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# Molecular and Cellular Mechanisms of Ecstasy-Induced Neurotoxicity: An Overview

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**Abstract** "Ecstasy" [(±)-3,4-methylenedioxymethamphetamine, MDMA, XTC, X, E] is a psychoactive recreational hallucinogenic substance and a major worldwide drug of abuse. Several reports raised the concern that MDMA has the ability to induce neurotoxic effects both in laboratory animals and humans. Despite more than two decades of research, the mechanisms by which MDMA is neurotoxic are still to be fully elucidated. MDMA induces serotonergic terminal loss in rats and also in some mice strains, but also a broader neuronal degeneration throughout several brain areas such as the cortex, hippocampus, and striatum. Meanwhile, in human "ecstasy" abusers, there are evidences for deficits in seronergic biochemical markers, which correlate with long-term impairments in memory and learning. There are several factors that contribute to MDMA-induced neurotoxicity, namely, hyperthermia, monoamine oxidase metabolism of dopamine and serotonin, dopamine oxidation, the serotonin transporter action, nitric oxide, and the formation of peroxinitrite, glutamate excitotoxicity, serotonin 2A receptor agonism, and, importantly, the formation of MDMA neurotoxic metabolites. The present review covered the following topics: history and epidemiology, pharmacological mechanisms, metabolic pathways and the influence of isoenzyme genetic polymorphisms, as well as the acute effects of MDMA in laboratory animals and humans, with a special focus on MDMA-induced neurotoxic effects at the cellular and molecular level. The main aim of this review was to contribute to the understanding of the cellular and molecular mechanisms involved in MDMA neurotoxicity, which can help in the development of therapeutic approaches to prevent or treat the long-term neuropsychiatric complications of MDMA abuse in humans.

**Keywords** Ecstasy · MDMA · Drug abuse · Hallucinogen · Neurotoxicity · Mechanism of neurodegeneration

**Abbreviations**

| | |
|---|---|
| Amph | Amphetamine |
| AMPT | α-Methyl-*p*-tyrosine |
| ATP | Adenosine triphosphate |
| AUC | Area under the curve |
| $C_{max}$ | Maximum concentration |
| CNS | Central nervous system |
| COMT | Catechol-*O*-methyltransferase |
| CSF | Cerebrospinal fluid |
| CTX | Cortex |
| CYP | Cytochrome P450 |
| DA | Dopamine |
| DAT | Dopamine transporter |
| DHT | Dihydroxytriptamine |

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| | |
|---|---|
| DOI | (±)-2,5-Dimethoxy-4-iodoamphetamine |
| $EC_{50}$ | Effective concentration 50% |
| EU | European Union |
| GABA | Gamma-aminobutyric acid |
| GFAP | Glial fibrillary acidic protein |
| GLU | Glutamate |
| GSH | Glutathione |
| GST | Glutathione S-transferase |
| γ-GT | gamma-glutamyl transpeptidase or gamma-glutamyltransferase |
| 5-HIAA | 5-Hydroxyindoleacetic acid |
| HIP | Hippocampus |
| HMA | 4-Hydroxy-3-methoxyamphetamine, 3-*O*-Me-α-MeDA |
| HMMA | 4-Hydroxy-3-methoxymethamphetamine, 3-*O*-Me-*N*-Me-α-MeDA |
| $HO^{\bullet}$ | Hydroxyl radical |
| $H_2O_2$ | Hydrogen peroxide |
| 5-HT | 5-Hydroxytriptamine, serotonin |
| 5-HTT | Serotonin transporter |
| HVA | 4-Hydroxy-3-methoxyphenylacetic acid, homovanillic acid |
| i.p. | Intraperitoneal |
| i.v. | Intravenous |
| $iCa^{2+}$ | Intracellular calcium |
| ICV | Intracerebroventricular |
| $K_e$ | Elimination constant |
| KO | Knockout |
| MAO | Monoamine oxidase |
| MDA | (±)-3,4-Methylenedioxyamphetamine |
| MDMA | (±)-3,4-Methylenedioxymethamphetamine, "ecstasy" |
| α-MeDA | α-Methyldopamine, 3,4-Dihydroxyamphetamine, HHA |
| *N*-Me-α-MeDA | *N*-methyl-α-methyldopamine, 3,4-Dihydroxymethamphetamine, HHMA |
| Meth | Methamphetamine |
| MK-801 | Dizocilpine |
| NAC | *N*-acetylcysteine |
| NE | Norepinephrine |
| NET | Norepinephrine transporter |
| NMDA | *N*-methyl-D-aspartic acid |
| L-NAME | $N_\omega$-nitro-L-arginine methyl ester |
| L-NNA | $N_\omega$-nitro-L-arginine |
| NO | Nitric oxide |
| $NO^{\bullet}$ | Nitric oxide radical |
| $O_2^{\bullet-}$ | Superoxide anion |
| $ONOO^-$ | Peroxynitrite |
| p.o. | Per os |
| PBN | α-Phenyl-*N*-*tert*-butyl nitrone |
| PND | Postnatal day |
| PET | Positron emission tomography |
| PKC | Protein kinase C |
| R-96544 | (2R,4R)-5-[2-[2-[2-(3-Methoxyphenyl) ethyl]phenoxy]ethyl]-1-methyl-3-pyrrolidinol hydrochloride |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| s.c. | Subcutaneous |
| –SH | Sulfhydryl |
| SPECT | Single photon emission computed tomography |
| SULT | Sulfotransferase |
| $t_{1/2}$ | Elimination half-life |
| $T_{max}$ | Median time to maximum concentration |
| T-4,5-D | Tryptamine-4,5-dione |
| TPH | Tryptophan hydroxylase |
| UGT | UDP-glucuronosyltransferase |
| VMAT | Vesicular monoamine transporter |
| WT | Wild type |

## General Introduction

### History of "Ecstasy"—From the Clinics to the Streets

3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy", XTC, E, X) is a ring-substituted amphetamine derivative structurally related to the hallucinogenic compound mescaline, to amphetamine, and also to the monoamine neurotransmitters (Fig. 1). It was first synthesized and patented in 1912 by the German pharmaceutical company Merck under the name of "methylsafrylamin" [1]. At that time, MDMA was not intended for therapeutic use, but only as a precursor for therapeutically active compounds. Freudenmann et al. [1] assured in their examination of the Merck company archives that it had no intentions of using therapeutic MDMA as an apetite suppressor, as many times erroneously has been written. The first proven pharmacological tests with MDMA occurred in the year of 1927 in Merck's laboratory and the substance was again tested in 1959, but there is only indication of animal testing [1]. The toxicology of MDMA was examined in the year 1953, together with other similar compounds, at the University of Michigan in a classified research program sponsored by the USA military, presumably as part of a chemical warfare program [2]. The research, concerning the behavioral and toxicological effects, was declassified in 1969 and published in 1973 [2].

In the year 1976, MDMA was used for the first time in the clinics as an adjuvant to psychiatric treatment by Leo Zeff. A Californian chemist, Shulgin, who is seen by some as the "stepfather" of "ecstasy," synthesized and tested the drug and was the first to describe that MDMA was a psychoactive drug in humans [3]. Both Shulgin and Leo Zeff presented MDMA to professional therapists as a

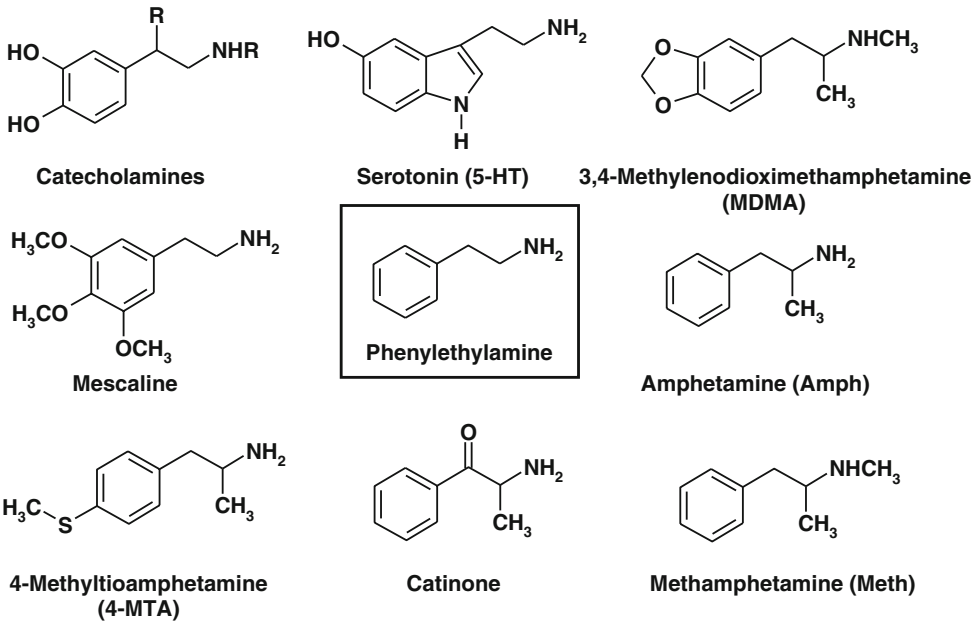


**Fig. 1** Chemical structures of MDMA ("ecstasy") and related amphetamines. Amphetamines are phenylethylamine derivatives chemically related to the monoamine neurotransmitters and to the naturally occurring hallucinogenic compounds, like mescaline and catinone. Mescaline is found in the peyote cactus that grows in North America. Catinone in present in the leaves of the Khat plant, native to the sub-Sahara, which is chewed in many Arabic countries to combat starvation and fatigue during the Ramadan period or even on a daily basis in social occasions

valuable adjunct to psychotherapy in therapeutic settings. By the early 1980s, over a thousand private psychotherapists in the USA were using MDMA under the name of "ADAM" in their clinical practice [2]. MDMA was believed to increase patient self-esteem and facilitate therapeutic communication. In those practices, it was administered orally (75–175 mg) and noted to produce acute sympathomimetic effects, such as increased heart rate and blood pressure, and transient anxiety [4, 5]. Meanwhile, in 1977, the UK classified MDMA as a class A schedule 1 drug, meaning it is illegal to possess, sell, or give away [6]. In the USA, since the early 1980s, it became popular in the streets as a recreational drug, as a "fun drug" that was "good to dance". In San Francisco, drug dealers sold MDMA under the name of "ecstasy", which they invented for commercial purposes [2]. In 1985, the USA Drug Enforcement Administration classified MDMA as a schedule 1 drug due to its high abuse potential, lack of clinical application, lack of accepted safety for use under medical supervision, and evidence that it could be neurotoxic [2, 7, 8]. This classification was severely criticized by some psychotherapists who realized that their research and medical use of MDMA could not continue [2]. Still, nowadays, some argue that MDMA can have a medical application, as well as other psychedelics, and is being studied as a treatment for anxiety and posttraumatic stress disorder with the reasoning that MDMA can be used by specially trained psychotherapists [9–11]. However, there are many drawbacks for the medical use of MDMA, as recently reviewed by Parrot [12]. In our view, given the massive evidence of MDMA-related toxic events, extensively discussed in this review, as well as the abuse liability, there are no safe clinical applications for MDMA.

MDMA's USA fame spread across the Atlantic. "Ecstasy" became associated with the birth of "acid house" music in the Spanish touristic resort of Ibiza. By the summer of 1986, Ibiza was popularly known as "XTC island" [2]. Returning tourists and disc jockeys took the message back home, spreading the use of "ecstasy" across Europe and the world [7]. The UK "rave" scene was then born and continues very active today. As a consequence, across European countries, MDMA was considered an illegal substance [13].

Globally, the European Union (EU) remains the main center of "ecstasy" production, although its relative importance appears to be declining, as "ecstasy" manufacture has spread to other parts of the world in recent years, notably to North America and east and southeast Asia [14]. Conceivably, 80% of MDMA is manufactured illegally by clandestine labs in central EU, especially in The Netherlands and Belgium [14, 15].

## "Ecstasy" Tablets

"Ecstasy" is almost exclusively sold and consumed orally in the form of tablets (rarely capsules), which frequently contain symbols (logos) and are colored. MDMA is often taken at "rave" or "techno" parties, or simply called "parties," particularly at large dance clubs. Thus, "ecstasy" is used mainly over the weekend, when dance clubs are actively open and "parties" occur [16].

MDMA content of "ecstasy" tablets varies greatly from batch to batch (even among those tablets with the same logo) both between and within countries. Also, occasionally, tablets that are sold as "ecstasy" do contain drugs other than MDMA, or even none at all. Other psychoactive substances found in tablets sold as "ecstasy" included

mostly other amphetamines, such as 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxyethylamphetamine (MDE), paramethoxyamphetamine (PMA), 2,5-dimethoxy-4-bromoamphetamine (DOB), and 4-methylthioamphetamine (4-MTA) [14, 17]. However, the problem of MDMA purity was predominantly a phenomenon of the mid to late 1990s when many tablets were impure. The latest reports suggest that purity levels of "ecstasy" tablets, in terms of MDMA content, lie between 90% and 100% [17]. In 2005, EU surveys showed that the average or typical content of MDMA per tablet ranged from 2 to 130 mg, although the average was between 30 and 80 mg of MDMA. In the same year, the average or typical retail cost of MDMA tablets in the EU ranged from less than 3€ to 15€ per tablet [14].

### "Ecstasy" Epidemiology

The United Nations estimates that global "ecstasy" use affects some nine million people of the population aged between 15 and 64 years. There are more than three million "ecstasy" users in Europe, accounting for some 36% of ecstasy users worldwide. About 90% of them are located in West and Central Europe. "Ecstasy" abuse has also spread to Asia and Oceania. The annual prevalence rate of "ecstasy" use is estimated at 0.9% of the population aged 15–64 in West and Central Europe, exceeding the levels reported from North America (0.8%). While drug use trends of Western Europe are largely stable, "ecstasy" use in several East and Southeast European countries continues to grow [15].

In the EU, the European Monitoring Centre on Drugs and Drug Addiction estimates that more than one million adults take "ecstasy" every month. In the UK alone, it is estimated that every weekend, 500,000 young people consume the drug. Traditionally, population surveys have shown that after cannabis, amphetamines are the most commonly used illegal substances. This pattern now appears to be changing in many countries, with "ecstasy" overtaking the other amphetamines and getting the second place, after cannabis, in both recent general population and school surveys [14]. Based on general population surveys, it has been estimated that almost 8.5 million Europeans have tried "ecstasy", and almost three million have used it in 2005 [14].

The USA National Institute on Drug Abuse publishes annual results concerning a study conducted by the University of Michigan's Institute for Social Research, entitled "Monitoring the Future Study." This study examines trends of drug abuse within different populations—school children, college students, and adults aged 19 to 45 [18, 19]. Among tenth graders, annual prevalence has risen from a recent low of 2.4% in 2004 to 3.5% in 2007, while in 12th graders, it has risen from a recent low of 3% in 2005 to 4.5% in 2007 [20]. While none of the 1-year increases were statistically significant for 2007, a clear pattern of gradually rising use is discernable in the upper grades, and their cumulative increases over the past couple of years are statistically significant [18, 19].

## Pharmacology of MDMA

### MDMA: An Indirect Monoaminergic Agonist

MDMA affects peripheral and central nervous system (CNS) functions by acting mainly on the monoaminergic system. The evaluation of monoamine release after MDMA intake has not been studied in humans or non-human primates, but data obtained from research in laboratory animals, namely in rats, proved that MDMA is an indirect monoaminergic agonist [21]. Presented in a chronological manner, initial studies conducted in vitro with rat brain tissue showed that MDMA stimulates the efflux of preloaded [$^3$H]5-HT, serotonin (5-HT), and, to a lesser extent, [$^3$H]DA, dopamine (DA) [21, 22]. More recent findings revealed that MDMA interacts with monoamine transporters to stimulate non-exocytotic release of 5-HT, DA, and norepinephrine (NE) in the rat brain [23, 24].

#### *Inhibition of DAT, NET, and 5-HTT by MDMA*

MDMA is known to inhibit the DA transporter (DAT), NE transporter (NET), and 5-HT transporter (5-HTT) [25–27]. Recently, a study analyzed the potencies of racemic MDMA to inhibit human and mouse DAT, NET, and 5-HTT transiently expressed in cultured intestinal 407 cells. In these cells expressing the human transporters, the potency order of racemic MDMA to inhibit the monoamine transporters was NET > 5-HTT > DAT [27]. In intestinal 407 cells expressing the mouse transporters, the order of potencies for MDMA to inhibit the monoamine transporters was 5-HTT > NET > DAT, which is in accordance with the data obtained in rats [27]. In rat synaptosomes, it was found that the rank potency order of racemic MDMA to inhibit the monoamine transporters was 5-HTT > NET > DAT [25]. The rank order of potencies for MDMA inhibition of the uptake of the three monoamine transporters of humans, rats, and mice in comparison with (+)-amphetamine is shown in Table 1. Data show that the major consequence of the methylenedioxy ring introduction in the molecule is the substantial increase of MDMA's potency to inhibit 5-HTT while reducing its potencies to inhibit DAT and NET when compared to methamphetamine (Meth) or amphetamine (Amph). Consistent with in vitro results, in vivo microdialysis experiments in the rat demonstrate that MDMA increases extracellular 5-HT and DA levels in the brain, with effects on 5-HT being greater in magnitude [28–30].

**Table 1** (±)-MDMA and (+)-Amph potencies to inhibit the human, rat, and mouse 5-HTT, DAT, and NET transporters based on the $K_i$ values (equilibrium dissociation constants)

| Drug (substrate) | Experimental model | 5-HTT $K_i$ (nM) | DAT $K_i$ (nM) | NET $K_i$ (nM) | Reference |
|---|---|---|---|---|---|
| (+)-Amph | Cloned human transporters | 38.46±3.84 | 0.64±0.14 | 0.07±0.01 | [27] |
| | Cloned mouse transporters | 23.82±1.71 | 0.56±0.11 | 0.12±0.02 | |
| | Rat synaptosomes | 3,830±170 | 34±6 | 38.9±1.8 | [25] |
| (±)-MDMA | Cloned human transporters | 2.41±0.73 | 8.29±1.67 | 1.19±0.13 | [27] |
| | Cloned mouse transporters | 0.64±0.05 | 4.87±0.65 | 1.75±0.51 | |
| | Rat synaptosomes | 238±13 | 1,572±59 | 462±18 | [25] |

Data from [27] is presented as mean ± SEM, and the values from the work of [25] as mean ± SD

*MDMA: A Substrate for Monoamine Transporters*

MDMA and other Amph derivates act as substrate-type releasers. They bind to the plasma membrane monoamine transporters, being transported and translocated into the cytoplasm, stimulating neurotransmitter release via the transporter [25, 31–33]. Specifically concerning MDMA-induced 5-HT neurotransmitter release, these studies have revealed that it occurs by two mechanisms: (1) transmitter molecules exit the cell along their concentration gradients via reversal of normal 5-HTT function and (2) cytoplasmic concentrations of transmitter are increased due to drug-induced disruption of vesicular storage [23, 24, 28, 30, 34–36]. The rapid enhancement of 5-HT release from the storage vesicles by MDMA occurs via a carrier-mediated exchange mechanism. MDMA is a substrate for vesicular monoamine transporter (VMAT) and possibly enters the vesicles via VMAT and depletes vesicular neurotransmitter storage by reversal of transporter activity [36]. Amphetamines can also deplete vesicular biogenic amine content by disrupting the pH gradient via a weak base effect that powers the transporter [37]. Accordingly, studies using cultured raphe neurons showed that the calcium-independent 5-HT release is blocked by fluoxetine, a drug that inhibits 5-HTT [23, 35]. In addition, MDMA might also increase extracellular levels of monoamines by partially inhibiting brain monoamine oxidase (MAO) activity, as evaluated in rat brain homogenates [38]. Detailed insights into the mechanism of MDMA action on the serotonergic neuronal terminal and synapse are represented in Fig. 2.

Recent studies have enlightened the mechanisms by which amphetamines interact with monoamine transporters, regulating their activity. 5-HTT expression can be rapidly modulated by receptor stimulation, second messenger production, and kinase activation. Suppression of 5-HTT activity accompanying protein kinase C (PKC) activation arises from a loss of 5-HT uptake capacity ($V_{max}$) [31, 39]. The loss in 5-HT uptake capacity correlates with a loss of surface-expressed 5-HTTs. Furthermore, PKC activation increases 5-HTT basal phosphorylation, and p38 MAPK inhibition decreases 5-HTT basal phosphorylation. 5-HTT contains consensus sites for PKC and other kinases, suggesting that alterations in 5-HTT phosphorylation state may dictate transporter localization either to surface microdomains or intracellular compartments [31, 39, 40]. PKC-mediated phosphorylation of 5-HTT occurs on the plasma membrane during the initial phase of rapid transporter

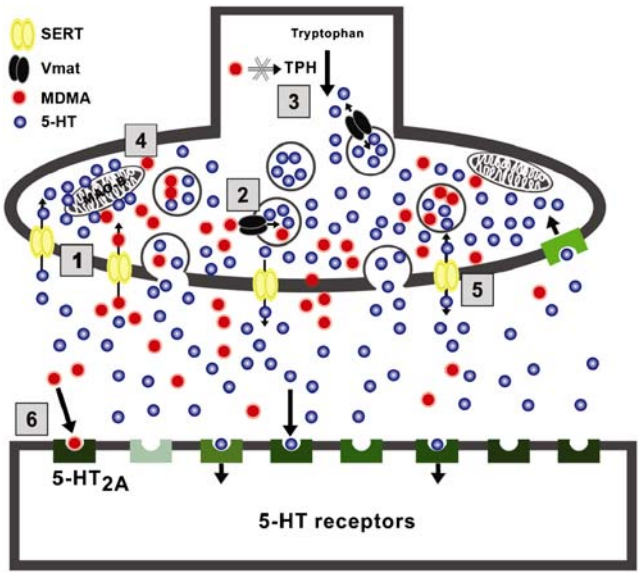


**Fig. 2** MDMA pharmacological mechanism of action at the neuronal serotonergic terminal and synapse. *1* MDMA, like serotonin (*5-HT*), is a substrate of the serotonin transporter (*5-HTT*) and uses the transporter to enter inside the neuronal terminal, although at high concentration, it may also enter by diffusion. *2* Once inside, MDMA produces an acute and rapid enhancement in the release of 5-HT from the storage vesicles, possibly by entering the vesicles via the vesicular monoamine transporter (*VMAT*) and depletes vesicular neurotransmitter stores via a carrier-mediated exchange mechanism. *3* MDMA also inhibits tryptophan hydroxylase (*TPH*), the rate-limiting enzyme for 5-HT synthesis. *4* Monoamine oxidase B (*MAO-B*), located in the outer membrane of the mitochondria of serotonergic neurons, is the enzyme responsible for 5-HT degradation and its activity is partially inhibited by MDMA. *5* Due to the increase in the free cytoplasmatic pool of 5-HT, MDMA promotes a rapid release of intracellular 5-HT to the neuronal synapse via reversal of the 5-HTT activity. *6* MDMA hallucinogenic properties depend on the agonist activity at the $5\text{-HT}_{2A}$-receptor

inhibition, and later, the phosphorylated 5-HTT enters the intracellular pool [40]. The 5-HTT substrates D-amphetamine and fenfluramine reduced 5-HTT phosphorylation to a similar extent as 5-HT. Activation of PKC results in a loss of transport capacity, sequestration of transporter proteins, or phosphorylation of multiple members of the $Na^+/Cl^-$ coupled neurotransmitter transporter gene family. Amphetamines substitute for 5-HT in suppressing PKC-mediated 5-HTT phosphorylation [31]. The same results are to be expected with MDMA. Another amphetamine, Meth, stimulates DAT phosphorylation and down-regulation by a mechanism that requires PKC, but Meth-induced down-regulation can occur independently of direct transporter phosphorylation [39]. Overall, the modulation of transporter activity and sequestration seems to be a property of all monoamine transporter substrates.

In the rat, MDMA stereoisomers are substrates for 5-HTT, NET, and DAT, with (+) isomers exhibiting greater potency as releasers. In particular, (+) isomers of MDMA and MDA (another drug of abuse and a major MDMA metabolite) are much more effective DA releasers than their corresponding (−) isomers. When compared to Amph and Meth, (+)-MDMA induced the release of 5-HT about ten times more potently than (+)-Meth, whereas (+)-MDMA releases DA about six times less potently than (+)-Meth [25, 36]. In the mouse brain, MDMA administration causes an acute release of 5-HT, as indicated by a rapid decrease in the 5-HT content [41]. There is also evidence of DA release after MDMA administration to the mouse, as the striatal contents of DA and its metabolites, homovanillic acid (HVA, 4-hydroxy-3-methoxyphenylacetic acid) and 3,4-dihydroxyphenylacetic acid, were reduced 3 h after the last of three doses of intraperitoneal (i.p.) MDMA (30 mg/kg, 3 h apart) [41].

## Hallucinogenic Properties of MDMA—Agonism at the 5-$HT_{2A}$ Receptor

Hallucinogens are considered psychoactive substances that powerfully alter perception, mood, and a multitude of cognitive processes. Agonism at the 5-HT 2A receptor (5-$HT_{2A}$ receptor) is associated with the hallucinogenic effects of substituted amphetamines and ergolines, which share some structure similarities, as it can be seen in Fig. 3 [42, 43]. Nowadays, there is a general consensus on the molecular mechanism of hallucinogens action, being their effects mainly mediated by the stimulation of the 5-$HT_{2A}$ receptor [42].

Like other hallucinogenic compounds, MDMA was found to possess an affinity for the 5-$HT_2$ receptor located in rat and human cortical neurons [43], with an estimated affinity $K_i$=5 μM [44]. Additionally, an agonist role at this receptor was established for the first time through the finding that MDMA induces phosphatidylinositol turnover in cells expressing 5-$HT_{2A}$ and 5$HT_{2C}$ receptors [45].

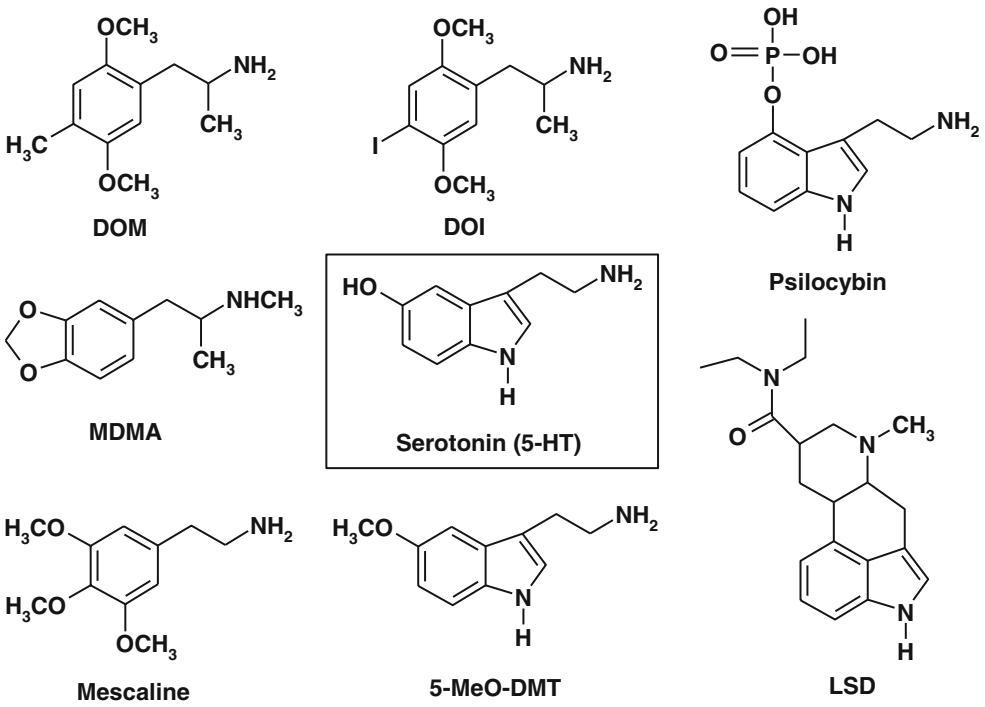


**Fig. 3** Chemical structures of hallucinogenic compounds. These structures are closely related to the endogenous neurotransmitter 5-HT. *DOI* 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, *DOM* 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane, *5-MeO-DMT* 5-methoxy-*N*,*N*-dimethyltryptamine, *LSD* D-lysergic acid diethylamide

In animal behavioral studies, the hallucinogenic properties of MDMA are well established. (+)-Lysergic acid diethylamide (LSD) did substitute for (−)-MDMA in rats trained to discriminate (−)-MDMA from saline [46, 47]. MDA, a major MDMA metabolite, was also found to have hallucinogenic properties, which further strengthens the notion that the methylenodioxy ring is important for the hallucinogenic actions of these two amphetamines. In rats trained to discriminate either (−) MDA or (+) MDA from saline, the hallucinogens LSD and (±)-2,5-dimethoxy-4-methylamphetamine (DOM) substituted for (−) MDA; additionally, LSD also substituted for (+) MDA [48]. In a latter study conducted in rats that used a different paradigm of discrimination, a three-choice drug discrimination procedure, the isomers R(−)-MDA and R(−)-MDMA, produced nearly complete substitution for mescaline; also, rats could partially substitute for LSD with both isomers of MDMA and S(+)-MDA and also near-complete substitution with R(−)MDA for LSD [49].

### MDMA Affinity for Other Receptors

The ability of MDMA to directly bind to other receptors is of physiological relevance, at least in animal models, since micromolar concentrations are attained in the brains of rats or mice after administration of this drug [50, 51].

MDMA binding affinities for the classical neurotransmitter receptors can be divided into three groups on the basis of $K_i$ values. With relative high affinity (bellow 10 μM), it binds to $\alpha_2$-adrenergic ($K_i$=4 μM) histamine 1 ($H_1$) receptors ($K_i$= 6 μM) and muscarinic 1 ($M_1$; $K_i$=6 μM). MDMA displays in the 10- to 100-μM range affinity for $M_2$ muscarinic, $\alpha_1$-adrenergic, β-adrenergic and 5-$HT_1$ receptors and above 100 μM for dopamine $D_1$ and $D_2$, opioid receptors, and benzodiazepine sites [44]. Later studies have confirmed these early findings of Battaglia and co-workers and added further significance to the interaction with these receptors for MDMA-mediated actions. In striatal brain slices and prostatic portions of the rat vas deferens, MDMA was proven to have significant $\alpha_2$-adrenoceptor agonist actions at all three receptor subtypes ($K_i$=5 μM), which may contribute to its abusive potential and cardiovascular and autonomic side effects [52]. Another study, using rat striatal slices, proved that MDMA direct stimulation of the $H_1$ receptor promoted acetylcholine release, enlightening a role of this receptor in the direct MDMA brain actions [53].

More recently, it has been demonstrated that MDMA has high affinity for brain nicotinic acetylcholine receptors (nAChR), more specifically for α7 nAChR heteromeric receptors with a $K_i$ of about 0.7 μM, which is practically the same for the 5-HT transporter ($K_i$~0.61 μM), its main physiological target [54]. According with the same authors, the nAChR receptor may account for MDMA-induced neurotoxicity, cholinergic neurotransmission, and in processes related to addiction and dependence. Therefore, a direct interaction of MDMA at recreational doses with heteromeric nAChR, $\alpha_2$-adrenoceptor, and $H_1$ receptors for which the drug has been proven to have high affinity certainly contributes to MDMA-mediated actions.

## MDMA Pharmacokinetics

Throughout this section, we will focus on MDMA pharmacokinetics and toxicokinetics in laboratory animal models and in humans.

### MDMA Pharmacokinetics: Similar Pathways in Humans and Experimental Animals

MDMA and related amphetamine compounds are weak bases with p$K_a$ values around 9.9, low molecular weight, low protein binding (around 20%), and high volume of distribution [55]. These properties confer easy diffusion across cell membranes, lipid layers, and to tissues or biological matrices with a more acidic pH compared to blood. MDMA in humans is well absorbed when taken orally in the form of tablets [56]. As far as our knowledge goes, MDMA's absolute bioavailability in humans has not been determined.

Several studies have been performed to evaluate human pharmacokinetics of MDMA in controlled settings. Following oral ingestion of MDMA by humans, maximum concentration ($C_{max}$) appears at 1.5–3 h [56]. After the administration of five different doses of MDMA (50, 75, 100, 125, and 150 mg), it could be seen that the $C_{max}$ and area under the curve (AUC) for 24 h increased according to the administered dose. Meanwhile, for the 150 mg dose, the increase in MDMA kinetic parameters was not proportional to the dose, which clearly implies nonlinear pharmacokinetics [56]. This finding was explained by a possible saturation of MDMA metabolism as well as by interaction of MDMA metabolites with the enzymes involved in its own metabolic pathways. In vitro studies suggest that MDMA can act as inhibitor of cytochrome P450 (CYP) 2D6 isoenzymes, in a time- and concentration-dependent manner, through mechanisms that include a competitive interaction and/or the formation of a metabolic intermediate complex between MDMA and this enzyme [57–59].

Farré et al. [60] have evaluated the kinetics of MDMA after administration of two repeated doses to humans (24 h apart). They found that following a second MDMA dose, the plasma concentrations increased disproportionately in comparison to the first administration, as it was seen in the increase of the pharmacokinetic parameters AUC and $C_{max}$ 77% and 29%, respectively. The observed disproportionate

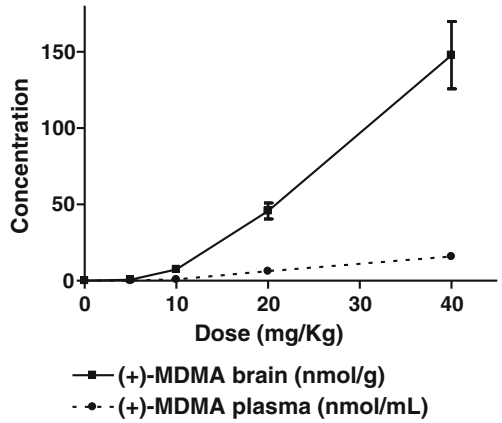


**Fig. 4** (+)-MDMA concentration time-curves in the plasma and brain as a function of the (+)-MDMA dose given to Sprague–Dawley rats. MDMA concentrations rise in a nonlinear manner with the increase of MDMA dose. The expected brain concentrations of MDMA, assuming a linear trend of concentrations with increasing doses, would be much lower than the ones actually found. The rate of MDMA conversion to 3,4-methylenedioxyamphetamine (*MDA*) by *N*-demethylation, the main metabolic pathway in rats, becomes saturated at higher dosage of MDMA. Data were taken and reworked from [50]

increase in plasma concentrations of MDMA and MDA was most likely due to the ability of MDMA to autoinhibit its own metabolism, which lasts at least 24 h [60]. The pharmacological effects after the second dose were also slightly higher than those observed after the first one in the majority of parameters including blood pressure, heart rate, cortisol concentrations, and most subjective effects [60]. The decreased contribution of CYP2D6 to MDMA clearance, as a consequence of autoinhibition, is also consistent with the relatively small inhibitory effect (about 30%) of paroxetine, a known CYP2D6 inhibitor, on MDMA clearance in humans [61].

Similarly to humans, the nonlinear pharmacokinetics of MDMA can also be found in rats. For instance, MDMA concentrations in the rat brain increase nonlinearly with the dose [50]. In Fig. 4, parallel MDMA concentration–time curves in plasma and brain according to the dose given to Sprague–Dawley rats are shown. The rate of conversion of MDMA to MDA, the main metabolic pathway in rats, which is dissimilar to humans, is linear up to doses of 10 mg/kg. However, at higher doses of MDMA, hepatic metabolism becomes saturated, via enzymatic inhibition, and the rate of *N*-demethylation slows [50]. The expected brain concentrations of MDMA in rats, assuming a linear trend of concentrations with increasing doses, would be much lower than the ones really found. MDMA properties of easy diffusion across cell membranes and lipid layers, in addition to its accumulation inside the serotonergic neurons via 5-HTT, explain its high brain concentrations relatively to plasma or even other tissues. Also, in rats, there are sex and strain differences in MDMA disposition [50]. These sex and strain differences have been attributed to the polymorphism among the hepatic CYP isoenzymes [50, 62–64]. In several rat strains, CYP2D1 is equivalent to the human CYP2D6, and this isoenzyme is absent in Dark Agouti female rats [62]. This helps to explain the higher brain concentrations of MDMA found in this strain, which is a model for a CYP2D poor metabolizer. Data corroborating sex and strain differences of MDMA disposition in rats can be visualized in the examples inserted in Tables 2 and 3.

In humans, MDMA was shown to have low protein binding (<20%) [56]. This means that almost the total amount of drug is available in the plasma and may diffuse to the extravascular compartment. A distribution volume of 452±137 L (6.4 L/kg) can be attained following 100-mg oral administration of MDMA [56]. Of note, the formulation of MDMA tablets is composed of the racemate, 1:1 mixture of MDMA enantiomers. Regarding MDMA enantiomers, the more extensive distribution of the more active (S)-(+)-MDMA enantiomer supports the suggestion of enantioselective disposition of MDMA in humans [65, 66]. Using human liver microsomes, which were baculovirus-infected insect cell microsomes containing various cDNA-expressed human CYP isoenzymes conclud-

**Table 2** Gender differences in brain disposition of (+)-MDMA and its metabolite MDA in Sprague–Dawley rats

| Dose (mg/kg) | (+)-MDMA (nmol/g) | | MDA (nmol/g) | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| 5 | 1.34±0.13 | 0.74±0.53 | 2.88±0.37 | 0.75±0.34** |
| 10 | 9.29±2.79 | 7.40±1.78 | 14.51±3.80 | 3.82±1.78** |
| 20 | 43.66±23.34 | 45.68±10.62 | 35.74±5.49 | 15.85±3.83** |
| 40 | 121.79±41.36 | 147.65±44.06 | 75.72±14.3 | 28.47±4.40** |

Data are presented as mean ± SD of four animals and were obtained from the work of [50]. Rats received a single subcutaneous (s.c.) administration of MDMA and were killed 3 h later

**$p<0.01$ male vs female

**Table 3** Strain differences in brain disposition of (+)-MDMA and its metabolite MDA in female Sprague–Dawley and female Dark Agouti rats

| Dose (mg/kg) | (+)-MDMA (nmol/g) | | MDA (nmol/g) | |
|---|---|---|---|---|
| | Dark Agouti | Sprague–Dawley | Dark Agouti | Sprague–Dawley |
| 5 | 11.77±2.46 | 0.74±0.53** | 4.73±0.73 | 0.75±0.34** |
| 10 | 19.99±6.42 | 7.40±1.78** | 7.59±1.52 | 3.82±1.78* |
| 20 | 63.79±7.02 | 45.68±10.62* | 18.05±2.56 | 15.85±3.83 |
| 40 | 114±19.74 | 147.65±44.06 | 30.03±8.42 | 28.47±4.40 |

Rats received a single s.c. administration of MDMA and were killed 3 h later. Data are presented as mean±SD of four animals and were obtained from the work of [50]

**$p<0.01$, *$p<0.05$ Sprague–Dawley vs Dark Agouti

ed that the enantioselective disposition of MDMA is mainly mediated by the CYP2C19 and CYP2D6 isoforms [67].

In humans and rats, MDMA is mainly metabolized in the liver. In humans, its major metabolic pathway includes *O*-demethylenation to 3,4-dihydroxymethamphetamine (HHMA, *N*-methyl-α-methyldopamine, *N*-Me-α-MeDA), which is a reaction mainly regulated by CYP2D6 followed by *O*-methylation to 4-hydroxy-3-methoxymethamphetamine (HMMA, 3-*O*-Me-*N*-Me-α-MeDA), a reaction regulated by catechol-*O*-methyltransferase (COMT). It has been shown that the contribution of human liver isoenzymes CYP2D6 for the *O*-demethylenation accounts for less than 60% of this metabolic step; other isozymes involved in this reaction are CYP1A2 and, to a lesser extent, CYP2B6 and CYP3A4 [68]. At a lower rate, MDMA is *N*-demethylated to MDA (a reaction mainly regulated by CYP2B6), which is further metabolized to the catechol intermediate, 3,4-dihydroxyamphetamine (HHA, α-methyldopamine, α-MeDA) and finally *O*-methylated to 4-hydroxy-3-methoxyamphetamine (HMA, 3-*O*-Me-α-MeDA) [67, 69–71]. Studies in rats suggested that *N*-Me-α-MeDA and α-MeDA can undergo oxidation to the corresponding *ortho*-quinones, which can form adducts with glutathione (GSH) and other thiol-containing compounds [72–75]. These GSH conjugates remain redox active and, as already shown in rats, can undergo the addition of a second molecule of GSH, yielding a 2,5-bis-glutathionyl conjugate [72, 76, 77]. The systemic formation of GSH conjugates of α-MeDA and *N*-Me-α-MeDA in the liver is followed by their distribution into other organs, and their uptake into the brain is well established [78]. GSH conjugates can undergo further metabolism via the mercapturic acid pathway by γ-glutamyl transpeptidase (γ-GT) and dipeptidase to the corresponding cysteine conjugates and finally by *N*-acetyltransferase to the corresponding *N*-acetylcysteine (NAC) conjugates [76–78]. A detailed pathway of MDMA metabolism is represented in Fig. 5.

Concerning the clearance of MDMA in humans, about 80% of the drug is transformed metabolically through the liver, with about 20% of the dose excreted unaltered in urine [56, 70]. Urinary excretion rate of MDMA, after administration of different doses, appeared to be rather constant. On the contrary, non-renal clearance was shown to be dose-dependent. The oral administration of 75 mg MDMA resulted in a non-renal clearance of 74.0±71.1 L/h, meanwhile for 125 mg MDMA resulted in a non-renal clearance of 38.1±13.3 L/h, which suggests an impairment in MDMA hepatic metabolism [56]. After controlled administration of different MDMA doses, the urinary recovery is approximately 60%, independently of the dose administrated. A similar recovery has been reported for *N*-Me-α-MeDA. Higher recovery rates have been detected for HMMA, the main metabolite found in urine (>20%), with less than 2% of the dose excreted as MDA [56, 70]. The elimination half-life ($t_{1/2}$) of MDMA was found to be in the range of 6–9 h [70]. An enantioselective clearance of MDMA has been described in humans. Recently, (S)-MDMA has been shown to have a short $t_{1/2}$ (4.8 h), and this component of the racemate is thought to be associated with its subjective effects and psychomotor performance. In contrast, (R)-MDMA has a much longer $t_{1/2}$ (14.8 h) in humans and is thought to be associated with mood and cognitive effects experienced on the next days after MDMA use [66]. The pharmacokinetic parameters of MDMA and its metabolites in humans after a single dose are presented in Table 4.

The importance of MDMA metabolism for its toxicity, namely, the neurotoxic events, will be addressed on a later section concerning the mechanisms of MDMA neurotoxicity ("MDMA Metabolism Produces Neurotoxic Metabolites").

## Influence of Human Polymorphisms in Metabolic Enzymes to MDMA Pharmacokinetics

Individual risk towards xenobiotic-induced toxicity can be a consequence of genetic characteristics, non-genetic variables, and of gene–environment interactions. Non-

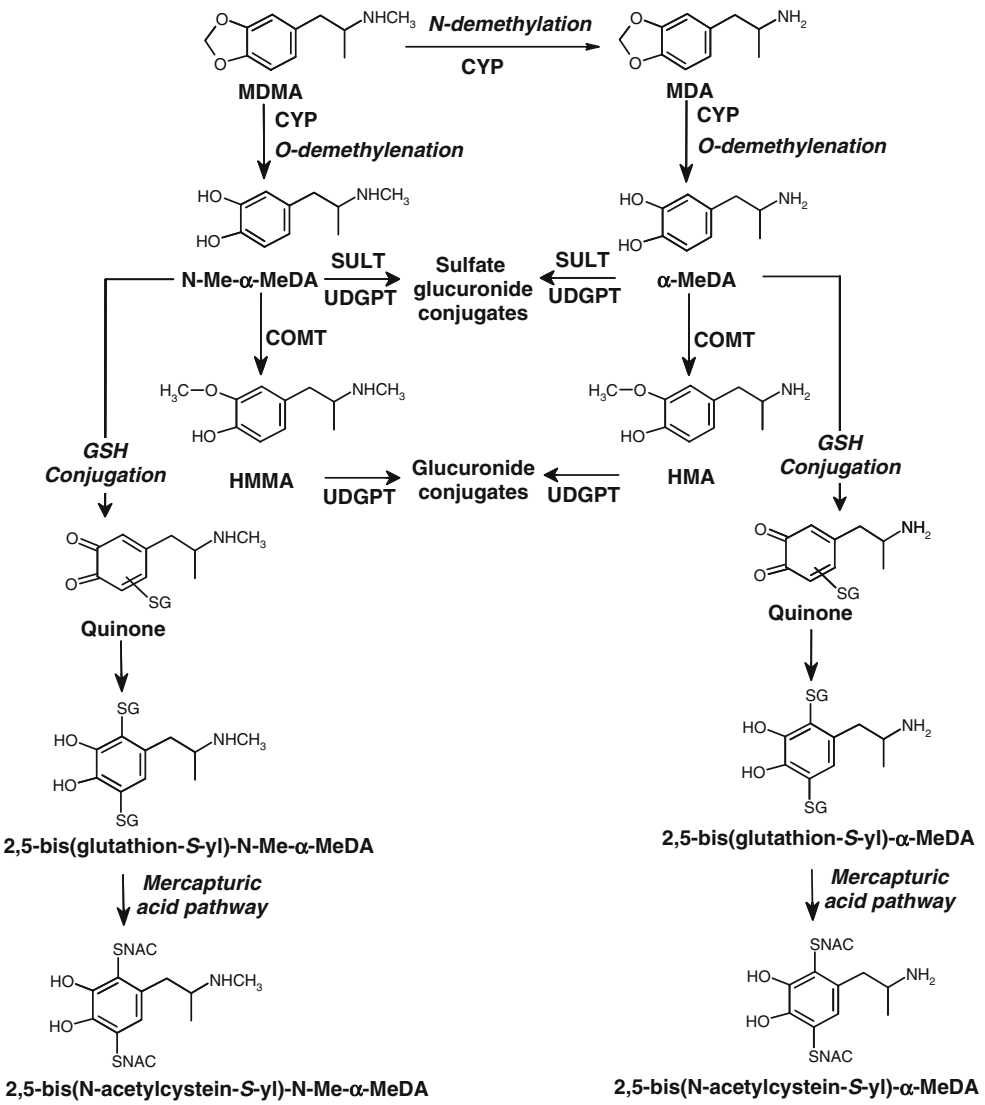


**Fig. 5** Major pathways of MDMA metabolism. The parent compound is *N*-demethylated to form MDA and *O*-demethylenated to form *N*-Me-α-MeDA, which is further *O*-methylated to HMMA. In rats, *N*-demethylation to MDA is one of the main metabolic pathways, whereas in humans, *O*-demethylenation to *N*-Me-α-MeDA predominates. Isoenzymes of the cytochrome P450 (*CYP*) are involved in the *N*-demethylation and *O*-demethylenation metabolic reactions. MDA is *O*-demethylenated to form α-MeDA. Both *N*-Me-α-MeDA and α-MeDA can undergo *O*-methylation to HMMA and HMA, respectively, a reaction regulated by catechol-*O*-methyltransferase (*COMT*). *N*-Me-α-MeDA, α-MeDA, HMMA, and HMA can suffer sulfation and glucuronidation reactions by the action of sulfotransferase (*SULT*) and uridine diphosphate glucuronosyl transferase (*UDPGT*), respectively. *N*-Me-α-MeDA and α-MeDA can undergo oxidation to the corresponding *ortho*-quinones, which can form adducts with GSH. GSH conjugates can undergo further metabolism via the mercapturic acid pathway by γ-GT and dipeptidase to the corresponding cysteine and finally by *N*-acetyltransferase to *N*-acetylcysteine (*NAC*) conjugates

genetic factors include age, gender, diet, drug therapy, drug interactions, health condition, and many others, depending on life style such as tobacco and alcohol use. All these act together with the genes of each individual that code for the pharmacokinetic and pharmacodynamic determinants that can substantially alter the disposition and effects of the xenobiotic [79, 80]. Unlike the non-genetic determinants of the toxicological response, those that are inherited tend to be stable throughout the lifetime of the individual [81]. Differences among individuals or groups of individuals towards the adverse effects as a result of any xenobiotic exposure can be attributed to stable inherited changes that occur in the human genome. Stable mutations that occur in the population at a higher frequency than 1% are termed polymorphisms. Genetic variability in metabolizing enzymes accounts for significant pharmacokinetic alterations [79]. It is reasonable to assume that some individuals may be more prone to the adverse effects of specific

**Table 4** Pharmacokinetic parameters of MDMA and its metabolites in humans

| Drug | Isomer | $C_{max}$ (μg/L) | $t_{max}$ (h) | $AUC_{0-48h}$ (μg h/L) | $t_{1/2}$ (h) | $K_e$ ($h^{-1}$) |
|---|---|---|---|---|---|---|
| MDMA | Racemate | 208.7±17.1 | 1.6±0.4 | 3,108.5±329.8 | 11.8±4.4 | 0.07±0.03 |
| | (*R*) | 116.7±14.3 | 3.5±2.2 | 2,158.8±297.5 | 14.8±9.2 | 0.06±0.04 |
| | (*S*) | 88.8±17.0 | 1.9±0.5 | 773.0±83.3 | 4.8±1.7 | 0.16±0.07 |
| MDA | Racemate | 13.0±2.3 | 6.6±1.9 | 308.4±73.1 | 17.7±6.2 | 0.04±0.01 |
| HMMA | Racemate | 163.8±71.4 | 2.8±0.8 | 2,293.2±881.5 | 10.4±2.4 | 0.07±0.01 |
| | (*R*) | 65.5±26.1 | 2.9±0.7 | 868.9±453.3 | 13.5±4.1 | 0.06±0.02 |
| | (*S*) | 62.1±21.6 | 2.6±0.6 | 585.3±216.6 | 5.9±1.0 | 0.12±0.02 |
| HHMA | (*R*) | 38.9±12.4 | 2.4±1.9 | 653.5±22.2 | 42.6±56.3 | 0.06±0.05 |
| | (*S*) | 90.9±38.8 | 2.3±1.8 | 999.2±459.0 | 7.9±2.7 | 0.10±0.04 |

Data are presented as mean ± SD of seven human volunteers after administration of a racemic (1:1) mixture of (R,S)-MDMA (100 mg). Data are taken from the work of [66]

$C_{max}$ maximum concentration, $t_{max}$ maximum time, $AUC_{0-48\ h}$ area under the curve from 0 to 48 h, $t_{1/2}$ elimination half-time, $k_e$ elimination rate constant

xenobiotics. These genotypes are frequently associated with idiosyncratic intoxications [82].

Microsomal cytochrome P450 isoenzymes are determinant for phase I oxidative metabolism of MDMA. Many of the phase I metabolites are subsequently conjugated by COMT, sulfotransferase (SULT), or UDP-glucuronosyltransferase (UGT) phase II enzymes and preferentially excreted as the respective conjugates. Most of these enzymes are polymorphically expressed and as such may influence the pharmacokinetics and, consequently, the toxicity of MDMA and of amphetamines in general.

### *Polymorphisms in Phase I CYP2D6 Enzyme*

Among all cytochrome P450 isoenzymes, CYP2D6 is one of the most studied in terms of genetic variability. Variability in CYP2D6 activity in the human liver is mainly attributed to genetic polymorphism [83], and many of the genetic variants responsible for changes in the activity of the enzyme have been extensively characterized.

Allele *CYP2D6*4* is the most frequently associated with poor metabolizer phenotype, with an allele frequency between 12% and 21% in Caucasian populations [84]. It contains seven mutations relative to the wild-type *CYP2D6*1* allele. This allele is practically absent in the oriental populations, which explains the low incidence of the low metabolizer phenotype among these populations [84]. In contrast, allele *CYP2D6*5*, which corresponds to a total deletion of the *CYP2D6* gene, has a very similar frequency between populations of different ethnic origin [84].

Besides these two alleles, a number of rare alleles associated with the poor metabolizer phenotype have been identified. The expression of allele *CYP2D6*9* originates an enzyme with lower catalytic activity [85]. Allele *CYP2D6*10* is particularly prevalent among Asian populations (with a frequency around 50%) [86], and for this reason, these populations have, in average, a lower metabolic capacity for this isoenzyme in spite of the lower incidence of the poor metabolizer phenotype (around 1%) compared to the Caucasian populations. This variant originates a deficiency in the tertiary and quarternary structures of the protein and, consequently, a significantly diminished expression of the functional enzyme [86]. Allele *CYP2D6*2* is associated with the intermediate metabolizer phenotype in Caucasian populations [87] with a frequency between 25% and 35% [88, 89]. The presence of this allele is also elevated in some African populations, mainly in Ethiopia (prevalence of 10–29%). Allele *CYP2D6*17* was identified in a population from Zimbabwe [90]. Carriers of this allele show lower metabolic capacity for CYP2D6. Variant *CYP2D6*17* seems to be more frequent in black African and Afro-American populations (prevalence around 34%) [88, 90].

Alleles with two, three, four, five, and 13 copies of the *CYP2D6* gene have also been described, and the number of individuals carrying these multiple gene copies is higher in populations from Ethiopia and Saudi Arabia where up to one third of the population carries this genotype [84]. Interestingly, the incidence of deficient CYP2D6 alleles is very low in these geographical regions [84]. These variants show a 29% frequency in Ethiopians [91] and around 1% to 5% in Caucasians [84]. An incidence of around 10% in this ultrarapid metabolizer phenotype was demostrated in Italian and Turkish populations, while it seems to be almost absent in northern European populations [84]. Data from the western European population point to an overall incidence of 5.5% of this phenotype [92].

The influence of CYP2D6 polymorphism in the pharmacokinetic changes of MDMA has been reported both in

vitro [49, 88, 93–95] and in vivo [96]. Tucker et al. [93] showed that MDMA demethylenation was substantially compromised in liver microsomes obtained from an individual whose genotype indicated he was a poor metabolizer for the isoenzyme. The formation of the *N*-Me-α-MeDA metabolite in these microsomes was significantly lower than that observed in the other microsomal preparations obtained from extensive metabolizers. Another study was performed in vitro using a baculovirus expression system. The authors compared the catalytic activity of wild-type *CYP2D6*1* and the allelic variant *CYP2D6*10* towards several substrates and/or inhibitors, including MDMA and *p*-methoxyamphetamine [94]. The mutated CYP2D6*10 enzyme showed a significantly lower capacity for MDMA metabolism (by opening of the methylenedioxy ring followed by the catechol formation) and also for *p*-methoxyamphetamine (by demethylation of the methoxy group) when compared to the wild-type CYP2D6*1 enzyme ($V_{max}/K_m$ CYP2D6*1/CYP2D6*10 ratio of 34 for *p*-methoxyamphetamine demethylation and 123 for MDMA demethylenation) [94]. MDMA also inhibited the wild-type enzyme more efficiently ($K_i$ CYP2D6*10/$K_i$ CYP2D6*1 ratio of 21), which indicates a decrease in the affinity of this substrate towards the mutated enzyme variant [94]. The same group later investigated the demethylenation of MDMA in microsomes expressing variants CYP2D6*2, *10, and *17, all of them associated with a decrease in enzyme activity relative to the wild-type CYP2D6*1 [88]. Variants *2, *17, and *10 showed a reduction in MDMA metabolism towards the CYP2D6*1 enzyme of 15-, 13-, and 135-fold, respectively [88]. Beyond the marked differences in the catalytic activity of the different variants, the interactions of MDMA with ten different CYP2D6 inhibitors, which were likely to be consumed together with the drug, were also tested. Among those, fluoxetine and paroxetine (selective serotonin reuptake inhibitors antidepressant drugs) and cocaine strongly inhibited MDMA demethylenation, which anticipates the possibility of pharmacological interactions with repercussions in MDMA toxicity arising from possible metabolism reduction and increase in MDMA plasma concentrations [88].

Lin and co-workers conducted an in vitro study where a decrease in MDMA demethylenation was observed in liver microsomes obtained from a poor metabolizer relative to four other microsomal preparations obtained from extensive metabolizers [49]. The formation of GSH adducts with the MDMA and MDA catechol metabolite, α-MeDA, in human liver microsomes was also dependent on the CYP2D6 activity of the tested donors that were previously phenotyped with the substrate bufuralol [95].

These pharmacokinetic changes, observed in vitro, were more recently evaluated in vivo [71]. During a clinical trial where MDMA was repeatedly administered in two 100-mg doses with a 24-h interval period, one of the genotyped participants was found to carry two non-functional CYP2D6 alleles (*CYP2D6*4/*4*) and therefore identified as a poor metabolizer. The remaining nine participants in the trial were all identified as extensive metabolizers. Among these, three were also carriers of the *CYP2D6*4* allele (*CYP2D6*1/*4* genotype). When the pharmacokinetic parameters of MDMA were compared between the individuals homozygous for the *CYP2D6*1*, heterozygous for the *CYP2D6*1/*4* alleles, and homozygous for the *CYP2D6*4* allele, it was concluded that the pharmacokinetics of MDMA and its main metabolites including the catechol, *N*-Me-α-MeDA, and its mono-*O*-methylated derivative originated by COMT varied markedly according to the genotype [71]. It was observed that the MDMA plasma concentrations were significantly higher for the poor metabolizer, while the demethylenated metabolite production was significantly decreased. For the individuals genotyped as wild-type for CYP2D6, the time for the maximal plasma concentration of the *O*-methylated-*N*-Me-α-MeDA metabolite was lower than that of MDMA. This was not observed for the poor metabolizer, indicating that in this case, first passage metabolism was annulled [71]. The pharmacokinetic parameters of MDMA and metabolites were also markedly different in the case of those individuals that were genotyped as heterozygous for the *CYP2D6*1/*4* alleles and presented intermediate values between those observed with the *CYP2D6*1/*1* and *CYP2D6*4/*4* genotypes, with statistically significant differences towards both groups. Other interesting observations in this study was the increase in body temperature observed in the poor metabolizer when compared to the other nine participants, as was the lack of an increase in prolactin release in response to the MDMA administration that was observed in the extensive metabolizers. It was proposed that these effects could be related with the genotype, and as such, a higher risk for the occurrence of hyperthermia could be anticipated in individuals with the *CYP2D6*4/*4* genotype [71]. The pharmacokinetic differences among the genotypic groups were only observed during the 24 h after the first administration of the drug. After the second MDMA dose, and likely due to the CYP2D6 inhibition, the differences among different genotypes were abolished [71].

All these in vitro and in vivo studies allowed the anticipation that pharmacokinetic differences, resulting from the polymorphic expression of CYP2D6, could strongly influence the acute and chronic toxicity of MDMA. It was suggested that the poor metabolizers could be at an increased risk for the MDMA direct toxic effects that are associated with abnormally elevated MDMA plasma concentrations, including the hyperthermic and cardiovascular effects of the drug. However, the concern

that the accumulation of MDMA in the blood circulation as a result of a deficient metabolic clearance of the drug would result in the increase of toxic acute reactions was not supported by three studies that attempted to associate the poor metabolizer phenotype and corresponding genotype to the occurrence of such intoxications [97–99]. The retrospective study conducted by O'Donohoe et al. [98], where two different mutations for the *CYP2D6* gene were identified in a control population of 160 individuals and in a small population of seven individuals intoxicated with MDMA, revealed that none of the intoxicated individuals were homozygous for any of the mutations. Only one of the individuals was heterozygous carrying a mutated allele. This study, for which the principal limitations were the small population of MDMA intoxicated individuals and the low number of the investigated mutations, did not allow to conclude about the possible influence of the CYP2D6 genotype in the toxicity induced by MDMA. The authors suggested that the absence of correlation between the MDMA ingested dose and the occurrence of intoxications could be better explained by the possible presence of potentially toxic contaminants in the formulations ingested or other environmental and/or physiological factors that remained to be determined [98]. The authors of a similar study in which three individuals presenting severe hepatotoxicity caused by MDMA alone (other possibilities for this clinical condition were all ruled out) likewise concluded that there was no association between the CYP2D6 genotype of the individuals and the toxic reaction [99]. In this study, the genotyping was more comprehensive than that performed in the former study and included the investigation of five functional alleles (*CYP2D6*1*, **2*, **2xN*, **9*, and **10*) and seven non-functional alleles (*CYP2D6*3*, **4*, **6*, **7*, **8*, and **16*). All three individuals were characterized as extensive metabolizers (genotypes **1/*4*, **2/*5*, and **1/*1*) [99]. However, the presence of the **4* allele in one of the individuals and of the **2* and **5* alleles in another could be associated with the intermediate metabolizer phenotype or even with the low metabolizer phenotype for the **2/*5* genotype. Again, the limited number of the individuals in the study hindered the establishment of a causal relationship between genotype and toxicity. In a study performed later by Gilhooly and Daly [97], 15 samples of fatally intoxicated individuals were analyzed (14 samples of liver tissue and one blood sample). Thirteen of these samples could be genotyped for both deficient alleles *CYP2D6*3* and **4* and another one only for allele **4*. None of the samples belonged to a homozygous for the deficient alleles. Five of these individuals expressed one of the deficient alleles (four individuals with **1/*4* genotype and one individual with **1/*3* genotype) [97]. The allele frequency for *CYP2D6*3* and **4* found for these 15 individuals was similar to that found in a control population of 662 individuals [97]. Also, the frequency of the heterozygous genotype among the study and control populations did not differ. Therefore, in spite of the number of intoxicated individuals tested in this study being still considered low, a lack of correlation between CYP2D6 genotype associated with deficient metabolism and the occurrence of MDMA intoxications was once again noted. This lack of association noted in three independent studies [97–99] indicates that the expression of mutated variant enzymes responsible for a low metabolic capacity of CYP2D6 is not likely to be responsible for the increase in susceptibility towards the acute toxic effects of MDMA as a result of the increase in the plasma concentrations of the drug.

An alternative proposal to explain the lack of correlation between the amount of MDMA ingested and the magnitude of the produced toxic reactions was the nonlinear pharmacokinetics of MDMA that results from the mechanism-based inhibition of CYP2D6 through the formation of a complex with the enzyme [57, 59]. This was observed in two clinical trials where MDMA was administered in either a single dose [100] or in two doses separated by a 24-h period between administrations [96]. In both cases, the rise in the plasma concentrations of the drug was not proportional to what could be expected when different doses were tested, or when the second dose was administered, and could not be explained by the simple accumulation of the drug. Also, the magnitude of the cardiovascular, neuroendocrine, and subjective effects recorded in these studies was not proportional to the increases in plasma concentration [96, 100]. This strongly suggests that other factors beyond plasma concentration influence MDMA-related intoxications. Additionally, as CYP2D6 regulates the biotransformation of many therapeutic drugs, it may also be the source of a number of drug interactions with MDMA [101]. Therefore, it is particularly important regarding human toxicity to pay special notice to the concomitant use of other drugs with MDMA, including prescribed pharmaceuticals that may interact with the CYP isoenzymes. Concomitant intake of drugs has the potential to be the source of MDMA-related toxic events.

It is therefore reasonable to assume that the metabolic bioactivation can be crucial for the toxic effect of this drug of abuse, since it was suggested that ultrarapid metabolizers could be at an increased risk associated with highly cytotoxic metabolite formation. Additional in vitro studies were performed to shed some light on the possible implications of pharmacokinetic changes in the toxic effects of MDMA. These studies used genetically modified V79 fibroblasts for the expression of the wild-type and mutated variants of CYP2D6 and included two control V79 cell lines without cytochrome P450 activity and another cell line expressing CYP3A4 (the most abundant xenobiotic

metabolizing enzyme). These V79 cell lines provide a good in vitro model for the study of the consequences of the human polymorphic expression of metabolizing enzymes in the cytotoxic effects of their substrates [102]. In these studies, it was clearly demonstrated that CYP2D6 participates in the bioactivation of MDMA by producing highly toxic metabolites. In fact, when the cells were transfected with the wild-type *CYP2D6*1* allele and had a higher metabolic capacity, the cytotoxic effects of the drug were much higher than those observed in the cell lines transfected with the less active variants (*CYP2D6*2*, **9*, **10*, and **17*) and also in the control and CYP3A4 cell lines [103]. Similar results were obtained with another amphetamine drug of abuse, 4-methylthioamphetamine (chemical structure in Fig. 1) [104]. It was shown that the CYP2D6 polymorphism could greatly influence toxicity through the increase in the formation of cytotoxic metabolites. For MDMA, the metabolite responsible for the enhanced toxicity was the catechol metabolite *N*-Me-α-MeDA. This metabolite proved to be 100-fold more toxic than the parent drug MDMA when tested under the same experimental conditions [103]. The cytotoxic concentrations and effects of MDMA in the V79 fibroblasts were very similar to those observed previously in other cellular models including mouse hepatocytes [105, 106]. The in vitro model used by Carmo and co-workers accounts only for the CYP2D6 catalyzed production of the oxidative metabolites. However, in vivo, other enzymes including CYP1A2, CYP2B6, and CYP3A4 [68, 69] may also contribute to this cytotoxic mechanism.

### *Polymorphisms in Phase II Enzymes COMT, SULT, UGT, and GST*

The inter-individual variability in the susceptibility to the toxic effects of MDMA may not be only a consequence of the enhanced metabolite formation of toxic phase I metabolites but also of a decrease in their detoxification and/or of the reactive species that are formed alongside through phase II reactions. These multiple polymorphisms that affect different proteins in different metabolic reactions can act together to increase predisposition towards toxicity or, on the contrary, in an antagonistic manner resulting in a normal phenotype. In fact, in spite of the major influence of single polymorphisms in key proteins that regulate the toxic mechanism of a given substance, the toxicological response results from the interaction of several different genes that code for proteins that are involved in multiple pathways of the disposition and effects of the substance. It is therefore reasonable to assume that a rare combination of different genotypes can be responsible for rare, but frequently more severe, adverse reactions that occur with the ingestion of MDMA. Besides their polymorphic expression, the possibility of saturation of these conjugating phase II enzymes should also be considered. The in vivo increase in the release of the endogenous catecholamines as the result of the biological action of MDMA can saturate the metabolic capacity of the conjugating COMT, SULT, and UGT enzymes, thus increasing the plasma concentration of the metabolites.

A G1947A substitution in the *COMT* gene that results in the valine108methyonine substitution in the soluble COMT and in the valine158methyonine substitution in the membrane-bound COMT produces an enzyme with low catalytic activity [107–109]. Among Caucasians, COMT activity varies substantially with 25% of the population showing a high activity phenotype, while another 25% present a reduced enzyme activity phenotype [110]. The activity of the enzyme is substantially different for different ethnic origins. COMT activity is lower in Caucasian than in African, Afro-American, and Asian populations, which can be explained by a higher frequency of the mutated alleles in Caucasians [110–114]. Given the difference in enzyme activity with the polymorphic expression of COMT, it can be anticipated that it could be of consequence for the toxicity of MDMA. The decrease in the capacity of the metabolic inactivation of the endogenous catecholamines that are released as a consequence of MDMA can contribute to increased toxicity due to a sustained period of action and also by facilitating the chemical autoxidation of the catechols, which form highly reactive species that are deleterious for the cells. Such an assumption was supported by a study that compared the effect of D-amphetamine in the brain of individuals that were genotyped for the val/met mutation and grouped according to their genotype (val/val, val/met, or met/met) [115]. The results of this study, where the effects of D-amphetamine were monitored with brain imaging techniques, showed that the individuals homozygous for the mutation (genotype met/met) were more prone to the neurotoxic effects of the drug [115].

The human SULT enzymes that conjugate both the catechol metabolites of MDMA [69] and the endogenous neurotransmitter catecholamines are also polymorphically expressed [116, 117]. Several polymorphisms have been described for different isoenzymes of the SULT family, including SULT1A (four variants) [117], SULT1C1 (three variants) [116], and SULT2A (three variants) [117]. Although there are no studies that prove the importance of these polymorphisms in the detoxification of MDMA or any other amphetamine, some of these polymorphisms seem to be functionally important, affecting both the stability and the catalytic activity of these isoenzymes [116–118]. Their clinical relevance remains to be elucidated [118]. However, in the case of amphetamines and of MDMA in particular, it can be anticipated that the decrease in the enzymatic activity resulting from these polymor-

phisms could be probably associated with increased susceptibility towards the adverse effects of the drug due to a prolonged lifetime of the toxic metabolites that are less efficiently eliminated.

The same observation can be made in relation to other enzymes involved in the detoxification of the MDMA metabolites through glucuronide conjugation. The enzyme activity of the UGT shows a great inter-individual variability among the population [118, 119]. Since this is the most important phase II reaction in human metabolism, this variability has been associated with some pathologies including the Gilbert's syndrome (as a result of the deficient bilirubin glucuronidation) and to the decreased metabolism of several drugs (e.g., paracetamol) and the consequent increase in susceptibility towards their adverse effects. The polymorphisms described for UGT are a consequence of either a TA base insertion in the TATA-box that affects the levels of the enzyme expressed [118, 119] or of single nucleotide polymorphisms that code for amino acid substitutions that modify the catalytic activity of the enzyme [118, 120–124]. As with the SULT enzymes, there are still no studies that address the influence of this polymorphic expression of the cytotoxicity of MDMA. However, it is reasonable to assume that the expression of the less active polymorphic variants can increase the risk towards the occurrence of intoxications given the importance of this enzyme in the metabolic clearance of the reactive MDMA oxidative metabolites.

Other enzymes involved in phase II metabolism, that play a crucial role in the detoxification of reactive species formed due to oxidative stress, are glutathione S-transferase (GST) that catalyzes the conjugation of electrophilic compounds with GSH. This enzyme also binds directly to the substrates, hindering their cellular damage in a process that can inhibit the enzyme or not. It is also inducible and polymorphically expressed. These polymorphisms consist either in gene deletions (for GSTM1 and GSTT1 enzymes) that cause loss of enzyme activity [125, 126] or single nucleotide polymorphisms (GSTP1) that result in a decreased enzymatic activity [118, 127]. The genetic duplication of the GST has also been documented and consists in the expression of a rare allele that consequently increases the enzymatic capacity [128]. While the decrease in the enzyme activity of the GST enzymes can be associated with an increased risk towards toxicity because of their role in the detoxification of the electrophilic species resultant from the oxidative metabolism of MDMA, the increase in this detoxifying potential through the genetic amplification mechanism is expected to have a protective effect against these reactive species. In a study conducted in Japan in which the frequency of the mutated allele for the GSTP1 enzyme was compared between a population of individuals consuming Meth and a control population, a significant difference in the frequency of the mutated and wild-type alleles between these two populations was detected [129]. A correlation between the genotype frequency and the psychosis induced by the drug was also found [129]. It was proposed that this polymorphism could contribute to a higher vulnerability to the occurrence of psychosis induced by Meth abuse in the Japanese population [129].

Contrary to what is expected with the decrease in enzyme activity of phase II enzymes involved in the detoxification of the reactive MDMA oxidative metabolites that seem to be, at least partially, responsible for its toxicity (see "MDMA Metabolism Produces Neurotoxic Metabolites"), the decrease in the catalytic activity of phase I enzymes that can contribute to the in vivo formation of these metabolites can be regarded as protective against the toxic action of the drug. Besides CYP2D6, other CYP enzymes including CYP1A2, CYP2B6, and CYP3A4 can potentially influence the metabolic bioactivation of MDMA [68]. Contrary to CYP2D6, these enzymes are highly inducible, and therefore, in what their genetic variability is concerned, they are not only affected by the mutations in the genes that are responsible for their expression but also by those coding for the proteins involved in the complex process of enzyme induction [68].

From what has been exposed, it can be concluded that the genetic variability can be responsible for the great inter-individual variability towards the biological and toxic effects of MDMA and other amphetamines, including its neurotoxic potential. The high variability and rarity of severe acute intoxications as compared to the wide universe of abusers can be, at least partially, explained by a polygenic component of the underlying toxicity mechanisms. The possibility of a rare combination of genotypes that results in a highly susceptible phenotype cannot be overlooked only for the neurotoxic action of the drug but also for the occurrence of severe and frequently fatal acute reactions, which seem to be independent from the dose ingested and the plasma concentrations attained. However, the genetic variability is not only influenced by gene interactions but also by non-genetic and environmental factors. There is also a strong possibility of drug interactions associated with the pattern of MDMA abuse (with multiple drug ingestion including several other amphetamines, alcohol, cannabis, cocaine, opiates, and therapeutic drugs, among many others), which contribute to the variability in the pharmacokinetics and to the toxicological response. On the other hand, the behavioral and environmental factors associated with amphetamine abuse (e.g., elevated ambient temperature, loud music, intense physical effort) may also potentiate the toxicity of the drug. For all these reasons, the issue of variability in the toxicological response towards MDMA remains open. However, the

unraveling of the mechanisms that underlie this variability at the genetic level may in the future translate into an important tool for the comprehension and prediction of the higher or lower likelihood of developing a toxic reaction upon MDMA consumption or enhanced predisposition towards the long-lasting neurotoxic effects of the drug. Based upon this knowledge, molecular diagnosis tests can be developed for the identification of the more susceptible individuals according to their genetic determinants.

### Interspecies Differences in MDMA Pharmacokinetics

Comparing MDMA metabolism in humans and laboratory experimental animals, with the remarkable exception of mice, their major metabolic reactions are qualitatively similar. However, the rate and importance of those metabolic pathways show relevant quantitative differences [96]. In non-human primates, namely squirrel monkeys, the typical nonlinear pharmacokinetics for MDMA was established using doses of 0.4, 0.8, 1.6, and 2.8 mg/kg, which are equivalent to human doses [130]. Additionally, studies using this animal model indicate that nonlinear MDMA accumulation occurred at plasma MDMA concentrations of 100 to 300 ng/ml and above [130]. Also, in adult rhesus monkeys administrated with 10 mg/kg of MDMA, twice daily, for four consecutive days, MDMA plasma concentrations increased by 30%, and MDA plasma concentrations increased by 200% compared with levels present following the first dose of MDMA [131].

In contrast to nonlinear pharmacokinetics, which seems to occur both in laboratory animals and humans, the importance of the metabolic pathways *N*-demethylation and *O*-methylenation of MDMA differ among species. In the aforementioned study using adult rhesus monkeys, MDA concentrations were as high as 18% of the MDMA concentration [131]. In contrast, MDA plasma concentrations in humans are usually less than 5% of the MDMA concentration following oral MDMA administration [56]. Following MDMA administration to mice, MDMA is the main chemical species observed in both plasma and brain [132]. In contrast, although MDMA is observed in the plasma of rats and humans following its administration, *N*-Me-α-MeDA and HMMA metabolites are also present in high concentrations [96]. In rats, the *N*-demethylation of MDMA leading to the formation of MDA is one of the main metabolic pathways at low doses [50], whereas in humans, the *O*-demethylenation of MDMA to *N*-Me-α-MeDA predominates at any tested dose [56].

In humans, the enantioselective step in MDMA metabolism is the *O*-demethylenation, mainly regulated by CYP2D6, while in rats, it is associated with *N*-demethylation, a metabolic pathway that accounts for only 8% to 9% of the MDMA concentration in humans [56]. Moreover, the MDMA metabolizing liver CYP isoenzymes differ between rats and humans [69]. For instance, the human CYP2D6 is equivalent to the rat CYP2D1, and this isoenzyme is absent in Dark Agouti rats [62]. Figure 6 presents the CYP isoenzymes responsible for the *O*-demethylenation and *N*-demethylation steps of MDMA metabolism in rats and humans. The differences in MDMA metabolism between laboratory experimental animals and humans bring difficulties to the extrapolation of experimental data from investigation models to the human situation.

## MDMA Acute Effects

### MDMA-Induced Acute Effects to Experimental Animals

The acute effects of MDMA to the more studied laboratory animals, namely mice, rats, and non-human primates, include, most importantly, hyperthermia, hyperactivity, and the 5-HT behavioral syndrome.

#### *MDMA-Induced Hyperthermia to Experimental Animals*

MDMA was shown to cause acute dose-dependent hyperthermia in many laboratory animals, namely, in mice [51, 133–141], rats [142–156], in rabbits [157, 158], guinea pigs [159], and pigs [160]. In unrestrained non-human primates, MDMA was also shown to dose-dependently increase body temperature, leading to hyperthermia [161–164]. The acute hyperthermia produced by MDMA in laboratory experimental animals is one of the few effects that can be directly compared to humans.

The issue of MDMA-induced hyperthermia is complex. Though of clear importance, the fundamental biological mechanisms involved in heat production and progression to hyperthermia after MDMA exposure are not clearly

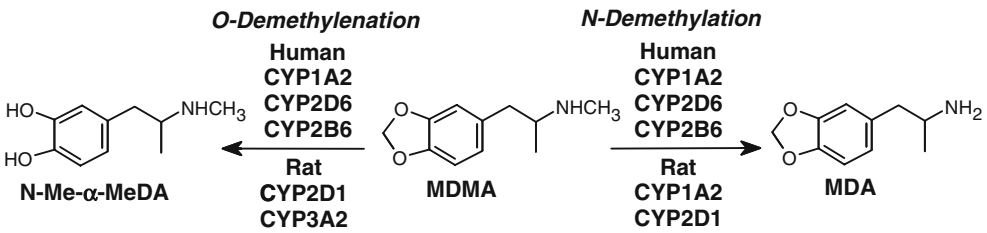


**Fig. 6** Major CYP isoenzyme-dependent MDMA metabolic pathways in humans and rats. Data are taken from [69] and [55]

understood. Furthermore, we do not fully comprehend the associations between hyperthermia and many of the pathological changes induced by MDMA. In experimental laboratory animals, there are reports confirming that ambient temperatures can influence the effect of MDMA and other amphetamines on body temperature. That has been confirmed for mice [133, 165], rats [142–144], and was also recently established in monkeys [162–164]. In particular, rats treated with 20 mg/kg MDMA and exposed to low environmental temperature (less than 22°C) tend to develop hypothermia, meanwhile, under high environmental temperature (higher than 28°C) can reach life-threatening hyperthermia [143]. C57BL/6J mouse injected with 20mg/kg MDMA developed hyperthermia under the environmental temperature of 22°C, but showed hypothermic temperatures under conditions of 15°C [165]. In contrast to results in rodents, rhesus macaques administrated intramuscularly with 0.56–2.4 mg/kg MDMA displayed a similar degree of hyperthermia across a range of environmental conditions (18°C to 30°C) [162]. These findings point out to differences among species regarding temperature responses to MDMA challenge. Overall, when evaluating the thermoregulatory effects of MDMA, one must take into account not only the animal model species but also the environmental temperature at which the experiment is conducted.

*Role of 5-HT and DA Receptors in MDMA-Induced Hyperthermia* The mechanisms of MDMA-induced hyperthermia are multifactorial and appear to involve a combination of both the serotonergic, dopaminergic, and also the adrenergic function. It was shown that administration to rats of the selective 5-HT uptake inhibitor, fluoxetine, almost totally inhibited the increase of extracellular 5-HT, as measured by in vivo microdialysis but, had no effect on the hyperthermic response in those animals, which accounts for a possible DA role [149, 166, 167]. Furthermore, the observation that the dopamine $D_1$ receptor antagonist, SCH 23390, dose-dependently inhibits MDMA-induced hyperthermia in mice [168] and rats [148] also suggests a role of DA in this effect. Agents with 5-HT receptor antagonist properties have been used, including selective and non-selective 5-$HT_{2A}$ and 5-$HT_{2C}$ antagonists, and proven to block MDMA-induced hyperthermia in rats, indicating that 5-HT may also be involved in the rat hyperthermic process [148, 157, 158]. Spinal 5-$HT_{2A}$ receptors contribute to the cutaneous vasoconstricting action of MDMA and clozapine and olanzapine, which have 5-$HT_{2A}$ receptor antagonist properties, and could reverse hyperthermia and cutaneous vasoconstriction occurring both in rats and rabbits [157, 158, 169]. Administration of risperidone before and after MDMA administration to rats (10 mg/kg, s.c.) significantly suppressed MDMA-induced hyperthermia in a dose-dependent manner [154]. Also, this recent study by Shioda et al. [154] sought to determine whether the blocking of MDMA-induced hyperthermia in rats by risperidone was dependent on the inhibition of the 5-$HT_{2A}$ or $D_1$ receptors. Rats' pretreatment with ritanserin, ketanserin, or R-96544, all of which are specific 5-$HT_{2A}$ receptor antagonists, significantly prevented MDMA-induced hyperthermia. Furthermore, pretreatment with WAY-100635 (a 5-$HT1_A$ receptor antagonist), SB 206553 (a mixed 5-$HT_{2B/2C}$ receptor antagonist), or SB 242084 (a specific 5-$HT_{2C}$ receptor antagonist) did not prevent MDMA-induced hyperthermia, which agrees with the mechanistic dependence from the 5-$HT_{2A}$ receptor activation [154]. On the other hand, rats' pretreatment with haloperidol, which blocks both the DA receptors $D_2$ and $D_1$, significantly prevented, meanwhile sulpiride and L-741626, which are $D_2$ receptor blockers, did not prevent MDMA-induced hyperthermia. In agreement with previous works in rats and mice [148, 168], pretreatment with SCH 23390 (a $D_1$ receptor antagonist) significantly prevented MDMA-induced hyperthermia [154]. Together with previous studies on MDMA hyperthermic effects, results demonstrate that the mechanism underlying MDMA-induced hyperthermia is clearly dependent on 5-$HT_{2A}$ and $D_1$ receptors activation. Drugs like risperidone or olanzapine, with 5-$HT_{2A}$ and $D_1$ receptor antagonist properties, could be of great clinical value in the therapeutic management of human MDMA hyperthermic actions.

*Role of Adrenoreceptors in MDMA-Induced Hyperthermia* The importance of the thermogenesis mediated through the activation of α- and β-adrenoreceptors in MDMA-induced hyperthermia has been recognized. Fischer 344 rats that suffered prior adrenalectomy (1 week beforehand) or were pretreated (30 min before each of the two injections of MDMA 10 mg/kg, s.c.) with the ganglionic blocker chlorisondamine (2.5 mg/kg) showed a reduction in the immediate hyperthermic effect induced by MDMA administration [155]. In the same report, supplementation of adrenalectomized rats with corticosterone almost reinstated the immediate hyperthermic effect of MDMA [155]. Sprague–Dawley rats' concomitant pretreatment with both $\alpha_1$-adrenoreceptor plus $\beta_3$-adrenoreceptor antagonists abolished MDMA-induced hyperthermia [156, 170]. Also in rats, neither propranolol nor nadolol (nonselective $\beta_1$- and $\beta_2$-adrenoreceptor antagonists) when administered 30 min before MDMA affected the thermogenic response; in contrast, carvedilol (nonselective $\alpha_1$- and β-adrenoreceptor antagonist), either injected 15 min before or after MDMA, prevented its hyperthermic response [171]. More recently, it was shown that more than one subtype of $\alpha_1$-adrenoceptor is involved in a component of the hyperthermic response to MDMA in mouse, probably mediated by both $\alpha_{1A}$- and

$\alpha_{1D}$-adrenoceptors [172]. Additionally, MDMA was able to produce significant hyperthermia in $\alpha_2$-adrenoceptor knockout (KO) mice [138]. This interesting result must be regarded in the light that MDMA is an $\alpha_2$-adrenoceptor agonist and $\alpha_2$-adrenoceptor agonists such as clonidine produce hypothermia [52, 138].

*Role of Thyroid Hormone and UCP in MDMA-Induced Hyperthermia* Sprague et al. [156] support that the mechanisms of thermogenesis induced by MDMA result from an interaction between the hypothalamic–pituitary–thyroid axis and the sympathetic nervous system, leading to core and skeletal muscle hyperthermia. Thyroid hormone is the primary endocrine regulator of metabolism and thermogenesis, and studies suggest that it might be linked to the hyperthermic response to MDMA. Sprague–Dawley rats treated with MDMA (40 mg/kg, s.c.) responded with a significant increase (maximal at 1 h) in rectal and skeletal muscle temperatures that lasted for at least 3 h posttreatment [156]. Hypophysectomized and thyroparathyroidectomized rats treated with the same dose of MDMA did not become hyperthermic and in fact displayed a significant hypothermia. When thyroid hormone was replaced to thyroparathyroidectomized rats, they returned the hyperthermic response, arguing for a role of the hypothalamic–pituitary–thyroid axis in the hyperthermic effects of MDMA [156]. At the cellular level, thyroid hormone seems to play both a permissive and synergistic role in NE-mediated thermogenesis, which might be mediated by a family of mitochondrial uncoupling proteins (UCP) [156]. The activation of the skeletal muscle thermogenic protein, UCP-3, demonstrated to have an important role in MDMA-induced hyperthermia [139]. Mice deficient in a mitochondrial protein, known as UCP-3 (for "uncoupling protein-3"), have a diminished thermogenic response to MDMA and therefore are protected against its toxic effect [139]. Additionally, it seems that rat Sprague–Dawley females are less sensitive to the hyperthermic effects of MDMA (20 mg/kg, s.c.) than rat males, this event being also related to a lower UCP3 expression in the skeletal muscle of females [173].

*Role of Aggregation in MDMA-Induced Hyperthermia* MDMA was also shown to produce aggregate toxicity in mice, an event related to MDMA-induced hyperthermia [140]. The term aggregate toxicity refers to the finding that the toxicity and lethality of MDMA increases in injected animals housed in groups compared to those housed singly. MDMA aggregate toxicity is closely related to its hyperthermic effects, especially in crowded settings. In accordance, induction of a hypothermic state in mice via reduction of the ambient temperature to 4°C results in a complete abolition of the MDMA lethal effects [140]. The potentiation of the lethal effects of MDMA in humans following ingestion of the drug in crowded settings may be analogous to the aggregate toxicity of MDMA [174]. Aggregate toxicity methodologies in animal studies of MDMA-induced toxic effects can be used to model the environment in which the drug is often administered by humans [140].

### *MDMA-Induced Hyperlocomotion and 5-HT Syndrome to Experimental Animals*

Not surprisingly, given the evidence that MDMA administration results in a major release of 5-HT in several brain regions, this compound also produces in rats an acute, dose-dependent, hyperlocomotor response [29, 175–177] together with the appearance of all the major behavioral features of the 5-HT syndrome [29, 175–180]. The characteristics of 5-HT behavioral syndrome produced in rats include enhanced locomotor activity, reciprocal forepaw treading, head weaving, piloerection, hind limb abduction, proptosis, ataxia, penile erection, ejaculation, salivation, defecation, subsequent dose-dependent convulsions, and possible death [178–180]. The complex motor effects of MDMA are dependent upon monoamine release followed by activation of multiple monoamine receptors presumably in the brain.

In non-human primates, namely, in rhesus monkeys, MDMA administered in doses similar to human recreational use does not stimulate significant locomotor activity under normal laboratory housing conditions in the first few hours after dosing [162, 163]. These findings may also point to particular differences in the response of primates versus rodents.

### *MDMA Effects in the Cardiovascular System of Experimental Animals*

In rats, MDMA is well known to produce a range of effects on the cardiovascular function in doses either single or binge administrated ranging from 10 to 30 mg/kg. As previously mentioned, MDMA promotes an enhancement of cutaneous vasoconstriction both in rats and rabbits [157, 158, 169, 181]. In a recent study, it was shown in Sprague–Dawley rats that the effects of MDMA (9.19 mg/kg, i.p.) on the heart rate, as well as locomotor activity, are dependent on the environmental temperature at which animals are exposed to the drug. Rats at an elevated ambient temperature (30±1°C) showed higher heart rate as well as core temperature and locomotor activity than animals exposed at a lower room temperature (21.5±1.5°C) [182]. MDMA can produce a dose-dependent increase in mean arterial pressure and a range of other effects on the rat cardiovascular function due to its cardiac stimulant effects, resulting in tachycardia and arrhythmia

[145, 183, 184]. Importantly, MDMA and MDA, its main metabolite in rats, produce a prolonged increase in both systolic and diastolic pressures, with MDA causing the most marked rise [185].

*MDMA Anxiogenic Effects to Experimental Animals*

MDMA has been shown to induce anxiogenic effects both in mice and rats. Using an animal model of anxiety, the elevated plus maze test in male Quackenbush Swiss mice MDMA (4 mg/kg, i.p.) [186] or in albino male OF.1 strain MDMA (15 mg/kg, i.p.) [187] decreased the percent of open arm entries and increased enclosed entries, indicators of anxiogenic effects [186, 187]. The effects of MDMA and amphetamine on the mouse's responses were comparable [186]. Male Lister hooded rats treated with MDMA (10 mg/kg×3, i.p.) [188] or male Wistar rats treated with MDMA (15 mg/kg, i.p.) [189] revealed a clear anxiogenic-like behavioral profile with a reduction of open arm entries and suppression of explorative behaviors [188, 189]. Other studies reported that Wistar rats at postnatal day (PND) 28 administrated MDMA (5 mg/kg i.p., four doses daily for 2 days) showed "anxiety-like" behavior in the social interaction test, accompanied by a long-lasting reduction in specific 5-$HT_{2A}$-receptor-mediated behavior [190]. Additionally, MDMA administration to adolescent rats was also shown to reduce social interaction [191]. The anxiogenic effect seems to be dose-dependent, since low doses (4 to 7.5 mg/kg) produce anxiogenic effects, meanwhile high doses (10 to 15 mg/kg) were found to be "anxiolytic-like" both in mice and rats [189]. MDMA-induced generalized anxiety to rats, as measured by the emergence test, seems unlikely to involve the 5-$HT1_A$, 5-$HT_{1B}$ or 5-$HT_{2A}$, 5-$HT_{2B}$ or 5-$HT_{2C}$ receptors, since antagonists targeted to these receptors did not prevent this effect [180].

*MDMA Effects on the Neuroendocrine System of Experimental Animals*

MDMA has been reported to promote neuroendocrine changes, namely, it has the ability to promote prolactin and corticosterone secretion in animals. Nash et al. [146] were the first to report that i.p. injections of 1–3 mg/kg of MDMA stimulate prolactin and corticosterone secretion in Sprague–Dawley rats and that both fluoxetine and 5-$HT_2$-antagonists pretreatment could blunt corticosterone secretion. Additionally, rats previously exposed to MDMA (2 or 20 mg/kg, s.c.) show a diminished corticotropin and enhanced prolactin responses to a 5-$HT_1$-agonist [192]. Also, more recently, it was shown that previous exposure to MDMA may reduce the hormonal response of a later MDMA dose, since Sprague–Dawley rats pretreated with 7.5 mg/kg×3 (i.p.) displayed significant reductions in evoked prolactin and corticosterone secretion when challenged 1 week later with a new dose of MDMA [193]. The mechanisms of MDMA-induced changes in hormones prolactin and corticosterone secretion still remain to be clarified, but certainly, both hyperthermia and serotonergic depletion might have a major role.

*MDMA Effects on the Immune System of Experimental Animals*

MDMA administration has also been associated with alterations in the immune function of animals [194]. Acute administration of MDMA (20 mg/kg, i.p.) to female Sprague–Dawley rats produced a rapid (within 30 min) suppression of concanavalin-A-induced lymphocyte proliferation and a profound reduction in the total leucocyte count that persisted for at least 6 h following administration [195]. These alterations in immune function were accompanied by a significant increase in plasma corticosterone concentrations 30 min post-MDMA administration [195]. Additionally, acute MDMA (20 mg/kg, i.p.) administration to female Sprague–Dawley rats impairs interleukin-1 beta and tumor necrosis factor-alpha secretion following an in vivo lipopolysaccharide challenge [196]. More recently, the ability of MDMA to alter a viral-induced macrophage response was demonstrated. Bone-marrow-derived macrophages were exposed in vitro to 500 μM MDMA for 24 h and then infected with murine gammaherpesvirus-68. MDMA promoted reductions in the virus-stimulated monokine mRNA expression in a dose-dependent manner [197]. Altogether, these data come to agree with the ability of MDMA administration to increase the susceptibility to infectious diseases.

*Reinforcing and Behavioral Sensitization Properties of MDMA in Experimental Animals*

Studies that analyzed the ability of animals to self-administrate MDMA demonstrate that the drug serves as a reinforcer and suggest that MDMA has indeed abuse liability.

Male Sprague–Dawley rats will self-administer MDMA at doses ranging from 0.25 to 1.0 mg/kg (i.v.), indicating that these doses possess reinforcing efficacy [198, 199]. Additionally, MDMA (1.0 mg/kg, i.v.) displays similar reinforcing potency in Sprague–Dawley and Long–Evans rat strains [200]. Studies show that the percentage of rats that acquired MDMA self-administration was lower than the percentage of rats that acquired cocaine, but prior experience with cocaine self-administration facilitates the acquisition of MDMA self-administration [198].

The study of the mechanism underlying the reinforcing effects of MDMA was conducted in 5-HTT [201] and also in cannabinoid receptor 1 ($CB_1$) KO mice [202]. 5-HTT and $CB_1$ KO mice failed to self-administer MDMA at any of the tested doses (0.03, 0.06, 0.125, and 0.25 mg/kg per infusion, i.v.) [201, 202]. Both works provide evidence that 5-HTT and the serotonergic system, as well as the $CB_1$ cannabinoid receptors, are essential in the acquisition of an operant behavior to self-administer MDMA.

The reinforcing effect of MDMA has been investigated in rhesus monkeys. It was evaluated whether the animals would self-administer MDMA and its stereoisomers [203, 204]. These studies demonstrated that MDMA (dose range between 0.01–0.56 mg/kg/injection, i.v.) functions as a reinforcer, although its reinforcing efficacy appears to be less than that of cocaine or Meth. Also, the chronic self-administration of the drug attenuated the reinforcement effects of MDMA [205]. The 5-$HT_{2A}$ antagonists ketanserin and MDL 100,907 attenuated the reinforcing effect of MDMA in rhesus monkeys, suggesting a role for this receptor in this effect [204].

Behavioral sensitization is the enhanced motor-stimulant response that occurs with repeated exposure to psychostimulants. The mesocorticolimbic DA system, which arises in the ventral tegmental area and innervates the nucleus accumbens, has been implicated in this process [206]. Behavioral sensitization evoked by single and repeated Amph pretreatment has been shown in rats [207]. This phenomenon has been considered, for many years, a useful animal model for the development of psychosis [208]. As for Amph, MDMA was also was shown to elicit long-term behavioral and neurochemical sensitization in rats after repeated exposure [152, 178, 208–210]. In male Sprague–Dawley rats, locomotor activity in response to MDMA at three doses (2.5, 5.0, 7.5 mg/kg, i.p.) increased in a dose-dependent manner. Locomotor behavior was augmented on subsequent testing after injection of the same MDMA dosage in subsequent days, indicating behavioral sensitization [178]. In another study, male Sprague–Dawley rats pretreated with repeated injections of one of two doses of MDMA (5 or 20 mg/kg, s.c.) demonstrated an augmented increase in motor activity upon exposure to MDMA 12 days after the last repeated injection [209]. Furthermore, microdialysis was conducted in the nucleus accumbens, and the capacity of MDMA (5 mg/kg, s.c.) to elevate extracellular DA content was augmented in rats pretreated with repeated MDMA. These data reveal that repeated MDMA administration produces behavioral sensitization and enhanced DA transmission in the nucleus accumbens of rats [209]. In male Wistar rats, MDMA-induced behavioral sensitization can be blocked by 5-$HT_{2C}$ receptor agonist, but not by blockade of medial prefrontal cortex (CTX) $D_1$ receptors [208]. A very recent study analyzed which areas are involved in the mechanisms of behavioral sensitization to MDMA [210]. In male Sprague–Dawley rats, the sensitized behavioral response was related to changes in Fos expression in the lateral shell of the nucleus accumbens, the central nucleus of the amygdala, and the anteromedial part of the lateral habenula [210]. Regarding gender differences to MDMA-induced behavioral sensitization, female rats seem more sensitive than males. In a recent study, male and female Sprague–Dawley rats were treated with MDMA (15 mg/kg, two times per day for 4 days, i.p.). Females exhibited markedly greater locomotor stimulation after acute MDMA and also showed sensitization to an acute challenge, 2 weeks later. MDMA elicits substantially greater locomotor activation in female rats than in males, but persistent effects on anxiety and 5-HT content were found to be similar in males and females [152].

## MDMA-Induced Acute Human Clinical Symptoms

### *MDMA-Induced Acute Psychological Human Clinical Symptoms*

MDMA became established as a dance drug because of its mood-enhancing properties, conveyed in the "3 Es" expression: energy, empathy, and euphoria [211]. Human studies on the behavioral and psychological effects of MDMA (oral doses ranging from 50 to 150 mg) demonstrated that MDMA induces a state of enhanced mood, peaceful experience with enhanced insight, feelings of increased closeness to others, euphoria, heightened sensory awareness, and symptoms of sympathetic arousal including tremors, effects described by some as "entactogenic" [212–215]. In accordance, the hallucinogenic characteristics of the drug are well documented in humans. The hallucinogenic effects of MDMA are reported to be decreased by pretreatment with ketanserin (a 5-$HT_{2A}$ antagonist) [214] and also by selective 5-HTT inhibitors like citalopram, paroxetine, and fluoxetine [216, 217].

Supporting the general notion that MDMA has high abuse liability, Tancer and Johanson [218] reported that 1 and 2 mg/kg of MDMA have reinforcing properties in humans that resemble those of (+)-Amph. In fact, only agents who have the ability to release DA like MDMA or Amph seem to have reinforcing effects in humans, meanwhile metachlorophenylpiperazine (a 5-HT releasing agent) does not.

### *MDMA-Induced Acute Human Peripheral Symptoms, a Special Focus in Hyperthermia*

Peripheral effects of MDMA to humans, in typical oral recreational doses ranging from 50 to 150 mg, include enhanced energy that drives physical activity, increased blood

pressure, tachycardia, palpitations, hyperthermia, and also nausea, sweating, and hyperflexia [212–215, 217, 219, 220].

Hyperthermia represents a clinically relevant aspect in MDMA abusers. It has been reported that body temperature may reach up to 43°C, which can be fatal [221–224]. Misuse of MDMA in crowded conditions with a high ambient temperature, physical activity, and dehydration, i.e., under conditions that MDMA is often used at "dance parties", may all contribute to increase the hyperthermic response. The main contributing factors for the hyperthermic effect associated with MDMA are summarized in Table 5. In individuals at a "dance party", physiological parameters, such as blood pressure, heart rate, and body temperature, were moderately increased in subjects with the highest MDMA plasma concentrations [221]. In controlled laboratory settings, MDMA administration to human volunteers increases modestly the body temperature, and usually, observed increases do not exceed 0.4°C [56, 212, 220]. These studies indicate that there is an interaction between the environmental variables and the effects of MDMA on body temperature. As remarked in a review of the clinical effects of MDMA by Cole and Sumnall [16], the observed hyperthermia and the adverse reactions to MDMA ingestion are probably conditioned by the environmental circumstances of raves and nightclubs. However, unlike findings in rodents, MDMA increased core body temperature regardless of ambient temperature in humans, and these increases appeared related to increases in metabolic rate, which were substantial. A study by Freedman et al. [219] evaluated the effects of MDMA on core body temperature in humans at cold (18°C) and warm (30°C) ambient temperatures in a temperature- and humidity-controlled laboratory. MDMA (2 mg/kg, p.o.) produced significant elevations in core body temperature and metabolic rate in both warm and cold conditions. A similar degree of hyperthermia was reported in rhesus monkeys administered MDMA (dose range 0.56–2.4 mg/kg, intramuscular) across a wide range of ambient temperature conditions (18°C up to 30°C), corroborating that this animal model is closer to humans [162]. Altogether, these reports account for differences regarding MDMA-induced hyperthermia in animal models and humans.

**Table 5** Summary of the factors contributing to MDMA-mediated hyperthermia in human recreational abusers

- Warm environment
- Repetitive physical activity (dancing)
- Peripheral vasoconstriction
- Loss of thermoregulatory mechanisms at the CNS
- Loss of body signals perception: thirst, exhaustion
- Euphoria
- Increased muscle tone
- Heat production

The mechanisms involved in MDMA-induced hyperthermia have also been investigated in human volunteers. Pretreatment with the 5-$HT_{2A}$ antagonist ketanserin (50 mg, p.o.) attenuates the MDMA-induced (1.5 mg/kg, p.o.) raise in body temperature [214]. The increase in blood pressure and heart rate produced by MDMA (1.5 mg/kg, p.o.) was attenuated by citalopram pretreatment (40 mg, i.v.), while body temperature was not modified [220].

### *MDMA-Induced Acute Human Neuroendocrine Changes*

MDMA intake by humans, in typical oral recreational doses ranging from 50 to 150 mg, has been shown to increase plasma levels of adrenocorticotropic hormone, cortisol, prolactin, oxytocin, arginine, and vasopressin, with a decrease in plasma $Na^+$ concentrations [56, 225, 226]. The neuroendocrine changes induced by MDMA are compatible with the effects of serotonergic drugs on the hypothalamic–pituitary–adrenal axis [225]. Additionally, the rave environment may also produce similar physiological effects, as normal volunteers listening to "techno music" experience increase in heart rate, systolic blood pressure, stress, and plasma levels of noradrenaline, adrenaline, prolactin, cortisol, growth hormone, adrenocorticotropic hormone, and β-endorphin compared to volunteers listening to "classical music" [227]. Listening to fast music alters the glucocorticoid response to exercise and increases endurance, which may explain the preference for loud, fast music at night raves [227]. It is possible that the rave music may contribute to the profile of MDMA effects in human users [16].

### *MDMA-Induced Acute Human Immunosuppression*

Human exposure to MDMA could be regarded as a "chemical stressor" on the immune system, inducing immunosuppression [194]. In human male volunteers, repeated administration of 100 mg of MDMA at either short (4 h) or long (24 h) intervals, compatible with the consumption patterns among users, was followed by rapid and sustained changes of certain immunological and neuroendocrine parameters. MDMA administration led to a decrease in circulating T-helper cells (CD4) with a consequent decline in the CD4/CD8 T cell ratio and simultaneous increase in number of NK cells, representing this fact an immunosuppressive action [228]. These effects showed a parallelism with both MDMA plasma concentrations and MDMA-induced cortisol stimulation kinetics [228]. It seems that following MDMA consumption, there is a critical period in which immunocompetence is highly impaired. However, studies conducted in the laboratory

setting contrast with the environment associated with the recreational use of MDMA where crowded conditions, stressed physical activity without control of food intake, and association with other drugs could potentiate MDMA-induced immunosuppression [194, 228]. Altogether, MDMA abusers have an enhanced susceptibility to infection and immune-related disorders. MDMA abuse is strongly associated with high-risk sexual behaviors, which, in addition to MDMA-induced immunosuppression, increase the susceptibility of users to sexually transmitted diseases [229].

## "MDMA"—Human Acute Toxicity and Lethal Intoxications

### *MDMA—Human Acute Toxicity*

The immediate acute adverse effects of MDMA oscillate from minor symptoms to those potentially life-threatening. The reported acute adverse effects of MDMA by human users include nausea, vomiting, jaw clenching (trismus), teeth grinding (bruxism), hypertension, palpitations, headaches, hyperreflexia, difficulty in walking (ataxia), urinary urgency, muscle aches and tension, midriasis resulting in vision difficulties, and dry mouth [230, 231]. Other physiological symptoms that have been reported during the first few hours following ingestion of MDMA include tachycardia, coagulopathy, thrombocytopenia, delayed leukocytosis, acidosis, hypoglycemia, pulmonary congestion, edema, and hepatitis [174, 211, 232]. Adverse psychological effects that follow MDMA ingestion include depression, irritability, panic attacks, visual hallucinations, and paranoid delusions [16, 230, 231]. Visual hallucinations and paranoid delusions, which can persist for days or weeks, have also been reported by some users of MDMA [230]. The principal features of MDMA-induced acute adverse effects are summarized in Table 6.

**Table 6** Minor acute adverse symptoms after MDMA use

| Central nervous system symptoms | Autonomic nervous system symptoms |
|---|---|
| | • Tachycardia |
| • Panic attacks | • Hypertension |
| • Hallucinations | • Mydriasis |
| • Confusion | • Dry mouth |
| • Impaired judgment | • Sweating |
| • Insomnia | • Ataxia |
| • Irritability | • Nystagmus |
| | • Bruxism |
| | • Trismus |

More severe acute effects include acute panic disorder and psychosis, arrhythmias and cardiovascular collapse with histological postmortem changes ranging from contraction band necrosis to individual myocyte necrosis, severe hyperpyrexia, liver failure accompanied by foci of individual cell necrosis to centrilobular necrosis and microvascular steatosis, acute renal failure with histological tubular necrosis, rhabdomyolysis and multi-organ failure, disseminated intravascular coagulation, hyponatremia with cerebral edema, the 5-HT syndrome, and sudden death [174, 211, 222, 224, 232, 233]. In addition, potentially fatal neurological effects can occur following MDMA ingestion, including subarachnoid hemorrhage, intracranial hemorrhage or cerebral infarction, and cerebral venous sinus thrombosis [174, 211, 231].

More delayed neuropsychiatric adverse effects, within the following days after weekend intake, include "midweek blues" (normally at Tuesday and Wednesday) characterized by a prolonged "hangover" that may last up to 5 days [231]. More chronic neuropsychiatric complications after MDMA abuse include panic disorder, psichosis, aggressive behavior, flashbacks, major depressive disorder, and memory or cognitive disturbance [16, 213, 231].

### *MDMA—Human Lethal Intoxications*

It is clear that despite large-scale consumption of MDMA, when compared to other drugs of abuse, serious acute illness remains relatively rare. Nonetheless, when complications occur, they can be life-threatening [211]. It is important that clinicians, in particular those working in Intensive Care and Emergency Medicine Units, have the knowledge to handle MDMA-related emergencies. "Ecstasy"-related emergencies are even more problematic in cases of multiple drug ingestion [232].

The most prominent adverse reaction to MDMA intoxication is the fulminant hyperthermia, with core temperatures higher than 42°C, which usually precedes disseminated intravascular coagulation, rhabdomyolysis, (multiple) organ failure, and acute renal failure [211]. Although there are a few cases of patients surviving a core temperature of 42°C, the majority of adverse reactions with core temperatures of 42°C and above are fatal [16, 222–224, 234, 235]. Indeed, hyperthermia represents a clinically relevant aspect in MDMA toxicity, since high temperatures potentiate all the multi-organ toxic events [174]. MDMA-related fatal complications have been secondary to cardiac abnormalities, massive neurological disturbances, and multiple organ failure [174, 211, 224, 231, 232].

Scarce data are available concerning the number of emergencies and deaths related to "ecstasy" intake. In the USA, the number of deaths involving MDMA rose

dramatically from six in 1998 to 76 in 2001 according to the Substance Abuse and Mental Health Services Administration [236]. The increase in reports of MDMA-related deaths matched the increases in Emergency Room visits [236]. Figures from the annual report published by the EU Drug Monitoring Center confirm that emergencies have increased in recent years. Additionally, there were 78 MDMA-related deaths reported in the EU during 2005 [14]. Deaths related to "ecstasy" started to be reported in Europe during the 1990s, as the drug became popular and cause considerable concern, since they often occur unexpectedly among socially integrated young people. Of note is that some of these "ecstasy"-related deaths occur in first time MDMA users. The death risk for first time users has been estimated to lie between one in 2,000 and one in 50,000 [237]. EU reports suggest that deaths involving "ecstasy" are rare, especially deaths involving MDMA alone [14]. The description "ecstasy-related death" could mean that "ecstasy" was mentioned on the death certificate or that it was found in the toxicological analysis (often along with other drugs). However, the number of "ecstasy"-related deaths is underestimated due to deficient toxicological analysis after death and the lack of reporting to the national authorities [14].

## MDMA Long-Term Effects—Neurotoxicity

### MDMA-Induced Neurotoxicity—Evidences from Animal Models

There are more than 250 published papers indexed in Pubmed showing that administration of single or multiple doses of MDMA to mice, rats or non-human primates results in neurotoxicity to the serotonergic or dopaminergic systems, predominantly to the 5-HT-containing neurons. The rat is by far the laboratory animal more used to study MDMA-induced neurotoxic actions. Therefore, we start the revision of the findings on MDMA-induced neurotoxicity in the rat model, subsequently the data on non-human primates, and finally in the mice model.

#### *MDMA-Induced Neurotoxicity to the Rat—Biochemical and Histological Findings*

Almost certainly, the first report proving that MDMA induces neurotoxicity to the serotonergic system was conducted in rats and was published in 1986 by Schmidt et al. [238]. In the following year, there were already, at least, six papers corroborating MDMA-induced serotonergic neurotoxicity to rats [21, 239–243]. However, in 1985, 1 year before the first report on MDMA-induced neurotoxicity, another study had reported that MDA, a major metabolite of MDMA and also a hallucinogenic drug of abuse, was neurotoxic to rat serotonergic neurons [244]. After these early reports, many others continued to prove both biochemically and histologically, using state-of-the-art techniques, that MDMA produces neurotoxicity to serotonergic terminals and also to other neurons of rats [142, 152, 166, 245–265].

*Loss of Serotonergic Terminals in MDMA-Administrated Rats* The studies in rats have shown that the neurotoxic effects of MDMA to serotonergic neurons appear 24 h to 2 weeks following MDMA administration. Neurochemical measurements report long-term reductions in biochemical markers of the serotonergic system, namely, decreased levels of 5-HT and its major metabolite, 5-hydroxyindoleacetic acid (5-HIAA), and decreased number of 5-HTT binding sites. Especially, these studies confirmed that the most severe reductions of the 5-HT and 5-HIAA levels occurred in the rat neocortex, striatum, and hippocampus (HIP). Furthermore, binding of [$^3$H]paroxetine to the presynaptic 5-HTT is decreased and high affinity uptake of [$^3$H]5-HT is reduced, which indicate neurodegeneration of 5-HT terminals (see references above). Additionally, there are long-term decreases in tryptophan hydroxylase (TPH) activity, the rate-limiting enzyme of 5-HT synthesis, in the neostriatum, HIP, hypothalamus, and CTX [252, 257, 266, 267]. These abnormalities are reported to last for months or even years after drug administration [253, 268]. Importantly, in rats, S(+)-MDMA has been shown to be more neurotoxic than R(−)-MDMA [269], which correlates with the fact that (+) isomers of MDMA are more potent releasers of monoamines [25]. In Table 7 are gathered data from several studies including MDMA dosage regimens that produce 5-HT neurotoxicity measured by 5-HT depletion in cortical and hippocampal areas.

The vast majority of studies that evaluate the neurotoxic effects of MDMA to the serotonergic system do it 1 or 2 weeks after the drug administration. With the established serotonergic neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT), it is necessary to wait 4 to 12 days after administration to rats to observe the minimum 5-HT levels, this time window seeming important for the degeneration of fibers [270, 271]. It seems that neuronal axotomy of serotonergic fibers (partial or total dendritic loss) with cell bodies being spared, which is known as the "pruning effect," follows the pattern of retrograde degeneration, the typical dying back phenomena [246, 270, 271].

Most of the studies on MDMA neurotoxicity rely on the evaluation of the decrease in the biochemical markers of 5-HT neurons to evaluate the extent of toxicity. Studies have been performed to evaluate the time course of brain 5-HT levels after MDMA treatment. Cortical 5-HT depletion following a single dose of MDMA (10 mg/kg) showed two

**Table 7** Differences among rat strains towards MDMA-induced toxicity to 5-HT neurons

| Rat strain | MDMA dosage regimen (mg/kg) | CTX 5-HT % depletion | HIP 5-HT % depletion | Survival interval | Reference |
|---|---|---|---|---|---|
| Sprague–Dawley | 20, single dose, s.c. | 52 | 50 | 1 week | [147] |
| | 10×4 doses, i.p. | 75 | 70 | 2 weeks | [274] |
| | 7.5×3 doses, i.p. | 56 | 50 | 2 weeks | [280] |
| Dark Agouti | 10, single dose, i.p. | 40 | 41 | 1 week | [272] |
| | 6×3 doses, i.p. | 62 | 72 | 1 week | [151] |
| Wistar | 15, single dose, i.p. | 53 | 58 | 1 week | [264] |
| | 5×4 doses for 2 days, i.p. | 22 | 18 | 9 weeks | [190] |
| Lister Hooded | 20, single dose, i.p. | 28 | 28 | 4 days | [254] |
| | 15×2 doses for 3 days, i.p. | 16 | 20 | 4 weeks | [265] |

The differences can be attributed to metabolic differences within the rat strains. It seems that the Dark Agouti strain needs lower MDMA doses to acquire the same level of 5-HT depletion in the cortex (CTX) and hippocampus (HIP) presented by the other three strains. However, other factors such as the employed MDMA dosage, regimen administration routes (s.c. or i.p.), and the survival interval until the 5-HT content is measured, also contribute to the differences among studies, after a neurotoxic MDMA regimen

clearly distinguishable phases of response [239]. In the first phase, within 3 h after drug treatment, 5-HT was significantly depleted, with the concentrations being 16% of control values between 3 and 6 h post-drug administration. Between 6 and 24 h, however, a sharp recovery was observed and the 5-HT concentration returned to control values 1 day later. The second phase of depletion was apparent 1 week posttreatment, 74% of control values, with 5-HT levels gradually declining during the period between 1 and 7 days. Other studies demonstrated a dose-dependent reduction in the concentration of 5-HT and 5-HIAA in the frontal CTX during the subacute phase, 18 h after multiple doses of MDMA [267, 268].

The fact that the serotonergic system biochemical markers are reduced after MDMA administration does not necessarily imply that neurodegeneration has indeed occurred, since these neurochemical measures are indirect. Absolute direct identification of neurotoxic damage can only be made with histological analysis in the brain of MDMA-administrated animals. Several studies demonstrated, after performing histochemical and/or immunohistochemical analysis, that MDMA causes long-term neurodegeneration in the brain. The techniques used in the early studies were mainly histochemical and included silver staining. Using this technique in rat striatal slices, 13 to 16 h after high doses of MDMA (80 mg/kg, s.c., twice daily, 4 days), the presence of argyrophilic deposits in MDMA-treated rats was demonstrated, which were absent in control animals [240]. Primary somatosensory CTX slices contained shrunken, argyrophilic neuronal cell bodies, fragmented dendrites, and degenerating axon terminals [240]. Other studies, using 5-HT, 5-HTT and TPH antisera for immunohistochemical analysis of regional brain sections, 1 or 2 weeks after MDMA administration, demonstrated a reduced intensity of the staining in MDMA-treated brain slices, reflective of a marked reduction in serotonergic axonal density [246, 251]. These changes were particularly apparent in the neocortex, striatum, thalamus, HIP, septum, and amygdala [246, 251]. The terminal portions of axons were 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, while fibers of passage and raphe cell bodies were unaffected [251, 252]. Also, the loss of the 5-HT terminals in rats is both biochemically and histologically comparable to the one that occurs following exposure to the classic 5-HT neurotoxin, 5,7-DHT [246, 270]. These histological observations match the biochemical measurements and therefore confirm the legitimacy for the use of biochemical parameters to evaluate MDMA-induced serotonergic neurotoxicity.

One must keep in mind, when evaluating the reports on MDMA-induced neurotoxicity in rats, that the different strains of rats used by different investigators have different sensitivities to both the acute and the long-term neurotoxic effects of MDMA. In Table 7 are presented results from several studies showing differences among rat strains towards MDMA-induced toxicity to 5-HT neurons. Both the dose required and the extent of the obtained neurotoxicity was shown to be strain-dependent. The most obvious example is the Dark Agouti strain, which requires a single dose (10–15 mg/kg) of MDMA to produce a clear 30% to 50% or greater loss in cerebral 5-HT content [272]. However, several doses of MDMA, often of 20 mg/kg or more, are usually required to produce a similar loss in Sprague–Dawley, Hooded Lister, and Wistar rats [249, 254, 273]. The variation in the metabolism of MDMA among these rat strains should account for the differences towards MDMA-induced neurotoxic effects, as previously described in the MDMA pharmacokinetics section.

Several studies have addressed the possible recovery of the 5-HT neurochemical markers in rats following MDMA neurotoxic doses. These studies indicate that there might be a recovery of these markers between 6 months and 1 year after MDMA treatment, but this event was only complete in particular brain regions [253, 268, 274–277]. One study measured the regional brain content of 5-HT, 5-HIAA, and [$^3$H]paroxetine-labeled 5-HT uptake sites in Sprague–Dawley rats and performed immunocytochemical analysis of 5-HT-containing nerve fibers for up to 1 year after MDMA treatment (10 mg/kg, four times every 2 h, i.p.) [274]. All examined brain regions showed complete recovery of 5-HT within 1 year of drug treatment, and similar patterns were observed in the recovery of 5-HIAA content. Furthermore, [$^3$H]paroxetine binding values in the CTX and striatum had returned to control levels within 32 weeks, while hippocampal binding was still 29% below control values at 52 weeks posttreatment. Despite the recovery of 5-HT markers in some brain areas, immunocytochemical techniques demonstrated that most animals still presented a significant reduction in 5-HT axon density in the parietal CTX 52 weeks posttreatment [274]. Fifty-two weeks after exposure to MDMA (10 mg/kg, four times every 2 h, i.p.), Sprague–Dawley rats presented a reinnervation pattern that was highly abnormal, reinnervated, or hyperinnervated (e.g., amygdala, hypothalamus), meanwhile some brain regions remained denervated (e.g., dorsal neocortex) [253]. Another study investigated the extent of recovery of both regional brain 5-HT content and [$^3$H]5-HT uptake in striatal and hippocampal synaptosomes of Sprague–Dawley rats up to 1 year after MDMA administration (20 mg/kg, s.c., eight times at 12-h intervals) [275]. Although, 1 year after MDMA treatment there were no signs of [$^3$H]5-HT uptake decrease, which were seen 2 and 8 weeks, brain 5-HT concentrations were still significantly reduced in the frontal–parietal and occipital–temporal CTX, while some hyperinnervation was observed in the hypothalamus at 52 weeks. Meanwhile, in the frontal CTX and striatum, 5-HT levels showed complete recovery by 52 weeks posttreatment. These data indicate that MDMA-induced serotonergic neurotoxicity and possible later recovery are region-dependent [275]. Another more recent study by Kovács et al. [277] evaluated the effect of a single dose of MDMA (15 mg/kg, i.p.) on serotoninergic fiber density, defined by TPH and 5-HTT immunoreactivity, in the spinal cord and brain areas of Dark Agouti rats 7 and 180 days after applications. Densities of TPH and 5-HTT-immunoreactive fibers of rats were reduced 7 days after a single injection of MDMA by 57.6%, 60.4%, and 40.8% in the frontal CTX, caudate–putamen, and HIP CA1 area, respectively. Seven days after MDMA treatment, the authors observed also reductions of the same type of fibers in several regions of the spinal cord. However, the densities of TPH- and 5-HTT-expressing fibers in all the above regions were similar in the saline- and MDMA-treated rats 180 days after the treatment. Furthermore, TPH- and 5-HTT-expressing fibers revealed no blockade of the fast axonal transport; however, they presented up-regulation of 5-HTT mRNA expressions in 5-HT cell bodies 7 days after treatment, which was absent at 180 days after administration. These events may reveal a compensatory mechanism of the rat to the neurotoxic insult [277]. Overall, these data agree with previous studies and indicate that MDMA-induced serotonergic neurotoxicity and possible later recovery are region-dependent [253, 275, 277].

A few authors, in contrast to the general notion among the scientific community, do not regard MDMA-induced serotonergic biochemical marker deficits as neurotoxicity but rather as brain neuroadaptations [277–280]. Kovács et al. [277] suggested that even the well-documented, long-term decrease in brain 5-HT and/or 5-HIAA concentration does not necessarily mean axonal degeneration. In recent years, Rothman and Baumann have been attributing MDMA-induced serotonergic deficits to an alleged neuroadaptation process [278–281]. They argue that a dosing regimen of MDMA that depletes brain 5-HT does not necessarily produce detectable axotomy. In three works from this group, they killed male Sprague–Dawley rats 2 weeks after MDMA (7.5 mg/kg, i.p., every 2 h×three doses) [278, 280, 281]. Using this protocol, a decrease in tissue 5-HT in CTX, HIP, and caudate by about 50% was observed at the end of 2 weeks after MDMA exposure. However, MDMA treatment had no significant effect on the expression level of serotonin transporter (5-HTT) and glial fibrillary acidic protein (GFAP) in any brain region tested. In contrast, the widely accepted serotonergic neurotoxin, 5,7-DHT (150 μg/rat, i.c.v.), reduced tissue 5-HT by more than 90%, decreased 5-HTT protein expression by 20–35%, and increased GFAP by 30–39% [280]. Furthermore, MDMA did not alter 5-HTT expression, meanwhile 5,7-DHT decreased 5-HTT expression by 33% [278]. In another report of this group, rats exposed to the same regimen of MDMA were treated with L-5-hydroxytryptophan, the 5-HT precursor, plus benserazide, aromatic L-amino acid decarboxylase inhibitor 2 weeks later [281]. Some restoration of MDMA-induced 5-HT depletion by the administration of L-5-hydroxytryptophan was seen in several brain areas. The authors postulated that for the restoration of 5-HT content, serotonergic terminals must have remained intact after MDMA exposure. However, in the same study, restoration of the 5-HT content, after the same protocol of administration of L-5-hydroxytryptophan, was observed in brain areas previously lesioned with the established neurotoxin 5,7-DHT [281]. This result necessarily means that despite axotomy produced by the neurotoxic regimen

of 5,7-DHT, restoration of 5-HT content can be achieved. Thus, lasting serotonergic deficits after MDMA are probably due to neuronal axotomy rather than neuroadaptation.

*Toxicity to Non-serotonergic Neurons in MDMA-Administrated Rats* The loss of serotonergic terminals in MDMA-administrated rats is by far the most studied neurotoxic event. However, several reports emphasize that MDMA-induced neurotoxicity is not only limited to the axons of serotonergic neurons and that a broader neurodegeneration occurs in the brains of MDMA-treated animals. Studies that analyzed the localization of MDMA-induced neuronal degeneration throughout the entire rat brain have reported neuronal degeneration in different brain areas, such as the parietal CTX, the insular/perirhinal CTX, the ventromedial/ventrolateral thalamus, and the tenia tecta [240, 245, 250, 255, 282–284]. A study by Schumued [250] that analyzed the localization of MDMA-induced neuronal degeneration throughout the entire rat brain, after a single MDMA dose of 20 mg/kg, reported that degenerating neurons were found in the parietal CTX, insular/perirhinal CTX, the ventromedial/ventrolateral thalamus, and the tenia tecta. Of note is that the extent of the observed neuronal degeneration generally correlated with the degree of hyperthermia achieved. Importantly, MDMA binge administration to rats (eight injections of 5 mg/kg at 6-h intervals) was shown to significantly decrease the survival rate of cells incorporated in the granular layer of the HIP dentate gyrus of about 50% and of those remaining in the subgranular layer by approximately 30%, thereby affecting the neurogenesis process [285]. Also, in the rat brain, astroglial activation and Hsp27 overexpression, a molecular chaperone, were found in the HIP CA1 region after a single dose of 30 mg/kg MDMA, which may indicate a particular vulnerability of this region [286]. The presence of strong Hsp72 immunopositivity in neurons of certain brain areas may reflect additional effects of MDMA on non-serotonergic neurons. The findings on markers of neuronal degeneration in the rat brain after MDMA treatment are summarized in Table 8. In addition to studies conducted in animals, several in vitro studies have reported that MDMA, and related amphetamines, could induce neuronal apoptosis in cultured rat cortical and cerebellar granule neurons [76, 77, 287–290].

Astrocyte hypertrophy can occur as a result of neuronal injury and can lead to the enhanced expression of GFAP, which can be used as a marker of neuronal damage, detected by immunohistochemical analysis of brain slices. There are few studies reporting the use of this marker to examine MDMA-induced damage, which demonstrate GFAP reactivity increase in the HIP of MDMA-pretreated rats paralleling 5-HT damage [286, 291]. However, in general, data referring to glial activation after neurotoxic regimens of MDMA are not consistent. A few studies report an increase in GFAP expression in the context of MDMA-induced neurotoxicity [286, 292, 293], whereas others do not [280, 294, 295]. Also, with the established 5-HT neurotoxicant, 5,7-DHT, data from different studies are also not consistent, with some studies revealing increased GFAP expression after neurotoxic regimens [280, 296, 297], whereas others do not [295, 298]. It has been suggested that a lack of GFAP expression increase may be due to an insufficiently strong signal and that the use of this parameter for detecting degeneration of serotonergic termi-

**Table 8** MDMA-induced neurotoxicity to rats goes beyond the serotonergic system

| Neurodegeneration marker and brain area | MDMA dosage regimen | Survival interval (days) | References |
|---|---|---|---|
| Silver-positive staining of degenerating neurons in the parietal CTX, neocortex and striatum | 80 mg/kg, s.c., twice daily, 4 days | 2 | [240] |
| | 25–150 mg/kg, s.c., four doses, 1 day | 2 | [284] |
| Astrogliosis measured by increased GFAP staining in the HIP | 20 mg/kg, i.p., single dose | 7 | [291] |
| | 7.5–30 mg/kg, i.p., single dose | 3 | [286] |
| Fluoro-Jade B staining of degenerating neurons in the parietal CTX, insular/perirhinal CTX, thalamus and the tenia tecta. | 20–40 mg/kg, i.p., single dose | 2 | [250] |
| Caspase-3 immunoreactive neurons in the cingulate CTX and HIP | 10 mg/kg, s.c., twice daily, 4 days | 1 | [282] |
| Decreased binding of [$^3$H]-Flunitrazepam binding to GABAergic neurons in the HIP, ventral medial thalamus, anterior retrosplenial CTX, habenula, posterior retrosplenial CTX and dorsal raphe | 40 mg/kg/day, s.c., (implanted osmotic minipumps), 5 days | 2 | [255] |
| Increased caspase-3 activity in the amygdala and HIP | 5 mg/kg, i.p., three doses, 1 day | 7 | [283] |
| Activated calpain-1 and caspase-3 increases could be localized to neurons in the CTX | 10 mg/kg, i.p., four doses, 1 day | 1 | [245] |
| Decreased survival of neuronal precursors incorporated in the dentate gyrus of the HIP | 5 mg/kg, i.p., eight doses, 1 day | 14 | [285] |

The table summarizes the findings on markers of neuronal degeneration in the rat brain after MDMA treatment. The doses and MDMA administration routes are included

nals may have limitations [296]. Additional research is needed to fully characterize the determinants, timing, nature, and role of glial responses in the context of MDMA neurotoxicity.

*Neurotoxicity of MDMA at Perinatal and Early Postnatal Developmental Stages of Rats* Several studies have addressed the perinatal and early postnatal MDMA neurotoxicity in rats. Studies examining these effects of MDMA exposure in rats have resulted in inconsistent data. The discrepancies among reports using animals at this developmental stage have been related to MDMA pharmacokinetics, namely, the poor ability to metabolize and eliminate MDMA, and to the immaturity of brain neuronal systems [299]. A study that evaluated the effect of MDMA administration to rats during pregnancy (20 mg/kg, s.c., twice daily, days 14–17 of the gestation period) demonstrated that the born pups had no changes in brain 5-HT or 5-HIAA concentrations despite the use of dosing regimens known to be neurotoxic in adult rats [300]. Similar results were later reported in a study using a similar experimental design [301]. However, recent investigations have demonstrated that exposure of prenatal rats to MDMA results in significant and persistent changes in the developing brain. Additionally, it was proven that MDMA administration to pregnant rats results in its passage to the fetal compartment, with MDMA and its metabolite MDA being measurable in the amniotic fluid and fetal brain [302]. In contrast to previous negative results, another report showed that twice-daily administration of 15 mg/kg MDMA to pregnant rats during embryonic days 14–20 led to reduced 5-HIAA levels in the offspring at PND 3 and reduced 5-HT turnover in the nucleus accumbens at PND 21 [303]. Another study reported that rats prenatally exposed to MDMA (10 mg/kg, s.c.) from 13–20 days of gestation showed a twofold decrease of whole brain levels of 5-HT and 5-HIAA at PND 0 [304]. These studies suggest that prenatal exposure to MDMA may also produce long-term neurochemical modifications in the serotonergic function.

Regarding exposure of neonatal rats to MDMA, variable results also have been reported. Some studies found no lasting 5-HT neurotoxic effects of MDMA treatment in pups at PND 10 [142, 301], while others using pups administered MDMA from PND 10–20 did obtain evidence for short- or long-term deficits in serotonergic markers [305–308]. Newborn rat pups (PND 1–4) administered MDMA (10 mg/kg, s.c., twice daily) suffered apoptotic neuronal death in the HIP and CTX and also deficits in serotonergic markers in the HIP and neocortex at PND 25 and/or PND 60, which subsequently led to a reorganization of the forebrain serotonergic innervation [282, 309]. Additionally, MDMA exposure (10 or 20 mg/kg, s.c.) on PND 11–20 resulted in dose-related impairments of sequential learning, spatial learning, and memory, whereas neonatal rats exposed on days 1–10 showed almost no effects [306]. The decreases in serotonin in regions important for learning and memory in conjunction with elevated levels of corticosterone during a period of stress hyporesponsiveness suggest that these initial responses to MDMA may contribute to the long-term learning and memory deficits following neonatal MDMA exposure [308]. Considering that the neonatal period in rats (PND 10 to 20) is thought to model the third trimester of fetal brain development in humans, these results indicate that infants whose mothers take MDMA during late pregnancy are at risk of multiple neurodevelopmental abnormalities, namely, long-term learning and memory deficits [282, 306].

### *MDMA-Induced Neurotoxicity to the Rat—Relevant Associated Functional Deficits*

MDMA-induced neurotoxic damage may lead to important long-term functional deficits. MDMA-induced neurotoxicity leads to the loss of rat's ability to thermoregulate. Rats previously exposed to a neurotoxic dose of MDMA present impaired thermoregulation when exposed to high ambient temperature, which can be a consequence of serotonergic deficit [150, 179, 182, 310]. In fact, rats exposed to a single neurotoxic dose of MDMA (12.5 mg/kg) [150] multiple doses, 10 mg/kg once per day for 4 days [310], or 40 μmol/kg for 3 days [182] displayed an impaired thermoregulation when exposed to high ambient temperature 4 to 6 weeks after the drug administration.

Studies seem to agree that the anxiogenic effects and reduction on social interaction are developed in the long term following MDMA exposure, which results from an impaired serotonergic function [190, 191]. Additionally, it has been shown that acute MDMA treatment induces learning deficits in rats. Intermittent MDMA exposure during the adolescent period exposure to relatively moderate doses produces later changes in behavior [179, 311]. Male Sprague–Dawley rats received s.c. injections of 10 mg/kg of MDMA or saline twice daily with an interdose interval of 4 h, the administrations being given every fifth day from PND 35 to PND 60 [311]. Five days after the last dose, rats displayed an impairment of object recognition memory and reduced anxiety. The authors concluded that MDMA exposure during the adolescent period of development can influence subsequent cognitive and affective functioning in the absence of severe serotonergic damage [311]. Another study performed in male Sprague–Dawley rats exposed to MDMA (5 mg/kg, hourly for a period of 4 h every fifth day from PND 35 to 60) showed that animals 4 days after the final MDMA dose displayed several deficits [179]. MDMA altered habituation to the open field,

increased locomotor activity in the elevated plus maze, decreased attention in the novel object recognition test, and reduced serotonin transporter binding in the neocortex [179]. Adult male Lister Hooded rats treated twice daily with ascending doses of MDMA (10, 15, and 20 mg/kg, i.p.) over 3 days showed 5-HT neurotoxicity and a lasting cognitive impairment in the long term [312]. Spatial learning and memory can be tested utilizing a Morris water maze, which is especially sensitive to lesions of the HIP, the primary brain region believed to be involved in spatial learning and memory processing [313]. MDMA exposure was also reported to produce a disruption of sequential and spatial memory-based learning [312–315]. Studies demonstrated that hippocampal lesions induced by MDMA may be linked to a reference memory deficit in rats. The nature of these deficits may be based on deficits of the monoamines 5-HT and DA, induced by MDMA in the HIP [313, 314]. However, the nature of MDMA-induced learning deficits in rats could also be linked to the MDMA-induced neuronal death in the hippocampal neurons [282, 283].

### MDMA-Induced Neurotoxicity to Non-human Primates

Similar to rats, in non-human primates, serotonergic depletion and neuronal damage after exposure to MDMA have been documented by several reports [131, 253, 316–321]. Indeed, monkeys seem to be very susceptible towards MDMA neurotoxicity. After administration of MDMA doses ranging between 2.5 and 5.0 mg/kg to squirrel monkeys (s.c., twice daily, for four consecutive days), a dose-dependent reduction in the 5-HT content of the CTX, caudate nucleus, putamen, HIP, hypothalamus, and the thalamus was proven [317]. In one study, MDMA was administered at doses of 2.5 or 10 mg/kg, twice daily for 4 days, to rhesus monkeys. It produced selective and significant decrease in cerebrospinal fluid (CSF) levels of 5-HIAA and brain 5-HT and 5-HIAA concentrations, with the higher dose producing a selective decrease of 5-HT uptake sites [319]. Also, the long-lasting deficits characteristic of MDMA-induced neurotoxicity were evidenced by the fact that squirrel monkeys presented reduced serotonergic innervation and reduced 5-HT levels 7 years following exposure to the drug [320]. The reorganization of 5-HT projections has also been reported to occur in the brains of non-human primates. Squirrel monkeys, previously lesioned with MDMA (total dose 40 mg/kg, s.c.), showed substantial serotonergic axonal sprouting and a highly abnormal reinnervation pattern 18 months after MDMA treatment [253]. The route of the 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. When MDMA was administered in a dose of 5 mg/kg, twice daily for four consecutive days, it produced 86% depletion of frontal CTX 5-HT when given s.c. compared with a 42% depletion when given orally [322]. Since a single 5-mg/kg oral dose in monkeys has been proposed to be equivalent to a 1.4-mg/kg dose in a 70-kg human, based on interspecies dose scaling made by the authors, these data may indicate a possible risk of serotonergic damage in humans even after a single dose [322, 323]. Additionally, plasma MDMA levels in squirrel monkeys highly correlated with regional brain 5-HT deficits observed 2 weeks after administration [324]. These results indicate that plasma concentrations of MDMA shown to produce lasting serotonergic deficits in squirrel monkeys overlap those reported by other laboratories in some recreational "ecstasy" consumers and are two to three times higher than those found in humans administered a single 100- to 150-mg dose of MDMA in a controlled setting [324].

### MDMA-Induced Neurotoxicity to Mice

Unlike rats or non-human primates, the mouse, which corresponds to a regular experimental animal model in the lab, has proven to be a notable exception regarding the pattern of MDMA-induced neurotoxicity. It is well established that MDMA administration to mice produces a different neurotoxic profile, producing rather dopaminergic neurotoxicity in contrast to 5-HT neurotoxicity in rats or non-human primates. MDMA administration to mice induces depletions of DA and its metabolites in several brain regions, but in particular produces long-term degeneration of striatal DA nerve terminals [41, 51, 135, 136, 243, 293, 325–328]. The normal dosage regimens of MDMA used in those studies range from single administration of 15 mg/kg, to four binge injections of 20 mg/kg every 2 h. However, depending on MDMA dosage and mouse strain, it can cause dual DA/5-HT neurotoxicity [326, 329, 330]. Mice administered with MDMA (50 mg/kg×2, 2 h apart) presented 1 week later both 5HT and DA depletion in various brain regions, namely, cerebellum, frontal CTX, HIP, hypothalamus, striatum, and substantia nigra [326]. A neurotoxic regimen of MDMA (15 mg/kg×2, 8 h apart, for 2 days) in Swiss–Webster mice was shown to cause marked depletion of striatal DA and DAT binding sites as well as depletion of 5-HT in the striatum and HIP and depletion of 5-HTT binding sites in the frontal CTX. The depletion of striatal DAT and frontocortical 5-HTT binding sites in mice was demonstrated to be long-lasting, as it was observed 82 days following exposure to MDMA [329]. Therefore, strain differences in mice, possibly through metabolic variations, account for the observed dissimilar neurotoxic profile of MDMA.

A few studies have analyzed microglia activation and GFAP expression as markers of neurotoxicity in mice exposed to MDMA. Similar to rats, some conflicting data have been reported regarding this marker. Female C57BL/6

mice administrated with MDMA (20 mg/kg, four times at 2-h intervals, s.c.) presented increased expression of GFAP accompanied by microglial activation in the striatum 48 h after treatment [331]. Another study, where the same mice strain received a neurotoxic regimen of D-MDMA (15.0 mg/kg, s.c., as the base every 2 h, four times) showed an elevation of striatal GFAP [51]. However, a recent study reported that four injections of 30 mg/kg at MDMA 2-h intervals do not induce microglial effects in the striatum of male NIH Swiss mice [327].

Several studies also indicate that MDMA-induced neurotoxic effects in mice also go beyond the dopaminergic system, as neurodegeneration could be found in other neurons. MDMA administration (30 mg/kg×3, 2 h apart) to C57 Black mice induced degeneration of striatal GABA neurons, which were found to have neuronal inclusions [332, 333]. In another study, the same mice strain injected with MDMA (5 mg/kg×4, 2 h apart), besides decreasing nigrostriatal DA innervation and 5-HT loss, produced neuronal inclusions within nigral and intrinsic striatal neurons consisting of multilayer ubiquitin-positive whorls extending to the nucleus of the cell, as well as oxidation of DNA bases followed by DNA damage [330]. Additionally, mice treated chronically (25 days) or for 5 days with daily injections of MDMA (2.5 mg/kg) presented neuronal degeneration accompanied by acute oxidative stress and DNA single- and double-strand breaks, which persist together with long-lasting metabolic changes in the hippocampal formation [334, 335]. These findings demonstrate that MDMA-induced alterations extend beyond axon terminals loss and involve subcellular changes at the level of DA and GABA cell bodies.

Several studies also describe MDMA-induced learning deficits in the mice model. A recent study conducted in mice showed that acute MDMA administration (30 mg/kg 1 h before different active avoidance training sessions) modified the acquisition and execution of active avoidance of a previously learned task. Meanwhile, repeated pretreatment with MDMA (30 mg/kg twice a day over 4 days before training) impaired acquisition and recall of this task [336]. Authors did not correlate the learning deficits observed in mice with dopaminergic neurotoxicity since non-neurotoxic low doses of MDMA, which did not reduce DAT binding, also produced deficits. Therefore, even low MDMA doses have the ability to produce learning deficits and cognitive impairment in mice, similar to those seen in the rat model. In mice administered with MDMA at cumulative doses of 10–50 mg/kg, increases in tau protein phosphorylation were produced, which are typically associated with Alzheimer's disease and other chronic neurodegenerative disorders [337]. Additionally, the same authors found that the impairment in HIP-dependent spatial learning was induced by doses of MDMA that increased tau phosphorylation [337]. Another recent study showed that 4 weeks after the last injection of MDMA (20 mg/kg twice a day for 4 days) to C57BL/6J mice showed a 30% decrease in BrdU labeling of proliferating hippocampal cells and increased immobility duration in the forced swim test, suggesting a depressive-like behavior [338]. All the described effects were abolished in 5-HTT KO mice, which also did not present 5-HT depletions in the anterior raphe area, HIP, striatum, and CTX brain areas that were seen in wild-type (WT) animals [338]. Authors related the observed defects in mice to the impairment of the serotonergic function produced by MDMA.

## "Ecstasy"-Induced Neurotoxicity—Evidences in Humans

Results obtained in animal models are very important for the prediction of the neurotoxic potential in human recreational users of MDMA; however, only studies with humans can provide indication of doses or frequency regimes that may put "ecstasy" users at risk of neurotoxicity. In addition to the known difficulties of extrapolating data from animal models to the human situation, MDMA has also been shown to comprise some particular pharmacological differences in humans relatively to the animal models normally used to evaluate MDMA neurotoxicity. The use of high doses and different routes of administration in animal studies, diverse of recreational human use, has led to the suggestion that the animal data may relate more to a "heavy" user of MDMA [96, 339]. Also, the role of pharmacokinetic and metabolic differences and factors such as age, sex, and polydrug use by users of MDMA comprehend some of the variables that are different in the human and animal situation, which may affect the long-term actions of the drug [96, 339]. The differences between animal models and humans concerning the metabolism of MDMA have already been addressed in this text ("Interspecies Differences in MDMA Pharmacokinetics"). Although animal models have inherent limitations, these studies are important means to evaluate the mechanism and the neurotoxic actions of MDMA. Yet, they should be supplemented with the data found in human "ecstasy" users in order to confirm the animal findings. This stresses the striking importance of the scarce human studies in the context of MDMA-induced neurotoxicity.

There is a single report on a direct measurement of brain striatal 5-HT and 5-HIAA in the brain of a MDMA user. In a 26-year-old male, who had taken MDMA regularly for 9 years, severe depletions (50–80%) of striatal 5-HT and 5-HIAA were found in the brain (measured 21 h postmortem). However, the subject was a polydrug user and had also taken cocaine and heroin in the previous months before his death. Since none of these drugs has previously been demonstrated to alter striatal 5-HT concentration, the

authors suggested that the observed postmortem data were most likely to be due to chronic use of MDMA [340].

Studies on the long-term effects of MDMA on serotonergic neurotransmission in humans are based on indirect methods, such as the measurement of 5-HT metabolites in the CSF or in neuroimaging studies using 5-HTT ligands. In the CSF of recreational users of MDMA, defined by the intake of "ecstasy" on more than 25 occasions and on control subjects, the metabolite concentrations of 5-HT, 5-HIAA, and DA HVA were measured [341, 342]. MDMA users had significantly lower levels of CSF 5-HIAA than control subjects, the reduction being greater in females (46%) than in males (20%). An apparent negative correlation between CSF 5-HIAA levels and the number of MDMA exposures was found, though there was no correlation between CSF 5-HIAA levels and the duration or frequency of MDMA use. There was no overall difference in CSF HVA concentrations between the two groups [341, 342]. The apparent negative correlation between CSF 5-HIAA levels and MDMA consumption was, however, statistically significant in another study in which the mean concentration of 5-HIAA in the CSF of MDMA users was lower than in the control group. The CSF 5-HIAA levels decreased at higher MDMA doses [343]. Additionally, MDMA users displayed multiple regions of gray matter reduction in the neocortical, bilateral cerebellum, and midline brainstem brain regions, potentially accounting for previously reported neuropsychiatric impairments in MDMA users [344].

Neuroimaging techniques have also been applied to the study of the 5-HT system in the brains of humans with a history of MDMA use. Positron emission tomography (PET) used in combination with a 5-HTT ligand found a lower density of brain 5-HTT sites in MDMA users, which correlated with the previous MDMA use [345–347] and also showed reduced 5-HTT activity following MDMA exposure [348–350]. Additionally, there are indications that the observer 5-HTT reductions play a role in memory deficits in individuals with a history of recreational MDMA use [347]. Single photon emission computed tomography (SPECT) has also been used with a 5-HTT ligand, showing that human MDMA users have reductions in 5-HT binding sites [351, 352]. Another study by Reneman et al. [353] has evaluated the acute and chronic effects of MDMA on cortical 5-$HT_{2A}$ receptors in rat and human brains. In rats, a decrease followed by a time-dependent recovery of cortical 5-$HT_{2A}$ receptor densities was observed. In recent MDMA human users, postsynaptic 5-$HT_{2A}$ receptor densities were significantly lower in all cortical areas studied. There have been some critics claiming that brain 5-HT neuronal damage data obtained by neuroimaging could have methodological flaws and also that some studies included polydrug users [354]. Nevertheless, the PET and SPECT data obtained from the studies in humans have been validated for the measure of MDMA-induced neurotoxicity, and these studies are important and essential to study human MDMA users [355].

Some studies have demonstrated that females are more sensitive towards MDMA neurotoxicity than male human subjects. Female users show higher depression scores than male users or male or female control subjects several days after an acute dose of MDMA [356]. Furthermore, using SPECT imaging, binding of 123iodine-2beta-carbomethoxy-3beta-(4-iodophenyl) tropane ([123I]beta-CIT), a radioligand that binds with high affinity to 5-HTT, is lower in female "heavy" MDMA users than in male "heavy" MDMA users or than controls, "heavy" use being defined as previous use of more than 50 tablets. This effect was not related to greater MDMA use, which suggests that females may be more sensitive to the neurotoxic effects of MDMA [351]. Furthermore, biochemical evidence supports this suggestion, as CSF 5-HIAA levels following MDMA ingestion have been measured and the reduction in females is significantly greater than that observed in males (the reduction in 5-HIAA levels in females was 46%, while in males was 20% compared to the control values) [342]. These differences can be attributed to possible sex differences in the brain serotonergic system or can be also due to gender differences on MDMA metabolic profile.

Complementary to the studies on brain serotonergic markers, reports have shown long-term impairments in memory and learning of human MDMA abusers, in particular, working memory, planning ability, executive control, and cognitive impulsivity, which persist long after cessation of drug use [231, 341–343, 357–367]. Occasionally, visual hallucinations and paranoid delusions that form part of the acute effects of the drug may persist for days or weeks along with depression, panic disorder, increased impulsivity, cognitive impairment, and other alterations in behavior [231, 342, 343, 350, 358, 359, 368, 369]. Cognitive deficits are more apparent in tasks sensitive to temporal functioning [361]. Verbal and visual memory have been shown to be impaired by MDMA use and are related to the CSF concentration of 5-HIAA, indicating that serotonergic damage may be the cause of these deficits [343]. Moreover, MDMA use in adolescence may be associated with cognitive impairments and dysfunction of inhibitory circuits in the HIP, indicating that similar behavioral changes occur in this age group, as those observed in adults [365]. A longitudinal study was conducted by Daumann et al. [370] with a follow-up period of 18 months in MDMA users which had used "ecstasy" regularly over at least 6 months, with a minimum frequency of twice a month or had used "ecstasy" on at least 20 occasions. This study revealed that altered cortical activation patterns in MDMA users persist, reverse, or intensify

after prolonged use or abstinence. "Ecstasy" use appears to be associated with greater activation in the parietal CTX during working memory processing, which seems to intensify with continued use and may not remit after a prolonged time of abstinence. An altered activation pattern might appear before changes in cognitive performance become apparent and, hence, may reflect an early stage of neuronal injury by "ecstasy" [370].

Studies point out a correlation between memory impairment and altered 5-HT neuronal function [341, 347, 371]. Reports also indicated that primary memory dysfunction in "heavy" "ecstasy" users may be related to a particularly high vulnerability of the HIP to the neurotoxic effects of MDMA [360]. A lower retrieval-related and more spatially restricted activity in the left anterior HIP was verified in MDMA abusers by functional magnetic resonance imaging, probably due to neurotoxic effects of the drug [366]. However, the mechanism and origin of the MDMA-related cognitive disturbance remain to be fully elucidated.

The influence of 5-HTT polymorphism in the context of MDMA neurotoxicity has been evaluated. Human variability in the 5-HTT expression seems to be due to a polymorphism that consists of a repetition element upstream of the transcription site of the *SCL6A4* gene which codes for the transporter [372]. Most of the alleles correspond to either a repetition element of 14 units (short allele) or 16 units (long allele) [372]. The homozygous expression of the short allele is associated to a lower expression of the 5-HTT, to a diminished capacity for the 5-HT uptake, and to a deficiency in the serotonergic neurotransmission [372, 373]. The expression of the short allele has also been associated with neurologic adverse reactions in MDMA abusers, such as cognitive impairment and emotional dysfunction, as a result of a higher susceptibility of the serotonergic system to the deleterious effects of MDMA [373, 374].

Not all studies support the notion that MDMA abuse is associated with cognitive deficits. Some studies point out that a low dose of MDMA (one to two tablets containing the usual amount of MDMA) has neither effects on human cognitive brain function in the domains of working memory, selective attention, and associative memory nor indications for structural neuronal damage [375, 376]. Some authors suggest that "ecstasy" is a risk factor for earlier onset and/or more severe decline of age-related memory deficits in later years. It has also been suggested that primary memory dysfunction is more associated with "heavy" "ecstasy" use ("heavy use" was defined as use on 30–1,000 occasions) [360].

There are clear evidences of cognitive dysfunctions in humans following long-term MDMA use, although not always straightforward. Most studies on the effect of recreational use of "ecstasy" have necessarily been done retrospectively. Furthermore, studies are confounded by the possibility that the drug taken as "ecstasy" might not have been MDMA (in the mid to late 1990s, MDMA tablets sometimes contained a wide range of other psychoactive drugs) and that many users also took additional drugs, namely, cannabis and alcohol, which indicates that some of the effects of MDMA can be attributed or promoted by the use of other drugs [377]. In at least one study where polydrug use has been controlled, many of the deleterious effects attributed to "ecstasy" have been found at similar levels in the polydrug users [378]. Additionally, in "ecstasy" users where psychiatric illnesses have been identified, it was difficult to determine if the development of these problems are a result of taking MDMA or a natural development of a psychiatric disorder. Despite these difficulties in conducting studies in human drug abusers, the vast majority of studies provide data to support the idea that MDMA alone has deleterious effects on brain cognitive function.

## Molecular and Cellular Mechanisms of MDMA-Induced Neurotoxicity

Regardless of more than two decades of studies on MDMA neurotoxicity, the underlying mechanisms remain to be fully elucidated. In the following topics, we intend to provide an overview on the factors and mechanisms that have been experimentally established to explain MDMA-induced neurotoxicity. The order of the items' appearance was selected not necessarily taking into account the relevance but rather the order which, in our belief, would provide a better understanding for the readers.

### Hyperthermia Induced by MDMA Treatment

Many in vivo studies indicate that hyperthermia plays a major role in MDMA-induced neurotoxicity. Malberg and Seiden [143] demonstrated the importance of ambient temperature in the long-term depletion of 5-HT and 5-HIAA following MDMA (20 or 40 mg/kg) administration to rats. These authors found no significant depletions in any examined brain regions when MDMA had been administered at ambient temperatures of 20°C, 22°C, or 24°C. However, under environment temperatures of 26°C, 28°C, or 30°C, significant depletion was observed. Therefore, small changes in ambient temperature were shown to produce marked changes in the degree of serotonergic neurotoxicity. In another study employing binge dosing to rats (three doses given 3 h apart of 2, 4 and 6 mg/kg, i.p.) to simulate recreational users that take several doses in one session, it was possible to verify the potentiation of MDMA-induced 5-HT neurotoxicity in a hot environment compared to normal

temperature conditions [151]. On the other hand, surgical treatments to animals that prevent hyperthermia revealed neuroprotection. Hypophysectomized and thyroparathyroidectomized rats treated with MDMA (40 mg/kg, s.c.) did not become hyperthermic. In fact, these animals revealed a significant hypothermia and did not display serotonergic neurotoxic effects assessed by 5-HT measurements 4 to 7 days later in the striatum and HIP brain areas [156].

Hyperthermia has also been shown to enhance MDMA-induced DA and 5-HT release in the rat. In a study on MDMA-induced DA and 5-HT efflux in the shell of nucleus accumbens of freely moving rats, using microdialysis, MDMA (2.5 or 5 mg/kg, i.p.) evidenced a substantial increase in extracellular DA and 5-HT, which was more marked in this brain region at high room temperature (30°C) [379]. These results show that high ambient temperature enhances MDMA-induced locomotor activity and monoamine release in a region involved in the incentive motivational properties of drugs of abuse, which suggests that the rewarding effects of MDMA may be more pronounced at high ambient temperature [379]. Enhancement of DA and 5-HT efflux under hot environmental conditions can, as well, potentiate the neurotoxic events promoted by MDMA.

It seems that hyperthermia is required for the neurotoxic events to occur when a single MDMA dose is administrated. However, repeated administration of low doses of MDMA (4 mg/kg, twice daily, for 4 days) did not produce hyperthermia, but did induce 5-HT long-term depletion [263]. Even when hypothermia was promoted to animals by placing them in a cold environmental temperature (4°C or 10°C) after MDMA administration (20 mg/kg, i.p.) to female Sprague–Dawley rats or 12.5 mg/kg, i.p. to male Dark Agouti rats, long-term reductions in 5-HT content and 5-HT reuptake sites were still observed, demonstrating that neurodegeneration can still occur even under normo- or hypothermic conditions [142, 257]. Thus, hyperthermia can potentiate MDMA-induced neurotoxic events, although it is not mandatory for the long-term neurotoxicity that follows after MDMA administration. On the other hand, as previously stated, unlike rats, MDMA administration promoted always an increase in core body temperature in humans [219] and also in rhesus macaques [162] regardless of ambient temperature, providing evidences for interspecies differences regarding MDMA temperature effects.

It was shown that prevention of MDMA-induced hyperthermia decreases the neurotoxicity and that many drugs that protect animals against MDMA-induced serotonergic neurotoxicity also decrease the body temperature [149, 380]. Compounds that were initially reported to be neuroprotective in animals against MDMA-induced neurotoxicity have confirmed to afford protection not because of a specific neurochemical action but because of an indirect effect by lowering body temperature. In fact, administration of a drug that prevents rats' hyperthermia will produce neuroprotection [149, 381]. The *N*-methyl-D-aspartic acid (NMDA) glutamate (GLU) receptor antagonist MK-801 (dizocilpine) was reported to be neuroprotective against MDMA-induced damage to 5-HT nerve endings [254, 260, 382]. However, it was later shown that co-administration of MK-801 and MDMA produced a hypothermic response and that when the temperatures of these animals were kept elevated, the neuroprotective effect of MK-801 was lost [149, 380]. Furthermore, another NMDA antagonist, S-(+)-α-phenyl-2-pyridine ethanamide dihydrochloride (AR-R15896AR), does not attenuate MDMA-induced hyperthermia to rats and did not produce neuroprotection [381]. Other compounds, namely, 5-$HT_{2A}$ receptor antagonists, like ketanserin and ritanserin [147, 383] and also α-methyl-*p*-tyrosine (AMPT) [384], were shown to protect against MDMA-induced damage to 5-HT nerve endings of rats. However, it was also shown that in co-administration with MDMA of ketanserin, ritanserin, R-96544, or even risperidone, all 5-$HT_{2A}$ receptor antagonists inhibited the MDMA-induced hyperthermia in rats [149, 154]. When the temperatures of ketanserin or AMPT-treated animals were kept elevated, the neuroprotective effect of these drugs against MDMA serotonergic neurotoxicity was lost [149]. On the other hand, drugs that enhance MDMA-induced hyperthermia can enhance neurotoxicity. MDMA abuse by humans commonly occurs in hot overcrowded environments in combination with caffeine. Co-administration of caffeine with MDMA or MDA to rats resulted in and enhanced hyperthermic response, which promotes the acute and long-term 5-HT toxicity associated with MDMA and MDA [259].

Another mechanism by which MDMA-induced hyperthermia can contribute to neurodegeneration is through the enhancement of free radical formation in the brain. Several studies have measured brain reactive oxygen species (ROS) formation after MDMA administration [249, 385, 386]. Free radical formation can be evaluated by implantation of a microdialysis probe into the brain area under study, which allows the measurement of 2,3- and 2,5-dihydroxybenzoic acid (2,3-DHBA and 2,5-DHBA) formed from salicylic acid perfused by the probe [249, 385, 386]. Administration of MDMA (15 mg/kg, i.p.) to Dark Agouti rats increased the formation of 2,3-DHBA in the HIP for at least 6 h, which was prevented by the free radical scavenging agent alpha-phenyl-*N*-*tert*-butyl nitrone (PBN; 120 mg/kg, i.p.) 10 min before and 120 min after MDMA administration [385]. Other study by the same authors proved that MDMA (15 mg/kg, i.p.) increased the formation of 2,3-DHBA and 2,5-DHBA from salicylic acid perfused through a microdialysis probe implanted in the HIP [386]. The drug clomethiazole (50 mg/kg, i.p.), a gamma-aminobutyric acid ($GABA_A$) agonist that has no free radical scavenging

activity, administered 5 min prior and 55 min post-MDMA prevented both the acute MDMA-induced hyperthermia and the rise in 2,3- and 2,5-DHBA. However, the authors also show that when the temperature of the MDMA +clomethiazole-treated rats was kept elevated with a homeothermic blanket, there was no inhibition of the MDMA-induced increase in 2,3-DHBA or 2,5-DHBA. The authors concluded that free radical formation is inhibited when the acute MDMA-induced hyperthermia is prevented [386]. Therefore, hyperthermia has a major role in the production of free radicals in the brain of MDMA-treated rats. However, drugs that do not interfere with MDMA-induced hyperthermia can increase MDMA-induced ROS formation in the brain of animals, for instance ethanol. MDMA (5 mg/kg)-promoted increase in hydroxyl radical ($HO^{\bullet}$) production in the HIP is more marked in rats pre-exposed to ethanol (4-day ethanol regimen leading to plasma ethanol levels of around 450 mg/dl), a fact that enhances long-term 5-HT neurotoxicity [258].

Despite these reports, it should be considered that animal experiments performed in the regular laboratory might increase the core temperature up to 1°C due to the handling of the animals [387]. In addition, there are a variety of physiological changes that accompany modifications of body temperature [289]. Hence, in animal studies, researchers are faced with a variety of factors affecting body temperature, which is a variable of major importance regarding MDMA neurotoxicity. Our group has used primary cultured cortical neurons under tight temperature-controlled conditions to circumvent these problems. Animal brain temperature can be simulated by cultivating cortical neurons both at the normal temperature of 36.5°C and at a temperature of 40°C after addition of MDMA [76, 77, 289, 290]. This procedure has the advantage of tight temperature control throughout the whole experiment without the interference of other factors that are associated with in vivo experiments. The incubation of neuronal cortical cultures both at normal (36.5°C) or high (40°C) temperature following addition of MDMA or MDMA metabolites to cultures (concentration range 100–400 μM) leads to a time- and concentration-dependent neuronal apoptotic death [76, 77, 289, 290]. Furthermore, these studies demonstrated that the toxicity of MDMA and its metabolites to cortical neurons was significantly higher under hyperthermic conditions (40°C) than that obtained under normothermia (36.5°C). These studies were the first conducted in cortical serum-free neuronal cultures, lacking 5-HT producing cells, demonstrating that there is a concentration-, time-, and temperature-dependent neuronal death mediated by MDMA and its metabolites to non-serotonergic neurons [76, 77, 289, 290]. As the misuse of MDMA as a recreational drug (particularly in crowded and hot environments) can induce hyperthermia, the obtained results corroborate the serious concern previously reported in the literature. Furthermore, our studies prove that several drugs that were shown to protect against MDMA's serotonergic neurotoxicity via a hypothermic effect could attenuate MDMA-induced toxicity to cortical neurons in a temperature-independent manner. The protective effect afforded by the selective 5-$HT_{2A}$ receptor antagonist ketanserin, the GLU receptor antagonist MK-801, and a NOS inhibitor $N_{\omega}$-nitro-L-arginine (L-NNA) against MDMA-induced cortical neuronal death occurred both under normal and hyperthermic temperatures [289, 290]. Therefore, our studies, unlike previous animal reports, showed that these antagonists afford protection against MDMA-induced neurotoxicity in a temperature-independent manner.

In summary, hyperthermia is an important factor in MDMA-induced neurotoxic events. The hyperthermia that follows MDMA administration potentiates MDMA neurotoxicity, and drugs that block hyperthermia attenuate or avoid it. Although reports clearly suggest a link between hyperthermia and subsequent neurotoxicity, not all agents that prevent MDMA-induced neurotoxicity necessarily do so by blocking the hyperthermic response, as we shall revise in the following text. Therefore, the long-term neurotoxicity that follows MDMA neurotoxic regimens is not directly related to hyperthermia.

## MAO-Mediated Metabolism of Monoamine Neurotransmitter

Monoamine oxidase (flavin-containing) [amine: oxygen oxidoreductase (deaminating; flavin-containing); MAO] is an enzyme present in the outer mitochondrial membrane which catalyzes the following reaction: $RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2$. MAO acts on primary amines and also on some secondary and tertiary amines [388]. It is involved in the metabolism of important monoamine neurotransmitters, such as dopamine, noradrenaline, adrenaline, and serotonin. Two isoforms of MAO exist, MAO-A and MAO-B. In the brain, MAO-A is expressed predominantly in catecholaminergic neurons, whereas MAO-B is expressed in serotoninergic neurons, astrocytes, and glia [388]. The oxidative deamination of monoamine neurotransmitters by MAO produces hydrogen peroxide ($H_2O_2$), which subsequently may be converted into the highly reactive $HO^{\bullet}$, and consequent oxidative stress-related damage may occur [388, 389].

The relevance of MAO in the context of MDMA-induced neurotoxicity results from the fact that after MDMA administration to experimental animals, there is a phase of abrupt increase of the extravesicular levels of monoamine neurotransmitters inside nerve endings, mainly NE, 5-HT, and DA, which are mainly metabolized by MAO

[248, 389–391]. Oxidative-stress-related neurotoxicity is known to occur due to the production of high levels of $H_2O_2$, as mentioned above. Another consequence of MAO-mediated metabolism of DA and 5-HT is the generation of the reactive aldehyde intermediates 3,4-dihydroxyphenylacetaldehyde (DOPAL) and 5-hydroxyindole-3-acetaldehyde (5-HIAL), respectively, before their conversion into the more stable metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and hydroxyindole-3-acetic acid (5-HIAA) by aldehyde dehydrogenase (ALDH; Fig. 7). At least DOPAL is a neurotoxicant known to contribute to MAO-related damage [392]. The elegant hypothesis of MAO-related damage has been supported by various studies where inhibition of MAO-B prevented serotonergic neurotoxicity and oxidative damage to the mitochondria induced by MDMA. Inhibition of MAO-B, either by pharmacological agents (L-deprenyl also termed selegiline) or antisense oligonucleotide targeted at MAO-B, was shown to be protective [248, 391, 393]. Furthermore, MAO-B-deficient mice were also shown to be protected against MDMA (50 mg/kg×2, 2 h apart)-promoted serotonergic damage, which occurred in WT mice [326].

A recent study conducted by our group has shown that the exposure of an adolescence rat model (PND 40, which corresponds to the human adolescence period) to a neurotoxic binge administration of MDMA (four times, 10 mg/kg, i.p., every 2 h) induces oxidative stress to whole brain mitochondria [389]. Additionally, this MDMA neurotoxic regimen produced deletion of mitochondrial DNA and subsequent impairment in the genes required for protein synthesis, with the correspondent lower protein expression, which are essential complexes of the mitochondrial respiratory system required for energy production [389]. The deleted portion of the mitochondrial genome includes codons for NADH dehydrogenase and cytochrome c oxidase proteins [394, 395]. Inhibition of MAO-B by selegiline (2 mg/kg, i.p.) 30 min before MDMA did not reduce hyperthermia but reversed MDMA-induced effects in the oxidative stress markers, mitochondrial DNA, and related protein expression [389]. On the other hand, MAO-A inhibition by the specific inhibitor clorgyline (1 mg/kg, i.p.) 30 min prior to the same MDMA dose resulted in synergistic effects on 5-HT-mediated behavior and body temperature, provoking high mortality [396]. A mechanistic hypothesis has been postulated based on these findings. Since MAO is localized in the outer membrane of the mitochondria, the formation of $H_2O_2$ resulting from the enzyme metabolism of MDMA-released monoamine neurotransmitters (mostly NE, 5-HT, and DA) will mostly affect the mitochondria itself. When $H_2O_2$ removal pathways are lacking or inactivated, $H_2O_2$ accumulates and is converted into the highly reactive $HO^{\bullet}$, inducing intense oxidative stress in mitochondria. Rat mitochondrial DNA, like human mitochondrial DNA, is vulnerable to ROS because it lacks the protection of histones and DNA-binding proteins, and therefore, bursts of ROS are prone to produce mitochondrial DNA deletions [389]. Oxidative damage to the mitochondria can initiate the intracellular cascade of reactions leading to neurotoxicity. In a model shown in Fig. 7, we postulate a cascade of events associated with DA and 5-HT metabolism, which occur at the mitochondrial level leading to neurotoxicity via mitochondrial damage.

In particular, the damage to the serotonergic neurons by MDMA could be explained either by the increase in the cytoplasmatic non-vesicular 5-HT content or by the uptake of the excessive extracellular DA, which can as well undergo deamination by MAO-B inside the serotonergic nerve terminal. 5-HT has been shown to be metabolized in vitro by MAO-A ($K_m$, 178±2 μM) and MAO-B ($K_m$, 1,170 ±432 μM) [397]. Although 5-HT metabolism by MAO-B is only residual in the presence of MAO-A, it is fully effective in the absence of the latter, as it happens inside serotonergic neurons [388, 389]. Due to this massive amount of non-vesicular 5-HT, MAO-B metabolism of this amine can lead to massive production of ROS and reactive metabolite intermediates. The ensuing damage generated in the mitochondria will also occur inside the nerve terminal, which eventually degenerates.

## DA Substantial Release Promoted by MDMA

Evidences from several reports support a role for DA in MDMA-induced neurotoxicity. As mentioned earlier, MDMA elicits DA release both in vitro and in vivo. MDMA-induced release of DA in the brain of rats is also amplified by the subsequent increase in postsynaptic 5-HT through the activation of postsynaptic $5\text{-HT}_{2A}$ receptors, which enhances DA synthesis and release [30]. Additionally, it has been shown that rats, prior exposure to both MDMA (10 mg/kg×4, i.p.) and stress, can produce a long-term augmentation in mesolimbic DA transmission and potentiates DA release [398]. Probably, the most convincing evidences of the importance of DA in MDMA-induced neurotoxicity have been reported in studies inhibiting MAO-B, as it was described earlier. Nonetheless, other studies supported for a role of DA alone, since prior administration to rats of 6-hydroxydopamine, which destroys DA terminals, blocks MDMA-induced neurotoxicity [384, 399]. Meanwhile, pretreatment of rats with L-DOPA, a DA precursor, increases MDMA-induced neurotoxicity [399]. Furthermore, DAT inhibition, either by GBR 12909 and mazindol or by means of antisense oligonucleotide complementary to the mRNA translational start site of the rat dopamine transporter, prevents increase in DA levels and serotonergic neurotoxicity following treatment with MDMA [384, 400, 401]. Several other compounds that interfere with the DA

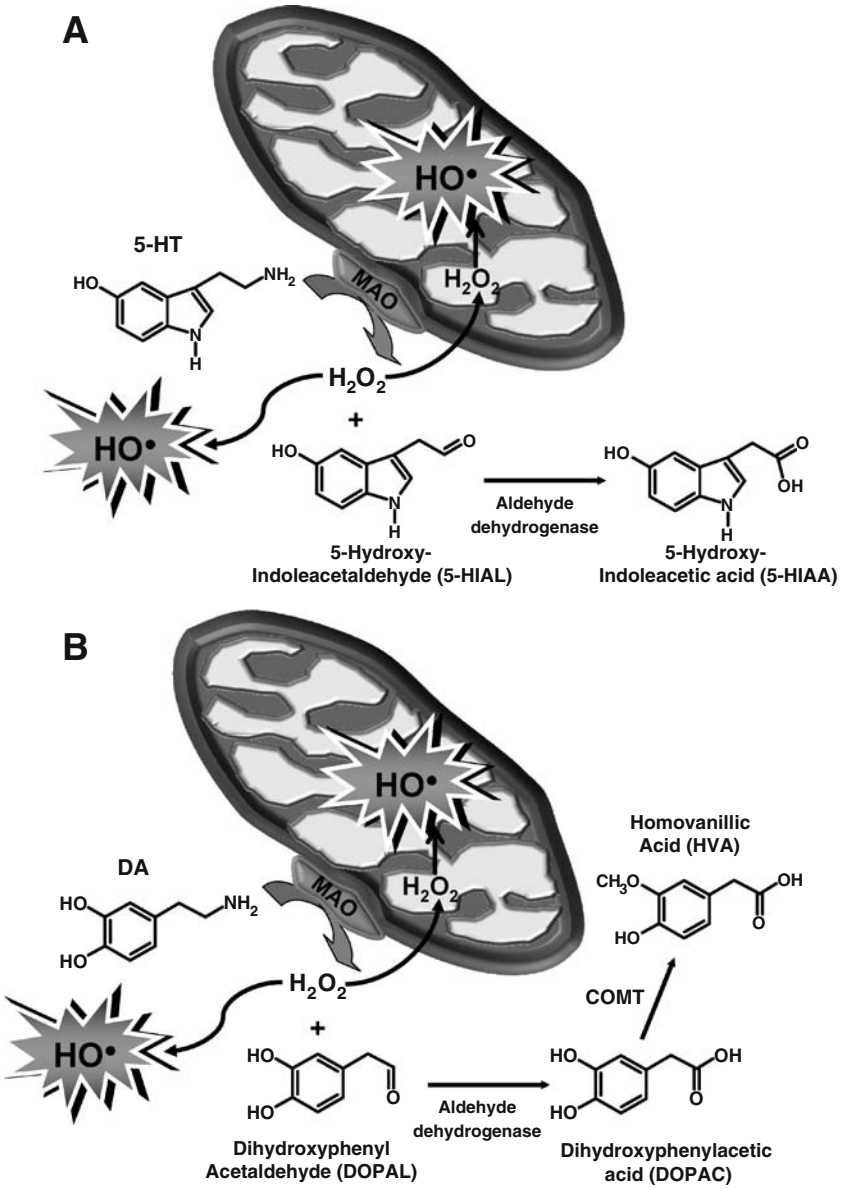


**Fig. 7** Monoamine oxidase (*MAO*) located in the outer membrane of the mitochondria of neurons mediates the metabolism of serotonin (*5-HT*) in **a** and of dopamine (*DA*) in **b**. MAO-mediated metabolism of DA and 5-HT leads to the generation of the reactive aldehyde intermediates 3,4-dihydroxyphenylacetaldehyde (*DOPAL*) and 5-hydroxyindole-3-acetaldehyde (*5-HIAL*), respectively, before their conversion into the more stable metabolites 3,4-dihydroxyphenylacetic acid (*DOPAC*) and hydroxyindole-3-acetic acid (*5-HIAA*) by aldehyde dehydrogenase (*ALDH*). Metabolism of both neurotransmitters leads to the formation of hydrogen peroxide ($H_2O_2$) as a metabolic by-product. This metabolic product promotes an increase in hydroxyl radical ($HO^{\bullet}$) formation, a highly toxic reactive oxygen species leading to the damage of cellular proteins, as well as mitochondrial DNA and proteins, which contribute to MDMA neurotoxic events

transmission, namely, reserpine (vesicular dopamine-depleting agent), AMPT, and haloperidol (DA receptor antagonist), can protect against MDMA-induced neurotoxicity, although these protection studies are confounded by the fact that in animals these agents do attenuate MDMA-induced hyperthermia [384, 402].

When considering DA involvement in the neurotoxic events of MDMA, one has to remember the oxidation chemistry of this catecholamine. The oxidation of catecholamines (DA, adrenaline and NE) at physiological pH seems to occur very slowly; however, it increases considerably by enzymatic or metal catalysis or in the presence of superoxide anion ($O_2^{\bullet-}$) [403–406]. In comparison with other catecholamine neurotransmitters, DA is more prone to suffer oxidation, with the consequent formation of quinones, which, in the presence of GSH, may form a glutathionyl conjugate that has the ability of redox cycling, promoting by this way the formation of free radicals [404–

407]. This particularity of DA has long been studied for explaining the dopaminergic neurodegeneration that occurs in Parkinson disease [407, 408]. Not only DA but also NE are massively released in the brain of animals after MDMA treatment, and the oxidation of both catecholamines can promote oxidative stress leading to neurodegeneration. DA oxidation certainly contributes to the increase of ROS formation in the brain after MDMA administration [249, 385, 386]. Several studies corroborate the hypothesis that the excess of cytoplasmatic and extracellular DA are important in the context of MDMA-induced neurodegeneration [330, 332, 333]. Administration of MDMA (30 mg/kg×3, 2 h apart) to male C57 Black mice leads to ultrastructural alterations of striatal postsynaptic striatal GABAergic cells consisting of neuronal inclusions shaped as whorls of concentric membranes [332], which were prevented by drugs reducing DA availability [333]. These whorls stained for ubiquitin, but not for synuclein, and represent the first morphologic correlation of striatal postsynaptic effects induced by MDMA [330, 332, 333]. Occurrence of ubiquitin-positive neuronal inclusions and DNA damage both in nigral and striatal cells suggests the involvement of nuclear and cytoplasmic components of the ubiquitin-proteasome pathway in MDMA neurotoxicity [330].

The GABA neuronal system, which is thought to serve as a modulator of dopaminergic activity, has been shown to modulate MDMA-induced neurotoxicity. It was demonstrated that clomethiazole, a $GABA_A$ agonist, also attenuated the serotonergic toxicity induced by MDMA treatment in rats [254]. Using microdialysis, it was possible to observe that MDMA-induced increase in extracellular DA was coupled with a decrease in extracellular GABA levels in the rat striatum, which could be reversed by the 5-$HT_{2A}$ receptor antagonist, ritanserin [409]. On the other hand, it was suggested that MDMA-mediated increases in DA within the rat nucleus accumbens shell are dampened by GABA increases in ventral tegmental area subsequent to the activation of 5-$HT_{2C}$ receptors in that brain area [410]. Interestingly, in mice where MDMA produces a different neurotoxic profile, pretreatment with clomethiazole was ineffective in protecting against MDMA-induced dopamine brain loss [136]. It is possible that MDMA interference with the GABAergic system via different pathways might enhance DA synthesis and release in rats, thus potentiating MDMA neurotoxic effects in the long term.

Other studies suggested a role for tyrosine, the amino acid precursor in DA biosynthesis, in the MDMA-induced depletion of serotonergic biomarkers [411]. In male Sprague–Dawley rats, extracellular tyrosine concentrations increased fivefold in the striatum and 2.5-fold in HIP during the administration of neurotoxic doses of MDMA (10 mg/kg×4, i.p., every 2 h) [411]. Local infusion of L-tyrosine into the striatum or HIP of rats, during MDMA administration, potentiated the long-term depletion of 5-HT, which could be attenuated by the inhibition of aromatic amino acid decarboxylase, the enzyme responsible for the conversion of L-DOPA to DA [411]. The authors postulated that MDMA depletes 5-HT by increasing tyrosine brain concentration, which can be converted to DA within 5-HT terminals [411]. Another study reported that MDMA (10 mg/kg×4, i.p., every 2 h) also promoted an elevation of extracellular tyrosine concentrations in the striatum of male Sprague–Dawley rats [412]. Posterior MAO metabolism of this monoamine would explain free radical formation inside the nerve terminal that would lead to degeneration. A more recent study also showed that plasma tyrosine levels in rats were higher after a neurotoxic MDMA dose (15 mg/kg, i.p.) vs. a non-toxic dose of MDMA (7.5 mg/kg, i.p.) [413]. Additionally, these authors showed that administration of a non-toxic dose of MDMA in combination with L-tyrosine (0.2 mmol/kg, i.p.) produced a similar increase in serum tyrosine levels to those found after a toxic dose of MDMA. However, no brain 5-HT neurotoxicity was found. Furthermore, the non-toxic dose of MDMA combined with a high dose of tyrosine (0.5 mmol/kg, i.p.) caused long-term 5-HT depletions in hyperthermic rats [413]. These authors concluded that although tyrosine may contribute to MDMA-induced toxicity, other factors like MDMA metabolism appear to be the limiting step for its neurotoxicity [413].

Based on the findings about the role of DA and MAO metabolism, Sprague et al. [390] elaborated an integrated hypothesis, with DA playing a major role in MDMA-induced neurotoxicity. This hypothesis states that MDMA induces the following sequence of events resulting in serotonergic neurotoxicity: (1) MDMA induces an acute release of 5-HT and DA. (2) This acute release is followed by depletion of intraneuronal 5-HT stores. (3) The initially released 5-HT activates postsynaptic 5-$HT_{2A/2C}$ receptors located on GABA interneurons, resulting in a decrease in GABAergic transmission and increased DA release and synthesis. (4) The excessive DA released may be transported via 5-HTT into the depleted 5-HT terminal. (5) DA is then deaminated by MAO-B located in the mitochondria, producing $H_2O_2$, located within the 5-HT terminal. This would result in free radical formation and the selective degeneration of the serotonergic axons and axon terminals [390].

Although DA alone may have an important role in MDMA neurotoxicity, there are several evidences demonstrating that it may not be decisive. DA alone could not account for the fact that 5-HT terminal damage is observed throughout the CNS even in areas of the brain with very little DA innervation, such as HIP. The role of DA in other brain regions where DA content is very low would imply

different mechanisms in different brain regions for MDMA-induced neurotoxicity [414]. On the other hand, studies supporting a role for tyrosine in MDMA-induced neurotoxicity could account for the previously mentioned stepback in the DA alone theory. As mentioned above, rats treated with reserpine, alone and in combination with AMPT to deplete vesicular and cytoplasmic stores of DA, revealed hypothermia. When hypothermia is averted (by raising ambient temperature), the 5-HT neuroprotective effects of reserpine and AMPT against MDMA-induced neurotoxicity were lost [411, 415]. Overall, reports argue for an important role of DA, though by itself might not be a determinant for the occurrence of MDMA neurotoxic events.

## 5-HT Metabolites Produced Under Oxidative Environment

5-HT metabolism in an oxidizing environment can lead to the formation of reactive toxic metabolites. Such conditions are thought to be generated by the action of substituted amphetamines such as MDMA. The oxidation of 5-HT, in vitro, by an $O_2^{\bullet-}$-generating system (xanthine/xanthine oxidase) in a buffered aqueous solution at pH 7.4 has been reported [416]. 5-HT was rapidly oxidized, initially originating a mixture of 2,5-, 4,5-, and 5,6-DHT, with the major reaction product being 2,5-DHT, which at physiological pH exists as 5-hydroxy-3-ethylamino-2-oxindole [416, 417]. Rapid autoxidation of 4,5-DHT originates tryptamine-4,5-dione (T-4,5-D), which is postulated to be a major metabolite under these conditions [417, 418].

A massive release of 5-HT induced by MDMA in the brain of animals appears to result in an energy impairment largely because of 5-HTT-mediated efflux [23, 35, 37, 419]. During the period of reduced serotonergic energy metabolism, endogenous or elevated extracellular levels of GLU and NMDA receptor activation would be expected to mediate intraneuronal $O_2^{\bullet-}$, nitric oxide radical ($NO^{\bullet}$), and then peroxynitrite ($ONOO^-$) generation. It has been postulated that under these conditions, oxidation of 5-HT in the cytoplasm of serotonergic neurons by such ROS and reactive nitrogen species (RNS) would be expected to form the toxic metabolite T-4,5-D [417]. T-4,5-D is rapidly conjugated with GSH and reacts with other –SH-containing groups, forming, initially, 7-*S*-substituted thioethers [420]. This putative cytoplasmic metabolite of 5-HT is extremely electrophilic and reacts with –SH residues of proteins and enzymes [418, 421]. It has been shown, in vitro, that T-4,5-D irreversibly inactivates rat brain TPH [421], mitochondrial NADH-coenzyme Q1 (CoQ1) reductase (complex I), and cytochrome c oxidase (complex IV) [418] through covalent modification of active site cysteine residues and inhibits the pyruvate dehydrogenase complex (the link of glycolysis to the Krebs cycle) and the $\alpha$-ketoglutarate dehydrogenase complex (the link of the Krebs cycle to GLU metabolism) [420]. In the event of T-4,5-D formation, its ability to uncouple mitochondrial respiration and to inhibit NADH-CoQ1 reductase, cytochrome c oxidase, PDHC, and KGDHC all together is expected to severely impair mitochondrial ATP production by serotonergic neurons [417, 420].

Whether the hypothesis of intraneuronal ROS/RNS-mediated oxidation of 5-HT to T-4,5-D or other toxic 5-HT metabolites is relevant to the serotonergic neurotoxicity of MDMA remains to be established. Additionally, efforts to detect free T-4,5-D in the brains of rats exposed to neurotoxic doses of MDMA are almost certain to be unsuccessful due to the very rapid reaction of this highly electrophilic species with proteins and low-molecular-weight nucleophiles like GSH [420].

## TPH Inhibition by MDMA

Since early studies, it was recognized that TPH could have a role in MDMA-induced neurotoxicity. As previously stated, after MDMA administration to rats, there are long-term decreases in TPH activity, the rate-limiting enzyme of 5-HT synthesis, in the neostriatum, HIP, hypothalamus, CTX, and dorsal raphe nucleus [147, 252, 257, 266, 267, 399, 422, 423]. The enzyme activity began to decline immediately following MDMA s.c. administration to rats [167]. A study that followed TPH activity up to 32 weeks in male Dark Agouti rats administered MDMA (12.5 mg/kg, i.p.) showed that the TPH activity in the CTX and HIP was similarly reduced up to 8 weeks, but had recovered at 32 weeks, while striatal TPH activity recovered after 1 week [257]. MDMA induced an irreversible inhibition because restoration of enzyme activity requires new enzyme synthesis [257, 266]. Enzyme inhibition is not thought to be caused directly by MDMA because the drug has no effect on TPH activity in vitro. However, MDMA metabolites can undergo redox cycling and interact with –SH groups of the enzyme conducting to its oxidation [402, 424]. Studies also indicate that NO inactivates TPH by selective action on critical –SH groups (i.e., cysteine residues) while sparing catalytic iron sites within the enzyme [425]. This may have relevance to MDMA-induced neurotoxicity, since the drug is known to increase the production of NO and ROS in vivo. In a recent study, rats were administered a neurotoxic dose of MDMA (20 mg/kg, s.c.) twice daily for 4 days and killed 14 days later for evaluation of tissue sections of the dorsal raphe nucleus by in situ hybridization histochemistry for measurements of the levels of TPH2 immunoreactivity and TPH2 mRNA [423]. TPH2 immunoreactivity levels were significantly decreased by 45% and 40% in the mid and the caudal dorsal raphe nucleus, respectively, in the MDMA-treated rats compared with saline-injected rats. In contrast, TPH2

mRNA levels were significantly increased by 24% and 12% in the mid and in the caudal dorsal raphe nucleus, respectively [423]. The authors postulated that the increase in TPH2 mRNA levels in both those areas of dorsal raphe nucleus of MDMA-treated rats may reflect a compensatory mechanism after injury to 5-HT neurons in order to increase TPH2 protein synthesis to cope with terminal damage [423].

In the rat model, prevention of the long-term decreases in TPH activity, after MDMA administration, was shown to be achieved by 5-HT uptake inhibitors [167] and also by MAO-B inhibition [391]. The decrease in TPH activity can contribute to MDMA-induced 5-HT deficits, since it is the rate-limitant enzyme involved in its synthesis process. However, in contrast to other MDMA-induced insults, neurons appear to be capable of recovering from this neurotoxic event.

### 5-HTT-Mediated Transport

The involvement of 5-HTT in the mechanism of MDMA-induced neurotoxicity has been evidenced by many studies. The basis for this hypothesis has initially been the fact that 5-HTT inhibitors, like fluoxetine or fluvoxamine, prevent MDMA-induced 5-HT neurotoxicity in rats without interfering with MDMA-induced hyperthermia [149, 166, 167, 249, 273, 294].

The activation of the 5-HTT by MDMA appears to have a critical role in the long-term toxicity of 5-HT terminals. The concomitant administration of fluoxetine (10 mg/kg×2, i.p.) or fluvoxamine (15 mg/kg×2, i.p.) to male Dark Agouti rats provided complete protection against MDMA-induced (15 mg/kg, i.p.) cortical, hippocampal, and striatum serotonergic neurotoxicity [166]. It is noteworthy that the administration of fluoxetine (10 mg/kg, i.p.) 4 h following the administration of MDMA (20 mg/kg, s.c.) afforded total protection against MDMA-induced 5-HT toxicity, while the same treatment, up to 6 h after MDMA, significantly attenuates MDMA-induced 5-HT neurotoxicity to adult male Sprague–Dawley [249]. Furthermore, fluoxetine administration to rats 2 and 4 days earlier provided complete protection, and a significant protection was also seen when given 7 days prior to MDMA administration [166]. The authors have linked these long-lasting protective effects of fluoxetine to a significant brain concentration of the fluoxetine metabolite, norfluoxetine. Norfluoxetine is a fluoxetine metabolite with a longer $t_{1/2}$ and equipotent 5-HTT inhibitory activity, which is still detectable in the brain of rats 7 days after fluoxetine administration [166, 426].

The protection afforded by fluoxetine can also result from a pharmacokinetic interaction between fluoxetine and MDMA. In humans, it is known that CYP2D6 inhibitors modify MDMA pharmacokinetics [217]. Paroxetine a 5-HTT inhibitor is also a known CYP2D6 inhibitor, which, in human subjects, leads to a 30% increase in MDMA plasma concentrations, as a result of its metabolic biotransformation inhibition [217]. Fluoxetine is extensively biotransformed by the hepatic CYP2D6 enzyme system to norfluoxetine, an active metabolite that is eliminated much more slowly than fluoxetine by humans [426]. In rats, MDMA is metabolized by CYP2D1 and fluoxetine and norfluoxetine are also potent inhibitors of CYP2D1 [166]. A study evaluated the effect of fluoxetine in MDMA brain concentrations in Dark Agouti rats given fluoxetine (10 mg/kg, i.p., two doses, 60 min apart). The animals received two days later a single dose of MDMA (15 mg/kg, i.p.) and the cortical concentration of MDMA was measured 60 min later. There was no difference between MDMA brain levels found in rats pretreated with fluoxetine and those injected with saline [166]. However, these authors did not evaluate MDMA and MDA levels in plasma or in the brain over time.

Unlike the findings by Sanchez et al. [166] which suggested that fluoxetine does not alter MDMA kinetics, a more recent study has documented the opposite [427]. In the latter study, Sprague–Dawley rats pretreated with fluoxetine (4 days, 10 mg/kg, i.p.) or saline, followed by MDMA (10 mg/kg, p.o.), were evaluated for both brain and plasma, MDMA, and MDA levels during 10 h post-MDMA administration. Fluoxetine pretreatment resulted in an increase in MDMA (1.4-fold) and MDA (1.5-fold) exposure in both brain and plasma. Furthermore, elimination half-life was increased for MDMA (2.4 vs. 4.9 h) and MDA (1.8 vs. 8.2 h) with fluoxetine pretreatment. The same authors also showed that if brain or plasma MDMA concentrations were evaluated using only the 60-min time point, there were no differences in both fluoxetine pretreated versus non-treated [427]. This apparent contradiction among the two studies might be related not only to the fact that the first study only evaluated one time point but also to the different rat strains used. Sanchez and co-workers used male Dark Agouti rats, meanwhile Upreti and co-workers used Sprague–Dawley rats. The different metabolic profiles of these strains are well documented [428, 429]. Dark Agouti rats exhibit quite low mRNA levels of CYP2D2 and low metabolic activities for the biotransformation of some typical human CYP2D6 substrates (debrisoquine, bunitrolol, alprenolol, and metoprolol) compared to Sprague–Dawley or Wistar rats [428]. In fact, the Dark Agouti strain is considered (CYP)2D1/2D2-deficient and the Sprague–Dawley strain (CYP)2D1/2D2-replete [429]. CYP2D isoenzymes, as previously referred, are very important in MDMA liver metabolism. Therefore, differences in the cytochrome P450 isoenzymes may account for the different outcomes of studies conducted with MDMA. Once again, we stress the fact that it is important to consider the metabolic profile of the rat strain when interpreting the findings of MDMA-induced

neurotoxic events (Table 7). Fluoxetine pretreatment may provide protection against MDMA-induced long-term neurotoxicity by decreasing the metabolic transformation of MDMA and MDA to neurotoxic metabolites. On the other hand, it might pose users to enhanced risk of MDMA acute toxic effects, since plasma MDMA concentrations increase.

5-HTT expression can be rapidly modulated by receptor stimulation, second messenger production, and kinase activation. The suppression of 5-HTT activity accompanies PKC activation, which induces a loss of 5-HT uptake capacity [31]. PKC activation leads to transporter phosphorylation, transporter redistribution from the cell surface (sequestration), and loss of functional uptake activity. Ligands that can permeate the transporter, such as 5-HT or amphetamines, prevented PKC-dependent 5-HTT phosphorylation. In contrast to 5-HT and amphetamines, antidepressants facilitate the 5-HTT phosphorylation and block 5-HT uptake [31, 40, 430]. Since MDMA is a substrate for 5-HTT, an internalization of cell surface transporter protein, and therefore a decrease in the density of transporter proteins in the cell membrane induced by the presence of 5-HTT inhibitors, could involve a significant and lasting blockade in the uptake of MDMA or most likely of a metabolic product to the presynaptic 5-HT nerve terminals [166]. In vivo and also in vitro, fluoxetine was also shown to totally inhibit MDMA's prolonged activation of PKC translocation within the 5-HT nerve terminal [431–433].

There is a single report using 5-HTT KO mice to evaluate MDMA-induced neurotoxic effects. Four weeks after the last injection of MDMA (20 mg/kg twice a day for 4 days), WT C57BL/6J mice presented 5-HT depletions in the anterior raphe area, HIP, striatum, and CTX brain areas [338]. The same regimen produced 30% decrease in BrdU labeling of proliferating hippocampal cells [338]. All the described effects were abolished in 5-HTT KO mice [338]. These results stress the importance of the 5-HTT function for the serotonergic defects observed in MDMA-treated animals.

One potential mechanism by which 5-HTT inhibitors avoid the MDMA-induced 5-HT neurotoxicity is by preventing the entry, into the 5-HT terminal, of reactive substances like MDMA metabolites that are capable of generating free radicals. This hypothesis will be addressed later in the section concerning the metabolites' role in the neurotoxic events. In summary, there are enough data to argue for an important role of 5-HTT function in the mechanism of MDMA-induced neurotoxicity, but it is clear that it is not 5-HTT itself that directly regulates those events.

## MDMA-Induced GLU Release Leads to Excitotoxicity

GLU is the most abundant excitatory amino acid in the CNS and is capable of producing neuronal damage in a variety of experimental paradigms [434]. This has led to the hypothesis that GLU excitotoxicity could play a role in MDMA-induced neurotoxicity.

One study evaluated the effect of repeated administration of either Meth (7.5 mg/kg, three doses every 2 h, i.p.) or MDMA (13.8 mg/kg, three doses every 2 h, i.p.) on the extracellular concentrations of GLU in awake, freely moving rats using in vivo microdialysis. Meth increased the extracellular concentration of GLU in the striatum, whereas MDMA did not increase GLU efflux in the same area following a dose that produced a long-term depletion of striatal 5-HT content [435]. Another study showed that a microiontophoretic application of MDMA in rats inhibited GLU-evoked firing of most of the nucleus accumbens cells that were tested [436]. These results indicate that MDMA has inhibitory effects on GLU-evoked neuronal firing in the nucleus accumbens and suggest that the inhibition is mediated by increased extracellular DA and 5-HT [436]. In accordance, it was also demonstrated that 5-$HT_{2A}$ receptor activation caused an increase in DA levels, which effected a decrease in GLU levels [437]. These studies indicate that MDMA may induce a decrease in GLU release in some brain areas.

The NMDA GLU receptor antagonist MK-801 was reported to be neuroprotective against MDMA-induced damage to 5-HT nerve endings of rats [254, 260, 382]. However, it was later shown that co-administration of MK-801 and MDMA produced a hypothermic response, and when the temperatures of rats were kept elevated, the neuroprotective effect of MK-801 was lost [149, 380]. In another study, the novel low affinity NMDA receptor channel blocker AR-R15896AR, given to male Dark Agouti rats 5 min before and 55 min after MDMA (15 mg/kg, i.p.), did not prevent the MDMA-induced hyperthermia and did not alter either the MDMA-induced neurodegenerative loss of 5-HT and 5-HIAA in CTX, striatum, and HIP or loss of [$^3$H]-paroxetine binding in the CTX 7 days later [381]. These results lead the authors to conclude that NMDA antagonists have no intrinsic protective effect against MDMA serotonergic neurotoxicity [381]. However, more recently, it was shown that memantine, an antagonist of NMDA-receptor and alpha-7 nACh receptors, prevents MDMA-induced serotonergic neurotoxicity in rats [262, 438]. Memantine inhibited ROS production induced by MDMA and decreased 5-HT uptake by about 40% in rat synaptosomes. The authors concluded that antagonism at both NMDA and alpha-7 nACh receptors may be involved in the neuroprotection afforded by this agent [262, 438]. Repeated intermittent MDMA administration to adolescent male Sprague–Dawley rats induces neuroadaptive changes in gene transcript expressions of glutamatergic NMDA and AMPA receptor subunits, metabotropic receptors, and transporters in CTX caudate putamen, HIP, and hypothal-

amus brain regions, which are important in the regulation of reward-related associative learning, cognition, and memory and neuroendocrine functions [439]. These alterations may be a consequence but also may contribute to MDMA-induced neurotoxic actions.

Our works in cultured rat cortical neurons demonstrated that MDMA toxicity was partially prevented by the NMDA receptor antagonist MK-801 in both normothermic and hyperthermic conditions [289]. The protective effect against MDMA-induced neurotoxicity to 5-HT nerve endings, previously reported in in vivo studies, could be dissociated from the hypothermic effect elicited by MK-801 treatment [254, 260, 380–382]. Our data suggest the involvement of excitotoxicity in MDMA-induced neuronal death to post-synaptic cells. Thereby, MDMA might elicit GLU release either directly or indirectly via 5-$HT_{2A}$-receptor-mediated cell death [289].

In light of these studies, it seems plausible that GLU excitotoxicity could be involved but is not essential for MDMA-induced neurotoxicity. However, more studies are required to clarify the importance of GLU in the context of the neurotoxicity elicited by MDMA.

## Nitric Oxide and the Formation of Damaging Peroxinitrites

Nitric oxide (NO) is synthesized by NO synthase, which is classified into three major isoforms: neuronal (nNOS or NOS-I), endothelial (eNOS or NOS-III), and inducible (iNOS or NOS-II). The first two are constitutively expressed and calcium-dependent and the third is present primarily in microglia and macrophages in the brain [440]. NO has a major role in interneuronal communication and, importantly, also in excitotoxic neuronal injury. Specifically, $ONOO^-$ formed by the reaction of NO with $O_2^{\bullet-}$ radicals is a major neurotoxin [441]. As previously written in "Hyperthermia Induced by MDMA Treatment," ROS formation has been detected directly in the brain after MDMA administration [249, 385, 386]. Based on this assumption, the role of NO in the context of MDMA-induced neurotoxicity has been a motive of study.

A report demonstrated that Sprague–Dawley rat brain NOS activities in the CTX regions were significantly elevated 6 h after MDMA administration (40 mg/kg, i.p.) [442]. Pretreatment with L-NNA, a NOS inhibitor, partially protected against long-term 5-HT depletion induced by MDMA in frontal CTX and parietal CTX. Moreover, it was also proven that pretreatment with a NOS inhibitor caused significant inhibition of brain NOS activity, but did not affect the acute 5-HT and DA changes or the hyperthermia induced by MDMA [442]. Additionally, Darvesh et al. [412] showed that the systemic administration of the NOS inhibitors, $N_\omega$-nitro-L-arginine methyl ester (L-NAME), *S*-methyl-L-thiocitrulline, and the $ONOO^-$ decomposition catalyst Fe(III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride attenuated the depletion of striatal DA and 5-HT induced by local brain co-perfusion of MDMA and malonate to rats. *S*-methyl-L-thiocitrulline alone also attenuated the depletion of 5-HT in the rat striatum produced by the systemic administration of MDMA without attenuating MDMA-induced hyperthermia. However, the protective effect has not always been shown to be dependent on their NOS inhibitory properties. The NOS inhibitor L-NAME prevents the 5-HT-depleting effects of MDMA in the rat, but it was confirmed that this effect was dependent on the prevention of MDMA-induced hyperthermia [327]. Hypothermia is known to prevent free radical formation, which is otherwise increased in rats undergoing hyperthermia as occurs in MDMA-administrated animals [443, 444]. On the other hand, the systemic administration of MDMA significantly increases the formation of NO and the nitrotyrosine concentration in the rat striatum [412]. Overall, these reports support the conclusion that MDMA produces RNS in the rat, which contributes to the 5-HT neurotoxicity.

In mice, the NOS inhibitors *S*-methyl-L-thiocitrulline and AR-R17477AR provided significant neuroprotection and had a minor effect on MDMA-induced hyperthermia [136]. MDMA (25 mg/kg, three doses every 3 h) was also shown to increase 2,3-dihydroxybenzoic acid formation from salicylic acid perfused through a microdialysis probe implanted in the striatum, indicating increased free radical formation. This increase was also prevented by AR-R17477AR administration. Since the authors demonstrated that AR-R17477AR had no radical trapping activity, this result suggests that MDMA-induced neurotoxicity was dependent on the production of radicals that combine with NO to form tissue-damaging $ONOO^-$ [136]. Importantly, the effect of MDMA neurotoxicity in nNOS KO and WT mice was investigated and MDMA (15 mg/kg×6) was shown to cause dual depletion of DA and 5-HT markers in all brain regions examined in WT mice [445]. The extent of 5-HT neurotoxicity in WT and KO mice was similar, suggesting that deletion of the *nNOS* gene does not protect against 5-HT depletion. However, dopaminergic neurotoxicity was partially attenuated in MDMA-treated KO mice compared to the WT mice [445]. Other studies have shown that nNOS KO mice are resistant to METH-induced dopaminergic neurotoxicity [446]. Additionally, there are several lines of indication that DA-dependent psychomotor and reinforcing effects of amphetamines are diminished by pharmacological blockade of nNOS or deletion of the *nNOS* gene [447].

Our group has also evaluated the importance of NOS activity to MDMA-induced neurotoxic effects using primary cultured rat cortical neurons exposed to MDMA concentration range 100–800 μM for 48 h under both

normal (36.5°C) and hyperthermic (40°C) temperatures [289, 290]. These studies showed that non-selective NOS inhibitors, L-NNA [289] and the specific iNOS and nNOS inhibitor, 1400W and $N_\omega$-propyl-L-arginine, respectively [290], protected against MDMA-induced cortical neurodegeneration in a temperature-independent manner. Therefore, it seems reasonable to believe that both iNOS and nNOS activities contribute, at least in part, to the MDMA-induced formation of RNS. Additionally, it is also known that the activation of NMDA receptors by GLU increases the levels of intracellular calcium ($iCa^{2+}$), which activates nNOS via calmodulin, thereby enhancing the production of NO [447]. It seems then likely that NO production may be linked to the excitotoxicity seen in MDMA-induced neurotoxicity. Additionally, our results in cultured neurons demonstrated that the free radical scavengers PBN (100 μM) [289] and NAC (1 mM) [290] both provided protection against MDMA-induced neurotoxicity in a temperature-independent manner. Accordingly, PBN attenuated MDMA-induced neuronal damage in vivo at a dose that did not modify hyperthermia but was sufficient to scavenge the produced $HO^\bullet$ radicals [247]. These findings suggest that ROS/RNS contribute to MDMA-induced neurotoxicity. Increased $iCa^{2+}$ levels elicited by activation of the 5-$HT_{2A}$ and NMDA receptors can as well lead to the production of ROS/RNS, which may further enhance MDMA-induced neurotoxic effects.

### MDMA Agonism at the 5-$HT_{2A}$ Receptor

As described in "Pharmacology of MDMA", MDMA possesses a binding affinity for the 5-$HT_{2A}$ receptor in the range of 1 to 10 μM (as $K_i$ values) [44]. In accordance, an agonist role has been confirmed in cells expressing 5-$HT_{2A}$ receptors [45]. Noteworthy is that the activation of 5-$HT_{2A}$ receptors by direct agonists, such as (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) and MDMA, raises rat body temperature [157, 158]. Additionally, 5-$HT_{2A}$ receptor antagonists ketanserin or ritanserin showed efficacy in preventing MDMA-induced 5-HT neurotoxicity in rats [147, 383]. However, it was also shown that co-administration of ketanserin, ritanserin, R-96544, or even risperidone, all 5-$HT_{2A}$ receptor antagonists, inhibited the MDMA-induced hyperthermia [149, 154]. When the body temperatures of the ketanserin-treated animals were kept elevated, the neuroprotective effect of the drug was abolished [149]. These reports point out a possible connection between MDMA agonism at the 5-$HT_{2A}$ receptor and neurotoxicity.

Reports on the neurotoxic effects of MDMA and other amphetamines are mainly focused on the damage of the serotonergic and dopaminergic systems. Nevertheless, several studies report a broader neuronal death induced by MDMA, namely, neuronal degeneration in different rat brain areas such as the parietal CTX, the insular/perirhinal CTX, the ventromedial/ventrolateral thalamus, HIP, and the tenia tecta [240, 245, 250, 255, 282–284]. Also, MDMA induces neurodegeneration in nigral, striatal, and hippocampal neurons of mice [330, 332–335]. Since these neurons are targeted by the serotonergic system and as postsynaptic neurons bear the 5-$HT_{2A}$ receptor [42, 448, 449], it seems logical to investigate an interdependence between MDMA and that receptor. Results from Capela et al. [289, 290] clearly demonstrated that MDMA-induced degeneration of cortical neurons is dependent on its 5-$HT_{2A}$ receptor agonist properties both under normothermic and hyperthermic conditions. Furthermore, the selective 5-$HT_{2A}$ receptor antagonists ketanserin and R-96544 [(2R,4R)-5-[2-[2-[2-(3-Methoxyphenyl)ethyl]phenoxy]ethyl]-1-methyl-3-pyrrolidinol hydrochloride] afforded protection in a temperature-independent manner. Using neuronal cultures, which express the 5-$HT_{2A}$ receptor, it was possible to conclude that these blockers afforded protection directly, beyond the indirectly mediated protection afforded via hypothermic effects that may be elicited by the drug in vivo [289, 290]. Additionally, ketanserin pretreatment afforded protection against MDMA-induced GSH depletion in cortical neurons, thereby preventing MDMA-mediated oxidative stress [290]. To further corroborate the fact that MDMA agonistic properties at the 5-$HT_{2A}$ receptor could produce neuronal death, DOI, a prototypical agonist of that receptor was added to cortical neurons [289]. DOI (10 to 100 μM for 24 or 48 h) also induced a dose- and time-dependent apoptotic cortical neuronal apoptosis, which was attenuated by ketanserin and R-96544 [289]. Ketanserin and R-96544 are competitive selective 5-$HT_{2A}$ receptor antagonists and only attenuated MDMA-induced cortical neurodegeneration. However, an antibody raised against the 5-$HT_{2A}$-receptor, an "irreversible" non-competitive 5-$HT_{2A}$ receptor blocker, prevented almost completely MDMA- and DOI-induced cortical neurotoxicity [289, 290]. Neuronal apoptosis mediated by MDMA is accompanied by activation of caspase 3, which could be blocked by the antibody raised against the 5-$HT_{2A}$ receptor [290]. Therefore, it is likely that DOI- and MDMA-induced neuronal apoptosis arises from direct stimulation of the 5-$HT_{2A}$ receptor [289, 290]. In accordance with these findings, MDMA was proven to induce neuronal apoptosis in cortical and cerebellar granule neurons [287, 288]. In these works, apoptosis was characterized by endonucleosomal DNA cleavage and differential expression of antiapoptotic and proapoptotic proteins (bcl-$X_{L/S}$ variants) accompanied by activation of caspase 3. In addition, in vivo caspase-1-like protease activity was found to be increased in the rat frontal CTX 3 h after MDMA injection [450]. Moreover, a marked induction of caspase-3 activity was demonstrated in the HIP of rats

previously administrated with a neurotoxic dose of MDMA [283]. Figure 8 presents a model for the global events leading to MDMA-induced cortical neurodegeneration.

The possible mechanism of neuronal apoptosis via 5-$HT_{2A}$ receptor activation by MDMA can also be supported based on other lines of evidence. The activation of the 5-$HT_{2A}$ receptor leads to the stimulation of the phosphatidylinositol-specific phospholipase C and also of the phospholipase $A_2$ pathway [42]. There is evidence for a role of phospholipase $A_2$ in neuronal death after ischemic injury [451]. Therefore, it seems likely that 5-$HT_{2A}$ receptor stimulation by MDMA could lead to the activation of the phospholipase $A_2$ apoptosis pathway [289, 290]. Moreover, 5-$HT_{2A}$ receptor is known to promote neuronal firing by enhancing sensitivity to glutamatergic AMPA receptors and increase $iCa^{2+}$ levels [452]. These actions may destabilize the internal cytoskeleton, which may be a major factor in the activation of neuronal apoptosis by 5-$HT_{2A}$ agonism [452].

The 5-$HT_{2A}$ receptor plays an essential role in cognitive processing, including working memory of the brain. In recent years, the 5-$HT_{2A}$ receptor has gained tremendous importance as a potential therapeutic target for various psychiatric disorders, such as schizophrenia and depression [42]. For example, there is extensive evidence for a

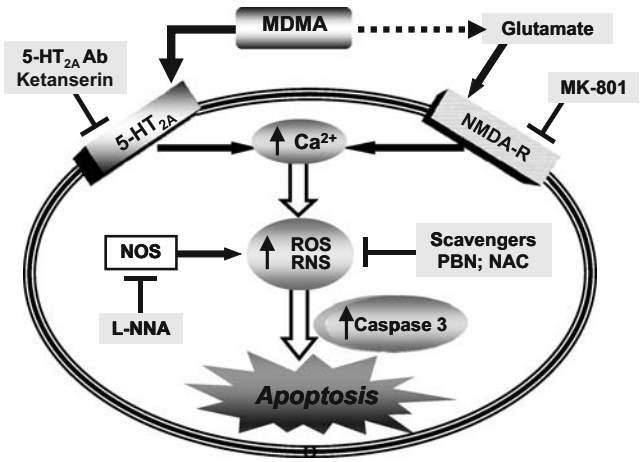


**Fig. 8** Postulated mechanism of MDMA-induced neurodegeneration in cortical neurons. Direct activation of 5-$HT_{2A}$-receptors by MDMA may induce neuronal death, which can be attenuated by the competitive antagonists ketanserin or R-96544 and completely blocked by the antibody (*5-$HT_{2A}$ Ab*) for that receptor. Moreover, MDMA might elicit GLU release either directly or indirectly via 5-$HT_{2A}$-receptor-mediated cell death, which will act on NMDA-receptors (*NMDA-R*), an effect that can be antagonized by MK-801. Both 5-$HT_{2A}$ and NMDA receptors activation will lead to an increase in $iCa^{2+}$ levels. This increase will activate NOS activity, leading to the overproduction of NO and subsequently to the formation of damaging peroxinitrite. Both nNOS and iNOs activity can be inhibited by selective and non-selective antagonists, like L-NNA. Under this scenario of oxidative stress, ROS/RNS can be scavenged by the antioxidants, NAC and PBN. Ultimately, neurons will undergo apoptotic death accompanied by caspase 3 activation

decrease in the density of cortical 5-$HT_{2A}$ receptors in schizophrenia, and it is now widely believed that 5-$HT_{2A}$ receptor antagonism may be a key pharmacological feature of atypical antipsychotic drugs [453]. As previously described, several studies have found significant correlations between MDMA-related alterations in cortical 5-$HT_{2A}$ receptor density and cognitive function [353, 371]. Importantly, in humans, pretreatment with the 5-$HT_{2A}$ antagonist ketanserin attenuates MDMA-induced perceptual changes, emotional excitation, and acute adverse responses, but has little effect on MDMA-induced positive mood, well-being, extroversion, and short-term sequelae [214]. In accordance with the involvement of the 5-$HT_{2A}$ receptor in neuronal cell death, ketanserin, as other 5-$HT_{2A}$ receptor antagonists, exhibit neuroprotective effects on hippocampal neurons in the Mongolian gerbil model of ischemia [454]. Activation of 5-$HT_{2A}$ receptors induces the closure of potassium channels and facilitates ischemia-induced release of excitatory amino acids, both effects being reduced by ketanserin [454]. Recently, it was reported that olanzapine and risperidone blocked Meth-induced schizophrenia-like behavioral abnormalities and accompanied apoptosis in the cortical brain area of male Sprague–Dawley rats [455]. In another recent study, rats injected with MDMA (10 mg/kg, s.c.) displayed increased levels of DA and serotonin (5-HT) in the anterior hypothalamus approximately tenfold and 50-fold, respectively, which were significantly suppressed by pretreatment with risperidone (0.5 mg/kg) [154]. Combining all the evidences, one might predict that 5-$HT_{2A}$ receptor antagonists like the clinically available atypical antipsychotic drugs (risperidone or olanzapine) could be valuable in the treatment of not only acute effects of MDMA-abuse like psychotic symptoms but also chronic effects based on long-term neurodegeneration [289, 290].

## MDMA Metabolism Produces Neurotoxic Metabolites

As discussed before, MDMA metabolism leads to the production of several highly reactive metabolites, namely, quinone intermediaries, as it can be seen in Fig. 9. Therefore, it would seem logical to think that those metabolites could mediate toxic events. Importantly, several studies failed to demonstrate serotonergic neurotoxicity when MDMA or MDA where injected directly into the rat brain [456, 457]. Since they could not reproduce the serotonergic neurotoxicity seen after the peripheral administration of the drugs, it was postulated that systemic metabolism is needed for the occurrence of neurotoxic events [96, 458]. Additionally, MDMA metabolites have been identified and measured in the brain of MDMA-administrated rats [78, 459]. Therefore, investigating the neurotoxic potential of MDMA metabolites and their role in neurotoxicity is the logical sequence. In accordance, several

investigations, described below, have confirmed that MDMA metabolism leads to highly neurotoxic metabolites.

Reports indicate that peripheral metabolism of *N*-Me-α-MeDA and α-MeDA, major MDMA metabolites, is required for metabolism-related neurotoxicity. Indeed, when these metabolites are centrally administrated, like the parent compound, they do not elicit neurotoxicity. In fact, α-MeDA was not neurotoxic following direct injection into the rat brain [460, 461]. Moreover, *N*-Me-α-MeDA intrastriatal injection in mice did not provoke long-term DA or 5-HT depletion [462]. However, they are neurotoxic when administrated peripherally. Subcutaneous administration of MDA metabolites, α-MeDA and HMA, can cause decreases in 5-HT concentrations in the rat frontal CTX [463]. Additionally, *N*-Me-α-MeDA i.p. injection in mice provoked long-term DA and 5-HT depletion in mice [462]. Studies have also shown that the direct injection of thioether MDMA metabolites in the rat brain produced neurobehavioral changes similar to those obtained after peripheral administration of MDA and MDMA [78, 95, 460, 464, 465]. Intracerebroventricular (ICV) injections of 5-(NAC)-α-MeDA and 5-(GSH)-α-MeDA into rats produced neurobehavioral changes characteristic of peripheral administration of MDMA/MDA, as well as acute increases in brain 5-HT and DA concentrations [460]. In addition to the effects observed after ICV administration of 5-(NAC)-α-MeDA and 5-(GSH)-α-MeDA, their direct injection into the striatum, CTX, and HIP produced prolonged depletions in 5-HT [465]. 2,5-(GSH)-α-MeDA also proved to decrease 5-HT levels in the striatum, HIP, and CTX 7 days after ICV injection to rats [464]. Recently, the metabolite 5-(NAC)-*N*-Me-α-MeDA was shown to produce acute behavior changes in rats similar to those seen with MDMA and other thioether conjugates of α-MeDA and also significantly decreased striatal and cortical concentrations of 5-HT and 5-HIAA in a dose-dependent manner [78]. In accordance,

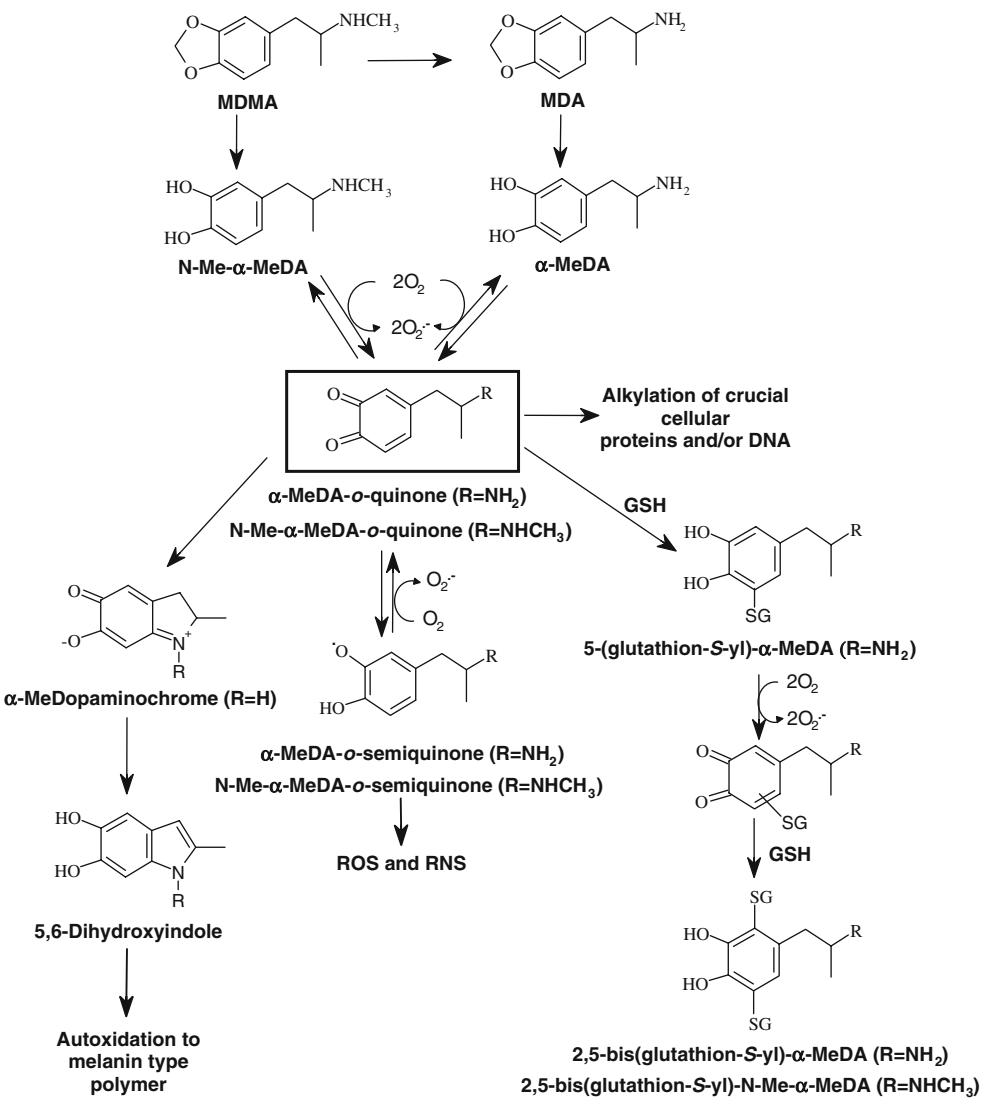


**Fig. 9** Postulated mechanisms for production of MDMA toxic derivatives like *ortho*-quinones, semiquinones, and amino-chrome. The catechols are readily oxidized to their corresponding *ortho*-quinones in the presence of catalytic amounts of transition metal ions, which can enter redox cycle and generate semiquinone radicals, leading to the formation of ROS and RNS. Alternatively, *ortho*-quinones can undergo adduction with nucleophiles like GSH, proteins, or even DNA. On cyclization, *ortho*-quinones give rise to the formation of amino-chromes and related compounds, such as 5,6-dihydroxyindoles, which can undergo further oxidation and polymerization to form brown or black insoluble pigments of melanin type. Adapted from [76]

5-(GSH)-α-MeDA and 2,5-(GSH)-α-MeDA were shown to produce ROS in SK-N-MC cells transiently transfected with the human 5-HTT [466].

*N*-Me-α-MeDA and α-MeDA are catechols and can undergo oxidation to the corresponding *ortho*-quinones [75–77, 458, 467]. These quinones are highly redox active molecules that can undergo redox cycling, which originates semiquinone radicals and leads to the generation of ROS and RNS [467]. As the reactive *ortho*-quinone intermediates are Michael acceptors, cellular damage can occur through alkylation of crucial cellular proteins and/or DNA [75–77, 468–470]. *Ortho*-quinones can form adducts with GSH and other thiol-containing compounds [72]. Moreover, *ortho*-quinones, aminochromes, and GSH conjugates are known to cause irreversible inhibition of enzymes that possess either a GSH binding site and/or cysteine residues critical for the enzymatic function. Likewise, inhibition of GSH reductase and GSH-*S*-transferase by quinones, as well as inhibition of GSH reductase, selenium-dependent GSH peroxidase, and GSH-*S*-transferase by aminochromes, was previously reported [471]. Conjugation of GSH with electrophiles, such as quinones, can result in the preservation or enhancement of their biologic (re)activity. GSH-conjugated MDMA metabolites can either be monoconjugated or undergo the addition of a second molecule of GSH, yielding a 2,5-bis-glutathionyl conjugate [78, 458, 459]. These systemically formed metabolites can be transported into the brain across the blood–brain barrier [459, 472–474]. These compounds can also undergo further metabolism to the NAC conjugates, which appear to be slowly eliminated and are persistent in the brain [78]. In fact, recent experiments using in vivo microdialysis have provided direct evidence that NAC conjugates of MDMA metabolites have a longer $t_{1/2}$ than GSH conjugates in the rat brain after in vivo s.c. administration of MDMA [78] and that they tend to accumulate after multiple MDMA dosing [459]. In Fig. 10, we present a postulated model for the delivery and metabolism of the catechol MDMA metabolites into the brain.

Our group has evaluated the neurotoxicity of nine MDMA metabolites obtained by chemical synthesis: *N*-Me-α-MeDA, α-MeDA, and their correspondent GSH and NAC adducts [76, 77]. The studies were conducted in rat cortical neuronal cultures for a short period of 6 h and also for longer periods of exposure 24 h under normal (36.5°C) and hyperthermic (40°C) conditions. The first studies aimed to verify the toxicity of MDMA catechol metabolites, *N*-Me-α-MeDA and α-MeDA (concentration range 100 to 800 μM) [76]. When incubated for a 24-h time period, they showed a higher toxicity than the parent compound MDMA. Both *N*-Me-α-MeDA and α-MeDA, like MDMA, induced programmed neuronal death during long periods of exposure (48 h) [76]. In subsequent studies, the neurotoxicity of GSH and NAC conjugates of the catechols *N*-Me-α-MeDA and α-MeDA was tested [77]. The results clearly showed that the non-conjugated catechol metabolites of MDMA (concentration range 100 to 400 μM) are not toxic to cortical neurons for a short period of exposure (6 h). However, for the same time of exposure and concentrations, their correspondent GSH conjugates, 5-(GSH)-*N*-Me-α-MeDA and 5-(GSH)-α-MeDA, were highly toxic to cultured cortical neurons. Particularly, the GSH conjugate of *N*-Me-α-MeDA was an extremely potent neurotoxin. Longer incubation periods (24 h) further confirmed that the GSH conjugation dramatically increases the neurotoxicity of the MDMA catechol metabolites [77]. Bridging with previous reports, it has been shown in vitro that the concentrations of *N*-Me-α-MeDA and α-MeDA decrease over time in biological media due to their oxidation to the correspondent aminochromes [75]. Aminochromes can also undergo further oxidation, leading to the formation of a melanin-type polymer [475]. The reactive intermediates, produced during the oxidation of these catecholamines into reactive *ortho*-quinones and/or aminochromes, can be conjugated with GSH to form the corresponding glutathionyl adducts or lead to the accumulation of toxic oxidation products, like quinoproteins [76, 77]. Additionally, it is important to consider that all the metabolites may appear in the brain simultaneously after administration of MDMA. Therefore, a synergistic or additive toxic effect of these metabolites may occur at lower concentrations than that of each compound isolated.

The results on the neurotoxicity of the nine MDMA metabolites offered the opportunity to propose a structure–toxicity–activity relationship. Monocojugated GSH metabolites, such as 5-(GSH)-α-MeDA and 2-(GSH)-α-MeDA, are more toxic than biconjugated metabolites, such as 2,5-(GSH)-α-MeDA. Moreover, 5-(GSH)-α-MeDA is more toxic than 2-(GSH)-α-MeDA. This correlated well with the lower ability of the GSH biconjugated adduct to enter the process of redox cycle [467]. NAC-conjugated MDMA metabolites retain the toxicity of their parent GSH conjugates. The presence of GSH conjugates in the brain is short, and therefore, the persistent metabolites are the NAC conjugates, which appear to be slowly eliminated from the brain [78, 459]. Consequently, the toxic potential of the NAC conjugates is higher, as they tend to accumulate in the brain. Table 9 summarizes the studies evidencing MDMA metabolites neurotoxic actions to both animals and cultured neurons.

It seems likely that the oxidation of thioether MDMA metabolites contributes, at least in part, to the ROS/RNS generated in the brain of MDMA-administrated animals [75–77, 466, 470]. Additionally, our results show that incubation of cortical neurons with thioether MDMA metabolites for a short time of exposure, either at

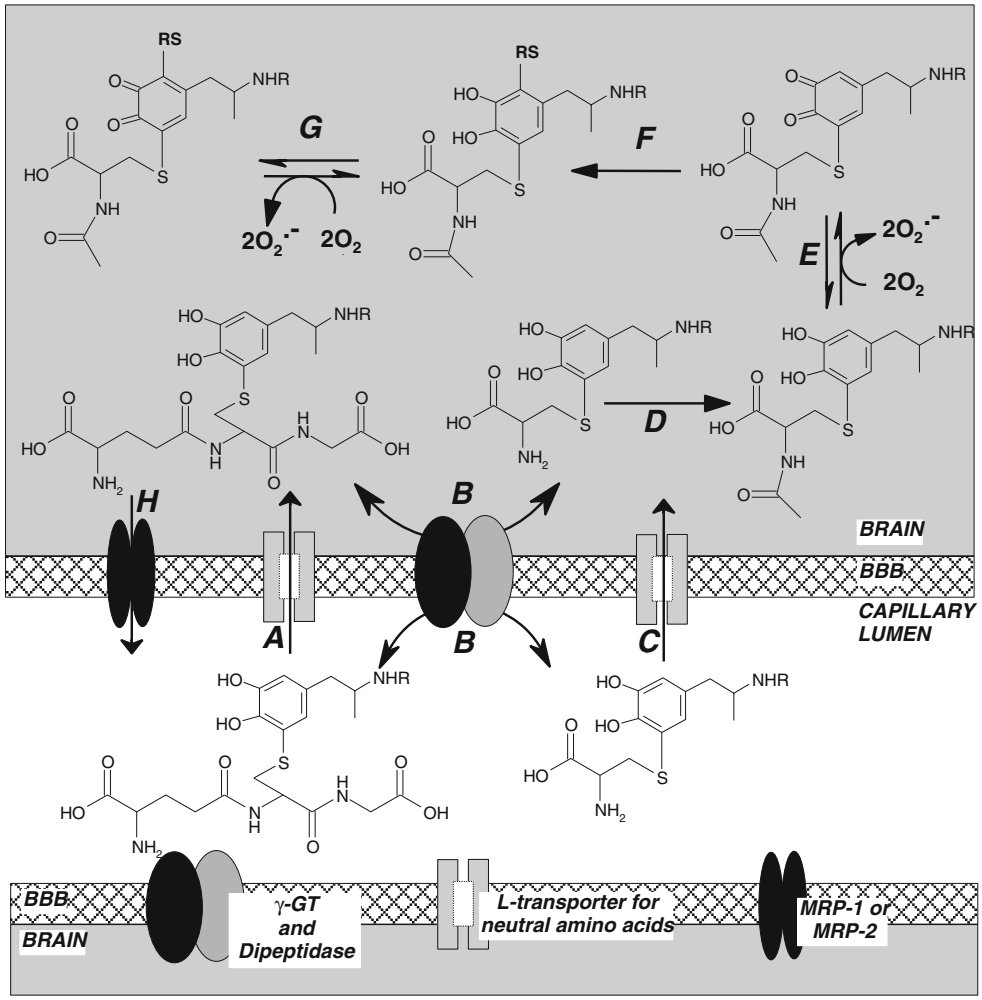


**Fig. 10** Postulated model for the delivery of GSH-conjugated MDMA metabolites into the brain. **a** Both 5-(GSH)-*N*-Me-α-MeDA (R=CH3) and 5-(GSH)-α-MeDA (R=H) can be transported into the brain by L-transporter for neutral amino acids located in the capillaries of the blood–brain barrier (BBB). **b** These conjugates can be metabolized by γ-GT and dipeptidase to their correspondent cysteine derivates either in the brain or even in the capillary lumen. **c** Cysteine conjugates formed in the capillary lumen can also be transported into the brain by the L-transporter for neutral amino acids. **d** By *N*-acetyltransferase action, cysteine conjugates can be further metabolized to their correspondent NAC conjugates. NAC conjugates appear to be slowly eliminated and tend to accumulate inside the brain; therefore, they can be major effectors of MDMA neurotoxic actions. **e** NAC conjugates can undergo redox cycling to their correspondent *ortho*-quinones in the presence of catalytic amounts of transition metal ions, releasing ROS that lead to oxidative stress. **f**, **g** *Ortho*-quinones can undergo the adduction with proteins, DNA, or other nucleophiles (–RS). **h** The accumulation of GSH-conjugated catechols in the brain can be compromised by the action of multiresistant drug protein transporters 1 and 2 (*MRP-1* or *MRP-2*), which are also located at the blood–brain barrier and were shown to mediate active extrusion of GSH adducts

normothermia or hyperthermia, resulted in intracellular depletion of GSH levels in a concentration-dependent manner [76, 77]. This depletion may lead to an increase of neuronal vulnerability to further oxidative injury mediated by the metabolites. Importantly, exposure of cultured neurons under hyperthermia, to simulate MDMA-induced hyperthermic effects, was shown to potentiate the neurotoxicity of thioether MDMA metabolites [76, 77]. It is noteworthy that plasma concentrations of MDMA metabolites were greatly enhanced in animals treated at 30°C [153]. Therefore, hyperthermia might potentiate neurotoxicity in two ways not only by enhancing oxidative stress but also by increasing the metabolic activity of enzymes, leading to the production of toxic MDMA metabolites.

Thioether MDMA metabolites induce in cortical cultured neurons a concentration-, time-, and temperature-dependent apoptotic neuronal apoptosis, accompanied by caspase 3 activation, which was fully prevented by NAC, an antioxidant and GSH synthesis precursor [76, 77]. NAC pretreatment prevented the formation of ROS/RNS and increased the intracellular GSH levels in neurons, thereby providing protection against the neurotoxicity mediated by thioether MDMA metabolites [76, 77]. In Sprague–Dawley rats, NAC pretreatment (326 mg/kg, i.p.) protect against

**Table 9** Reports on the neurotoxicity of MDMA metabolites

| MDMA metabolite | Neurotoxicity in vivo | Neurotoxicity in vitro |
|---|---|---|
| α-MeDA | ICV administrated was not neurotoxic [461]; s.c. administration produced long-term 5-HT neurotoxicity in rats [463] | Short term (6 h) not neurotoxic; only toxic during long term incubation periods (48 h) [76, 77] |
| *N*-Me-α-MeDA | i.p. administration was not neurotoxic; ICV administrated produced DA neurotoxicity in mice [462] | Short term (6 h) not neurotoxic, only toxic during long term incubation periods (48 h) [76, 77] |
| HMA | s.c. administration produced long-term 5-HT neurotoxicity in rats [463] | Not tested |
| HMMA | Not tested | Not tested |
| 5-(GSH)-α-MeDA | ICV administrated, did not induce long-term neurotoxicity [460, 464]; direct intrastriatal or intracortical administration caused long-term 5-HT neurotoxicity in rats [465] | Neurotoxic during short incubation period (6 h) [76, 77] |
| 5-(GSH)-*N*-Me-α-MeDA | Not tested | Highly neurotoxic during short incubation period (6 h) [77] |
| 5-(NAC)-α-MeDA | ICV administrated did not induce long-term neurotoxicity [464]; direct intrastriatal or intracortical administration caused long-term 5-HT neurotoxicity in rats [465] | Highly neurotoxic during short incubation period (6 h) |
| 5-(NAC)-*N*-Me-α-MeDA | direct intrastriatal administration caused long-term 5-HT neurotoxicity in rats [78] | Highly neurotoxic during short incubation period (6 h) [77] |
| 2,5-(GSH)-α-MeDA | ICV or direct intrastriatal or intracortical administrated, resulted in long-term 5-HT neurotoxicity in rats [464, 465] | Low toxicity during short incubation period (6 h) [77] |
| 2,5-(GSH)-*N*-Me-α-MeDA | Not tested | Not tested |
| 2,5-(NAC)-α-MeDA | Not tested | Not tested |
| 2,5-(NAC)-*N*-Me-α-MeDA | Not tested | Not tested |

Results obtained from in vivo experiments were conducted in mice or rats. The studies conducted in vitro were performed using primary rat cortical neuronal cultures

Amph (7.5 mg/kg, i.p.)-induced oxidative stress and dopaminergic toxicity without affecting the hyperthermia produced by the drug of abuse [476]. NAC is currently in clinical use as a mucolytic agent in respiratory disease and as a treatment for paracetamol (acetaminophen) poisoning, as a precursor for GSH synthesis in hepatocytes. By acting as a cysteine donor, NAC maintains intracellular GSH levels and is neuroprotective for a range of neuronal cell types against a variety of stimuli in vitro and in ischemic injuries in in vivo animal models [477–479]. Since protection against the metabolite-induced neurotoxicity was afforded by NAC and given the neuroprotective role of NAC, it would be important to study the potential of this drug to protect against MDMA-induced neurotoxicity in vivo. Previous works in animals have demonstrated the neuroprotective effect of antioxidants against MDMA-induced serotonergic deficits. In that respect, the work performed by Gudelsky [480] led the way to many others. In that report, the depletion of brain 5-HT (~30%) in male Sprague–Dawley rats, 7 days after administration of MDMA (20 mg/kg, s.c.), was abolished by treatments with sodium ascorbate (250 mg/kg, i.p.) and cysteine (500 mg/kg, i.p.) [480]. Although the author did not monitor the effects of the antioxidants in body temperature, these results are also consistent with the postulate that MDMA-induced neurotoxicity involves oxidative stress. Subsequent works in rats confirmed that other antioxidants were able to avoid MDMA-induced neurotoxicity without modifying MDMA-induced hyperthermia, namely, alpha-lipoic acid [291], ascorbic acid [481], and acetyl-L-carnitine [482].

Consistent with the importance of MDMA metabolism to the neurotoxic events is the fact that fluoxetine pretreatment may provide protection against MDMA-induced long-term neurotoxicity by decreasing the metabolic transformation of MDMA and MDA to neurotoxic metabolites [427]. Additionally, 5-HTT inhibitors can prevent MDMA-induced 5-HT neurotoxicity by preventing the entry, into the 5-HT terminal, of reactive substances like the MDMA metabolites [166].

Another factor that accounts for an important role of MDMA metabolites in the neurotoxic events is the different ability of MDMA to produce neurotoxicity in laboratory animals. Species differences in MDMA metabolism, namely, in the rate of *O*-demethylenation of MDMA and MDA, may underlie species and even strain differences in the neurotoxic response to these amphetamine analogs [96, 339]. The differences between several rat strains to MDMA-induced serotonergic neurotoxicity are shown in Table 7.

Bridging the findings in animals and cultured neurons on MDMA metabolite neurotoxicity to humans, although with

prudence, might prove to be important. *N*-Me-α-MeDA is known to be a main plasma metabolite of MDMA in humans, while in rats, the major metabolite is MDA [70, 96]. MDA further metabolism leads to the generation of α-MeDA [458]. Therefore, the neurotoxicity studies that use conjugated metabolites of α-MeDA might be more important to the rat case. Nonetheless, in particular, the neurotoxicity studies with the conjugated metabolites of *N*-Me-α-MeDA might be of high relevance for the extrapolation to the human situation [76, 77, 339].

Taking into account all the previous findings on the neurotoxicity of MDMA metabolites, we elaborated an integrated hypothesis with a sequence of events by which they exert their neurotoxic actions. (1) MDMA is metabolized in the liver to the catechols *N*-Me-α-MeDA and α-MeDA, which undergo further metabolization to their correspondent GSH conjugates (Fig. 5). (2) Either in the brain or inside the blood–brain barrier vessels, GSH metabolites can be further metabolized via the mercapturic acid pathway [458]. First, GSH conjugates are metabolized by the action of γ-GT and dipeptidase to the correspondent cysteine conjugates. In addition, these latter adducts can be metabolized to their correspondent NAC derivates via the action of *N*-acetyltransferase. Both GSH or cysteine conjugated metabolites can cross the blood–brain barrier through the L-transporter for neutral amino acids, which is located in this system and was shown to be involved in the transport of cysteine and GSH-conjugated compounds [459, 472–474]. However, the accumulation of these compounds in the brain can be counteracted by the action of multi-resistant drug protein transporters 1 and 2, which are also located at the blood–brain barrier and were shown to mediate active extrusion of GSH adducts, namely, GSH-conjugated catechols [483–485]. NAC adducts tend to accumulate most likely because of an absence of an active extrusion from the brain (Fig. 10). (3) Both GSH and NAC conjugates can exert their toxic actions to several types of neurons throughout the whole brain, but particularly can enter the serotonergic neuron via the 5-HTT transporter [468]. (4) Both GSH, but more importantly NAC-conjugated MDMA metabolites, are highly reactive, can undergo redox cycling, form quinones and produce ROS or arylate other molecules like proteins or even DNA once inside the cell [76, 77]. (5) MDMA metabolites, as well as other factors like monoamine metabolism, DA oxidation, and peroxinitrite formation can promote oxidative stress with which the neuron cannot cope and ultimately leads to serotonergic terminal loss and also to programmed neuronal death to other brain systems [389].

In summary, MDMA metabolism leading to reactive thioether MDMA metabolites, which elicit ROS/RNS and the formation of toxic oxidation products, might play an essential role in the neurotoxic events mediated by "ecstasy."

## Conclusions

The United Nations estimates that "ecstasy" use affects nine million people of the population aged among 15 to 64 worldwide. This raises the concern on the risk that those millions of people were or are currently exposed. There are no doubts that studies on MDMA-induced adverse toxic effects, namely, the more feared long-term effects of brain neurotoxic damage, are of striking importance. Despite more than two decades of studies on MDMA neurotoxic effects, the underlying mechanisms of neurotoxicity still remain to be fully elucidated. MDMA has been shown to induce neurotoxicity both in laboratory animals as well as long-term neurologic deficits in humans. Reports point that several factors are involved in MDMA-induced neurotoxicity, namely, hyperthermia, monoamine oxidase metabolism of dopamine and serotonin, the serotonin transporter action, nitric oxide and the formation of damaging peroxinitrite, glutamate excitotoxicity, $5\text{-HT}_{2A}$ agonism, and, importantly, the formation of MDMA neurotoxic metabolites. While the formation of toxic MDMA metabolites seems to be the most important mechanism, all the previous factors may act collectively in a synergistic manner conducing to neurotoxicity. Further studies are urged for the better knowledge of MDMA pharmacology and induced toxicity. Understanding the cellular and molecular mechanisms involved in MDMA neurotoxicity can help to develop therapeutic approaches that prevent or treat the long-term neuropsychiatric complications that result from human MDMA abuse.

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