”

(which is limited to three symptoms from above list) generally requires no direct medical intervention. Stronger responses (including four or more symptoms) would often necessitate medical supervision, and while severe reactions (including most of the symptoms from the list) could prove fatal. Nevertheless, in the light of the widespread use of MDMA, fatal intoxications still remain rare events (EMCDDA, Annual report on the State of the Drugs Problem in Europe, 2010). Exposure to MDMA could be regarded as a “chemical stressor” on the immune system, as it induces immunosuppression (Connor 2004; Connor et al. 2005), which may have a significant impact on the health of abusers. These effects may involve alterations of neutrophil phagocytosis, reduction of the production of inflammatory cytokines, suppression of the production of interferon γ (INT- γ), and reduction of the expression of MCH-II molecules at the level of neuronal dendrites and macrophages. Furthermore, MDMA reduces the number of circulating lymphocytes, especially of TCD4+, suppresses the proliferation of the T cell line, and diverts the production of cytokines by favouring the specialization of Th0 into Th2. The immunosuppressive effects of MDMA are likely not a result of a direct action of the substance on immune cells, but rather stem from the release of immunomodulatory endogenous substances (Boyle and Connor 2010).

MDMA can also cause a series of neurobehavioural disturbances that can appear during the first few hours from drug intake, and include depression, mania, psychosis, panic attacks, irritability, hallucinations, insomnia, tiredness, fatigue, and paranoid ideas (Cole and Sumnall 2003; Davison and Parrott 1997; Hall and Henry 2006; McCann et al. 1996). Hallucinations and paranoia can persist for days, or even weeks, after the intake of MDMA. In addition, cases of potentially fatal neurological effects, such as subarachnoid and intracranial hemorrhage and thrombosis, have been reported in MDMA consumers (Green et al. 2003).

In contrast to the acute effects described so far, the evaluation of the long term effects of MDMA, including neurotoxicity, is complex, and these effects should not be underestimated. Adverse neuropsychiatric effects have been described after chronic MDMA use and, most notably, some recreational MDMA users display cognitive deficits (Fox et al. 2001; Parrott and Lasky 1998) that become more marked in heavy drug users (Morgan 2000).

The existence of cognitive dysfunctions contributes to support the evidence of neurotoxicity induced by MDMA in the human brain. Indeed, functional magnetic resonance imaging (fMRI) studies have demonstrated that MDMA may induce serotonergic toxicity in brain areas involved in the regulation of cognition, mood and memory, such as the hippocampus and the PFC. A recent fMRI investigation that involved heavy abstinent MDMA users showed a significantly greater spatial extent of activation than controls in both the primary and secondary visual cortex,

suggesting that MDMA use may be associated with a long-lasting increase in cortical excitability, possibly through the loss of 5-HT input to cortical and subcortical regions (Bauernfeind et al. 2011). Positron emission tomography (PET) studies reported significant reductions in SERT binding compared to control subjects (McCann et al. 1998). In another PET study, Reneman et al. (2001) found an indication that women who heavy abused MDMA might be more susceptible to neurotoxic effects on serotonergic neurons than men who were heavy MDMA users, and that MDMA-induced neurotoxic changes in several brain regions of women who formerly abused MDMA are reversible.

Even though in humans the neurotoxicity of MDMA on the serotonergic system has been suggested by several studies on the one hand, on the other hand the effects of MDMA on dopaminergic neurons are less certain (Tai et al. 2011). In this regard, it is worth mentioning a recent study that analyzed the neurotoxicity of MDMA and its catechol metabolites, α -methyldopamine (α -MeDA) and N-methyl- α -methyldopamine (N-Me- α -MeDA) in human dopaminergic SH-SY5Y cells. In this investigation, α -MeDA and N-Me- α -MeDA were found to be neurotoxic to SH-SY5Y cells, leading to caspase 3-independent cell death in a concentration- and time-dependent manner, whereas MDMA did not show a concentration and time-dependent induction of cell death (Ferreira et al. 2013).

Abuse properties of drugs during adolescence

It must be considered that an increased risk of developing drug abuse and drug-related problems is often associated with the age of the consumers (Hawkins et al. 1992). Adolescents are likely to participate at “raves” and clubs parties, and it is during adolescence that most drug use and abuse patterns are initiated. In both humans and experimental animals, adolescence is a critical period during development (Casey et al. 2000), which is generally associated with the acquisition of mature survival skills that allow independence from parental care. Novelty-seeking, a temperamental/behavioral trait that is typical of this age period, might substantially contribute to both psychological and psychobiological vulnerability. In the light of preclinical studies suggesting that the behavioral responses to psychostimulants are the result of neurobiological adaptations that occur primarily in the mesolimbic dopaminergic system (Pierce and Kumaresan 2006), it is expected that exposure to MDMA during adolescence, as happen with other substances, would produce long-term changes in these systems that perpetuate in adulthood (Chambers et al. 2003; Morley-Fletcher et al. 2002; Sisk and Zehr 2005; Wahlstrom et al. 2009), and that the adverse peripheral reactions observed in adult would be more pronounced (Spear 2000).

Regarding cognitive deficits, little is known of brain functional modifications of MDMA use during adolescence. In the study of Jacobsen and co-workers (2004), adolescent MDMA users displayed significant delays in reaction time during simple, selective and divided attention tasks and abnormal function of the left hippocampus during high working memory load. Moreover, the study of Weinborn and collaborators (2011) support a deficit in prospective memory functioning amongst MDMA young users (18–30 years) for longer (15 min) ongoing task delays, as compared with shorter (2 min) delays.

1.2.2. Toxicology in experimental animals

The most important acute effects of MDMA depend on several factors, such as the dose administered, the age at administration, the species, the environmental temperature, the thermal conductivity of the animal housing, and the hydration status.

Non-human primates

MDMA suppresses locomotor activity in non-human primates following both intramuscular (i.m.) (Von Huben et al. 2007) and oral (Crean et al. 2007) administration. Differently from what observed in rats, the environmental temperature seems not to affect MDMA-induced hyperthermia in non-human primates, as suggested by data in rhesus macaques administered with doses ranging between 0.56 and 2.4 mg/kg that displayed a similar degree of hyperthermia across a wide range of environmental conditions (18 °C to 30 °C) (Von Huben et al. 2007). Nevertheless, MDMA induces acute hyperthermia in unrestrained rhesus monkeys, an effect that is much more pronounced than in rats, mice, pigs, rabbits and humans. Maximum and average temperature in the four hours interval post-dosing was elevated by 0.7–0.9 °C after racemic MDMA and either enantiomer (Taffe et al. 2006).

Non-human primates administered MDMA seem to be very susceptible towards the neurotoxic effects of this substance. A dose-dependent reduction in the 5-HT content in several brain areas has been observed in squirrel monkeys after the subcutaneous (s.c.) administration of MDMA at doses ranging between 2.5 and 5.0 mg/kg (Ricaurte et al. 1988a). In another study, MDMA was administered at the same doses, and significant decreases in cerebrospinal fluid (CSF) levels of 5-hydroxyindoleacetic acid (5-HIAA) and brain 5-HT and 5-HIAA concentrations were observed (Insel et al. 1989). In addition, the long-lasting deficits characteristic of MDMA-induced neurotoxicity were evidenced by the fact that squirrel monkeys presented reduced serotonergic innervations and reduced 5-HT levels seven years following exposure to the drug. The route of drug administration also seems to affect the degree of 5-HT depletion, as oral administration has been reported to be less toxic than s.c. injection. It is noteworthy that repeated MDMA

administrations at a dose of 5 mg/kg have been reported to produce an 86% depletion of frontal cortex 5-HT when given s.c., compared with a 42% depletion elicited by the same dose given orally (Ricaurte et al. 1988b). Furthermore, since a single 5 mg/kg oral dose in non-human primates has been suggested to be equivalent to a 1.4 mg/kg dose in a 70 kg human, based on interspecies dose scaling, these data may indicate a possible risk of serotonergic damage in humans even after a single dose (Ricaurte et al. 1988b).

There are few preclinical studies in non-human primates focused on MDMA ability to induce cognitive deficits. Nevertheless, Taffe and collaborators (2001) have demonstrated that rhesus monkeys, treated with a MDMA regimen able to induce a 50% reduction of 5-HIAA in CSF and persistent reductions of 5-HT content in several neocortical regions and hippocampus, and display overt deficits in a range of cognitive domains, like self-ordered spatial search, five-choice reaction time task, progressive ratio responding, and bimanual motor skill task that do not persist when animals are retested few months later.

Rodents

Administration of MDMA has profound effects not only on the cardiovascular and neuroendocrine systems, but also on the thermoregulatory system and the basal metabolism of rats (Gordon et al. 1991). Several studies have reported that rats housed at room temperature conditions (20–24 °C) display an acute hyperthermic response following administration of MDMA (Colado et al. 1999; Dafters 1994; Mechan et al. 2002; O’Loinsigh et al. 2001). Exposing rats to higher ambient room temperature conditions results in rats having a higher hyperthermic response to MDMA, while exposure to lower ambient temperatures is associated with a hypothermic response to MDMA (Malberg and Seiden 1998).

Another interesting point in MDMA toxicity is that MDMA tablets are often taken, in the “raves” and clubs parties, together with caffeinated beverages or with beverages contaminated with caffeine in varying amounts. Rat studies have shown that caffeine enhances hyperthermic and tachycardic responses induced by MDMA (Vanattou-Saïfoudine et al. 2010).

There is considerable evidence that MDMA administration produces a major release of both 5-HT and DA from their respective terminals in the forebrain (Johnson et al. 1986; Schmidt et al. 1987), and that MDMA-induced hyperthermia may be associated with an increased 5-HT (Shankaran and Gudelsky 1999). Several studies have demonstrated that MDMA causes long-term neurodegeneration in the rat brain, particularly in the neocortex, striatum, thalamus, hippocampus, septum, and amygdala, even though strain differences in this effect exist, with the Dark Agouti strain being more sensitive than other commonly used strains (Baumann et al. 2007; Colado et al. 1995; Logan et al. 1988). The terminal portions of axons have been shown to be

selectively vulnerable to MDMA-induced damage, as indicated by the reduced density of fine, arborized 5-HT axons and sparing of smooth, straight preterminal fibers (Molliver et al. 1990). A very relevant point in MDMA toxicity observed in rats is that this effect appeared particularly marked during adolescence. Thus, adolescent rats, repeatedly treated with MDMA at several time points, displayed a significantly reduced SERT-immunoreactive fibers density in the hippocampus, a deficit in the novel object-recognition task (NOR), an increased impulsivity in the elevated plus-maze, and a reduced sensitivity to a 5-HT_{1A} agonist challenge (Meyer et al. 2008). Paralleling to serotonergic fibers damage, other research groups displayed that astrocyte hypertrophy can occur as a result of neuronal injury after single or repeated MDMA treatment, and can lead to the enhanced expression of glial fibrillary acidic protein (GFAP) (Adori et al. 2006; Aguirre et al. 1999), the major protein of astrocyte intermediate filaments. In addition, it has been shown that acute MDMA treatment induces learning deficits in rats. Adolescent Sprague–Dawley rats treated with MDMA displayed, five days after the last dose, an impairment in NOR and increased open arm exploration in the elevated plus maze (Piper and Meyer 2004). This suggests that MDMA exposure during the adolescence may influence cognitive and affective functioning in the absence of severe serotonergic damage (Piper and Meyer 2004).

Similar to what observed in rats, MDMA administration produces a hyperthermic response in mice. The mice strain, the MDMA dose (Mann et al. 1997) and the housing conditions appear to influence the magnitude and features of the response detected. Fantegrossi and colleagues (2003) have demonstrated that racemic MDMA and the S(+)-MDMA enantiomer were approximately equipotent in terms of their lethal effects across singly housed mice or mice housed in crowded cages (twelve per cage), while the R(-)-MDMA enantiomer was approximately half as potent. MDMA lethality was reversed with the temperature decrease, as a cold environment significantly attenuated the lethal effects of racemic MDMA in singly housed mice, and completely abolished the lethal effects of racemic MDMA and S(+)-MDMA in mice housed in crowded cages. These effects could likely be explained by an inhibition of MDMA metabolism (Mueller et al. 2013).

It is now well established that MDMA produces in mice a neurotoxic profile different from the serotonergic one observed in rats or non-human primates, eliciting a significant dopaminergic neurotoxicity (O’Callaghan and Miller 1994). The basis for the variations in the profile of MDMA neurotoxicity across different species is unknown, but it has recently been suggested that differences in MDMA disposition and CYP metabolism may play a key role (Green et al. 2012). Nevertheless, MDMA can elicit dopaminergic neurotoxicity depending on its dosage (Itzhak et al. 2003).

Several studies have described MDMA-induced cognitive deficits in mice, but very few is known about cognitive deficits elicited by MDMA in adolescent mice (Ros-Simò et al. 2013; Vidal-Infer et al. 2012), indicating that further investigations are necessary to clarify whether neurochemical deficits induced by MDMA during adolescence correlate with behavioral and cognitive abnormalities.

1.2.3 Mechanisms involved in MDMA toxicology

Even though a large number of studies have been carried out to address the mechanisms involved in MDMA-induced neurotoxicity, these remain to be fully elucidated. Several lines of evidence indicate that multiple mechanisms can be involved, and that these mechanisms may differ depending on the specific toxic effect considered.

Hyperthermia

Several *in vivo* studies indicate that hyperthermia plays an important role in MDMA-induced neurotoxicity. As mentioned above, MDMA toxicity to 5-HT terminals during hyperthermic and hypothermic conditions can be enhanced and attenuated, respectively. The mechanism by means of which MDMA interferes with the regulation of body temperature is not fully understood. A study by Mechan et al. (2002) has demonstrated that the temperature of the tail was unaltered in rats following a dose of MDMA that produced a significant rise in rectal temperature. Since vasodilation of tail vessels is a major mechanism by which rats lose temperature (Grant 1963) these results suggest that MDMA could interfere with heat loss mechanisms. In this regard, it is worth mentioning the work by Gordon et al. (1991) who examined modifications in thermoregulatory mechanisms by MDMA by measuring metabolic rate, evaporative water loss and rectal temperature of rats housed at 10°, 20° and 30 °C. MDMA was able to produce a temperature-dependent increase in the metabolic rate and evaporative water loss. Hyperthermia by itself is not able to decrease the striatal levels of DA in rodents (Granado et al. 2011), but might interact with other known mediators of MDMA neurotoxicity, such as increased glutamate (GLU) neurotransmission and ROS production. A role for GLU in this effect is suggested by results showing that rats treated with the neuroprotective NMDA receptor antagonists memantine, MK-801, or CGS 19755 displayed a significantly decreased hyperthermia in response to MDMA (Nisijima et al. 2012). Moreover, it is noteworthy that MDMA-induced hyperthermia is associated with an increase in the formation of ROS and reactive nitrogen species (RNS) in Hep G2 cells (da Silva et al. 2013).

ROS production and oxidative stress

The hypothesis that substituted amphetamines may induce toxicity by inducing ROS production was proposed as early as 1989 by Stone and collaborators, after the observation that MDMA-induced inactivation of TPH was reversed by sulfhydryl-reducing compounds. This hypothesis was later supported by the findings that the neurotoxic effects of MDMA can be attenuated by either ROS scavengers and antioxidants or the over-expression of antioxidant enzymes (Cadet et al. 1994 and 1995a; Jahanthi et al. 1999).

Consistent evidence from several reports supports the idea that ROS are produced from four different pathways.

The first takes into consideration that MDMA elicits DA release both *in vitro* and *in vivo* (Cadoni et al. 2005), which may undergo over auto-enzymatic oxidation, resulting in the production of ROS catalyzed by Fe^{+++} via the Fenton reaction, mainly hydroxyl radical ($HO\bullet$), superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Siraki and O'Brien 2002), cytotoxic quinone (Stokes et al. 1999), and reactive aldehyde intermediates, such as 3,4-dihydroxyphenylacetaldehyde (DOPAL) (Marchitti et al. 2007). The role of elevation in DA release in MDMA-induced neurotoxicity has been substantiated by studies showing that the blockade of DAT (Kanthasamy et al. 2002) protect against neuronal damage induced MDMA.

The second pathway that lead to the production of ROS involve the GLU, that may contribute to MDMA-induced neurotoxic effects in three different ways. In the first, GLU activates N-methyl-D-aspartic acid (NMDA) receptor, leading to the increase in intracellular Ca^{++} levels, an effect that causes the activation of a variety of proteases and kinases and results in the breakdown of cytoskeletal proteins and the formation of ROS (Sattler and Tymianski 2000 and 2001). The second occur via a Ca^{++} -mediated activation of a synthase that produces nitric oxide (NO). Under physiological conditions, both the neuronal form of nitric oxide synthase (nNOS) and inducible form of nitric oxide synthase (iNOS), located in glial cells, produce significant amounts of NO, while levels of the $HO\bullet$ radical are kept in check by the abundance of superoxide dismutase (SOD). In pathological conditions, NO can either react with O_2^- to form peroxynitrite ($ONOO^-$), or with Fe^{++} and Cu^{++} to generate ROS (Lafon-Cazal et al. 1993; Radi et al. 1991). The mechanism through which NO and $ONOO^-$ mediate the toxicity of substituted amphetamines is not completely understood, however, the inhibition of nNOS, and the decrease of $ONOO^-$ levels, attenuate MDMA-induced depletions in striatal 5-HT (Darvesh et al. 2005). Moreover, it has been suggested that NO and $ONOO^-$ may oxidize tyrosine residues in proteins, producing nitro-tyrosine (Stamler and Hausladen 1998). Moreover, as mentioned before, MDMA-induced hyperthermia may interact with GLU, contributing to the neurotoxic effect.

The third pathway involves the demethylated metabolites of MDMA, such as MDA, that can promote the generation of cytotoxic species, as ROS and quinone intermediates.

The last mechanism involve the mitochondrial complex I. It has been demonstrated that MDMA administration determines the inhibition of mitochondrial complex I in the striatum of mouse, leading to ROS overproduction (Puerta et al. 2010)

In addition to these four pathways, the 5-HT released from MDMA may reacts with ROS and RNS, leading to the formation of a 5-HT-toxic metabolite, tryptamine-4,5-dione (T-4,5-D), which rapidly conjugates with glutathione (GSH) and reacts with other –SH-containing groups (Wrona and Dryhurst 2001). The role of elevation in 5-HT release in MDMA-induced neurotoxicity has been substantiated by the finding that blockade of 5-HT uptake by fluoxetine or citalopram facilitates the recovery of TPH activity following MDMA administration (Schmidt and Taylor 1987).

Taken together ROS, produced from the oxidation of DA, from GLU and from MDMA metabolites may contribute to mitochondria inactivation and oxidation of macromolecules such as lipids, DNA, and proteins, leading to neuronal death (Quinton and Yamamoto 2006).

Neuroinflammation

The role of neuroinflammation in the toxicity of amphetamine-related drugs is not as well defined as that of hyperthermia and oxidative stress; nevertheless, its importance in the toxicity of these drugs is highlighted by the fact that only neurotoxic amphetamines produce microglial activation (Thomas et al. 2004).

Microglial cells are the immune cells of the CNS (Kim and de Vellis 2005). They respond to insult with a reaction known as “microglial activation”. Many molecules and conditions can trigger a transformation of resting (or surveying) microglia to activated (alerted or reactive) states, included MDMA. There are few preclinical studies that have investigated the mechanisms by which microglial activation may contribute to MDMA-induced neurotoxicity, and all of them suggest that an increase in the expression of cytokines, such as interleukin-1 beta (IL-1 β), may promote neuroinflammation (Orio et al. 2004; O’Shea et al. 2005). Conversely, activation of microglial cells by MDMA seems not to depend on hyperthermia (Orio et al. 2004).

Astroglial cells hold neurons in place, get nutrients to them, and digest parts of dead neurons. Moreover, astrocytes can generate chemical signals in order to communicate with neurons: once activated, the levels of Ca⁺⁺ in the cytoplasm of astrocytes are increased, and gliotransmitters are released. Gliotransmitters are able to deliver their message in a process very similar to that used by neurotransmitters (Agulhon et al. 2012).

Activation of microglia and astroglia occurs at different stages, and, typically, activated microglia secretes pro-inflammatory cytokines (Allan and Rothwell 2001), which promote astrocytic activation. Among the various cytokines, IL-1 β is a pivotal mediator also in the neurotoxicity induced by activated astrocytes. IL-1 β is fast expressed in these pathological conditions, and determines the up-regulation of other inflammatory cytokines, such as IL-6 and tumour necrosis factor alpha (TNF- α) (Johnstone et al. 1999; Merrill and Benveniste 1996; Smith et al. 2012). MDMA administration to rodents elicits astrocytic hypertrophy in several brain areas, the hallmark of which is an enhanced expression of GFAP, that accompanies the rapid and persistent decline in DA and TH (O'Callaghan and Miller 1994; Granado et al. 2008a). Sharma and Ali (2008) have reported that MDMA administration induces the most massive activation of astrocytes, that is located in the edematous areas of the cortex, and that the magnitude and intensity of GFAP immunoreaction in the brain is more pronounced in mice than in rats.

Several studies have reported a dual effect of MDMA, on both microglial and astroglial activation. A research from Granado and collaborators (2008a) suggested for the first time that MDMA administration induces in mice DA toxicity in SNc and striatum, paired with an increase in neuroinflammation, by showing that MDMA produces a significant decrease in the number of TH-positive neurons in the SNc, and in TH and DAT-immunoreactivity in the striatum. Moreover, a recent study from our laboratory has demonstrated that administration of MDMA to mice, elicits a significant stereoselective activation of CD11b and GFAP immunoreactivity in the striatum, nucleus accumbens, motor cortex, and SNc, and correlates with an increase in body temperature and motility (Frau et al. 2013). The glial response is further potentiated by the co-administration of MDMA with caffeine (Khairnar et al. 2010).

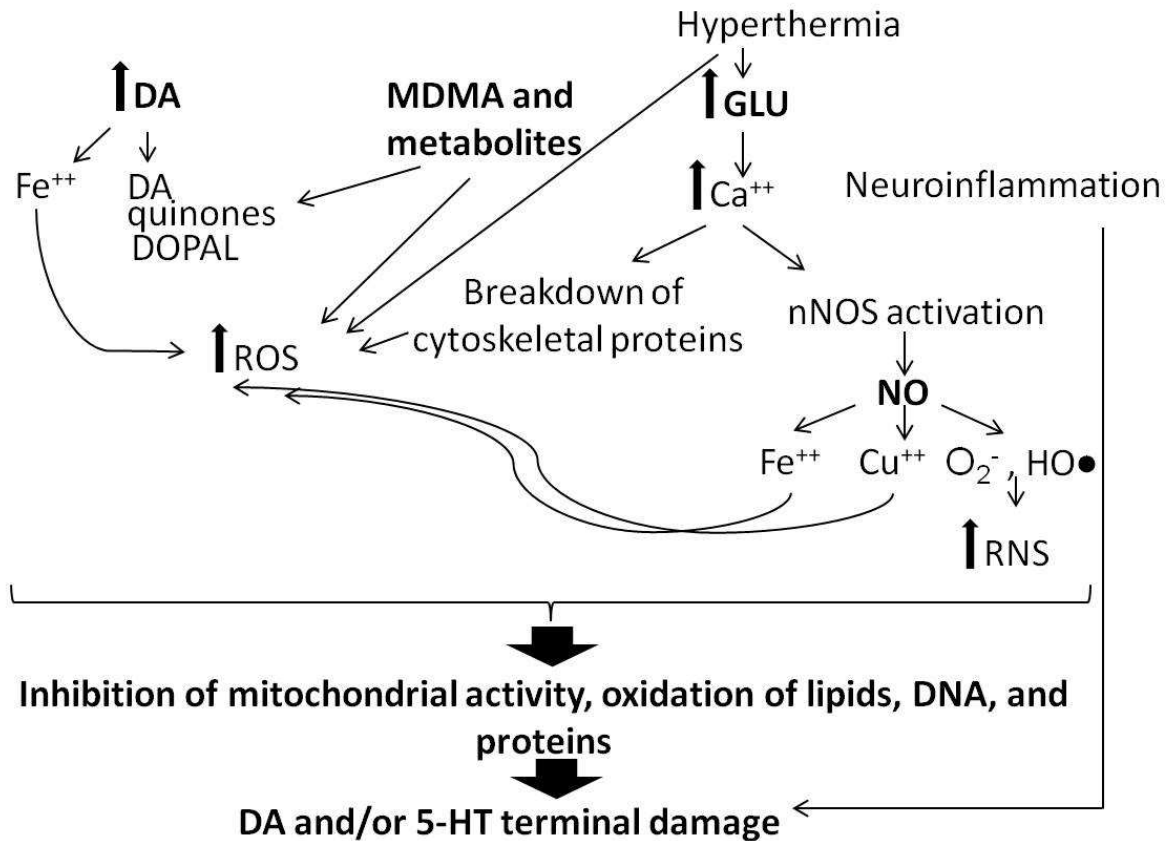


Figure 3. Schematic diagram of the possible causative factors and consequences of MDMA toxicity.

1.3 MDMA and Parkinson's disease (PD)

Parkinson's disease (PD) is a neurological disorder, characterized by the loss of dopaminergic neurons in the SNc (Hornykiewicz and Kish 1986). The etiology of the disease is unknown, but it is very likely that more than one factor, like genetic predisposition, oxidative stress, mitochondrial dysfunction, excitotoxicity, neuroinflammation and exposure to toxins, may play a role in the pathology (Marsden and Olanow 1998). Over the years, several clinical reports have found a higher rate of amphetamine-related drug exposure during the young age in patients diagnosed with neurodegenerative diseases, such as PD, compared with general population (Callaghan et al. 2010; Christine et al. 2010; Parrott et al. 2004). This has led to the hypothesis that amphetamine consumption may be a causative factor of PD, although some criticism has been raised to these studies, as amphetamine consumers are often polydrug abusers (Brust 2010). The study carried out by Garwood et al. (2006) employed a telephone survey conducted on patients with neurodegenerative diseases, including PD, peripheral neuropathy, and amyotrophic lateral sclerosis, who abused amphetamine-like drugs. The results obtained have shown that prolonged exposure to these substances is statistically more frequent in patients with PD than in

patients suffering from other neurodegenerative diseases; moreover, exposure to amphetamine occurred long before diagnosis in a significant percentage of individuals.

The causes of the dopaminergic cell loss in the SNc that underlies PD patients are not clear, but in the last decade solid evidence has linked it to intense neuroinflammation. PET studies revealed microglial activation in pons, basal ganglia including substantia nigra (SN), frontal and temporal cortical regions of individuals with PD, starting early in the disease process without significant longitudinal changes (Gerhard et al. 2006; McGeer et al. 1988; Ouchi et al. 2005). Further supporting a pathogenic role of neuroinflammation, post-mortem samples taken from PD patients have also shown infiltration of CD4+ and CD8+ T cells in the brain (Brochard et al. 2009). However, despite the progress made so far, the fundamental question remains whether immune-associated mechanisms are the main cause of the progressive loss of dopaminergic neurons, or are rather a consequence of the dopaminergic neurons.

Even though the typical symptoms of PD are motor (Fearnley and Lees 1991), non motor symptoms, including cognitive deficits, are increasingly being recognized in PD patients (Foltynie et al. 2004), and no studies have evaluated the influence of the exposure to exogenous substances (including amphetamine-related drugs) early in life on the onset of these symptoms.

1.4 MDMA and the nigrostriatal system in humans and experimental animals

The nigrostriatal system connects the SN to the striatum. It is one of the four DA pathways in the brain, and is usually more envisioned as involved in the production of movement, although fMRI and PET studies indicate that this pathway in the healthy modulates non-motor behavior and cognition (Cropley et al. 2006; Landau et al. 2009).

Despite the continuously increasing number of studies that are focused on MDMA-induced toxicity in the mice nigrostriatal system, few studies have addressed this issue in humans (Tai et al. 2011). As mentioned before, MDMA mostly affects the dopaminergic system in mice, rather than the serotonergic system (O'Callaghan and Miller 1994). MDMA administration depletes DA and its metabolites in several brain regions, but in particular produces long-term degeneration of striatal DA nerve terminals (Brodkin et al. 1993; Colado et al. 2001; Izco et al. 2007). A significant contribute to the elucidation of the effects of MDMA in the nigrostriatal system of mice comes from a recent study by Granado and co-workers (2008a), which shows that not all the dopaminergic systems are affected, because they found no TH or DAT fiber loss in the NAc, indicating that the dopaminergic neurotoxicity of MDMA is selective for the nigrostriatal pathway. In a later study, the same group demonstrated that the decrease in TH levels expression in mice treated with MDMA is accompanied by a reduction in DAT, which is considered an important marker of functional dopaminergic nerve terminals, suggesting that MDMA does not

reduce TH synthesis, but rather damages dopaminergic terminals, with an effect that appears more pronounced in the striosomal compartment than in the matrix (Granado et al. 2008b). Remarkably, a similar pattern of striosomal damage in the striatum has been observed following the administration of dopaminergic neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Iravani et al. 2005), and confirms earlier studies which described dopaminergic terminal loss in the mouse striatum following MDMA administration (Fornai et al. 2004).

1.5 MDMA and the limbic system in humans and experimental animals

The limbic system includes several brain areas such as the limbic cortex, hippocampus, parahippocampal gyrus, limbic midbrain areas, amygdala, anterior thalamic nuclei, fornix, column of fornix, mamillary body, septum pellucidum, habenular commissure, cingulate gyrus, and the olfactory bulbs. The hippocampus, which plays a prominent role in the consolidation of information from short-term memory to long-term memory and spatial navigation (Eichenbaum et al. 1996; Squire 1992), contains two main interlocking parts, where can be identified two major neuronal types: in the Cornu Ammonis the predominant neuronal type has a pyramidal morphology, whereas in the dentate gyrus, the predominant neuronal type is the granular cells. Moreover, in both the two areas, it has been identified tyrosine hydroxylase (TH)-labeled terminals and GABA-containing terminals (Bentivoglio and Morelli 2005; Milner and Bacon 1989).

As mentioned earlier, cognitive deficits are among the typical long-term effects observed in heavy MDMA users. These deficits depend on the effects of MDMA on the hippocampus, seem to be dose-related, and may vary according to complexity of the cognitive task.

Fox and colleagues (2001) have demonstrated that both medium and high MDMA users display cognitive deficits that depend on drug dosage but are not related to the extent of drug use. Previous studies have also associated consumption of MDMA, considered as a whole, with cognitive problems (Bolla et al. 1998; Parrott and Laskey 1998), whilst other have shown that impaired cognitive performance may depend on both the amount of MDMA consumed per session and the duration of MDMA use (Morgan 1999). Moreover, Brown and collaborators (2010) have demonstrated that memory tasks with high complexity reveal more marked deficits in MDMA users than memory tasks with a low complexity. Finally, PET studies have displayed differences in the activation of the hippocampus between MDMA users and healthy controls during working memory tasks (Becker et al. 2013; Daumann 2004 and 2005; Moeller et al. 2004).

Several studies have also described MDMA-induced learning and cognitive deficits in mice. Thus, acute and repeated pretreatment with MDMA modifies the acquisition and execution of an active avoidance task (Trigo et al. 2008). Moreover, repeated administration of MDMA at different doses is associated with deficits in the Morris water maze that persist even long after last MDMA administration (Busceti et al. 2008). Interestingly, the same doses of MDMA that induced spatial learning deficits in the Morris water maze were able to induce tau protein phosphorylation in the hippocampus, a biochemical hallmark of Alzheimer's disease, frontotemporal dementia, and other chronic neurodegenerative disorders characterized by a progressive cognitive decline (Alonso et al. 2001; Goedert 1993).

1.6 MDMA and the PFC in humans and experimental animals

The ascending mesostriatal and mesocortical dopaminergic systems widely modulate the cognitive functions supported by the PFC (Dalley et al. 2004; Ragozzino et al. 1999), a brain area enriched with dopaminergic terminals (Bentivoglio and Morelli 2005), and the striatal structures associated with it (Passingham and Sakai 2004). Thus, a wealth of evidence collected in drug abusers and individuals affected by various diseases, including PD patients (Bowen and Davison 1975; Downes et al. 1989) indicates that an altered dopaminergic function is associated with some of the cognitive impairments typically seen after PFC damage (Manes et al. 2002; Rogers et al. 1999). Moreover, even though most of the dopaminergic afferents to the PFC arise from the ventral tegmental area, some of them originate in the central area of the SNc (Albanese et al. 1986; Maurice et al. 1999; Middleton and Strick 2002) which can be very relevant for the changes in cognitive function associated with PD.

A PET study by McCann et al. (2008) showed that memory performance of recreational MDMA users was inversely associated with SERT binding levels, in the dorso-lateral PFC, orbitofrontal cortex, and parietal cortex. Moreover, a recent PET study has revealed glucose hypometabolism in the PFC and parietal cortex of chronic MDMA and other drugs users, which correlates with verbal learning and recall deficits (Bosch et al. 2013).

Consistent with these data are findings in rodents showing that chronic administration of amphetamine-related drugs can induce enduring reductions in monoamine levels in the striatum and PFC, and that, under at least some schedules of administration, neurotoxic and neuroinflammatory effects can occur (Atkins et al. 2009; Ball and Slane 2012; Ramos et al. 2005).

2. MPTP

In the late 1970s, an analogue of the synthetic opioid meperidine (Demerol®), with the chemical name of 1-methyl-4-phenyl-4-propionpiperidine (MPPP), and with an effect comparable to that induced by heroin, was discovered. A twenty-three years old graduate student, Barry Kidstone, set up a home laboratory to synthesize MPPP, but after four injections of what he thought to be MPPP, he experienced severe bradykinesia (Langston and Ballard 1983). Furthermore, similar to patients with idiopathic PD, he responded to treatment with L-3,4-dihydroxyphenylalanine (L-DOPA) and developed the same complications associated with L-DOPA therapy, including motor fluctuations and dyskinesias (Langston and Ballard 1983). Investigations were then conducted to clarify the etiology of his condition which found that MPPP was contaminated with MPTP, a byproduct of the synthetic reaction, which was later identified as the responsible of the degeneration of dopaminergic neurons in the SNc (Langston et al. 1983).

After this first documented episode, others young drug addicts developed an idiopathic parkinsonian syndrome after intravenous (i.v.) self-administration of MPPP (Ballard et al. 1985). Since its discovery, MPTP has been widely used to create animal models of PD in a variety of species (Jakowec and Petzinger 2004; Kopin 1987; Kurosaki et al. 2004), though the most used species are currently the non-human primates and the mouse. Susceptibility to MPTP varies across species, strain and age of animals (Giovanni et al. 1994a and b). Non-human primates are the species most sensitive and rats the lowest sensitive to MPTP neurotoxicity, whereas mouse strains widely vary in their sensitivity to the toxin, with the C57BL/6J being the most susceptible (Hamre et al. 1999; Sedelis et al. 2000). MPTP neurotoxicity has been found to be strongly age-dependent in all the species (Date et al. 1990; Irwin et al. 1992; Ovidia et al. 1995; Ricaurte et al. 1986). Rats have been generally not used for modeling PD with systemic MPTP administration like in other species, since the dosage required to induce a significant dopaminergic degeneration is associated with a high mortality rate (Giovanni et al. 1994a and b). However, stereotaxic injection of the toxic metabolite 1-methyl-4-phenylpyridinium (MPP⁺) has been used to model PD in rats (Heikkila et al. 1985a).

2.1 Toxicology

2.1.1 Toxicology in humans

Administration of MPTP to humans and experimental animals causes the degeneration of dopaminergic neurons in SNc and the depletion of DA in striatum. The severity of MPTP-induced lesion depends on the regimen and route of administration, and on the species considered.

With the exception of a single report (Ballard et al. 1985), all human cases of MPTP intoxication were caused by one, or few repeated administrations of the toxin (Langston 1987). A neuropathologic study of the brains of three MPTP-exposed addicts has revealed another important similarity with idiopathic PD (Langston et al. 1999), that is the loss of dopaminergic neurons restricted to the SNc, though not accompanied by Lewy bodies. The absence of Lewy bodies may be due to the young age at the onset of MPTP-induced parkinsonism, since age may be an important factor for development of the Lewy bodies (Gibb and Lees 1988). Moreover, PET studies using [^{18}F]-DOPA have revealed that MPTP-intoxicated individuals display a severely reduced DA uptake similar to that of late-stage idiopathic PD (Calne et al. 1985; Snow et al. 2000; Vingerhoets et al. 1994). Finally, the depletion of nigral dopaminergic neurons was found to be consistently present together with gliosis and clustering of microglia around nerve cells (Langston et al. 1999).

2.1.2 Toxicology in experimental animals

Non-human primates

Non-human primate models closely resemble the behavioral and neuroanatomical features of human PD and this species may be useful for exploring the neurological and pathological mechanisms of the disease (Fox and Brotchie 2010). Moreover, and similar to humans, non-human primates are susceptible to doses of MPTP lower than those that cause a nigrostriatal lesion in other species (Johannessen et al. 1985). Similar to humans, PET studies in cynomolgus and rhesus monkeys treated with MPTP have revealed a severe reduction in DA uptake (Pate et al. 1993). Thus, Moratalla and co-workers (1992), have reported that MPTP treatment in squirrel monkeys induces a higher loss of dopaminergic uptake-site binding in the putamen than in the caudatus, especially posteriorly. Moreover, Iravani and collaborators (2005) showed that subacute MPTP treatment is associated with a greater damage in striosomes than in matrix within the caudate nucleus. Administration of MPTP to non-human primates causes several parkinsonian-like symptoms, including bradykinesia, postural instability, freezing, stooped posture, and rigidity (Porrás et al. 2012). These symptoms may be also accompanied by cognitive impairment manifested in several tasks and reflecting a general impairment of attentional and executive functions (Decamp et al. 2004; Decamp and Schneider 2004 and 2006; Schneider and Kovelowski 1990; Schneider and Roeltgen 1993). Interestingly, MPTP-treated primates also suffer a dramatic disruption of sleep–wake architecture, with reduced sleep efficacy that persisted years after MPTP administration (Porrás et al. 2012), reminiscent of what observed in idiopathic PD patients (Comella 2008). Similar to idiopathic PD patients, MPTP-

lesioned non-human primates respond to anti-parkinsonian therapies such as L-DOPA and DA receptor agonists. The degree of clinical response to L-DOPA is dependent on the severity of the lesion and parkinsonian state.

Mice

To overcome the economic and moral limitations of experiments involving MPTP use in non-human primates, a MPTP model has been developed in mice. The C57BL/6J strain, in particular, is very susceptible to MPTP neurotoxicity, making an excellent conventional preclinical model for PD. In general, MPTP is administered to mice in either an acute or subchronic regimen (Heikkila et al. 1984; Sonsalla and Heikkila 1986). In these models, MPTP can produce death of dopaminergic neurons in SNc by at least the 40% in C57BL/6J mice and significant depletions in the striatal level of DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), along with a reduction in the striatal synaptosomal DA uptake (Ricaurte et al. 1986). However, if the survival time of mice is extended, the neurotoxic effects of MPTP may be reversible (Hallman et al. 1985). MPTP administration in mice induce a neuroinflammatory effect in the SNc, striatum (Czlonkowska et al. 1996; Kohutnicka et al. 1998; Kurkowska-Jastrzebska et al. 1999; Kurosaki et al. 2003), and hippocampus (Luellen et al. 2003). Bradykinesia, akinesia, altered balance and other motor features can be observed in MPTP-treated mice through various behavioral analyses (Fleming et al. 2013; Sedelis et al. 2001; Tillerson et al. 2002) although, despite the evidence of DA reductions, mice that receive MPTP acutely do not always exhibit motor dysfunctions or motor abnormalities (Heikkila et al. 1989; Meredith and Rademacher 2011). Whole-body tremor and postural abnormalities also have been reported, but chiefly in the first day after lesioning (Sedelis et al. 2001). Cognitive deficits after MPTP exposure in mice parallel the findings in non-human primates, even though with a much less effect (Tanila et al. 1998). In general, all these behavioral alterations tend to be highly variable, with some mice developing severe deficits whereas others exhibit little or no behavioral changes (Sedelis et al. 2001). This variability may be due to a number of factors, including the dose of MPTP, the mouse strain, the test sensitivity, and the period elapsed between lesioning and evaluation, when recovery may occur.

2.2 Pharmacological and toxicological mechanisms

The mechanism of MPTP toxicity is quite similar among humans, non-human primates and mice. MPTP is a highly lipophilic protoxin which rapidly crosses the BBB after systemic administration. Subsequently, MPTP is bioactivated by MAO type B enzymes to the unstable intermediate 1-methyl-4-phenyl-2,3-dihydropyridium (MPDP⁺), exclusively in non-

dopaminergic cells, especially in astrocytes (Ekblom et al. 1993). MPDP⁺ spontaneously oxidizes to MPP⁺ at least *in vitro* (Chiba et al. 1985; Fritz et al. 1985), whereas it is not clear if this reaction may occur *in vivo*. Another mechanism for MPDP⁺ oxidation to MPP⁺ involves HO• radicals (Castagnoli et al. 1985), which appears in line with the evidence showing that transgenic mice expressing high levels of superoxide dismutase are resistant to MPTP (Przedborski et al. 1992), and that dopaminergic neurons are very susceptible to the toxin. Once generated, MPP⁺ is released into the extracellular space by a mechanism that is still unknown. MPP⁺ is not able to enter dopaminergic neurons freely, and its uptake depends on active plasma membrane carrier systems, such as the DAT, for which MPP⁺ bears a high affinity (Chiba et al. 1985; Heikkila et al. 1985b). Interestingly, DAT is an absolute requirement for MPTP-induced neurotoxicity, since this effect is abolished in transgenic mice lacking this transporter (Bezard et al. 1999; Gainetdinov et al. 1997).

Once inside the cell, MPP⁺ can either be sequestered into synaptic vesicles via VMAT₂ (Del Zompo et al. 1993; Wimalasena et al. 2008) or enter into the mitochondria where it interferes with the mitochondrial complex I (Mizuno et al. 1987; Richardson et al. 2007). Both of these steps have been implicated in processes that either protect or kill the dopaminergic neurons. Staal and co-workers (2000) have demonstrated that rat striatal vesicles have a higher density of VMAT₂ and a greater ability to sequester MPP⁺, compared with those isolated from mice, thus justifying why rats are less sensitive to MPP⁺-induced toxicity. The primary reason for the neuroprotective effect of vesicular MPP⁺ sequestration is that less MPP⁺ can be accumulated into mitochondria, thus reducing mitochondrial damage.

Regarding the metabolism of MPTP, three major specie-related differences have been identified: 1) non-human primates, but not rodents, show a persistently high concentration of MPTP metabolites in the striatum, compared to other brain regions; 2) the rodent brain clears MPTP and its metabolites much more rapidly than what observed in non-human primates; 3) the predominant metabolite retained by the non-human primates brain is MPP⁺, while MPP⁺ cannot be detected in rodent brain for more than a few hours after injection (Johannessen et al. 1985). The presence of CYP2D6 in human and nonhuman primates and of the similar isoform CYP2D1 in rat brain indicates that this subfamily of CYP enzymes may be responsible for MPTP metabolism (Herraiz et al. 2006; Mann and Tyndale 2010). The persistence of MPP⁺ in the non-human primate brain may explain the heightened toxicity of MPTP in this species. The principal route of elimination of MPTP is through the urine (Lau et al. 1988).

The ability to interfere with mitochondrial respiration at the level of complex I is a key mechanism in the toxic effects of MPP⁺ (Nicklas et al. 1985; Suzuki et al. 1990). Importantly, the

cytotoxic effects of MPP⁺ are more marked in cells that are particularly sensitive to a deficiency in aerobic energy metabolism, a condition that applies to dopaminergic neurons, which have been found to be more vulnerable to inhibition of oxidative phosphorylation than other types of neurons (Marey-Semper et al. 1993 and 1995). However, the alterations in energy metabolism and generation of ROS peak within hours from MPTP administration, long before the occurrence of overt neuronal damage (Jackson-Lewis et al. 1995). Therefore, these events appear not to be a primary cause of neuronal death, but to initiate a cascade of events that eventually kill dopaminergic neurons (Gibson et al. 2010; Serra et al. 2002). One controversial aspect is whether MPTP induces neuronal death via apoptosis or necrosis. Przedborski and Jackson-Lewis (1998) have proposed that subchronic administration of MPTP is most likely to cause apoptosis, whereas necrotic cell death would be triggered by acute dosing. In this regard, it has however to be definitively clarified if during neurodegenerative conditions, such as PD, neurons degenerate by apoptosis or necrosis (Anglade et al. 1997; Kösel et al. 1997).

Further contributions to clarify the mechanisms of MPTP-mediated neurotoxicity apparently come from NO (Przedborski et al. 1996), the induction of GLU-mediated excitotoxicity, and the stimulation of neuroinflammation. Some studies have also suggested that MPP⁺ may act independently of inhibition of mitochondrial complex I (Choi et al. 2008; Gerlach et al. 1996).

Decline in ATP production and increase in ROS levels

As mentioned before, MPP⁺ blocks mitochondrial complex I (Nicklas et al. 1985; Suzuki et al. 1990), leading to a decline in adenosine triphosphate (ATP) levels. There is evidence of a good correlation between MPP⁺ concentrations, decline in ATP synthesis and cell death in hepatoma cells (Sandy et al. 1988). Furthermore, MPP⁺ has been shown to affect complex III (Mizuno et al. 1988), which leads to an increased production of ROS, HO● and O₂⁻ in particular.

In vitro experiments have shown that the interaction between MPP⁺ and the complex I may also induce the formation of ROS, and in particular HO● and O₂⁻ (Cleeter et al. 1992; Sriram et al. 1997). Interestingly, investigations in humans have found a decrease in complex I activity in various diseases, including PD (Lin and Beal 2006; Mizuno et al. 1989). Thus, low activity of complex I may translate to increased production of ROS, a depletion of SOD, and eventually an increased oxidative stress within the DA neuron. This idea is supported by experiments showing that, as long as sufficient stores of SOD are present, mice are protected against the effects of MPTP (Klivenyi et al. 1998). Moreover, neuronal stress could arise from a perturbation of DA homeostasis, as MPTP-induced DA release from cytoplasmic vesicular storage may trigger the formation of oxidizing metabolites and by-products, such as DA quinones and ROS (Graham 1978). In line with this, protection of neuronal damage by MPP⁺ has been achieved in cell

cultures treated with antioxidants (Perry et al. 1985), and resistance to MPP⁺ neurotoxicity has been demonstrated in SOD transgenic mice (Cadet et al. 1995; Przedborski et al. 1992).

Nitration and oxidative stress

Immunohistochemical studies (Dehmer et al. 2000; Liberatore et al. 1999) show that iNOS up-regulation occurs in microglia 24 hours after the administration of MPTP, an effect that lead to an increase in the production of NO and, subsequently, of OONO-. Both NO and OONO- may interfere with mitochondrial function (Cleeter et al. 1994; Radi et al. 1994), thus adding to the toxic effect of MPP⁺ on cellular energy supplies. Evidence for a role of NO in these effects comes from studies showing that inhibition of NOS can attenuate MPTP neurotoxicity in non-human primates (Hantraye et al. 1996) and mice (Przedborski et al. 1996; Smith et al. 1994).

An excitotoxic mechanism for MPTP neurotoxicity has also been proposed, based initially on the observation that intrastriatal administration of MPP⁺ to rats can lead to a marked increase in extracellular GLU and aspartate levels (Carboni et al. 1990). Subsequently, the neuroprotective effects of excitatory amino acid receptor antagonists have been described (Turski et al. 1991; Zuddas et al. 1992). Furthermore, overstimulation of GLU receptors can alter local Ca⁺⁺ homeostasis, and Ca⁺⁺ is known to up-regulate enzymes like phospholipase A₂, nNOS and xanthine oxidase, all of which are found in mitochondria and can elevate ROS production, eventually promoting mitochondrial dysfunction.

Neuroinflammation

The idea that glia may participate in MPTP-induced neurotoxicity is supported by findings showing that toxins activate microglia and promote the production of toxic compounds, and that astroglia is the major site where MPTP is metabolized to MPP⁺.

After the discovery of the presence of gliosis in post-mortem brains from MPTP-lesioned addicts, similar findings were later observed in the SNc of non-human primates (Barcia et al. 2004; McGeer et al. 2003) and mice (Czlonkowska et al. 1996; Kohutnicka et al. 1998; Kurkowska-Jastrzebska et al. 1999). The inflammation discovered in humans and experimental animals exposed to MPTP may be related to activated microglial cells, that express inflammatory mediators such NO and cytokines (IL-1 β and IL-6), as well as ROS, TNF- α , GLU, and arachidonic acid metabolites, which are toxic to dopaminergic neurons (Hanisch 2002). In line with this, an increase in both glial cells expressing iNOS and levels of proteins that contain nitrotyrosine have been detected in autaptic samples from the SNc of patients with idiopathic PD (Hunot et al. 1996). In this regard, Hirsch and Hunot (2000) suggested that MPTP acts directly on the induction of cytokines that activate iNOS, although elevation in striatal IL-1 β and IL-6

content seems to differ between idiopathic PD and MPTP mouse model of PD. The study of Mogi and colleagues (1994) in the caudate nucleus, putamen and cerebral cortex of idiopathic PD patients reported an increase in both IL-1 β and IL-6, compared with control subjects, but other studies in mice reported an increase of striatal IL-6 levels, and no increase in IL-1 β levels (Kaku et al. 1999; Luo et al. 2004). Further supporting a role of activated microglia in MPTP-induced neurotoxicity in mice, Liberatore and colleagues (1999) have shown that microglial cells not only increase in number after MPTP injection, but also can flood dopaminergic neurons with large amounts of NO and OONO-.

In contrast to the possible deleterious role of microglia, a neuroprotective role for activated astrocytes has been suggested in idiopathic PD as well as in MPTP-induced experimental PD. Astrocytes may reduce the cellular stress by metabolizing DA, since they express MAO type B and COMT (Ekblom et al. 1993; Huang et al. 2005). Additionally, astrocytes are capable to prevent NO-generated neurotoxicity by a GSH-dependent mechanism (Chen et al. 2004).

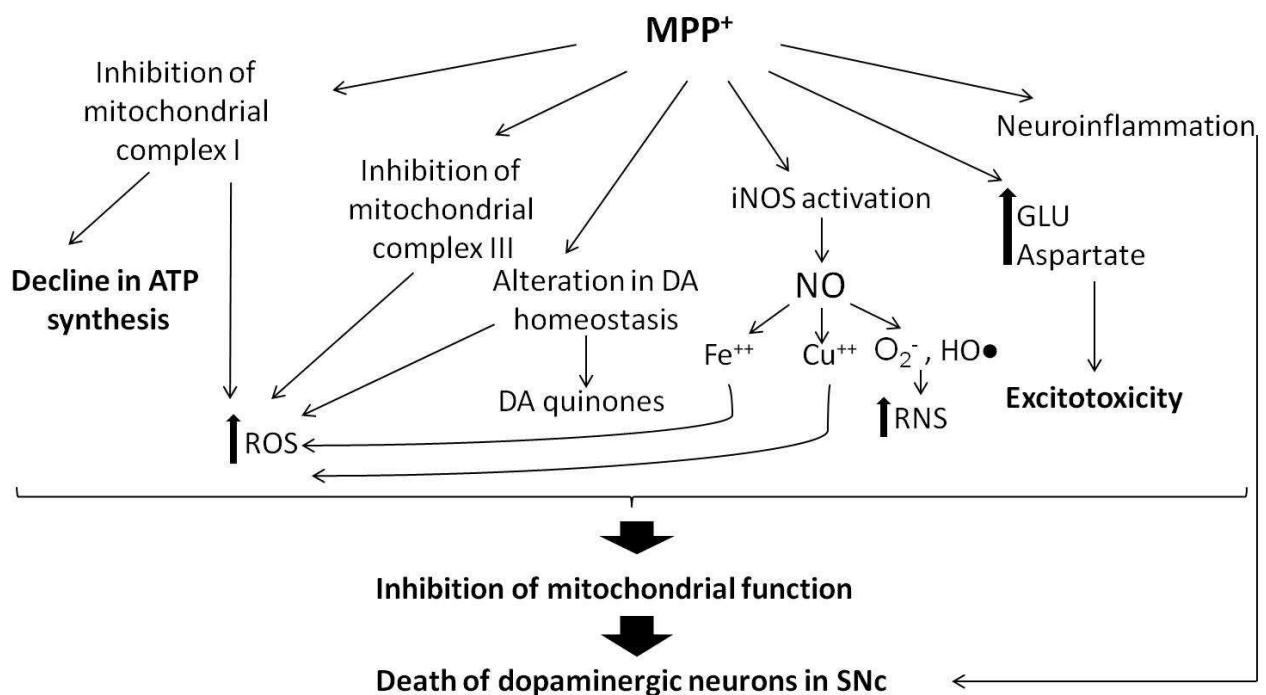


Figure 4. Mechanism of MPTP toxicity.

2.3 MPTP and the limbic system in humans and mice

Cognitive impairment is one of the most common and important non-motor aspects of PD, and varies from mild deficits to severe dementia (Dubois and Pillon 1997; Lees and Smith 1983; Owen et al. 1992; Svenningsson et al. 2012). Mild cognitive impairment is most common in the early stages of the disease, as up to a third of patients may have cognitive deficits at the time of

their diagnosis (Foltynie et al. 2004). Nigrostriatal dysfunction alone is probably not sufficient for the development of cognitive deficits in PD patients, and it is interesting to remark that activated microglia have been observed not only in the SNc and putamen, where DA loss is prominent, but also in the hippocampus of PD patients (McGeer et al. 1988 and 2001; McGeer and McGeer 2004). As mentioned earlier, activated microglia produce a variety of inflammatory cytokines (Allan and Rothwell 2001), which have been implicated in cognitive deficits in PD (Menza et al. 2010; Wilson et al. 2002). In addition, supporting a link between IL and cognitive performance, it is noteworthy that IL-2 immunotherapy of cancer patients is associated with pronounced cognitive disturbances (Caraceni et al. 1993; Denicoff et al. 1987), particularly in tests involving spatial learning and memory (Caraceni et al. 1993).

Data suggesting a link between neuroinflammation and cognitive deficits have also been obtained in mice, where MPTP treatment induces dopaminergic neurons loss in the SNc paired with a great inflammatory response (Frau et al. 2011; Miller et al. 2011) and cognitive deficits (Dluzen and Kreutzberg 1993; Deguil et al. 2010; Tanila et al. 1998). The increase of IL-6 in areas such as the hippocampus and hypothalamus induced by MPTP may be related to parkinsonian-like cognitive deficits (Wang et al. 2009), however, may be not the only responsible. Since the mechanism of cognitive deficits in PD has not been elucidated yet, the degree of striatal DA loss, the extent of neuroinflammation, as well as modifications in other brain areas related to cognitive processes may all potentially be involved and combine to elicit cognitive deficits observed in MPTP-treated mice.

2.4 MPTP and the PFC in humans and experimental animals

In idiopathic PD patients, abnormal cortical metabolic activity occurs in both the motor and non-motor regions (Eidelberg et al. 1994; Huang et al. 2007; Ridding et al. 1995). An hypothesis is that such changes could index the non-motor symptoms of PD thought to precede the more classic motor manifestations, such as depression, cognitive deficits and apathy (Cooper et al. 1991; Shen et al. 2013), and which involve brain areas, including the PFC, where was found a reduced DA content following treatment with dopaminergic neurotoxicant (Tadaiesky et al. 2008). Supporting a role of PFC in MPTP-induced cognitive deficits in experimental animals, a study by Storvik and co-workers (2010) has shown that specific transcriptomic changes occur in the PFC of parkinsonian MPTP-treated macaques, and that a large part of these changes may also be observed in human post-mortem samples from patients with neurodegenerative diseases, such as PD and Alzheimer disease, analysed by quantitative PCR. Similar results were obtained with a single intranasal infusion of MPTP in rats and mice, which produces progressive signs of PD

such as impairments in olfactory, cognitive, and motor functions, associated with a decrease in the levels of DA in the olfactory bulb, striatum, and PFC (Prediger et al. 2010).

Aims of the study

On the basis of epidemiological studies showing a higher incidence of PD in amphetamine-related drugs consumers, the present study evaluated in mice treated as adolescent with MDMA, and administered after MDMA washout with MPTP, the neuroinflammatory and neurotoxic effects in motor brain areas and the neuroinflammatory effects in non-motor brain areas. Moreover, cognitive deficits were evaluated by memory tasks.

The first aim of this study was to assess whether administration of MDMA to late adolescent mice may facilitate the neuroinflammatory and neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), in the nigrostriatal system of adult mice. Studies on the effects of amphetamine-related drugs in an experimental model of PD may, in fact, not only help to understand whether there is an increased risk of developing PD associated with the use of these substances during adolescence, but also clarify some of the mechanisms that promote the degeneration of DA neurons.

To pursue this first aim, the following experiments were carried out:

- Assessment of inflammatory response in SNc and striatum studied through immunohistochemical analysis of GFAP and CD11b, as markers of astroglia and microglia.
- Assessment of dopaminergic neuronal damage in SNc and striatum studied by TH immunohistochemistry.

The second aim of this study was to elucidate whether MDMA may amplify the neuroinflammatory effect elicited by MPTP in the hippocampus and in the mPFC. In the attempt to link neurochemical changes with behavioral modifications, this study investigated whether cognitive impairment took place in mice treated with MDMA, MPTP, and their combination.

To pursue this second aim, the following experiments were carried out:

- Assessment of inflammatory response in hippocampus and mPFC studied through immunohistochemical analysis of GFAP and CD11b.
- Assessment of NOR task and spontaneous alternation behavior in a Y-maze, which were performed both throughout the MDMA treatment and after MPTP administration.

Materials and methods

4.1 Animals

Male C57BL/6J mice (Charles River, Calco, Italy) weighing 20–23 g at the beginning of the experiments, were used. Mice were maintained at constant temperature ($21\pm 1^{\circ}\text{C}$) under a 12-h light/dark cycle with food and water ad libitum. Experimental procedures were approved by the Ethical Committee of the University of Cagliari, in compliance with Italian guidelines for care and use of experimental animals (DL 116/92) and European Communities Council Directive (2010/63/EEC). Efforts were made to minimize the number of animals used and maximize humane treatment.

4.2 Drugs

MDMA was synthesized at the Department of Environmental and Life Sciences of the University of Cagliari (Frau et al. 2013). MPTP was purchased from Santa Cruz Biotechnology, CA, USA. MDMA was dissolved in saline, whereas MPTP was dissolved in distilled water. Both drugs were administered intraperitoneally (i.p.) in a volume of 10 ml/kg.

4.3 Treatment

Late adolescent mice (8 weeks old) were treated with either MDMA (10 mg/kg i.p.) or vehicle according to a 9-week administration schedule (Fig. 5). In detail, each mouse received two administrations a day, separated by a 4–6-hour interval, twice a week, on the second and fifth day of each week, for a total of 36 MDMA, or vehicle, administrations. Experimental groups were composed as follows: MDMA-treated mice, $n=18-19$; vehicle-treated mice $n=17-18$. During MDMA treatment, mice performance in the NOR was evaluated at the following time points: week 1, week 4, and week 9 and then re-assessed two weeks after MDMA discontinuation. NOR performance was always measured on the sixth day of the week. The same mice were also evaluated for spontaneous alternation behavior in a Y-maze, 24 h after NOR (seventh day of the week) at the same time points indicated above. Two weeks after MDMA discontinuation, mice (19 weeks old) were distributed into four groups and challenged with either MPTP (20 mg/kg, i.p.) or vehicle, once a day for 4 consecutive days. Experimental groups were composed as follows: vehicle+vehicle, vehicle+MPTP, MDMA+vehicle, MDMA+MPTP ($n=5-10$). Mice were evaluated again for their NOR performance and spontaneous alternation in a Y-maze, respectively after 24 or 48 hours from the last MPTP or vehicle injection. The day after the completion of behavioral experiments, mice were anesthetized with chloral hydrate, transcardially perfused with 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4), and their brains were removed and processed for immunohistochemistry.

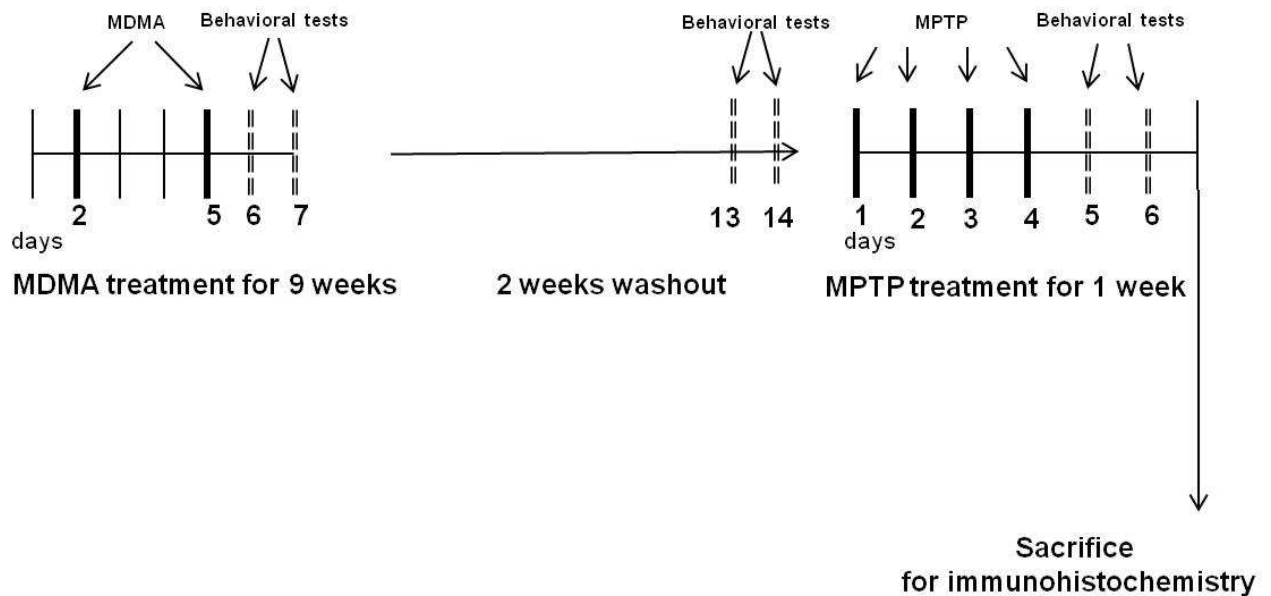


Figure 5. Experimental design.

4.4 Immunohistochemistry

For each mouse, three sections from: SNc (anterior–posterior: -2.92 mm to -3.52 mm from bregma); striatum (anterior–posterior: 1.34 mm to 0.74 mm from bregma); hippocampus (anterior–posterior: 1.46 mm to 1.82 mm from bregma) and mPFC (anterior–posterior: 2.22 mm to 1.78 mm from bregma), according to the atlas of Paxinos and Franklin (2008), were considered for each analysis.

Coronal brain sections (50 μm thick) were cut on a vibratome. Free-floating sections of SNc, striatum, hippocampus and mPFC were incubated overnight with CD11b, GFAP or TH antibodies (monoclonal rat anti-CD11b, 1:1000, Serotec, Oxford, UK; monoclonal mouse anti-GFAP, 1:400, Sigma, Milan, Italy; polyclonal rabbit anti-TH, 1:1000, Millipore, Milan, Italy). After incubation with primary antibodies, slices were incubate in proper biotinylated secondary antibodies (Vector, Peterborough, UK), and avidin-peroxidase protocol (ABC, Vector, Peterborough, UK) was applied for visualization, using 3,3'-diaminobenzidine (Sigma, Milan, Italy) as a chromogen. Sections were then mounted onto gelatine-coated slides to allow microscopy evaluation.

Analysis of CD11b immunoreactivity

Two portions of striatum (dorsolateral and ventromedial), two of SNc (lateral and medial), Cornu Ammonis regions of hippocampus (CA1, CA2 and CA3), and the whole prelimbic/infralimbic

area of mPFC were analyzed for both hemispheres. Images were digitized with a video camera (Pixelink® PL-A686, Ottawa, Canada), captured at $\times 20$ real magnification in gray scale, and then evaluated with the Scion Image analysis software (Scion Corp. Frederick, MD, USA). A threshold, set above the mean value \pm S.E.M. of the background, was applied for background correction. The area occupied by values above the threshold was automatically calculated inside each frame. No significant differences in CD11b levels were obtained between the two portions of SNc, the two portions of striatum or the three regions of the hippocampus, therefore the data obtained were pooled together. For each level of these areas, the obtained value was first normalized with respect to vehicle, and values from different levels were averaged thereafter.

Analysis of GFAP immunoreactivity

For the striatum, the hippocampus and the mPFC, GFAP analysis was performed similarly to what described for CD11b, whereas, for the SNc, the whole left and right area were analyzed. Images were captured and evaluated with the Pixelink® software. Absolute number of GFAP-positive cells was obtained separately for each level within each brain area, and no significant differences in the number of GFAP-positive cells were observed among the two hemispheres of SNc, the two portions of striatum or among the three regions of the hippocampus, therefore the data obtained were pooled together. Thereafter, the number of cells \times level from each mouse was normalized with respect to vehicle, and individual values from the levels were averaged to generate a mean value.

Stereological counting of TH-positive neurons

The total number (N) and density (D) of TH-positive neurons in SNc were measured stereologically on both hemispheres using a microscope (Leica, DMLB, Ballerup, Denmark) equipped with a projecting camera (Basler Vision Technologies, Ahrensburg, Germany) and a microscope stage connected to an xyz stepper (PRIOR ProScan) controlled by a computer equipped with new CAST Visiopharm software (Denmark).

Systemic uniform, random sampling was used to choose sections for analysis. The SNc region was carefully outlined under lower magnification ($\times 5$) according to the atlas Paxinos and Franklin (2008).

The number of labelled neurons was calculated under $\times 63$ magnification using randomized meander sampling and optical dissector methods. The cut thickness of sections was 50 μm and the optical dissector height was 12 μm . The sampling area covered 20% of the region of interest. The counting frame (8183, 4 μm^2) applied the exclusion and inclusion lines. In each sampled

area, the dissector position was adjusted. For further details see Frau et al. (2011) and Sterio (1984).

Analysis of TH-positive fibers

Images were digitized in gray scale, then TH-immunoreacted striata were captured at 20× real magnification in gray scale and evaluated using the image analysis software Scion Image. The average gray values from white matter were subtracted from each section to correct for background immunoreactivity.

For each level of striatum, the obtained value was first normalized with respect to vehicle, and values from different levels were averaged thereafter.

4.5 Memory tasks

NOR task

Evaluation of NOR performance is widely used for assessing non-spatial working memory in rodents (Ennaceur and Delacour 1988). NOR experiments were performed in a Plexiglas cage (length 25.5 cm, width 19 cm, height 14 cm) with the floor covered with sawdust. Objects to be discriminated were made of plastic, and differed as to their shape and color. Moreover, objects had no genuine significance, and had not been previously associated with either rewarding or aversive stimuli. Experimental procedure consisted of three phases: habituation (S0), acquisition (S1), and testing (S2). Habituation to the test cage (single 5 min trial) took place on the fifth day of the week during MDMA treatment, and on the fourth day during MPTP treatment, 6 hours after drug administration. Acquisition was performed the day after S0, by placing each mouse in the test cage together with two copies of the same object (familiar objects). Mice were left to freely explore the objects for 3 min. Testing phase took place 60 min after S1. Mice were exposed to a copy of the objects already presented in S1, plus a copy of an object that they had never experienced before (novel object).

Objects exploration was defined as the mouse sniffing, gnawing or touching the objects with the nose, whereas sitting on and/or turning around the objects were not considered exploratory behaviors. To avoid the presence of olfactory cues, objects were thoroughly cleaned after each session. Moreover, the combination of objects (novel vs. familiar) and their respective locations in the cage (right vs. left) were counterbalanced, to prevent biased preferences for particular objects and/or locations. Mice performance was videotaped, and the following parameters were evaluated: total amount of time spent by each mouse in exploring the objects during S1 and S2,

and percentage of time spent exploring the novel object over the total amount of time spent in exploring both objects (novel and familiar) during S2 (Sawyer et al. 2012; Simola et al. 2008).

Spontaneous alternation behavior in a Y-maze

Evaluation of spontaneous alternation behavior in a Y-maze is widely utilized to investigate spatial short-term memory and general cognitive function in rodents (Hughes 2004; Maurice et al. 1994; Yamada et al, 1996). The apparatus used was made of black PVC, and had three equal arms (length 40 cm, width 11 cm, height 20.5 cm). Arms converged onto a central triangular area, and the maze had his floor covered with sawdust. To avoid the presence of olfactory cues, the maze was thoroughly cleaned and the sawdust was replaced in between each trial. Mice were individually placed in the central area, and left free to explore the whole apparatus for a single 8 min trial, during which their performance was videotaped. Percentage of spontaneous alternation was calculated based on the sequence of arm entries, as reported elsewhere (Simola et al. 2008; Yamada et al. 1996).

4.6 Statistics

Statistical analysis was performed with Statistica for Windows (StatSoft, Tulsa, OK, USA). The statistical significance was assessed by Student's t-test, one, or two-way analysis of variance (ANOVA), followed by Newman–Keuls post-hoc test when appropriate. Three animals were excluded from the analysis of behavioral tests, because they did not perform the tasks assigned. Significance was set at $p < 0.05$. Results are expressed as mean \pm S.E.M. for every analysis performed.

Results

5.1 MDMA increases the vulnerability of mice to neuroinflammation and neurotoxicity induced by MPTP in the SNc and striatum

5.1.1 Immunohistochemistry

CD11b immunoreactivity in SNc and striatum

In vehicle+vehicle treated mice, resting microglia and very low levels of CD11b were observed, in both the SNc and the striatum. Similarly, mice exposed to chronic MDMA (10 mg/kg twice a day/twice a week) followed by vehicle administration did not show significant microglia activation, in both the SNc and the striatum. On the other hand, MPTP administration (20 mg/kg \times 4 administrations) to vehicle-pretreated mice elicited an elevation in microglia in the SNc, compared to vehicle+vehicle, and a higher elevation in microglia in striatum, compared to vehicle+vehicle and MDMA+vehicle treated mice. Finally, MPTP administration to MDMA-pretreated mice elicited a higher microglia activation in both the SNc and striatum, compared to all the other groups (Fig. 6).

In the SNc, one-way ANOVA showed a significant effect of treatment ($F_{3,19}=4.82$, $p<0.01$), and subsequent Newman-Keuls post-hoc test revealed that the levels of CD11b were higher in vehicle+MPTP mice compared with vehicle+vehicle treated mice. Moreover, MPTP administration in MDMA pretreated mice elicited a significantly higher microglia activation compared with all the other experimental groups (Fig.6A).

In the striatum, one-way ANOVA showed a significant effect of treatment ($F_{3,18}=20.05$, $p<0.01$), and subsequent Newman-Keuls post-hoc test revealed that the levels of CD11b were higher in vehicle+MPTP mice compared with vehicle+vehicle and MDMA+vehicle treated mice. Moreover, MPTP administration in MDMA pretreated mice elicited a significantly higher microglia activation compared with all the other experimental groups (Fig.6B).

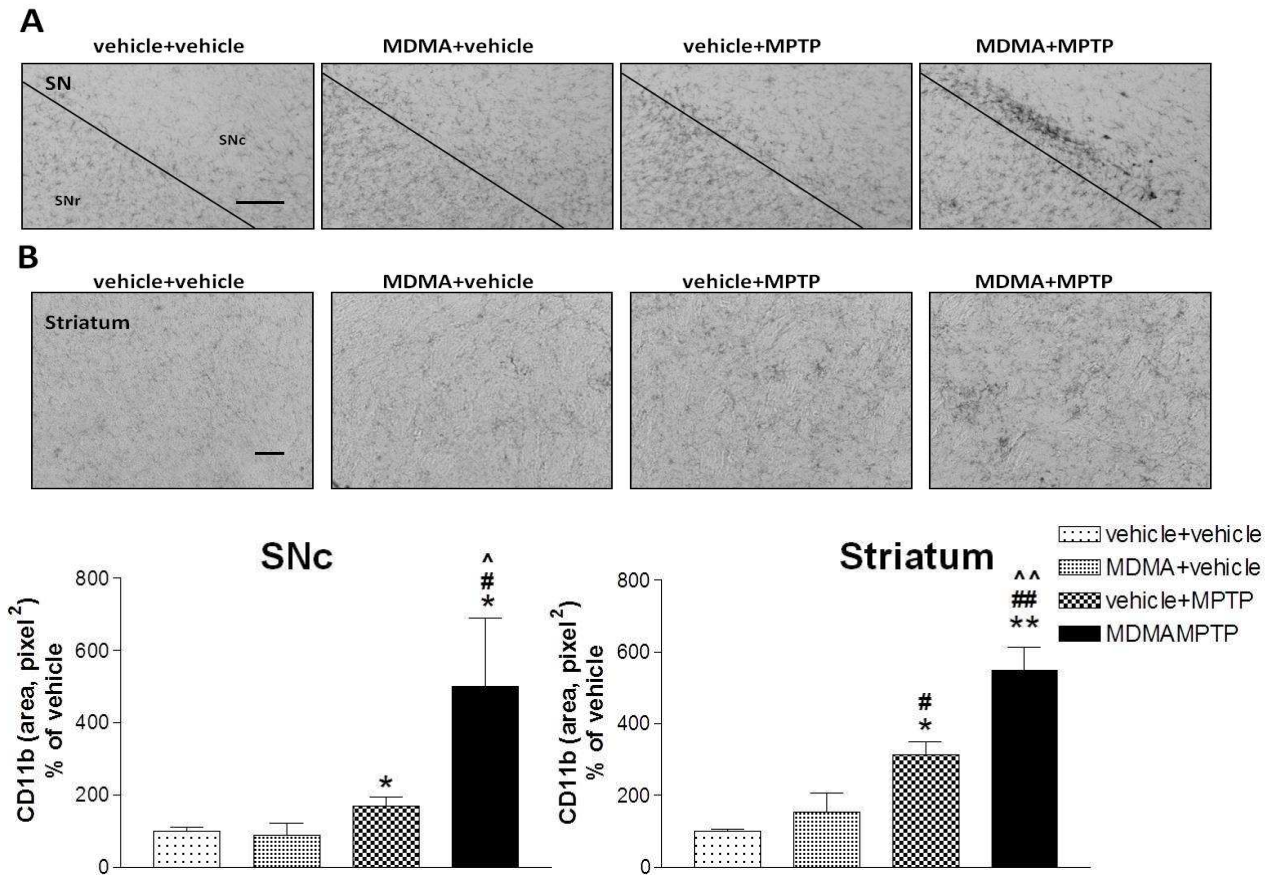


Figure 6. Representative sections and histograms of the SNc (A) and striatum (B) immunostained for CD11b are shown. Experimental groups: vehicle+vehicle (SNc: n=8, striatum: n=6); MDMA+vehicle (SNc: n=5, striatum: n=6); vehicle+MPTP (n=5 both for the SNc and striatum); MDMA+MPTP (n=5 both for the SNc and striatum). Scale bar: 50 μ m.

* $P < 0.01$, ** $P < 0.001$ vs vehicle+vehicle; # $P < 0.05$, ## $P < 0.001$ vs MDMA+vehicle; ^ $P < 0.05$; ^^ $P < 0.01$ vs vehicle+MPTP, by the Newman–Keuls post-hoc test.

GFAP immunoreactivity in SNc and striatum

Similar to what observed for CD11b, vehicle+vehicle mice displayed few astroglial cells, which showed a highly branched morphology with tiny processes and a small body, in both the striatum and SNc. Mice exposed to chronic MDMA (10 mg/kg twice a day/twice a week) followed by vehicle administration did not show significant astroglia activation, in both the SNc and the striatum. On the other hand, MPTP administration (20 mg/kg \times 4 administrations) to vehicle-pretreated mice elicited an elevation in astroglia in both the SNc and striatum, compared to vehicle+vehicle and MDMA+vehicle treated mice. Finally, MPTP administration to MDMA-pretreated mice elicited a higher astroglia activation in both the SNc and striatum, compared to all the other groups (Fig. 7).

In the SNc, one-way ANOVA showed a significant effect of treatment ($F_{3,22}=32.58$, $p<0.01$), and subsequent Newman-Keuls post-hoc test revealed that the levels of GFAP were higher in vehicle+MPTP mice compared with vehicle+vehicle and MDMA+vehicle treated mice. Moreover, MPTP administration in MDMA pretreated mice elicited a significantly higher astroglia activation compared with all the other experimental groups (Fig. 7A).

In the striatum, one-way ANOVA showed a significant effect of treatment ($F_{3,20}=441.1$, $p<0.01$), and subsequent Newman-Keuls post-hoc test revealed that the levels of GFAP were higher in vehicle+MPTP mice compared with vehicle+vehicle and MDMA+vehicle treated mice. Moreover, MPTP administration in MDMA pretreated mice elicited a significantly higher astroglia activation compared with all the other experimental groups (Fig. 7B).

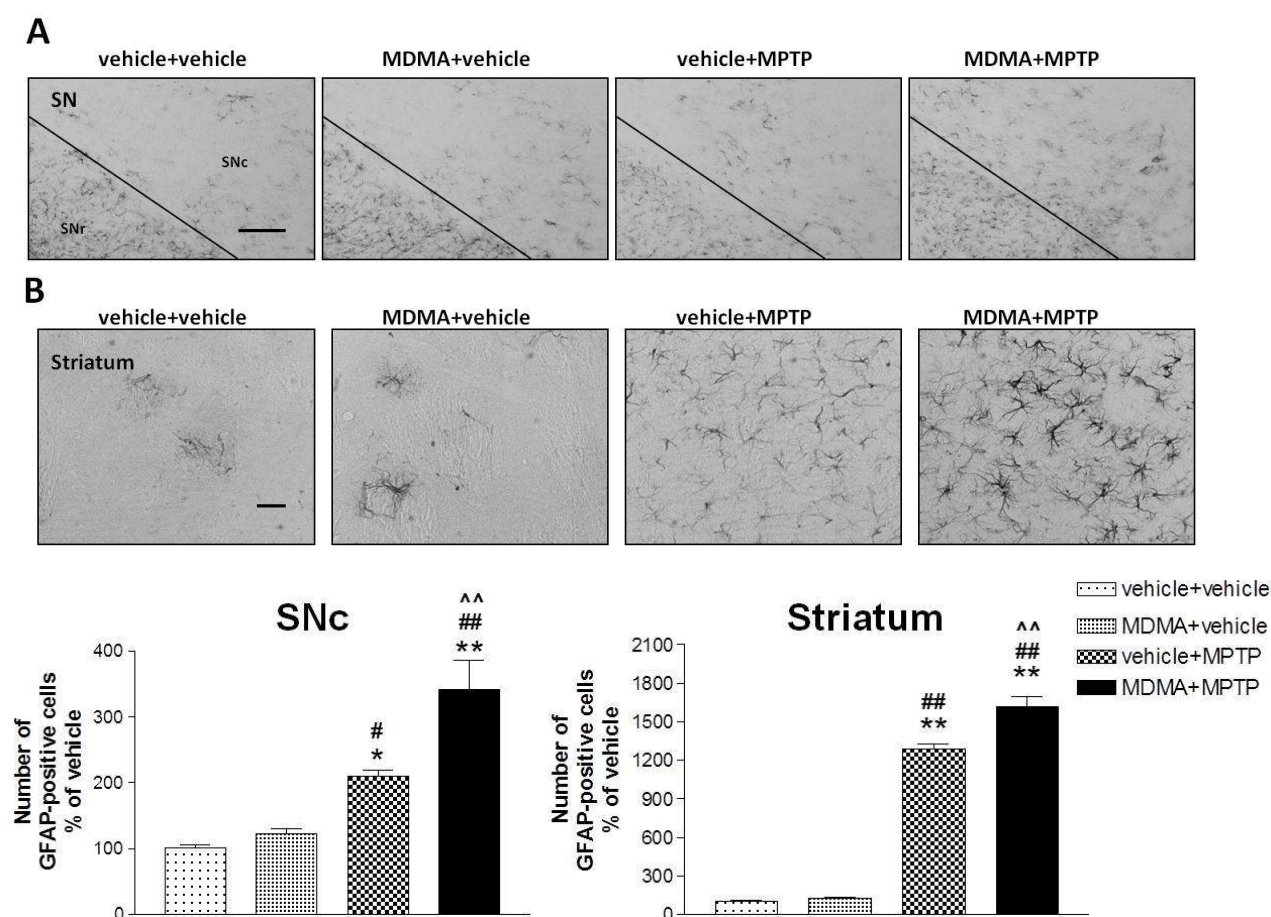


Figure 7. Representative sections and histograms of the SNc (A) and striatum (B) immunostained for GFAP are shown. Experimental groups: vehicle+vehicle (n=8 both for the SNc and striatum); MDMA+vehicle (SNc: n=8, striatum: n=6); vehicle+MPTP (n=5 both for SNc and striatum); MDMA+MPTP (n=5 both for the SNc and striatum). Scale bar: 50 μ m.

*P<0.005, **P<0.001 vs vehicle+vehicle; #P<0.005, ##P<0.001 vs MDMA+vehicle; ^^P<0.001 vs vehicle+MPTP, by the Newman–Keuls post-hoc test.

TH immunoreactivity in SNc and striatum

In vehicle+vehicle treated mice were not observed any decrease in both N and D of TH-positive neurons in the SNc, and in TH-positive fibers in the striatum. On the other hand, mice exposed to chronic MDMA (10 mg/kg twice a day/twice a week) followed by vehicle administration show a significant decrease in both the N and the D of TH-positive neurons in the SNc, whereas no significant decrease was found in the striatum. MPTP administration (20 mg/kg × 4 administrations) to vehicle-pretreated mice elicited a decrease in both the N and the D of TH-positive neurons in the SNc, and in TH-positive fibers in the striatum, compared to vehicle+vehicle treated mice. Finally, MPTP administration to MDMA-pretreated mice elicited a higher decrease in both the N and the D of TH-positive neurons in the SNc, and in TH-positive fibers in the striatum, compared to all the other groups (Fig. 8).

In the SNc, one-way ANOVA showed a significant effect of treatment (N: $F_{3,15}=21.09$, $p<0.01$; D: $F_{3,15}=21.35$, $p<0.01$). Subsequent Newman-Keuls post-hoc test revealed that the N and the D of TH-positive neurons of MDMA+vehicle treated mice were lower, compared to vehicle+vehicle treated mice. Moreover, as previously reported (Frau et al. 2011), administration of MPTP (20 mg/kg i.p. ×4 administrations) in vehicle-pretreated mice induced a decrease in both the N and the D values compared with vehicle+vehicle treated mice. Interestingly, chronic administration of MDMA followed by MPTP administration determined a further decrease in both N and D values compared with all other experimental groups (Fig. 8A). Examination of the VTA, did not show any decrease in TH-positive neurons (data not shown).

In the striatum, one-way ANOVA showed a significant effect of treatment ($F_{3,23}=10.52$, $p<0.01$). Subsequent Newman-Keuls post-hoc test revealed that administration of MPTP (20 mg/kg i.p. ×4 administrations) in vehicle-pretreated mice induced a decrease in the TH-positive fibers compared with vehicle+vehicle treated mice. Interestingly, chronic administration of MDMA followed by MPTP administration determined a further decrease in the TH-positive fibers compared with all other experimental groups (Fig. 8B). TH fiber loss was homogeneous along the rostrocaudal axis of the striatum.

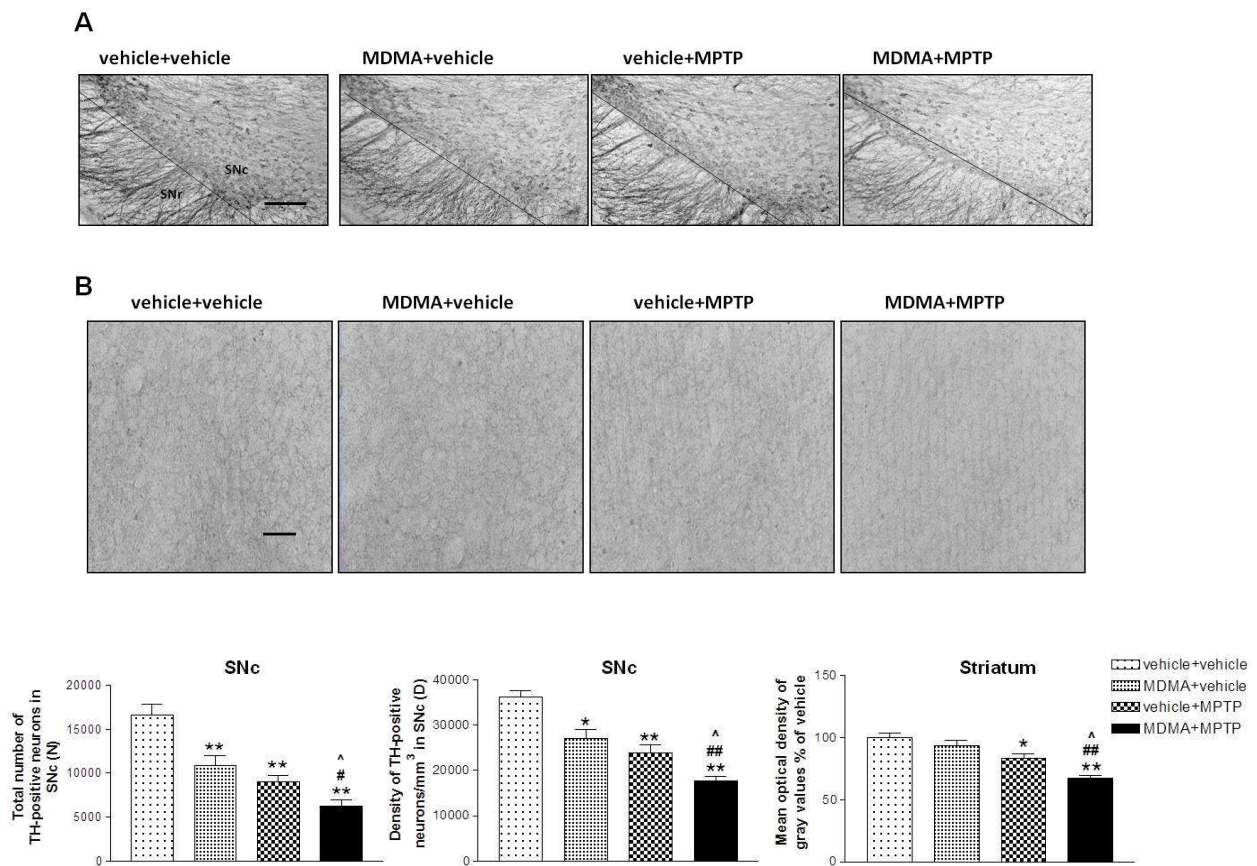


Figure 8. Representative sections and histograms of the SNc (A) and striatum (B) immunostained for TH are shown. Experimental groups: vehicle+vehicle (SNc: n=7; striatum: n=8); MDMA+vehicle (SNc: n=5; striatum: n=9); vehicle+MPTP (n=5 both for SNc and striatum); MDMA+MPTP (n=5 both for SNc and striatum). Scale bar: 50 μ m for SNc; 200 μ m for striatum.

* $P < 0.05$, ** $P < 0.001$ vs vehicle+vehicle; # $P < 0.01$, ## $P < 0.005$ vs MDMA+vehicle; ^ $P < 0.05$ vs vehicle+MPTP, by the Newman–Keuls post-hoc test.

5.2 MDMA increases the vulnerability of mice to the neuroinflammation induced by MPTP in the hippocampus and mPFC and is associated with cognitive deficits

5.2.1 Immunohistochemistry

CD11b immunoreactivity in hippocampus and mPFC

Vehicle+vehicle mice showed low levels of CD11b in both the hippocampus and mPFC. Similarly, mice exposed to chronic MDMA (10 mg/kg twice a day/twice a week) followed by vehicle administration did not show significant microglia activation. On the other hand, MPTP administration (20 mg/kg \times 4 administrations) to vehicle-pretreated mice elicited an elevation in

microglia in the hippocampus compared to vehicle+vehicle and MDMA+vehicle mice. Finally, MPTP administration to MDMA-pretreated mice elicited a higher microglia stimulation in both the hippocampus and mPFC, compared to all the other groups (Fig. 9).

In the hippocampus, one-way ANOVA showed a significant effect of treatment ($F_{3,25}=9.19$, $p<0.01$), and subsequent Newman-Keuls post-hoc test revealed that the levels of CD11b were higher in vehicle+MPTP mice compared with vehicle+vehicle mice and MDMA+vehicle mice. Interestingly, chronic administration of MDMA followed by MPTP administration determined a further increase in the levels of CD11b, compared with both vehicle+vehicle and MDMA+vehicle mice (Fig. 9A).

In the mPFC, one-way ANOVA indicated a significant effect of treatment ($F_{3,16}=6.89$, $p<0.01$), and subsequent Newman-Keuls post-hoc test showed that MDMA+MPTP mice exhibited CD11b levels significantly higher than those observed in all the other experimental groups (Fig. 9B).

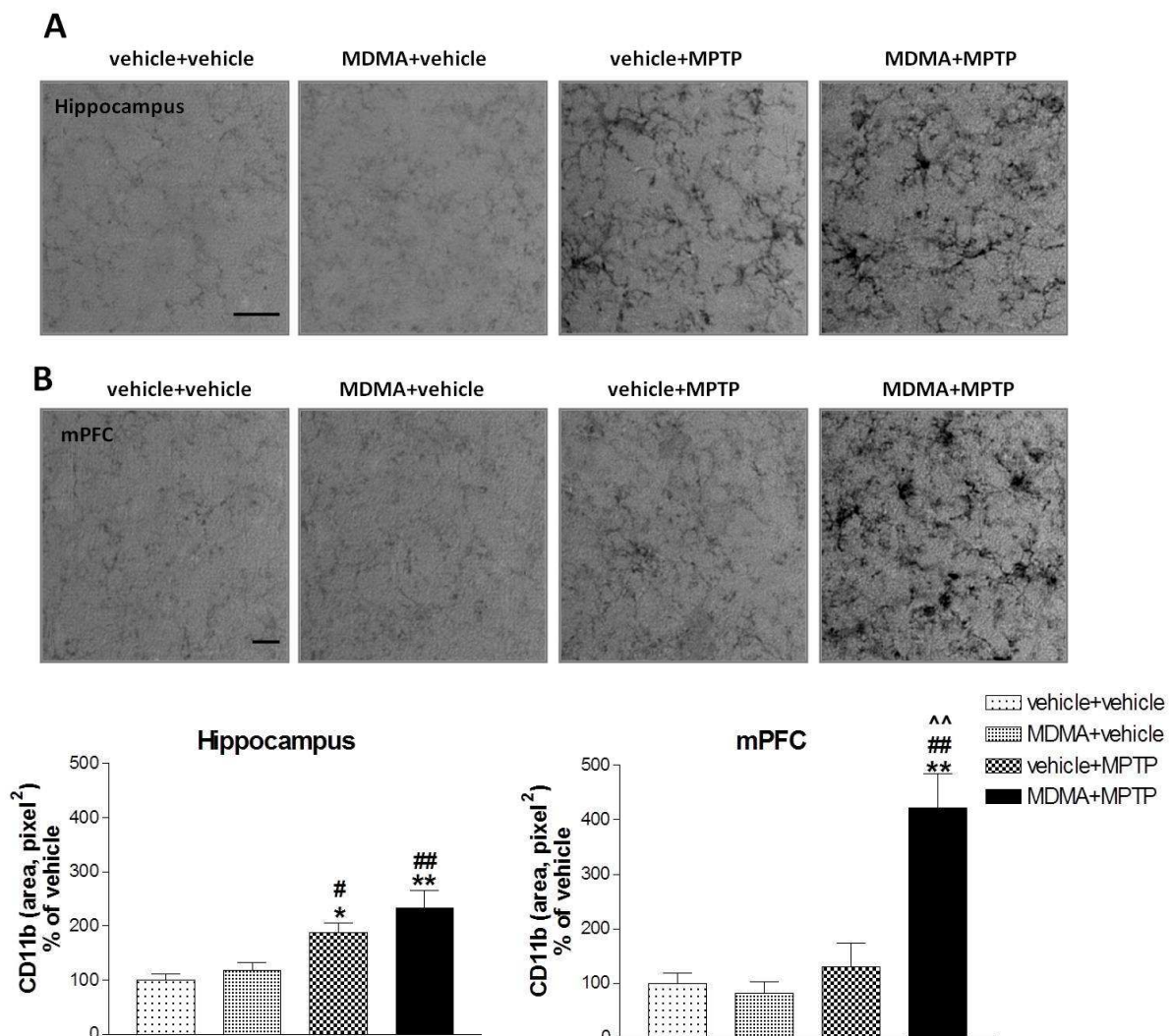


Figure 9. Representative sections and histograms of the hippocampus (A) and mPFC (B) immunostained for CD11b are shown. Experimental groups: vehicle+vehicle (hippocampus:

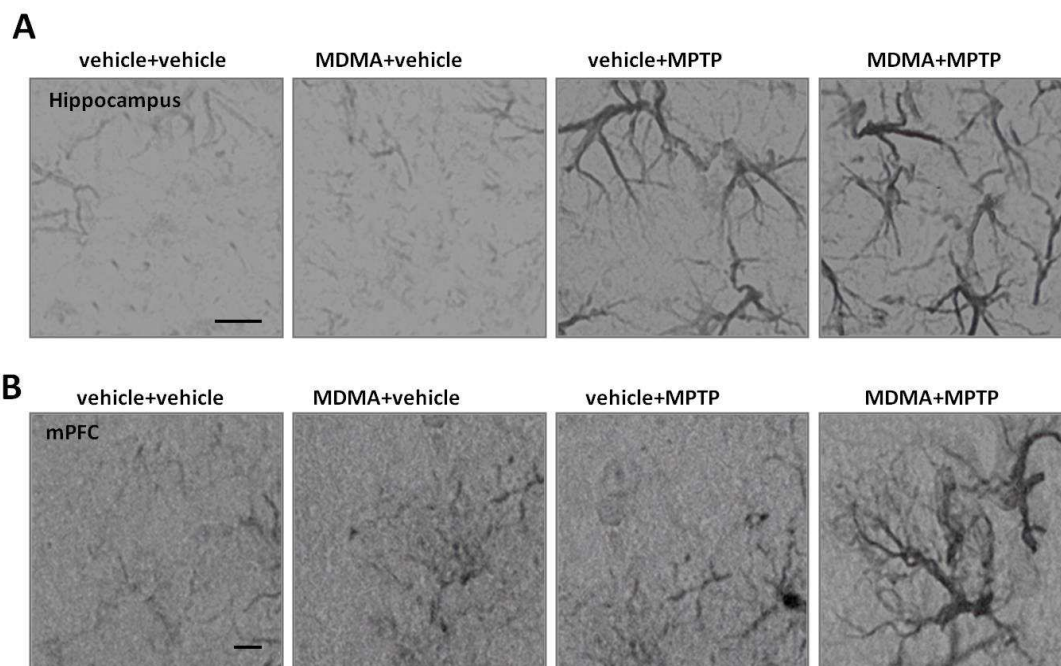
n=9, mPFC: n=5); MDMA+vehicle (hippocampus: n=5, mPFC: n=5); vehicle+MPTP (hippocampus: n=7, mPFC: n=5); MDMA+MPTP (hippocampus: n=8, mPFC: n=5). Scale bar: 50 μ m. *P<0.05, **P<0.001 vs vehicle+vehicle; #P<0.05, ##P<0.005 vs MDMA+vehicle; ^P<0.001 vs vehicle+MPTP, by Newman–Keuls post-hoc test.

GFAP immunoreactivity in hippocampus and mPFC

Vehicle+vehicle mice displayed astroglial cells with highly branched morphology, tiny processes and a small body in both the hippocampus and mPFC. Similarly, mice exposed to chronic MDMA (10 mg/kg twice a day/twice a week) followed by vehicle administration did not show a significant astroglia activation. On the other hand, MPTP administration (20 mg/kg \times 4 administrations) to vehicle-pretreated mice elicited an elevation in astroglia in the hippocampus compared to vehicle+vehicle mice. Moreover, MPTP administration to MDMA-pretreated mice elicited a higher elevation in microglia in the hippocampus and mPFC compared to all the other groups (Fig. 10).

In the hippocampus, one-way ANOVA revealed a significant effect of treatment ($F_{3,34}=9.42$, $p<0.01$), and subsequent Newman-Keuls post-hoc test showed that the number of GFAP-positive cells was higher in vehicle+MPTP mice compared with vehicle+vehicle mice. Interestingly, chronic administration of MDMA followed by MPTP administration determined a further increase in GFAP levels, compared with all the other experimental groups (Fig. 10A).

In the mPFC, one-way ANOVA showed a significant effect of treatment ($F_{3,16}=10.09$, $p<0.01$), and subsequent Newman-Keuls post-hoc test revealed that the number of GFAP-positive cells was higher in MDMA+MPTP mice, compared with all the other experimental groups (Fig. 10B).



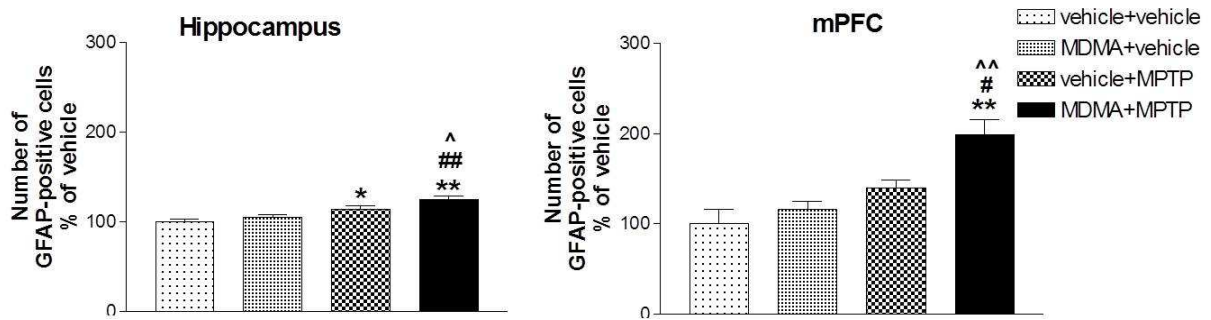


Figure 10. Representative sections and histograms of the hippocampus (A) and mPFC (B) immunostained for GFAP are shown. Experimental groups: vehicle+vehicle (hippocampus: n=9, mPFC: n=5); MDMA+vehicle (hippocampus: n=10, mPFC: n=5); vehicle+MPTP (hippocampus: n=9, mPFC: n=5); MDMA+MPTP (hippocampus: n=10, mPFC: n=5). Scale bar: 50 μ m. *P<0.05; **P<0.001 vs vehicle+vehicle; ###P<0.005 vs MDMA+vehicle; ^P<0.05; ^^P<0.01 vs vehicle+MPTP, by Newman–Keuls post-hoc test.

5.2.2 Memory tasks

NOR task

No significant modifications in NOR performance were observed during chronic administration of MDMA (10 mg/kg, i.p., twice a day/twice a week), compared with vehicle administration. Two-way ANOVA revealed no significant effect of chronic treatment ($F_{1,29}=0.04$, $p=0.84$), no significant effect of time ($F_{2,58}=1.09$, $p=0.34$), and no significant chronic treatment \times time interaction ($F_{2,58}=0.51$, $p=0.34$). However, when re-evaluated two weeks after treatment discontinuation, MDMA-treated mice showed an impairment in NOR performance compared with vehicle-treated mice, as shown by t-test ($df=32$, $t=4.10$, $p<0.01$) (Fig. 11A).

When NOR performance was re-evaluated in the same mice 24 hours after the last injection of MPTP (20 mg/kg \times 4 administrations), one-way ANOVA revealed a significant effect of treatment ($F_{1,31}=10.3$, $p=0.01$). Subsequent Newman-Keuls post-hoc indicated that both MDMA+vehicle and vehicle+MPTP mice had an impaired NOR performance, compared with vehicle+vehicle mice, and that MDMA+MPTP mice had a poorer NOR performance compared with all the other experimental groups (Fig. 11B). In all the NOR experiments performed, no significant differences in the total amount of time spent exploring the objects during either S1 or S2 were observed among the various experimental groups (data not shown).

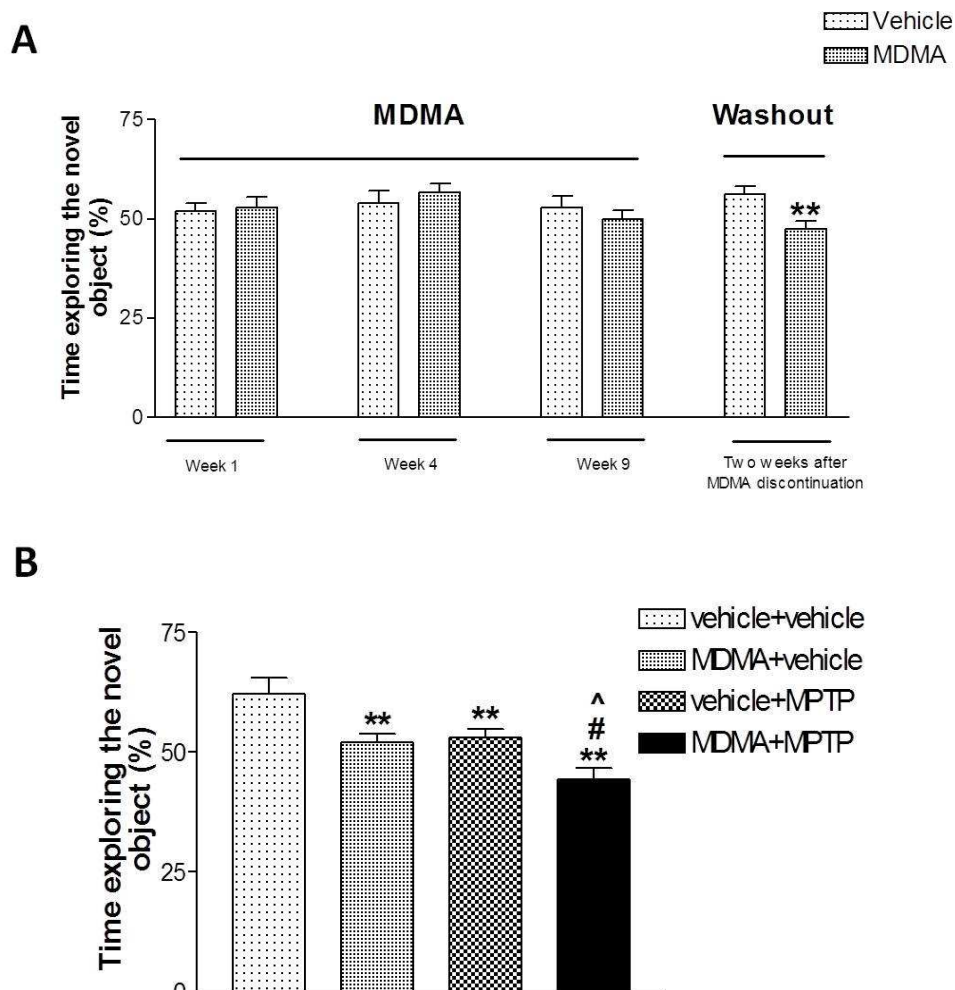


Figure 11. Representative histograms of NOR performance measured on week 1, week 4, and week 9 and then re-assessed two weeks after MDMA discontinuation (A). Representative histograms of NOR performance measured 24 hours after the last injection of MPTP (B). Experimental groups: MDMA (n=18); vehicle (n=18); vehicle+vehicle (n=9); MDMA+vehicle (n=10); vehicle+MPTP (n=9); MDMA+MPTP (n=8).

**P<0.005 vs vehicle+vehicle; #P<0.05 vs MDMA+vehicle; ^P<0.05 vs vehicle+MPTP, by t-test, or Newman–Keuls post-hoc test.

Spontaneous alternation behavior in a Y-maze

No significant changes in spontaneous alternation behavior were observed during chronic treatment with MDMA (10 mg/kg, i.p., twice a day/twice a week), compared with chronic vehicle administration (Fig. 12A). Two-way ANOVA revealed no significant effect of repeated treatment ($F_{1,29}=1.8$, $p=0.19$), no significant effect of time ($F_{2,58}=1.24$, $p=0.30$), and no significant repeated treatment \times time interaction ($F_{2,58}=1.2$, $p=0.31$). Similar results were observed when mice were re-evaluated two weeks after MDMA discontinuation, as shown by

Student's t-test ($df=32$, $t = -0.33$, $p=0.74$). Finally, administration of MPTP (20 mg/kg \times 4 administrations) did not affect spontaneous alternation behavior measured 48 hours after the last MPTP injection, as shown by one-way ANOVA ($F_{1,31}=0.08$, $p=0.97$) (Fig. 12B).

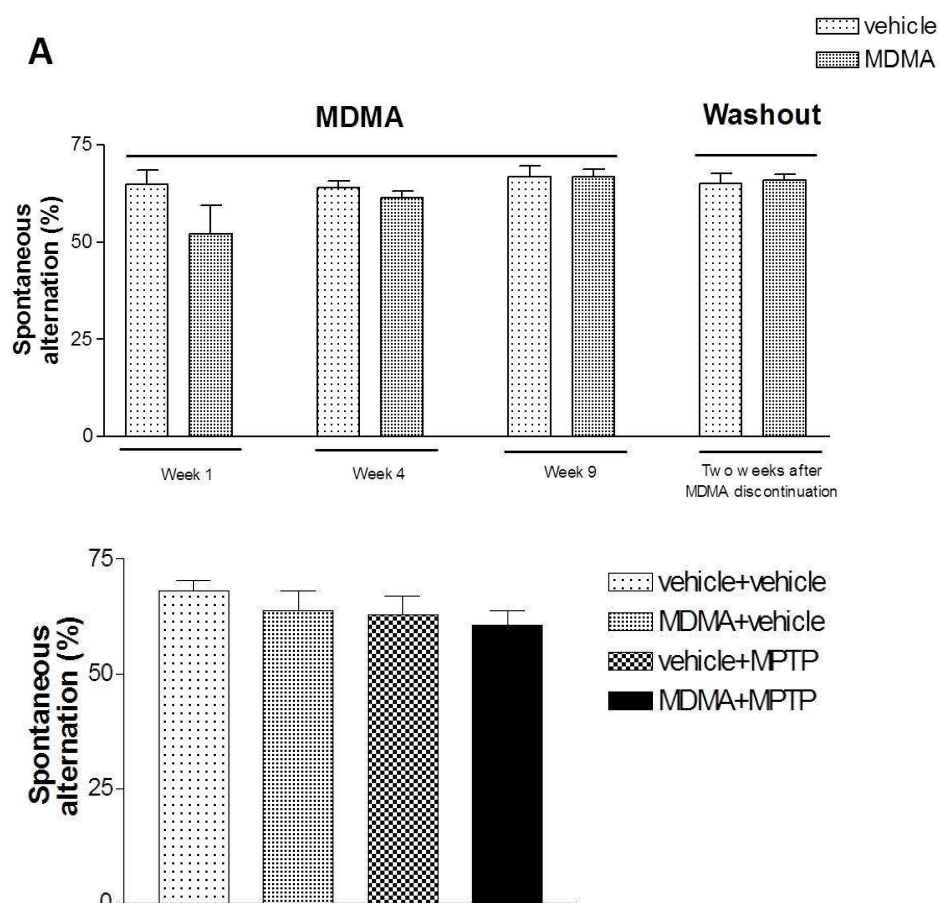


Figure 12. Representative histograms of spontaneous alternation behavior measured on week 1, week 4, and week 9 and then re-assessed two weeks after MDMA discontinuation (A). Representative histograms of spontaneous alternation behavior performance measured 24 hours after the last injection of MPTP (B). Experimental groups: MDMA (n=19); vehicle (n=17); vehicle+vehicle (n=8); MDMA+vehicle (n=10); vehicle+MPTP (n=9); MDMA+MPTP (n=9).

Discussion

In this work of thesis, late adolescent mice were treated as adolescent with MDMA, and administered as adults, after MDMA washout, with MPTP, a neurotoxin relevant for humans as it can induce a syndrome that reproduces the features of PD (Langston and Ballard 1983; Langston et al. 1983 and 1999), in order to study whether MDMA pre-exposure could modify the vulnerability to the noxious effects of MPTP. The results of this study demonstrate that the neuroinflammation in the nigrostriatal, limbic and cortical systems, as well as the neurotoxicity in the nigrostriatal system, elicited by MPTP, was exacerbated by chronic administration of MDMA to mice during adolescence. Moreover, MDMA administration during adolescence induces cognitive impairment, and amplified the detrimental effects of MPTP on cognitive performance measured at adulthood.

The study of how MDMA modulates the toxic effects of other substances appears a “hot topic” in neuropharmacology and neurotoxicology because this drug is largely consumed by adolescents (Strote et al. 2002), and adolescence is a critical period for the development of the brain (Casey et al. 2000). The adolescent brain appears particularly vulnerable to the long-term noxious effects of exogenous substances, including drugs of abuse (Cadoni et al. 2013; Daza-Losada et al. 2009). It has been demonstrated, in rats, that almost all drugs causing drug addiction increase the release of DA in the mesolimbic pathway (Pierce and Kumaresan 2006), and it has been demonstrated that amphetamine administration in adolescent rats induces a prominent sensitization of DA release, whereas no change was found in adult rats (Laviola et al. 2001). Therefore, it is becoming increasingly clear that MDMA should be regarded as harmful not only because its use may have implications for drug dependence (Fernandez-Serrano et al. 2011; Smirnov et al. 2013), but also because MDMA use could function as a “primer” for later brain insults.

Besides showing detrimental effects of MDMA itself, studies in experimental animals have shown that this substance may amplify the neuroinflammation and neurotoxicity elicited by other psychoactive/toxic substances, eventually leading to the exacerbation of neuronal damage. The potentiation of MPTP-mediated neuroinflammation in mice pretreated with MDMA during adolescence appeared very marked in striatum and mPFC, lower in SNc and hippocampus, involving both microglia and astroglia. It should be noted that MDMA alone did not appear to produce an increase in neuroinflammation in any brain areas evaluated. In this regard, it has to be considered that in the present study immunohistochemical evaluation of microglia and astroglia was performed three weeks after the last MDMA administration, and therefore it is

feasible that the acute neuroinflammatory effects of MDMA may have faded over time. Moreover, it has to be taken into account the differences in doses and protocols of MDMA administration between the present and previous studies (Frau et al. 2013; Granado et al. 2008a and 2011; Khairnar et al. 2009).

It is noteworthy that MPTP alone elicited both astrogliosis and microgliosis in the SNc, striatum and hippocampus, compared with vehicle+vehicle treated mice. At variance with these results, neither MDMA nor MPTP alone significantly stimulated both astrogliosis and microgliosis in the mPFC. This latter finding deserves consideration, since it suggests that the neurochemical modifications induced by repeated exposure to MDMA, even if subtle, may prime the brain for the effects of later noxious stimuli. In addition, MPTP administration in MDMA pretreated mice significantly stimulates astrogliosis, but not microgliosis, in the hippocampus, compared with MPTP alone. These results are confirmed by previous evidences in rodents studies reporting neuroinflammatory effects of MPTP in the SNc, striatum (Czlonkowska et al. 1996; Kohutnicka et al. 1998; Kurkowska-Jastrzebska et al. 1999; Kurosaki et al. 2003), and hippocampus (Kokovay and Cunningham 2005; Sy et al. 2010; Wang et al. 2009). The increased microgliosis and astrogliosis in hippocampus could be explained by the presence of a very high density of binding sites for MPP⁺ in this region (Del Zompo et al. 1992). As suggested by Thomas and co-workers (2004), the amphetamine-related drugs associated with DA or 5-HT nerve terminal damage also cause microglial activation, whereas non-neurotoxic amphetamine-related drugs do not have this effect.

MPTP administration in mice chronically pretreated during late adolescence with MDMA induced higher dopaminergic neuron degeneration than in MPTP or MDMA alone treated mice. Since, in these mice, the degree of neuronal degeneration induced by MPTP appears additive to that induced by MDMA, we speculate that dopaminergic degeneration produced by MDMA may create the basis for a degeneration which could reach the critical threshold to induce PD in presence of environmental toxins. Moreover, mice chronically treated with MDMA showed reduced N and the D of TH-positive neurons in the SNc three weeks after the interruption of MDMA treatment. This result, in line with previous reports showing that acute repeated MDMA administration produces dopaminergic neuron degeneration (Granado et al. 2008a and 2011), adds the important indication that also sporadic, but prolonged treatment of mice with this drug is toxic to dopaminergic neurons. Therefore, MDMA, like other recognized amphetamine-related drugs, such as methamphetamine (Albers et al. 1996; Sinchai et al. 2011), increases dopaminergic neuron vulnerability even when administered at low/medium doses and in an irregular way. Regarding the reduction in N and the D of TH-positive neurons in the SNc but not

in TH-positive fibers in striatum it can be hypothesized, as previously suggested (Rosenblad et al. 1999), that partial DA lesion might be associated to compensatory sprouting in the striatum. As mentioned in the introduction, MDMA and MPTP have several common mechanisms able to induce toxicity and this could underlie the exacerbation of MPTP-mediated neuroinflammatory and neurotoxic effects observed in MDMA pretreated mice. MDMA has been reported to inhibit the CYP2D6 (O'Mathùna et al. 2008), which has been shown to convert MPTP into non-toxic metabolites (Herraiz et al. 2006; Mann and Tyndale 2010). In addition, it is possible that prolonged exposure to MDMA result in an elevation of intracellular oxidative stress, due to several mechanisms, that would eventually render neurons more sensitive to the effects of MPTP, which acts itself by promoting the generation of ROS (Cleeter et al. 1992; Sriram et al. 1997). Thus, it has been demonstrated that MDMA, as MPTP, can inhibit mitochondrial complex I in mice (Nicklas et al. 1985; Puerta et al, 2010; Suzuki et al. 1990), leading to mitochondria inactivation and oxidizing macromolecules such as lipids, DNA, and proteins. Moreover, MDMA, could amplify oxidative stress also by promoting the release of DA (Cadoni et al. 2005), which can undergo auto-oxidation, and in turn generate free radicals and ROS (Marchitti et al. 2007; Siraki and O'Brien 2002; Stokes et al. 1999), eventually leading to the exacerbation of neuroinflammatory and neurotoxic phenomena triggered by a later noxious insult. This latter mechanism could be particularly relevant to the results of this study, as it might justify why MDMA pre-exposure was associated with a more marked exacerbation of MPTP-neuroinflammation in the striatum and mPFC, and neurotoxicity in SNc, which are highly enriched with dopaminergic terminals and neurons (Bentivoglio and Morelli 2005), than in the hippocampus, where dopaminergic innervation is less dense (Bentivoglio and Morelli, 2005; Milner and Bacon 1989).

In addition to neuroinflammatory and neurotoxic changes, mice treated with MDMA during adolescence displayed a persistent impairment in NOR performance after treatment discontinuation, and MDMA pretreatment was found to further aggravate cognitive impairment induced by MPTP. These results appear of great relevance since, despite the ever-increasing use of MDMA in humans, scarce information is available on the long-term effects on cognition elicited by the consumption of this drug during adolescence. Importantly, the impairment in NOR performance observed in this study appears clearly attributable to deficits in cognitive function. In fact, no differences were found between MDMA- and vehicle-treated mice as for the total amount of time spent in object exploration during both S1 and S2. This indicates that the regimen of MDMA used in this study is devoid of non-specific effects on object exploration. Moreover, MDMA seemed not to alter the ability of mice to discriminate between different

objects. In fact, mice could recognize novel objects at different time points during the MDMA treatment, but eventually loses this ability when re-evaluated at two weeks after treatment discontinuation, which would suggest the onset of progressive and long-term toxicity of MDMA. Striatum, hippocampus and mPFC have been shown to regulate item recognition in rodents (Cropley et al. 2006; Dalley et al. 2004; Eichenbaum et al. 1996; Landau et al. 2009; Ragozzino et al. 1999; Squire 1992). Therefore, the impairment in NOR performance observed here is likely attributable to the neuroinflammatory effect caused by MDMA and MPTP in these areas. Remarkably, a support to the existence of a causal link between neuroinflammation and cognitive impairment comes from previous studies which have employed the systemical or intrahippocampal injection of lipopolysaccharide (LPS) in rodents. Thus, LPS has been shown not only to stimulate the expression of inflammation-related molecules, including GFAP, iNOS, cyclooxygenase (COX)-2, IL-6 and IL-1 β (Herber et al. 2006; Jain et al. 2002; Pang et al. 2006; Sparkman et al. 2006) but also to engender cognitive impairment. Furthermore, METH, the abuse of which in humans is often associated with neurocognitive impairment (Nestor et al. 2011; North et al. 2013), causes an inflammatory response characterized by an upregulation of GFAP and CD11b levels, TNF- α , and TNF-receptor 1 protein levels (Gonçalves et al. 2010) in mice.

Interestingly, the cognitive impairment was narrowed to NOR, whilst no deficits were detectable in spontaneous alternation behavior. This lack of effect could be observed both during MDMA treatment and following MPTP administration, and no exacerbation of MPTP effects was present in MDMA-treated mice. These results could be explained in the light of previous studies that have shown how spontaneous alternation behavior may be relatively resistant to noxious brain insults that impair NOR performance, suggesting a different sensitivity of these two tasks to neuronal damage (Moriguchi et al. 2012; Simola et al. 2008). Moreover, it is interesting to consider that MPTP has been demonstrated to scarcely affect alternation behavior in mice (Tanila et al. 1998), which could justify the lack of an impairment in spontaneous alternation behavior in mice challenged with MPTP.

The present study, encompassing both neurochemical and behavioral findings, may significantly contribute to the elucidation of the interactions between MDMA and substances which are toxic for the brain, and appear also of potential relevance for humans. Several clinical and preclinical studies recognize that PD has multiple origins, from genes to toxins and several concomitant factors may contribute to its development (Marsden and Olanow 1998). Since one of the possibilities taken into account in PD pathology is the use of amphetamine-related drugs, the results of the present work of thesis support the hypothesis these substances might be part of the

multiple factors that lead to dopaminergic neuron degeneration and microglial activation associated with PD. Thus, a major conclusion that can be drawn from the present study is that the detrimental effects of MDMA, even if subtle, appear to be long-lasting, since they could be detected after treatment discontinuation. By showing an exacerbation of MPTP effects in several brain areas as SNc, striatum, hippocampus and mPFC of mice pre-exposed to MDMA, this study indicates that noxious MDMA-MPTP interactions are widespread in the brain. Moreover, it was observed that the abuse of MDMA creates the basis for a worsening in cognitive function, in agreement to data from several studies in adult MDMA consumers (Bolla et al. 1998; Brown et al. 2010; Fox et al. 2001; Morgan 2000; Parrott and Laskey 1998).

Conclusions

In conclusion, by demonstrating that mice repeatedly treated with MDMA during adolescence display an exacerbation in neuroinflammation, neurotoxicity and cognitive impairment induced by MPTP, a dopaminergic toxin relevant to humans, the present results underline the importance of exploring the long-term effects of amphetamine-related drugs, to clarify their potential as risk factors for the development of neurological and behavioral problems later in life.

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