Chapter 40

Mitochondrial Trails in the Neurotoxic Mechanisms of MDMA

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Abbreviations

5-(GSH)-α-MeDA 5-(Glutathion-*S*-yl)-α-methyldopamine
5-(GSH)-*N*-Me-α-MeDA 5-(Glutathion-*S*-yl)-*N*-methyl-α-methyldopamine
5-HT 5-Hydroxytryptamine or serotonin
5-(NAC)-α-MeDA 5-(*N*-acetylcysteine-*S*-yl)-α-methyldopamine
5-(NAC)-*N*-Me-α-MeDA 5-(*N*-acetylcysteine-*S*-yl)-*N*-methyl-α-methyldopamine
AIF Apoptosis-inducing factor
ATP Adenosine 5′-triphosphate
Bax Bcl-2-associated X protein
Bcl-2 B-cell lymphoma 2
COXI Cytochrome c oxidase subunit I
DA Dopamine
Drp1 Dynamin-related protein 1
ETC Electron transport chain
GSK3β Glycogen synthase 3β
H_2O_2 Hydrogen peroxide
IAPs Inhibitors of apoptotic proteins
MAO Monoamine oxidase
MDMA 3,4-Methylenedioxymethamphetamine or "ecstasy"
Mfn Mitofusin
mtDNA Mitochondrial deoxyribonucleic acid
***N*-Me-α-MeDA** *N*-methyl-α-methyldopamine
NDI Nicotinamide adenine dinucleotide dehydrogenase subunit I
NDII Nicotinamide adenine dinucleotide dehydrogenase subunit II
ROS Reactive oxygen species
Smac/DIABLO Second mitochondrial activator of caspases/Direct inhibitor of apoptotic protein-binding protein with low PI
$\Delta\Psi_m$ Mitochondrial membrane potential
α-MeDA α-Methyldopamine

INTRODUCTION

One of the most feared and debated issue concerning the use of 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") is related to its neurotoxic potential. In Europe, it is estimated that 10.6 million people have taken MDMA at some point in their lives (EMCDDA, 2014), which constitutes an important health issue. As such, studies analyzing the pathways and mechanisms mediating the neurotoxic effects of MDMA are of striking importance. Several factors have been suggested to be involved in the neuronal injury caused by MDMA abuse, the major ones being metabolism (Figure 1) and hyperthermia. Nevertheless, current and emerging studies have focused on the involvement of mitochondrial-dependent pathways in MDMA's neuronal effects, since mitochondria are key players of adenosine 5′-triphosphate (ATP) synthesis, calcium buffering, and apoptosis signaling in neurons. Once thought to be solitary and rigidly structured, mitochondria are presently acknowledged as highly dynamic organelles. The dynamic processes involved in regulating mitochondrial function enable mitochondrial recruitment to critical subcellular compartments, content exchange between mitochondria, mitochondrial shape control, mitochondrial communication with cytosol, and mitochondrial quality control. When mitochondrial function is disrupted, cellular dysfunction ensues, which ultimately may lead to neuronal damage and brain injury.

This chapter highlights evidence supporting the involvement of mitochondrial dysfunction in the acute and long-lasting neurotoxic effects of MDMA.

MITOCHONDRIAL ELECTRON TRANSPORT CHAIN FUNCTION

Mitochondria provides cellular energy by converting oxygen and nutrients into ATP, via oxidative phosphorylation along the electron transport chain (ETC), sited at the inner mitochondrial membrane. A schematic representation of the mitochondrial ETC is provided in Figure 2.

Studies Linking Mitochondrial ETC Impairment to MDMA-Induced Neurotoxicity

Increased evidence suggests that a fully functional mitochondrial ETC is important to prevent the neurotoxic effects of MDMA.

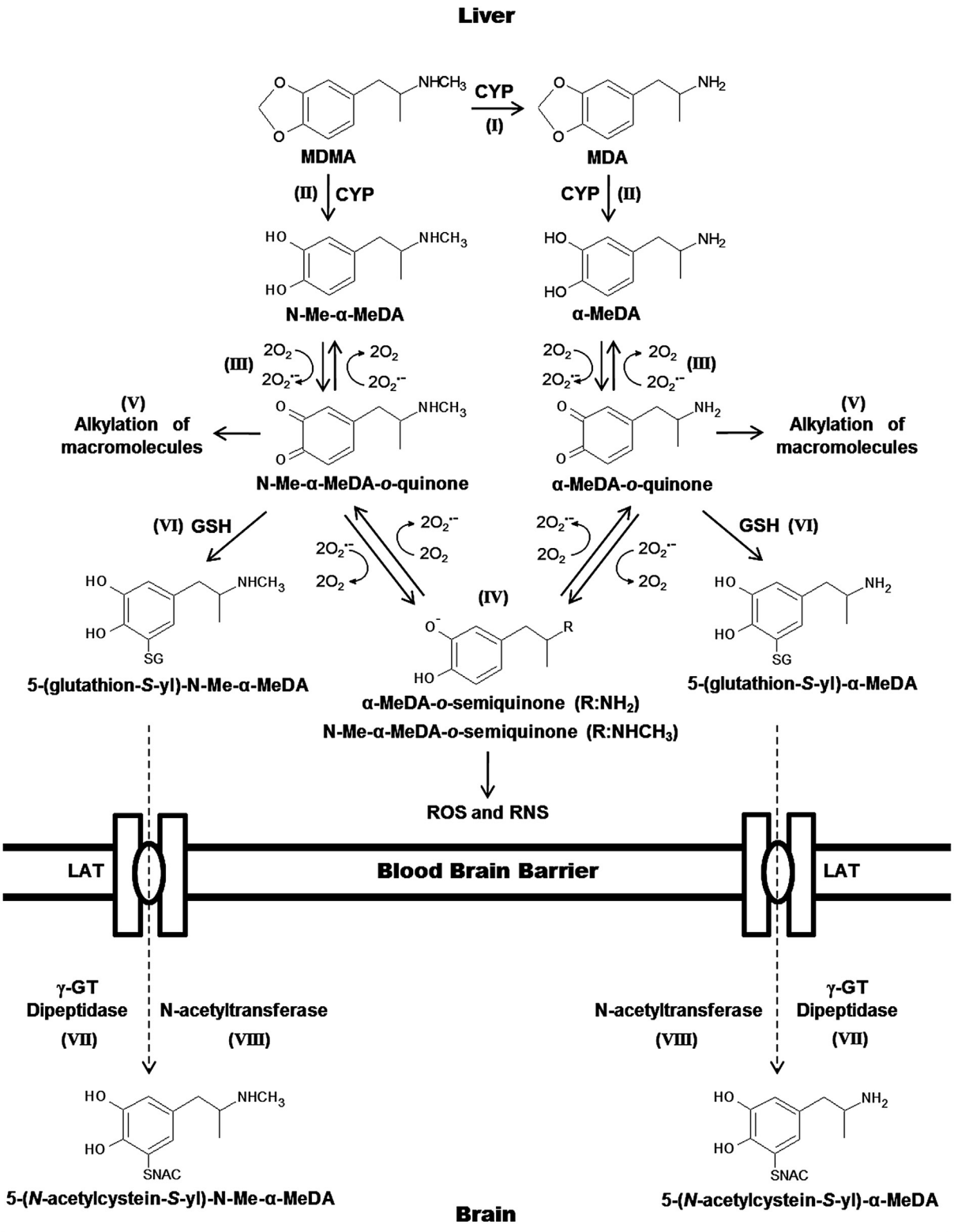

FIGURE 1 **Major pathways of MDMA metabolism.** The parent compound MDMA is *N*-demethylated to form 3,4-methylenedioxyamphetamine (MDA) (I). Both MDMA and MDA may be *O*-demethylenated to the catechol metabolites *N*-methyl-α-methyldopamine (*N*-Me-α-MeDA) and α-methyldopamine α-MeDA (II). In rats, *N*-demethylation to MDA is one of the main metabolic pathways, whereas in humans, *O*-demethylation to *N*-Me-α-MeDA predominates. The cytochrome P450 (CYP450) enzymes are involved in the *N*-demethylation and *O*-demethylation metabolic steps (I, II). Both *N*-Me-α-MeDA and α-MeDA can undergo oxidation to the corresponding ortho-quinones (III), which can enter into a redox cycle and generate semiquinone radicals (IV), thus leading to the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Alternatively, ortho-quinones can undergo adduction with nucleophiles, such as cellular macromolecules [deoxyribonucleic acid (DNA), lipids or proteins] (V) or reduced glutathione (GSH) (VI). GSH conjugates 5-(glutathion-*S*-yl)-*N*-methyl-α-methyldopamine (5-(GSH)-*N*-Me-α-MeDA) and 5-(glutathion-*S*-yl)-α-methyldopamine (5-(GSH)-α-MeDA) can be transported into the brain by *L*-transporter for neutral amino acids (LAT), located in the capillaries of the blood–brain barrier (BBB). These conjugates can be metabolized by γ-glutamyltransferase (γ-GT) and dipeptidase (VII) to their corresponding cysteine derivates, which, by *N*-acetyltransferase action (VIII), can be further metabolized to their corresponding *N*-acetylcysteine (NAC) conjugates. NAC-conjugated metabolites appear to be slowly eliminated and, consequently, persist in the brain. *Adapted from Capela et al. (2009).*

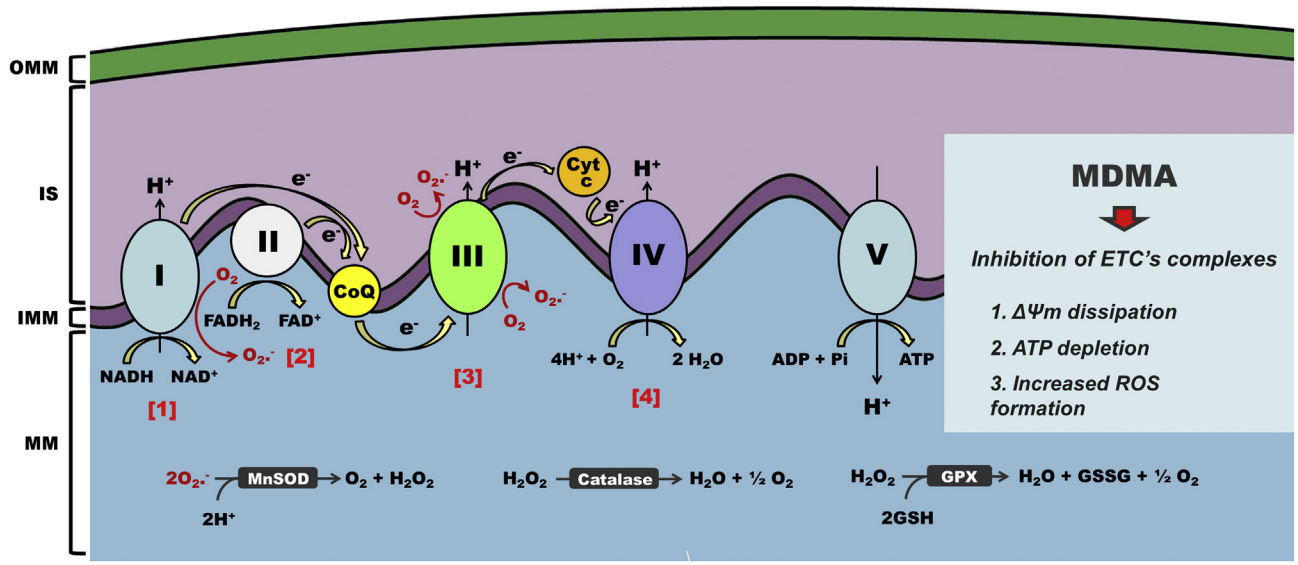

FIGURE 2 Modulation of mitochondrial electron transport chain (ETC) functioning by MDMA. The ETC, also called the mitochondrial respiratory chain, comprises five multisubunit protein complexes located in the inner mitochondrial membrane (IMM). The major sites of superoxide ($O_2^{\cdot -}$) generation include complexes I and III. Superoxide radicals are dismutated by manganese superoxide dismutase (MnSOD), generating hydrogen peroxide (H_2O_2). Hydrogen peroxide is then converted to H_2O by either catalase or glutathione peroxidase (GPX). In vivo studies have reported MDMA's ability to inhibit ETC's complexes I [1], II [2], III [3] and IV [4], which have been supported by additional in vitro settings. Inhibition of ETC functioning may trigger $\Delta\Psi_m$ dissipation, ATP depletion, and increased ROS formation. OMM, outer mitochondrial membrane; IS, intermembrane space; MM, mitochondrial matrix. *Adapted from Barbosa et al. (2015).*

This theory received particular attention after the observation that inhibitors of the ETC could enhance MDMA-induced neurotoxicity in rats (Nixdorf, Burrows, Gudelsky, & Yamamoto, 2001). The direct infusion of MDMA (100 µM, at 1.8 (Darvesh & Gudelsky, 2005) or 2 µl/min (Nixdorf et al., 2001), for 8 h) into the rat striatum did not affect dopamine (DA) or 5-hydroxytryptamine (5-HT or serotonin) tissue content, 5–7 days later (Darvesh & Gudelsky, 2005; Nixdorf et al., 2001). However, co-administration of MDMA with malonate (inhibitor of mitochondrial complex II) produced long-term depletion of both DA and 5-HT content (Darvesh & Gudelsky, 2005; Nixdorf et al., 2001). Other studies revealed that substrates of energy metabolism attenuated the neurotoxicity induced by MDMA (Darvesh & Gudelsky, 2005). Perfusion of nicotinamide (precursor for the electron carrier molecule nicotinamide adenine dinucleotide reduced form, 1 mM) or ubiquinone (an electron-carrying coenzyme of the ETC, 100 µM) in rat striatum, beginning 2 h prior to the first MDMA injection (10 mg/kg, i.p., every 2 h, four times), and ending 6 h after the last injection of MDMA, significantly attenuated MDMA-induced 5-HT depletion, as measured 5 days following exposure (Darvesh & Gudelsky, 2005).

MDMA was also shown to disrupt mitochondrial function. The first direct evidence that MDMA could interfere with the ETC function was provided by Burrows, Gudelsky, and Yamamoto (2000), describing a significant reduction of complex IV activity in substantia nigra, nucleus accumbens, and striatum of rats, 2 h after the last MDMA injection (15 mg/kg, i.p., four times, every 2 h). Following these studies, many other reports emphasized the potential of MDMA to interfere with mitochondrial ETC complex activity. MDMA (10 mg/kg, i.p., four times, every 2 h) was shown to cause an inhibition of complex I and II activity in rat striatum, 12 h after the last injection (Quinton & Yamamoto, 2006). In mouse striatum, high doses of MDMA (3 increasing doses of 10, 20, 30 mg/kg, i.p., 2-h interval) resulted in decreased activity of mitochondrial complex I, at 1, 3, 6, 12, or 24 h after the last MDMA injection (Puerta et al., 2010). Furthermore, a disruptive effect of MDMA in the expression pattern of mitochondrial complexes was also reported. Deletions in the genes coding for nicotinamide adenine dinucleotide dehydrogenase subunits I (NDI) and II (NDII) of the mitochondrial complex I and for cytochrome c oxidase subunit I (COXI) of the mitochondrial complex IV were found in isolated mitochondria from several rat brain areas, 2 weeks after MDMA administration (10 mg/kg, i.p., four times, 2-h intervals) (Alves, Binienda, et al., 2009; Alves et al., 2007). Furthermore, MDMA administration also resulted in decreased expression of NDII and COXI subunits of the mitochondrial complexes I and IV, respectively (Alves et al., 2007; Alves, Binienda, et al., 2009; Alves, Summavielle, et al., 2009). Notably, the effects of MDMA on mitochondrial deoxyribonucleic acid (mtDNA) or protein levels were almost completely rescued by co-administration of the monoamine oxidase (MAO)–B inhibitor selegiline (Alves et al., 2007) or acetyl-L-carnitine (Alves, Binienda, et al., 2009), but not by inhibiting MAO-A with clorgyline (Alves, Summavielle, et al., 2009). A further explanation of the mechanisms involved in this effect is provided below (see Section MDMA and Monoamine Oxidase: Implications for Drug Neurotoxicity). The ETC complexes targeted by MDMA are illustrated in Figure 2.

Changes in ATP Levels

One of the most expected consequence resulting from the impairment of mitochondrial ETC function is a decreased ATP generation.

In vitro studies have consistently reported the ability of MDMA metabolites to decrease neuronal ATP levels. In primary cultures of cortical neurons, the toxic effects observed after exposure to the MDMA's metabolite 5-(glutathion-*S*-yl)-*N*-methyl-α-methyldopamine (5-(GSH)-*N*-Me-α-MeDA, 400 μM), for 6 h resulted in decreased neuronal ATP, which was fully prevented by the antioxidant *N*-acetylcysteine (Capela, Macedo, et al., 2007). However, a role for mitochondrial ETC impairment has not been consistently provided, thus opening the possibility of an impairment of glycolytic pathways in that effect.

Production of Reactive Oxygen Species

The mitochondrial ETC, namely, complexes I and III, is considered the main site of superoxide production inside the cell. Under physiological conditions, superoxide produced in mitochondria has a very short half-life, as it is efficiently dismutated by manganese or copper/zinc superoxide dismutases, giving rise to the formation of hydrogen peroxide (H_2O_2). It is therefore reasonable to establish a key role for mitochondrial disruption in the pathogenesis of brain injury, by initiating and promoting oxidative stress (Adam-Vizi, 2005).

MDMA has been demonstrated to inhibit complexes within the ETC (see Section: Studies Linking Mitochondrial ETC Impairment to MDMA-Induced Neurotoxicity). This inhibition is likely to increase reactive oxygen species (ROS) generation and contribute to MDMA-induced neurotoxicity. Accordingly, inhibitors of the ETC increase ROS formation (Liot et al., 2009). These data suggest that inhibition of complexes within the ETC alters the balance between ROS formation and antioxidant systems, leading to a net increase in ROS accumulation, which might damage neurons.

Both in vivo and in vitro studies have documented increased ROS formation after MDMA exposure (Colado, O'Shea, Granados, Murray, & Green, 1997; Jiménez et al., 2004). Similar results have been shown for MDMA's *N*-demethylated analogue 3,4-methylenedioxyamphetamine, and MDMA's metabolites 5-(glutathion-*S*-yl)-α-methyldopamine (5-(GSH)-α-MeDA) and 2,5-bis(glutathion-*S*-yl)-α-methyldopamine in 5-HT or DA transporter–transfected SK-*N*-MC human cells, in a concentration- and time-dependent manner (Jones, Lau, & Monks, 2004). Subsequent studies established that the neurotoxic effects mediated by 5-(GSH)-α-MeDA and other MDMA metabolites, namely α-methyldopamine (α-MeDA), *N*-Me-α-MeDA, 5-(GSH)-*N*-Me-α-MeDA, 5-(*N*-acetylcysteine-*S*-yl)-α-methyldopamine (5-(NAC)-α-MeDA), and 5-(*N*-acetylcysteine-*S*-yl)-*N*-methyl-α-methyldopamine (5-(NAC)-*N*-Me-α-MeDA), in rat cultured cortical neurons (Capela, Macedo, et al., 2007) or mouse brain synaptosomes (Barbosa et al., 2012) relied on ROS formation. Notably, increased ROS formation was also demonstrated in SH-SY5Y differentiated cells exposed to the mixture of MDMA and six of its metabolites (α-MeDA, *N*-Me-α-MeDA, 5-(GSH)-α-MeDA, 5-(GSH)-*N*-Me-α-MeDA, 5-(NAC)-α-MeDA and 5-(NAC)-*N*-Me-α-MeDA), at in vivo–relevant concentrations (each compound at equimolar concentrations of 10 or 20 μM), during 6 h (Barbosa, Capela, Silva, Vilas-Boas, et al., 2014), thereby indicating that MDMA's toxic effects might result from a combined effect of the parent compound and metabolites. Nevertheless, MDMA's metabolites-induced ROS formation in mouse brain synaptosomes was independent on the mitochondrial polarization status (Barbosa et al., 2012), thereby indicating a role for ETC-independent pathways in that effect. Other pathways, including MAO metabolism of monoamine neurotransmitters (see Section: MDMA and Monoamine Oxidase: Implications on Drug's Neurotoxicity), DA auto-oxidation, hyperthermia, or glutamate excitotoxicity (Capela et al., 2009), have also been established as major contributors to MDMA-induced ROS formation.

THE OUTER MITOCHONDRIAL MEMBRANE ENZYME MAO

MAO is a family of flavin adenine dinucleotide–containing enzymes located in the mitochondrial outer membrane. It is involved in the metabolism of endogenous or exogenous monoamines, playing a crucial role in regulating the intracellular pool of monoamine neurotransmitters. In the brain, metabolism of monoamines by MAO constitutes the main source of H_2O_2, which, in the presence of transition metals, can be converted, through the Fenton reaction, to the highly reactive and toxic hydroxyl radical (Alves et al., 2007).

Two isoforms of MAO enzymes, MAO-A and MAO-B, are present in most mammalian tissues, which are distinguished by their substrate specificities and sensitivities to the acetylenic inhibitors (Youdim, Edmondson, & Tipton, 2006).

MDMA and MAO: Implications for Drug Neurotoxicity

The administration of MDMA to laboratory animals results in a phase of abrupt increase of the extravesicular levels of monoamine neurotransmitters inside nerve endings, mainly 5-HT, DA, and noradrenaline, which are essentially metabolized by MAO (Alves et al., 2007; Barbosa et al., 2012; Capela et al., 2009). MDMA was also shown to competitively inhibit 5-HT metabolism by rat brain MAO-A (IC_{50} value of 44 μM) and MAO-B (IC_{50} value of 370 μM) activities, showing a selective inhibition of MAO-A (Leonardi & Azmitia, 1994). Consistently, studies with human recombinant MAO reported a highly selective inhibition of MAO-A (IC_{50} = 34 μM) with a lower affinity for MAO-B inhibition (IC_{50} = 110 μM) (Matsumoto et al., 2014). As such, it is of the general consensus that MDMA shows a selective inhibition to MAO-A, with a lower inhibitor activity against MAO-B.

Probably the most convincing evidence toward a role for MAO in MDMA-induced neurotoxicity comes from studies using MAO inhibitors. Mitochondrial toxicity evaluated 2 weeks after MDMA administration (10 mg/kg, i.p., four times, 2-h intervals), including lipid peroxidation, protein carbonylation, and deletions in the genes coding for NDI and NDII subunits of the mitochondrial complex I and COXI subunit of the mitochondrial complex IV, in an adolescent rat model (postnatal day 45), were almost completely abolished by MAO-B inhibition with *L*-deprenyl (2 mg/kg, i.p., 30 min before the first MDMA injection) (Alves et al., 2007). The particular vulnerability of the hippocampus to MDMA-induced mtDNA deletions revealed in this study (Alves et al., 2007) was further supported by recent work in which MDMA-induced increased expression of MAO-B in rat brain was restricted to the hippocampal region (Cuyas et al., 2013). Furthermore, in rats, MAO-B inhibition with *L*-deprenyl (2 mg/kg, i.p., 30 min before MDMA injection) (Sprague & Nichols, 1995) or by

using an antisense oligonucleotide targeted at MAO-B (0.5 µl/h of a 50 µM solution (600 pmol/day), for 7 days before MDMA administration) (Falk, Cook, Nichols, & Sprague, 2002) also attenuated the 5-hydroxytryptaminergic neurotoxicity caused by MDMA (40 mg/kg, s.c.), in a similar extension to those observed in MAO-B–deficient mice following MDMA exposure (2×50 mg/kg, 2 h apart) as measured 1 week later (Fornai et al., 2001). This indicates a major role for MAO-B in MDMA-induced 5-hydroxytryptaminergic neurotoxicity.

Studies evaluating the role of MAO-A in MDMA-induced neurotoxicity have shown dissimilar results from those observed for MAO-B. Inhibition of MAO-A by clorgyline (1 mg/kg, i.p., 30 min before the first MDMA injection) had no protective effect on the alterations induced by MDMA (10 mg/kg, i.p., four times, every 2 h) in rat brain mitochondria. In particular, it even intensified MDMA-induced decreased expression of COXI subunit (Alves, Summavielle, et al., 2009). It has been suggested that the neurotoxicity induced by MDMA may be explained by the following sequence of events: (1) depletion of 5-HT from 5-hydroxytryptaminergic neurons; (2) increased DA synthesis and release, in part attributable to the stimulation of 5-HT_{2A} receptors; (3) increase in extracellular DA; (4) transport of DA into 5-HT nerve terminals by the 5-HT transporter; (5) deamination of DA inside 5-HT nerve terminals by MAO-B, with the consequent formation of H_2O_2 and related oxidative stress, leading to the selective 5-HT neuronal degeneration (Sprague & Nichols, 1995). Considering that MAO-A is expressed predominantly in catecholaminergic neurons (Alves et al., 2007), it might be hypothesized that its inhibition will increase the availability of DA for an uptake by 5-hydroxytryptaminergic neurons and subsequent MAO-B metabolism. This hypothesis may explain the lack of protective effects, or even potentiation of toxicity, mediated by MAO-A inhibition against MDMA-induced mitochondrial neurotoxicity (Alves, Summavielle, et al., 2009). However, this sequence of events fails to explain the neurotoxicity in brain areas where DA innervation is sparse and DA levels are low, for instance in the hippocampus, which is known to be very sensitive to MDMA-evoked neurotoxicity. Taking into account the above findings, the consequences of MAO inhibition at synaptic terminals are presented in Figure 3.

Microdialysis studies have also evaluated the role of both MAO-A inhibition and temperature in MDMA-induced changes in 5-HT brain levels. At a room temperature of 22°C, the co-administration of MDMA with MAO-A inhibitors (clorgyline (10 mg/k, i.p., 24 h before MDMA) or moclobemide (20 mg/kg,

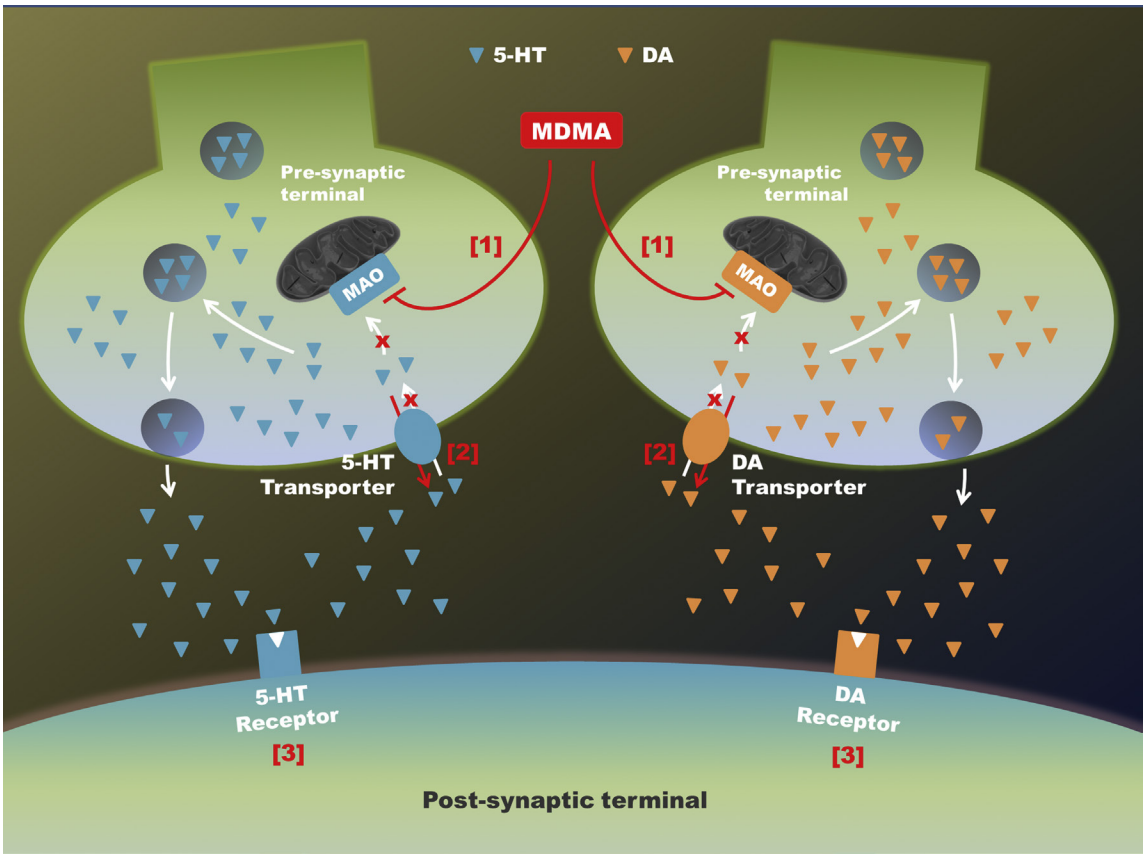

FIGURE 3 **Consequences of MAO inhibition by MDMA to synaptic terminals.** Monoamine oxidase located in the outer mitochondrial membrane is involved in serotonin (5-HT) and dopamine (DA) metabolism; its activity is inhibited by MDMA, leading to increased free cytoplasmic content [1]. As a result of the increased free cytoplasmic pool of neurotransmitters, MDMA promotes rapid release of neurotransmitters to the neuronal synapse via reversal of the neurotransmitter transporters' activity [2], leading to an overactivation of postsynaptic receptors [3]. The agonist activity on the serotonin 2A (5-HT_{2A}) receptor is described to be involved in both MDMA's hallucinogenic and neurotoxic properties (Capela et al., 2007).

i.p., 60 min before MDMA)) potentiated the increase in extracellular 5-HT levels caused by MDMA (10 mg/kg, i.p.) in rat brain (Freezer, Salem, & Irvine, 2005; Hewton, Salem, & Irvine, 2007). At an elevated room temperature of 30°C, similar results were found by inhibiting MAO-A with moclobemide (20 mg/kg, i.p., 60 min before MDMA at 10 mg/kg, i.p.) (Stanley, Salem, & Irvine, 2007), thereby suggesting that the effect of MAO-A inhibition was independent on the hyperthermic response associated with MDMA.

Some cases of human deaths have been reported as a consequence of the concomitant ingestion of MDMA and MAO-A inhibitors, which have been attributed to the 5-HT syndrome (Pilgrim, Gerostamoulos, & Drummer, 2011). In rats, the coadministration of MDMA (10 mg/kg, i.p., four times, every 2 h) and clorgyline (1 mg/kg, i.p., 30 min before the first MDMA injection) results in high mortality [about 57% of the animals injected with MDMA plus clorgyline (four out of seven rats) died or were euthanized before the last administration of MDMA as the pre-defined humane end points were met]. The death of these animals was preceded by 5-HT syndrome characteristic behavior features, namely head weaving, forepaw treading, tremor, flat body posture and backward movement (Alves, Summavielle, et al., 2009). Given that MDMA is an MAO-A inhibitor, further inhibition by another drug would reduce the activity of the enzyme to extremely low levels resulting in high levels of free 5-HT. Intraneuronal high levels of 5-HT would result in an increase in the release of this neurotransmitter promoted by MDMA, enhancing the toxicity and causing the 5-HT syndrome.

MITOCHONDRIAL FUSION/FISSION

Mitochondria are highly dynamic and complex organelles that undergo regulated movement throughout the cell and continuously alter their shape, ranging between two opposite processes, fusion and fission, in response to several stimuli and metabolic demands of the cell. These opposite processes are crucial in sustaining the robust structure and function of mitochondria (Chan, 2006).

In mammalian cells, mitofusins 1 (Mfn1) and 2 (Mfn2), located in the outer membranes of adjacent mitochondria, induce outer membrane fusion. Optic atrophy 1 protein, a protein residing in the intermembrane space, mediates fusion of the inner membranes (Chan, 2006). Mitochondrial fusion allows the exchange of mtDNA and other matrix components between neighboring mitochondria, including damaged or senescent mitochondria, thereby conferring protection against mtDNA mutations and maintaining a healthy oxidative phosphorylation (Chan, 2006; Ranieri et al., 2013).

Mitochondrial fission is mediated by dynamin-related protein 1 (Drp1). When mitochondrial fission is stimulated, Drp1 translocates from the cytosol to specific sites on the mitochondrial outer membrane and homo-oligomerizes, forming spiral chains around mitochondria, which, using guanosine 5′-triphosphate hydrolysis, constrict and twist tubules to initiate mitochondrial fission (Smirnova, Griparic, Shurland, & van der Bliek, 2001). Fission events permit mitochondrial separation to daughter cells during mitosis, control organelle size and shape for an efficient mitochondrial distribution in neuronal processes, allow the recovery of damaged mitochondria for degradation by mitophagy and regulate apoptosis through segregation of the most critically injured mitochondria (Guedes-Dias & Oliveira, 2013).

Studies Linking MDMA's Neuronal Effects to Alterations on Mitochondrial Fusion/Fission Equilibrium

Recent studies (Barbosa et al., 2014a; Barbosa et al., 2014b) showed a perturbation of mitochondrial fusion/fission equilibrium associated with MDMA. Exposure of cultured hippocampal neurons from mice to a high concentration of MDMA (1.6 mM) during a short incubation period of 90 min, resulted in increased fragmentation of axonal mitochondria, a phenotype closely correlated with impaired axonal mitochondrial trafficking (Barbosa et al., 2014a). Similar results were further described following an exposure to the mixture of MDMA and six of its major metabolites (α-MeDA, *N*-Me-α-MeDA, 5-(GSH)-α-MeDA, 5-(GSH)-*N*-Me-α-MeDA, 5-(NAC)-α-MeDA and 5-(NAC)-*N*-Me-α-MeDA), each compound at an equimolar concentration of 10 μM, for a 24 h-period (Barbosa et al., 2014b).

Studies have demonstrated that impaired mitochondrial fragmentation, resulting from a failure of mitochondrial quality control mechanisms, caused by some neurotoxicants, such as 1-methyl-4-phenylpyridinium, is associated with a collapse of mitochondrial network and neuronal injury or death (Wang et al., 2011). Accordingly, a reduction in mitochondrial fission results in longer and more interconnected mitochondrial tubules, thus preventing cell death (Barsoum et al., 2006). These data suggest a potential new mechanism underlying the neuronal effects of MDMA.

NEURONAL MITOCHONDRIAL TRAFFICKING

In neurons, essential constituents and mitochondria, which are generally synthesized or generated in the cell body, are delivered through the neuronal processes to their final destination, the synaptic terminals. The large size of many neurons (up to 1 m in humans) precludes rapid diffusion of ATP from one end of the cell to the other, implying that the energy production must be spatially matched to local energy usage (Sheng & Cai, 2012). Although mitochondrial biogenesis may occur locally in the axon, generation of new organelles occurs mainly within the cell body. Additionally, as the degradation of organelles, such as mitochondria, ensues in the cell body, dysfunctional mitochondria need to return to the soma for efficient degradation (mitophagy) through the autophagy–lysosomal system (Ashrafi & Schwarz, 2013). As such, neurons require highly efficient mechanisms for mitochondrial transport regulation from and to the cell body to enable the rapid redistribution of mitochondria to different areas, to supply increased metabolic requirements.

Kinesin-1 motor family, linked to mitochondrial Rho (Miro), through the adaptor proteins Trak1/2 drives "plus" end-directed mitochondrial transport along microtubule tracks. Cytoplasmic dynein, assisted by the multisubunit complex dynactin, typically mediates mitochondrial transport toward the "minus" ends of microtubules (Sheng & Cai, 2012). Microtubule-associated protein Tau, by assembly and maintenance of microtubules, plays a major role on mitochondrial trafficking control (Vossel et al., 2010).

Studies Linking MDMA's Neuronal Effects to Alterations in Mitochondrial Neuronal Trafficking

The apparent relevance of impaired neuronal trafficking of intracellular materials or organelles in the context of MDMA-induced neurotoxicity was first reported by Callahan, Cord, and Ricaurte (2001). In that study, a marked reduction was found in anterograde transport along ascending axonal projections, originating in the rostral *raphe nuclei* to various forebrain regions known to receive 5-hydroxytryptaminergic innervation, 3 weeks after MDMA exposure (20 mg/kg, s.c., twice daily, for 4 days). These transport perturbations were associated with lasting decrements in 5-HT axonal markers (5-HT and its metabolite 5-hydroxyindoleacetic acid content), thereby suggesting a role for impaired axonal transport in the mechanisms of neurotoxicity (Callahan et al., 2001). Nevertheless, this study did not establish any differentiation between neuronal mitochondrial trafficking and transport of other intracellular organelles or materials, not allowing, therefore, a clear appraisal of the real impact of MDMA on mitochondrial trafficking.

Subsequent studies reported an increased Tau phosphorylation in mouse hippocampus and striatum 24 h after an acute MDMA scheme of 2 × 25 mg/kg, i.p., every 2 h, or an accumulative dose of 2 × 15 mg/kg, i.p., every 2 h, for 6 days (Busceti et al., 2008). Later, other authors reported a disruption of microtubular system in 5-hydroxytryptaminergic axons of the frontal cortex of rats 3 days after a single MDMA dose of 15 mg/kg, i.p. (Ádori et al., 2011) following MDMA exposure.

Recently, two studies reported an alteration of mitochondrial movement in hippocampal neurons by the monoamine neurotransmitters 5-HT and DA. By modulating the protein kinase B-glycogen synthase 3β (GSK3β) signaling pathway, through the 5-HT_{1A} receptor subtype, 5-HT stimulates the axonal transport of mitochondria (Chen, Owens, Crossin, & Edelman, 2007). By contrast, DA and dopamine D_2 receptor agonists inhibited mitochondrial movement via the same protein kinase B-GSK3β signaling cascade (Chen, Owens, & Edelman, 2008). These observations highlight the hypothesis that 5-HT and DA might act as extracellular modulators in regulating neuronal ATP distribution by controlling axonal mitochondrial distribution, and suggests that the distribution of neuronal mitochondria may occur through a conserved regulatory mechanism. Considering that the acute and sustained release of monoamine neurotransmitters, including 5-HT and DA, from nerve endings is by far the most significant acute effect of MDMA in the brain (Capela et al., 2009), these studies strongly suggest that MDMA might affect neuronal mitochondrial trafficking through the release of monoamine neurotransmitters, although further studies are required to confirm this hypothesis.

Nevertheless, recent studies from our group provided direct evidence that MDMA targets neuronal mitochondrial trafficking (Barbosa et al., 2014a; Barbosa et al., 2014b). In one of these studies, MDMA (1.6 mM), after a short incubation period of 90 min, was shown to reduce the overall axonal mitochondrial movement in cultured neurons from mouse hippocampus in a time-dependent manner. This effect was closely associated with increases in intracellular free calcium levels, both in the cell body and in neuronal extensions, increased phosphorylation of Tau protein, at the Thr181 residue, and excessive fragmentation of axonal mitochondria (Barbosa et al., 2014a). Overexpression experiments with wild-type Miro1 or its mutant version (Miro1 ΔEF), lacking the EF-hand calcium-binding domains, which does not respond to calcium levels alterations, revealed an independence on Miro1 regulatory functions in MDMA's mitochondrial trafficking phenotype (Barbosa et al., 2014a). Nevertheless, experiments in Tau-null neurons showed a partial reversion of mitochondrial transport deficits caused by MDMA, in a similar fashion to those observed in wild-type neurons overexpressing a GSK3β-kinase death construct (GSK3β Aln9, which lacks kinase activity) (Barbosa et al., 2014a). Additionally, overexpression of wild-type Mfn2 or Drp1 K38A (mutant Drp1 construct that lacks fission properties) partially recovered MDMA's mitochondrial transport phenotype, although overexpression of Mfn2 R94Q (mutant Mfn2 construct lacking fusion and transport properties) did not (Barbosa et al., 2014a). This indicates that fully functional Mfn2 was required to reverse MDMA's effects on mitochondrial transport, and suggests that MDMA might alter fusion mechanisms.

Since MDMA and its metabolites have been shown to co-exist in the brain following peripheral administration of MDMA (Jones et al., 2005), the effects of the mixture of MDMA and six of its major in vivo metabolites (α-MeDA, *N*-Me-α-MeDA, 5-(GSH)-α-MeDA, 5-(GSH)-*N*-Me-α-MeDA, 5-(NAC)-α-MeDA, and 5-(NAC)-*N*-Me-α-MeDA), each compound at an equimolar concentration of 10 μM, on neuronal mitochondrial trafficking were further appraised. Using this experimental design, a reduction was shown in overall axonal mitochondrial motility of cultured hippocampal neurons following a 24-h period of exposure. Overexpression of wild-type Mfn2 or Drp1 K38A almost completely rescued the mitochondrial trafficking arrest caused by this mixture (Barbosa et al., 2014b). This indicates a major role for mitochondrial fusion/fission regulators and pathways in mediating the effects of MDMA and its metabolites on mitochondrial transport. Taking into account these findings, a mechanistic pathway for MDMA-induced deregulation of neuronal mitochondrial trafficking was postulated and is illustrated in Figure 4.

MITOCHONDRIAL REGULATORS AND APOPTOTIC PATHWAYS

The apoptotic-signaling cascade may be divided into two major pathways, extrinsic and intrinsic, which are triggered by soluble molecules that bind to plasma-membrane receptors or by mitochondrial stimuli, respectively. In the intrinsic pathway, also called mitochondrial pathway, mitochondria are involved and play a crucial role in integrating and circulating death signals, such as DNA damage, oxidative stress, hypoxia, or growth factor deprivation, which result in outer mitochondrial membrane permeabilization (Sinha, Das, Pal, & Sil, 2013).

Permeabilization of the outer mitochondrial membrane allows the release of at least five apoptogenic proteins/mediators from the mitochondrial intermembrane space: cytochrome c, second mitochondrial activator of caspases/Direct inhibitor of apoptotic proteins (IAPs)-binding protein with low PI (Smac/DIABLO), high temperature requirement protein A2 (HtrA2/Omi), apoptosis-inducing factor (AIF) and endonuclease G. Cytochrome c, Smac/DIABLO, and HtrA2/Omi are caspase-dependent factors, whereas AIF and endonuclease G constitute caspase-independent elements. The B-cell lymphoma 2 (Bcl-2) superfamily of proteins, which encompass two subcategories, anti-apoptotic and pro-apoptotic

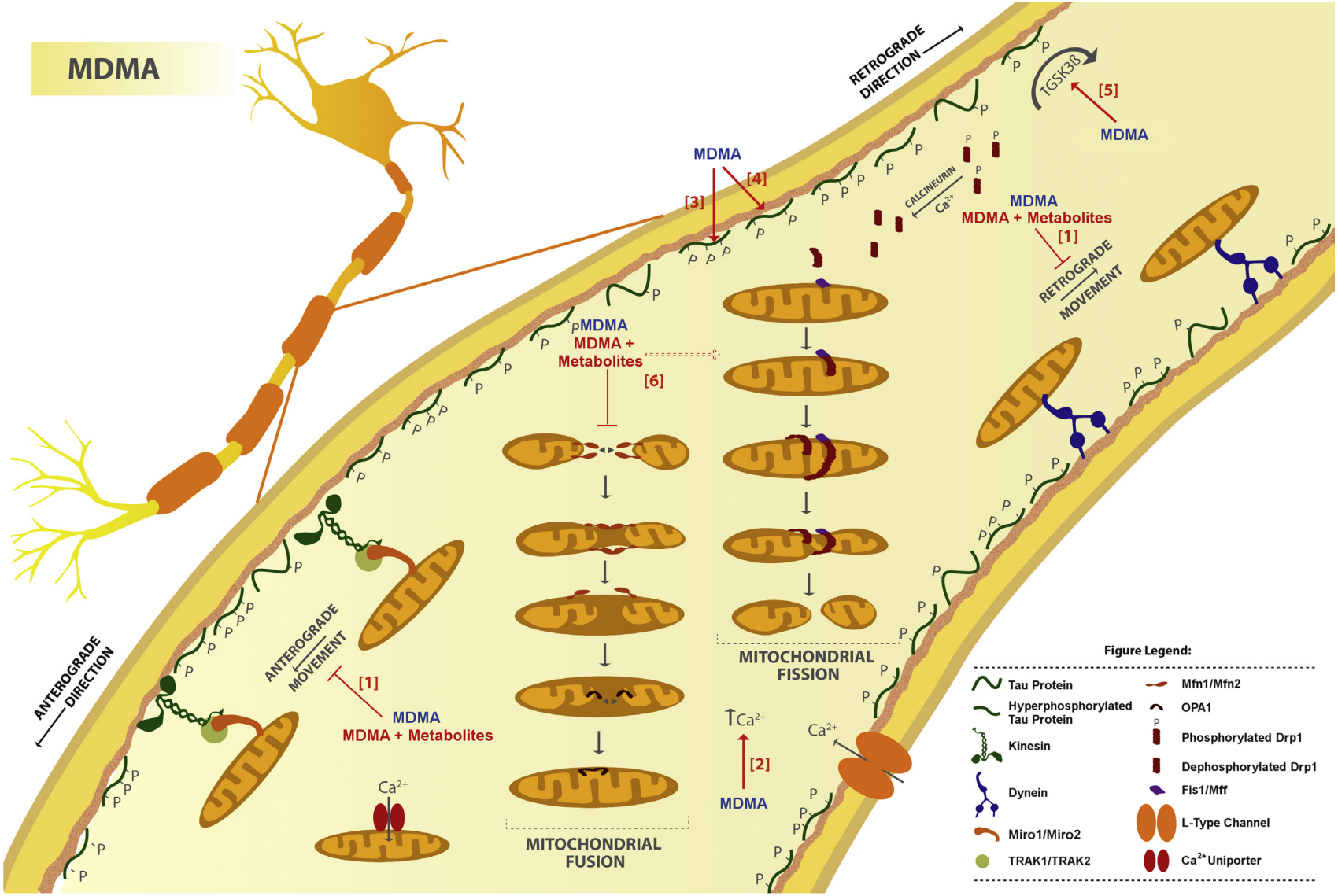

FIGURE 4 **Postulated mechanisms for the mitochondrial trafficking impairment induced by MDMA and its metabolites in cultured hippocampal neurons.** Both MDMA and the mixture of MDMA and its metabolites impair axonal transport of mitochondria in both anterograde and retrograde directions [1]. Intracellular calcium rises, both in the cell body and in neuronal extensions, are associated with the transport deficits induced by MDMA [2], although these effects most likely rely on Miro1-independent mechanisms. Nevertheless, MDMA increases Tau phosphorylation at Threonine181 residue [3], which contributes partially to its effects on mitochondrial neuronal trafficking, most likely due to microtubule destabilization [4]. This increase in Tau phosphorylation promoted by MDMA appears to be mediated by glycogen synthase 3β (GSK3β) [5]. Furthermore, both MDMA and the mixture of MDMA and its metabolites increase the fragmentation of axonal mitochondria. Overexpression of functional mitofusin 2 (Mfn2) or dynamin-related protein 1 (Drp1) K38A, a Drp1 construct that downregulates mitochondrial fission, partially rescue the trafficking deficits triggered either by MDMA or by the mixture of MDMA and its metabolites. Additionally, overexpression of Mfn2 R94Q, a Charcot-Marie-Tooth neuropathy 2A (CMT2A) mutant protein with impaired fusion and transport properties, does not recover the MDMA's and mixture's mitochondrial phenotype, indicating that MDMA, as well the mixture of MDMA and its metabolites, might alter mitochondrial fusion/fission mechanisms [6] and thus compromise mitochondrial transport. *Reproduced with permission from Barbosa et al. (2015).*

proteins, regulates the release of these apoptotic mediators (Ola, Nawaz, & Ahsan, 2011; Yuan & Akey, 2013).

Mitochondrial-Dependent Apoptosis Triggered by MDMA

Accumulating evidence indicates a major role for mitochondrial-dependent pathways in MDMA-mediated neuronal death. The involvement of the different mitochondrial proteins is detailed in the following sections.

Role of Bcl-2 Superfamily of Proteins in Mediating MDMA-Induced Neuronal Apoptosis

Anti-apoptotic Bcl-2 family multi-domain proteins include Bcl-2, Bcl-extra-long, Bcl-2-related protein A1, Bcl-w, and Bcl-2-related ovarian killer, which contain BH-(1–4) domains. Pro-apoptotic Bcl-2 protein family is divided into two subgroups according to the number of BH domains (Bcl-2-associated X protein (bax) and Bcl-2-associated death promoter) or proteins that have only the BH3 domain (e.g., BH3 interacting domain death agonist). Bcl-xS is not included in any of these two groups, since it has only BH3 and BH4 domains (Ola et al., 2011).

Studies in primary cultured cortical neurons showed that neuronal death induced by MDMA (500 μM) or 3,4-methylenedioxyamphetamine (500 μM) was closely associated with differential regulation of the Bcl-X splice variants, inhibition of the anti-apoptotic Bcl-extra-long-related proteins, and induction of the pro-apoptotic Bcl-xS variant, as ascertained 1 h, 24 h, or 96 h after exposure to drugs (Stumm et al., 1999). Following this initial report, increased observations have documented a crucial role for Bcl-2 superfamily members in MDMA-mediated apoptotic cascade. Studies in MDMA-exposed rats (5, 10, or 20 mg/kg, i.p., twice daily, for 7 days) revealed a dose-dependent up-regulation of Bax and down-regulation of Bcl-2 in the hippocampus, as ascertained 7 days after the last MDMA injection. Notably, these effects were evident by evaluating both messenger ribonucleic acid and protein expression levels (Soleimani Asl et al., 2012). Using an MDMA regimen of 10 mg/kg, once daily, for 7 days, another study showed a marked upregulation of Bax and downregulation of Bcl-2 in rat hippocampus 3 days after the last MDMA administration, as revealed by both messenger ribonucleic acid and protein expression levels (Mehdizadeh et al., 2012). This suggests that MDMA's effects on Bax and Bcl-2 expression patterns endure over time. A subsequent study also revealed an up-regulation of Bax and down-regulation of Bcl-2 genes, consistently traduced in altered expression levels of these proteins, in rat striatum, 2 weeks after MDMA administration (10 mg/kg, i.p., once daily, for 7 days) (Soleimani, Katebi, Alizadeh, Mohammadzadeh, & Mehdizadeh, 2012). These results indicate that MDMA's effects on the Bcl-2 superfamily of proteins are not restricted to the hippocampus region.

Cytochrome c and Caspase Activation in MDMA-Induced Neuronal Death

Cytochrome c is a 12.3 kDa nuclear DNA-encoded heme-containing protein located in the mitochondrial intermembrane space. As a mitochondrial response to apoptotic trigger stimuli, cytochrome c, along with other proteins, is released from mitochondria into the cytosol and forms a complex with apoptotic protease activating factor–1 and ATP/2′-deoxyadenosine 5′-triphosphate, the apoptosome, which promotes the activation of caspase-9. Once activated, caspase-9 processes executioner caspases, such as caspase-3, caspase-6, and caspase-7, thereby promoting the execution of apoptosis (Yuan & Akey, 2013). Nevertheless, IAPs may bind to active caspase-9 and -3, blocking the caspase cascade, thus inhibiting apoptosis (Bratton et al., 2001).

In vitro studies have demonstrated an apoptotic death profile in cerebellar granule cultured cells, characterized by chromatin condensation, caspase-6, caspase-9, and caspase-3 activation and mitochondrial cytochrome c release following MDMA exposure (4 mM for 24 h or 48 h) (Jiménez et al., 2004). Nevertheless, studies in hippocampal cultured neurons (400 μM for 24 h) found no changes in either cytosolic or mitochondrial cytochrome c content, but increased caspase-8–like and caspase-3–like activities after 48 h of exposure to MDMA (Capela et al., 2013), suggesting a role for the extrinsic pathway in apoptosis activation. Other reports in rat cultured cortical neurons or SH-SH5Y differentiated cells have also revealed increased caspase-3 activity following exposure to MDMA (200–800 μM for 48 h) (Capela et al., 2007) or its metabolites *N*-Me-α-MeDA (100 μM for 24 h) (Barbosa, Capela, Silva, Ferreira, et al., 2014), 5-(NAC)-α-MeDA, or 5-(NAC)-*N*-Me-α-MeDA (200 μM for 24 h) (Capela, Macedo, et al., 2007), or to the mixture of MDMA and six of its major in vivo metabolites (α-MeDA, *N*-Me-α-MeDA, 5-(GSH)-α-MeDA, 5-(GSH)-*N*-Me-α-MeDA, 5-(NAC)-α-MeDA, and 5-(NAC)-*N*-Me-α-MeDA), each compound at an equimolar concentration of 10 μM, for 24 h (Barbosa, Capela, Silva, Vilas-Boas, et al., 2014). Additionally, in cultured hippocampal neurons, only a partial prevention of MDMA-induced neuronal death resulted from caspase-3 inhibition (Capela et al., 2013), further supporting the existence of a combined effect between mitochondrial-dependent and -independent pathways in MDMA-mediated apoptotic neuronal response.

In vivo studies have also reported increased caspase-3 immunoreactivity in neurons of the cortex of rats exposed to MDMA (40 mg/kg, i.p.) for 24 h (Warren et al., 2007). In the rostral forebrain and hippocampus of neonatal rats, MDMA administration (2 × 10 mg/kg, s.c.) resulted in a twofold increase in the number of cleaved caspase-3–immunoreactive cells, as ascertained 4 days later (Meyer, Grande, Johnson, & Ali, 2004). In mice, MDMA (3 × 5 mg/kg, i.p.) also produced a marked induction of caspase-3 activity in the amygdala and hippocampus, as measured 7 days later (Tamburini et al., 2006).

Overall, these data suggest a major role for cytochrome c–mediated trails and activation of caspases in MDMA's neurotoxic effects.

Smac/DIABLO Involvement in MDMA-Induced Neuronal Apoptosis

Smac/DIABLO is a protein codified by a nuclear gene, released from mitochondria to cytosol after apoptotic stimuli. The Smac/DIABLO pro-apoptotic effect is mediated by its interaction with IAPs, releasing caspases from them, thus enhancing the

activation efficiency of caspases and apoptosis execution (Du, Fang, Li, Li, & Wang, 2000).

Although limited in scope, recently Smac/DIABLO activation was found to be associated with MDMA-induced apoptosis in rat liver (Cerretani et al., 2011). Nevertheless, the involvement of Smac/DIABLO in methamphetamine's neurotoxic effects has been consistently reported (Jayanthi, Deng, Noailles, Ladenheim, & Cadet, 2004), raising the possibility that it may also play a focal role in MDMA-induced neurotoxicity.

AIF Involvement in MDMA-Induced Neuronal Apoptosis

AIF, a mitochondrial flavin–adenine dinucleotide-containing flavoprotein oxidoreductase, is a caspase-independent element of the apoptosis cascade located in the mitochondrial intermembrane space, with its amino-terminal anchored in the inner mitochondrial membrane. Upon an apoptotic stimulus, AIF undergoes proteolysis and then translocates to the nuclei, leading to chromatin condensation and large-scale DNA degradation, in a caspase-independent manner (Sevrioukova, 2011).

Studies in cultured hippocampal neurons associated MDMA-induced neuronal death (400 μM for 24 h) with increased mitochondrial AIF subunit 67 kDa content, although no differences were found in the cytosolic compartment, as well as in the AIF processed subunit 57 kDa in mitochondria. Additionally, cytosolic AIF processed 57 kDa subunit and nuclear AIF remained undetectable following MDMA exposure (Capela et al., 2013). Therefore, it is likely that AIF plays a role in MDMA-induced neuronal apoptosis, although other regulators might also be determinant in mediating neuronal death.

Taking into account the above findings, the mitochondrial proteins involved in mitochondrial-dependent pathways of neuronal death described to be targeted by MDMA are depicted in Figure 5.

CONCLUDING REMARKS

MDMA's neurotoxicity is essentially characterized by damage to monoaminergic terminals, and accumulating evidence has clearly implicated mitochondria as a direct target. Mitochondrial damage promoted by MDMA includes inhibition of mitochondrial ETC, interference with mitochondrial dynamics, including mitochondrial fusion/fission and trafficking, as well as oxidative modifications in mitochondrial macromolecules. Additionally, other studies indicate that MDMA-induced neuronal toxicity and death rely on the activation of several mitochondrial pathways, whose roles have been well predictable in animal models and in in vitro settings. In this field, pathways involving the release of mitochondrial proteins with consequent activation of the caspase-mediated downstream cell death executioner pathway, closely regulated by Bcl-2 superfamily of proteins, have been implicated in MDMA's neurotoxic effects. Therefore, understanding the molecular mechanisms, at the mitochondrial level, involved in the neurotoxicity caused by MDMA can help in developing putative therapeutic approaches to prevent or to treat the acute and long-lasting neuropsychiatric complications associated with human MDMA abuse.

APPLICATIONS TO OTHER ADDICTIONS AND SUBSTANCE MISUSE

In addition to MDMA, the neuronal injury mediated by other drugs of abuse, including methamphetamine (Brown, Quinton, & Yamamoto, 2005), amphetamine (Cunha-Oliveira et al., 2006), ethanol (Chu, Tong, & Monte, 2007), cocaine (Cunha-Oliveira et al., 2006), and heroin (Cunha-Oliveira et al., 2007), have been closely associated with mitochondrial dysfunction. Indeed, inhibition of mitochondrial ETC and enzymes of the tricarboxylic acid cycle, perturbations of mitochondrial clearance mechanisms, interference with mitochondrial dynamics, as well as oxidative modifications in mitochondrial macromolecules have been consistently associated with these substances. Despite this, the relative contribution of mitochondrial dysfunction remains to be characterized. On one hand, although it is difficult to attribute a causal association of mitochondrial dysfunction to neuronal damage, it seems reasonable to consider that they might be closely interconnected. On the other hand, it is also clear that disrupted mitochondrial function may readily be secondary to other events, since it is difficult to attribute any neuronal injury caused by toxic substances to mitochondrial dysfunction as a primary cause. As such, the importance of these effects to neuropharmacological actions and toxicity caused by psychotropic drugs requires further research.

KEY FACTS ABOUT MITOCHONDRIA

- Mitochondria are critical for many cellular functions, including oxidative phosphorylation, intermediary metabolism, calcium buffering, and apoptosis regulation, which are important to all cells.
- Although the normal functions of mitochondria are critical for cell and organism viability, these same activities can pose serious challenges to cellular function when improperly regulated or disrupted.
- Mitochondrial deficits have been implicated in the pathogenesis of many neurodegenerative disorders.
- Advances in genetics and cell biology have provided valuable insights into the function of mitochondria and the contribution of defects of mitochondrial metabolism to human disease.

SUMMARY POINTS

- This chapter focuses on the role of mitochondria in MDMA-induced neuronal dysfunction and toxicity.
- MDMA is a major drug of abuse worldwide, known to elicit both acute and long-lasting neurotoxicity.
- In the field of MDMA research, there is a general consensus that mitochondrial-dependent pathways can provide a major understanding concerning pathological processes underlying the neurotoxicity mediated by this recreational drug.
- MDMA has been shown to disrupt the function of mitochondrial ETC complexes, to interfere with mitochondrial dynamics, and to cause oxidative modifications in mitochondrial macromolecules.
- Understanding the molecular mechanisms, at the mitochondrial level, that are involved in MDMA's neurotoxicity can help in defining target pathways and in developing putative therapeutic approaches to prevent or treat the acute and long-lasting neuropsychiatric complications seen in human users.

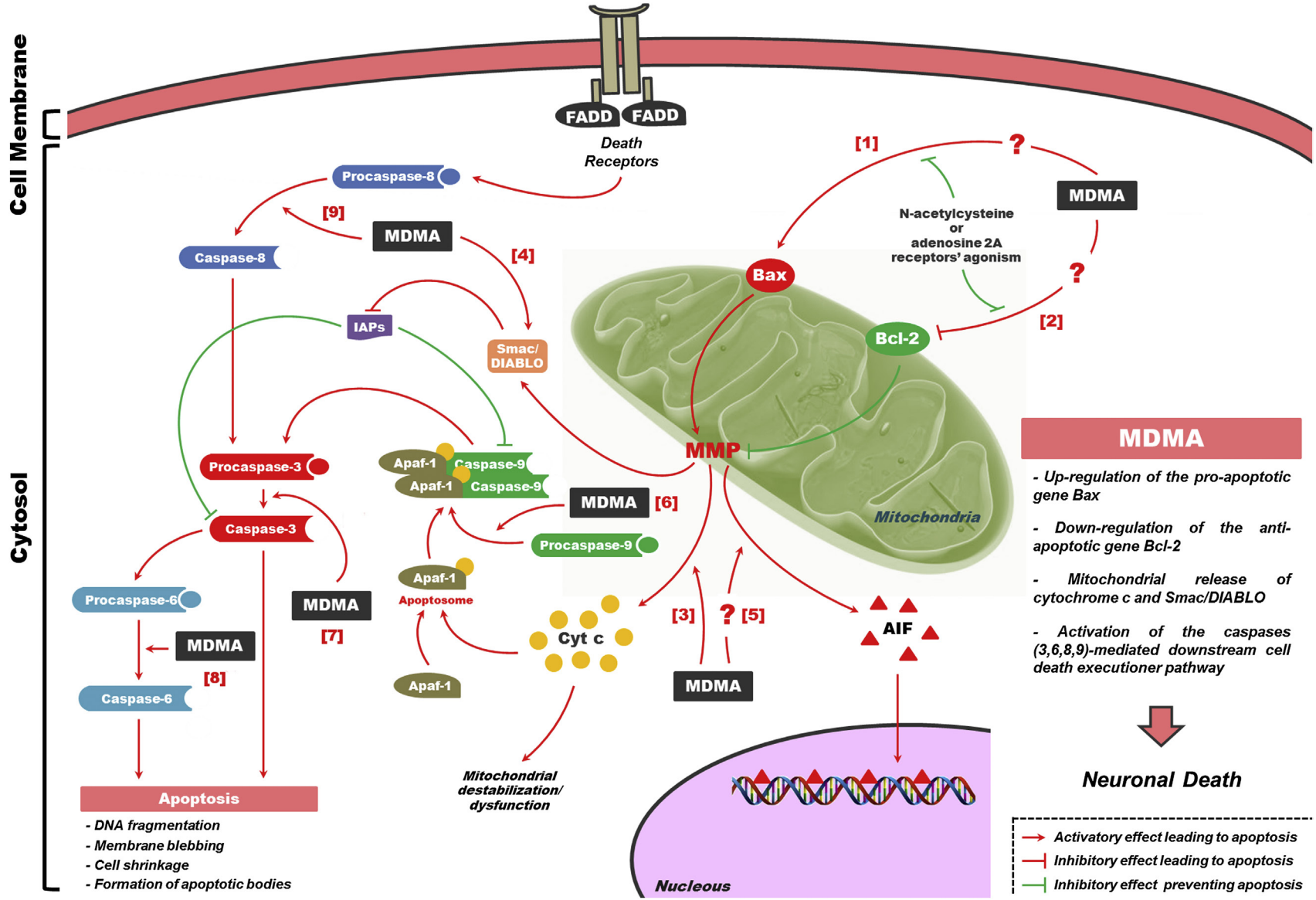

FIGURE 5 **Postulated players in MDMA-induced mitochondrial-dependent apoptotic neuronal death.** Mitochondrial-dependent apoptotic cell death is regulated by several proteins (anti-apoptotic and pro-apoptotic Bcl-2 family of proteins). After inducing mitochondrial membrane permeabilization (MMP), pro-apoptotic proteins allow the release of apoptogenic proteins/mediators from the mitochondrial intermembrane space. Both in vivo and in vitro studies have reported the MDMA's ability to up-regulate the pro-death gene bax [1] and to downregulate the anti-apoptotic gene bcl-2 [2], both of which have been consistently correlated with increased protein expression. These changes result in mitochondrial membrane permeabilization (MMP), with consequent release of cytochrome c [3] and second mitochondrial activator of caspases/direct inhibitor of apoptotic proteins (IAPs)–binding protein with low PI (Smac/DIABLO) [4]. At present, the involvement of apoptosis-inducing factor (AIF) [5] in MDMA-induced neuronal effects remains poorly understood. The release of these apoptogenic factors triggered by MDMA has been reported to cause caspases' activation, namely caspase 3 [7] and caspase 6 [8], leading to mitochondrial-dependent apoptotic cell death. Some studies have also reported MDMA's ability to trigger activation of caspase 8 [9], thus suggesting a tight regulation of mitochondrial-dependent and -independent apoptotic pathways in MDMA-induced neuronal death. *Adapted from Barbosa et al. (2015).*

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