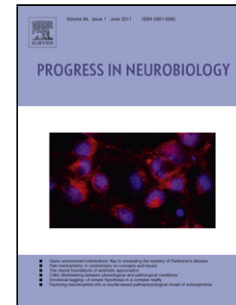


## Accepted Manuscript

Title: Amphetamine-related drugs neurotoxicity in humans and in experimental animals: Main mechanisms

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We revise the neurotoxic effects of MDMA and METH in different animal species

The toxic effect of MDMA is species-specific and damages the serotonergic system in non-human primates and rats, and the dopaminergic system in mice

The toxic effect of METH targets the dopaminergic system in all animal species studied including humans

The nigrostriatal system is more vulnerable than the mesolimbic system

Within the striatum the striosomes are more vulnerable than the matrix

METH kills dopamine neurons as demonstrated by reduced silver-staining in rodents

METH reduces DAT binding sites and motor skills in human addicts.

Accepted Manuscript

## **Amphetamine-related drugs neurotoxicity in humans and in experimental animals: Main mechanisms**

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**Abstract**

Amphetamine-related drugs, such as 3,4-methylenedioxymethamphetamine (MDMA) and methamphetamine (METH) are popular recreational psychostimulants. Several preclinical studies have demonstrated that, besides having the potential for abuse, amphetamine-related drugs may also elicit neurotoxic and neuroinflammatory effects. The neurotoxic potentials of MDMA and METH to dopaminergic and serotonergic neurons have been clearly demonstrated in both rodents and non-human primates. This review summarizes the species-specific cellular and molecular mechanisms involved in MDMA and METH-mediated neurotoxic and neuroinflammatory effects, along with the most important behavioral changes elicited by these substances in experimental animals and humans. Emphasis is placed on the neuropsychological and neurological consequences associated with the neuronal damage. Moreover, we point out the gap in our knowledge and the need for developing appropriate therapeutic strategies to manage the neurological problems associated with amphetamine-related drug abuse

**Keywords:** dopamine; ecstasy; methamphetamine; METH; 3,4-methylenedioxymethamphetamine; MDMA; mouse; neurodegeneration; neuroinflammation neurotoxicity; non-human primate; rat

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**List of abbreviations:**

AC, adenylyl cyclase

AcbSh, nucleus accumbens shell

AcbC, nucleus accumbens core

cAMP, cyclic adenosine monophosphate

CNS, central nervous system

CPu, caudate putamen

CREB, cAMP responsive element binding protein

CSF, cerebrospinal fluid

CYP, cytochrome P450 enzymes

DA, dopamine

DAT, DA transporter

5,7-DHT, 5,7-dihydroxytryptamine

L-DOPA, 3,4-dihydroxy-l-phenylalanine

DOPAC, 3,4-dihydroxyphenylacetic acid

DPCPX, dipropylcyclopentylxanthine, adenosine A<sub>1</sub> receptor antagonist

ERK, extracellular-signal-regulated kinase

FDA, Food and Drug Administration

fMRI, functional magnetic resonance imaging

GBR12909, vanoxerine, antagonist of DAT

GFAP, glial fibrillary acidic protein

GLU, glutamate

GSH, glutathione

HHMA, 3,4-dihydroxymethamphetamine

5-HIAA, 5-hydroxyindoleacetic acid

HPA, hypothalamus–pituitary–adrenal axis

5-HT, serotonin

HVA, homovanillic acid

HPLC, high-performance liquid chromatography

JAK, Janus kinase

JNK, c-Jun N-terminal kinases

ICV, intracerebroventricular

IL, interleukin

Mac-1, Macrophage-1 antigen

MAO-B, monoamine oxidase type B  
MAPK, mitogen-activated protein kinase  
 $\alpha$ -MeDA,  $\alpha$ -methyldopamine  
MDA, 3,4-methylenedioxyamphetamine  
MDMA, 3,4-methylenedioxymethamphetamine  
METH, methamphetamine  
MK-801, dizocilpine, NMDA receptor antagonist  
MOR-1,  $\mu$  opioid receptor  
a-MPT, alpha-methyl-para-tyrosine  
MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine  
MRI, magnetic resonance imaging  
NA, noradrenaline  
NAC, N-acetylcysteine  
NET, NA transporter  
7-NI, 7-nitroindazole  
NMDA, N-methyl-D-aspartate  
NO, nitric oxide  
3-NT, 3-nitrotyrosine  
nNOS, neuronal NO synthase  
6-OHDA, 6-hydroxydopamine  
PD, Parkinson's disease  
PET, positron emission tomography  
PKC, protein kinase C  
ROS, reactive oxygen species  
RNS, reactive nitrogen species  
SCH23390, dopamine D1 receptor antagonist  
SERT, 5-HT transporter  
SN, substantia nigra  
SNc, SN pars compacta  
SOD, superoxide dismutase  
SPECT, single photon emission computed tomography  
TH, tyrosine hydroxylase  
TNF, tumor necrosis factor  
VMAT<sub>2</sub>, vesicular monoamine transporter

VTA, ventral tegmental area

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## 1. Dopamine neurotransmission and neuroinflammation

The primary brain targets for the damage induced by both 3,4-methylenedioxymethamphetamine (MDMA) and methamphetamine (METH) are the striatum and the substantia nigra (SN). Thus, dysregulation of nigro-striatal dopaminergic system is the major cause of motor impairments induced by these drugs of abuse. Moreover, inflammatory effects of MDMA and METH play a significant role in the eventual dopaminergic dysregulation and symptom manifestation by these drugs. Hence, we will present a short overview on dopamine (DA) transmission and receptors and the role of neuroinflammation in neurodegenerative diseases with particular focus on these two drugs.

MDMA and METH may act as indirect DA agonists, producing their effects through DA receptors. These receptors are coupled to heterotrimeric G proteins and are classified into two families: D<sub>1</sub>-like (which includes D<sub>1</sub> and D<sub>5</sub> receptors in mammals) and D<sub>2</sub>-like (which includes D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub> receptors) receptor families. Initially, these two families were defined functionally based on their ability to modulate adenylyl cyclase (AC) activity and cAMP accumulation in cells, but later this classification was confirmed by molecular cloning (Beaulieu and Gainetdinov, 2011). The D<sub>1</sub>-like receptors are found exclusively post-synaptically and activate the G<sub>as/olf</sub> family of G proteins to stimulate AC and cAMP production. The D<sub>2</sub>-like family are expressed both pre- and post-synaptically and activate the G<sub>ai/olf</sub> family of G proteins to inhibit AC and cAMP production (Beaulieu and Gainetdinov, 2011). There exist two variants of D<sub>2</sub> receptors: D<sub>2</sub>-short (D<sub>2</sub>-S) and D<sub>2</sub>-long (D<sub>2</sub>-L) (Giros et al., 1989; Monsma et al., 1989). The D<sub>2</sub>-S variant is expressed mostly pre-synaptically and is involved in autoreceptor functions (e.g. control of DA release and regulation of extrasynaptic DA levels), whereas D<sub>2</sub>-L is mainly a postsynaptic isoform (Usiello et al., 2000; De Mei et al., 2009). In addition, D<sub>2</sub>-S potentiates DA transporter (DAT) activity via the formation of heteromeric protein-protein complexes with DAT localized in the dopaminergic terminals (Hadlock et al., 2010). Interestingly, the distribution of DA receptors is similar in humans and rodents (Ares-Santos et al., 2013).

The significance of DA receptors is reflected in the diverse action of DA in behavior and cognition, voluntary movement, motivation, punishment and reward, attention, learning and working memory (Granado et al., 2008a; Martín et al., 2008; Darmopil et al., 2009; Darvas and Palmiter, 2009; 2010; Ortiz et al., 2010; Murer and Moratalla, 2011; Espadas et al., 2012; Ruiz-DeDiego et al., 2015a, b). Moreover, DA receptors (e.g. D<sub>1</sub>-like receptors) may mediate the interactions between glutamatergic and dopaminergic systems (Rodrigues et al., 2007). Consistent with this broad

spectrum of activities, there is a wide expression of DA receptors in the brain. Both D<sub>1</sub>-like and D<sub>2</sub>-like receptor subtypes are present in all of the known DA projection fields in the CNS and their expression generally overlaps in most brain areas. Moreover, D<sub>1</sub>-like and D<sub>2</sub>-like receptors are highly expressed in the striatum, nucleus accumbens, olfactory bulb, amygdala, frontal cortex and SN and, at lower levels, in the hippocampus and ventral tegmental area (VTA) (Moratalla et al., 1996a; Beaulieu and Gainetdinov, 2011; Gangarossa et al., 2012). The striatum, one of the areas most affected by MDMA and METH, is the site with the highest concentration of DA in the brain. Although multiple DA receptor subtypes are present in the striatum, the D<sub>1</sub>-like and D<sub>2</sub>-like receptors are the most abundant in this area. Interestingly, D<sub>1</sub>- and D<sub>2</sub>-containing projection neurons are segregated in the striatum, as well as in the nucleus accumbens (Callier et al., 2003; Beaulieu and Gainetdinov, 2011; Suarez et al., 2014). D<sub>1</sub> receptor is selectively expressed in striatal projection neurons that form the direct projecting pathway to the SN, while D<sub>2</sub>-like receptors are selectively expressed in the striatal projection neurons that form the indirect projecting pathway to the SN by first projecting to the globus pallidus (Beaulieu and Gainetdinov, 2011; Suarez et al., 2014).

Loss of neurons and neuroinflammation are two connected responses in neurodegenerative diseases. Brain immunological response to insults such as infection, injury, toxic agents, or stress, is primarily mediated by the glia cells that remove or inactivate potentially damaging agents or damaged tissue. However, over-activation of this system, reflected in elevated levels of immunological markers such as pro-inflammatory cytokines, can result in neuroinflammation, leading to changes in brain structure and synaptic plasticity, and eventually neurodegeneration (Hayley et al., 2005; Leonard, 2007; Patterson, 2014).

It is of importance to note that chronic stress may exacerbate the release of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6 that can precipitate, or aggravate, neurological diseases like Alzheimer's disease (Wuwongse et al., 2010), amyotrophic lateral sclerosis, epilepsy, Huntington's disease, multiple sclerosis, and Parkinson's disease (PD) (Hemmerle et al., 2012). Thus, drugs that prevent or counteract the detrimental consequences of stress on inflammatory pathways may offer novel treatments for a variety of neurodegenerative pathologies (Hurley and Tizabi, 2013).

As mentioned above, glial cells, particularly microglia, are the primary modulators of inflammation in the CNS (Stoll and Jander, 1999; Block et al., 2007) as they constantly "survey" their

environment and utilize their constitutively expressed surface receptors to trigger or amplify responses to a given insult (Aloisi, 2001). Glial activation can quickly lead to the release of both pro- and anti-inflammatory cytokines, where the final effect is dependent on the balance between these opposing responses.

It is believed that a dysregulation, or breakdown, of the normal response triggered by any of the stimuli mentioned above can cause inflammation to become persistent and harmful (Gao and Hong, 2008). For example, inflammation induced by lipopolysaccharide (a compound derived from membrane of Gram-negative bacteria causing inflammatory-mediated damage) can result in long term increase in TNF- $\alpha$  from brain microglia months after it has subsided in the periphery (Qin et al., 2007). This increased pro-inflammatory response may result in a delayed and progressive loss in dopaminergic neurons in the SN, similar to that seen in PD, strengthening the suggestion that unregulated neuroinflammation could lead to neurodegeneration (Qin et al., 2007).

### **3,4-Methylenedioxymethamphetamine (MDMA; “ecstasy”)**

#### **2. History of MDMA**

MDMA, also known as “ecstasy”, is a ring-substituted amphetamine that belongs to the phenylisopropylamine class of substances, and has marked psychostimulant properties (Green et al., 2003; Lyles and Cadet, 2003).

MDMA was first synthesized and patented around 1912 by Köllisch at the German pharmaceutical company Merck. It was not tested pharmacologically at the time because it was only regarded as being an intermediate in a new synthetic pathway for hemostatic substances (Freudenmann et al., 2006). In 1953, a large toxicological study was performed at the University of Michigan, in the USA, where MDMA was administered to five animal species. These results were published in 1973 by Hardman and colleagues, and indicated, for the first time, the half lethal dose of MDMA in each species (Hardman et al., 1973). The next phase of research on MDMA began with the studies and work by Alexander Shulgin. According to his autobiography and interviews, in 1977 Shulgin introduced MDMA to Leo Zeff, a retired psychologist from Oakland (CA, USA), who was so impressed by the effects of MDMA that he decided to come out of retirement, and began to introduce MDMA to other psychotherapists across the USA (Benzenhöfer and Passie, 2010). The appeal of MDMA that has led to its use in psychotherapy is chiefly due to the particular profile of

action of this substance, which includes amphetamine-like stimulant effects together with feelings of increased emotional sensitivity and closeness to other individuals (Kirkpatrick et al., 2014). Similar to other amphetamine-related drugs, MDMA induces a state of “high”, mainly characterized by disinhibition in social relations, openness of spirit, increased empathy towards other people, increased self-esteem and self-confidence, euphoria, increased vigilance, improvement of mood, and abolition of fatigue (Downing, 1986; Greer and Tolbert, 1986; Kirkpatrick et al., 2014). MDMA has been popular as a recreational drug since the mid 1980s, and is often consumed in dance clubs because of its effects on mood and social relations (Hall and Henry, 2006).

### **3. General toxicity of MDMA**

Although MDMA generally elicits “positive” effects, up to the 25% of MDMA users report having had at least one adverse reaction to the substance (Davison and Parrott, 1997; Green et al., 2003; Morton, 2005). Acute toxicity elicited by MDMA in humans and experimental animals includes effects on the neuroendocrine and thermoregulatory systems, in particular induction of hyperthermia, and on the cardiovascular system (Gordon et al., 1991; Vollenweider et al., 1998; de la Torre et al., 2000). In this regard, it is worth mentioning that the typical environmental conditions featuring dance clubs, where music is deafening and room temperatures are high due to crowding, together with the fact that club-goers usually consume little water and considerable amounts of ethanol, are crucial to amplifying MDMA-induced hyperthermia (Green et al., 2003).

Hyperthermia is a major clinical problem associated with the use of MDMA, and the issue of MDMA-induced hyperthermia is complex, since the biological mechanisms involved in heat production and progression to hyperthermia after exposure to the drug are not clearly understood. It is feasible to hypothesize that the increased release of monoamines following MDMA administration may stimulate receptors involved in thermoregulation (Shankaran and Gudelsky, 1999). This would suggest that DA could also be involved in MDMA-induced hyperthermia (Green et al., 2003). Moreover, MDMA may induce vascular constriction by activating serotonin (5-HT) 5-HT<sub>1B</sub> and 5-HT<sub>2A</sub> receptors (Gudelsky et al., 1986), thus interfering with the peripheral thermoregulatory mechanisms of the body (Sprague et al., 2003).

MDMA affects neuroendocrine functioning by promoting the release of 5-HT and noradrenaline (NA), in turn stimulating the activity of the hypothalamus–pituitary–adrenal axis (HPA), and increasing the levels of cortisol (de la Torre et al., 2000; Harris et al., 2002; Farré et al., 2004). A recent article by Parrott and colleagues (Parrot et al., 2014) has reviewed the results obtained in

several earlier studies (Parrott et al., 2001, 2007, 2008), and reported that MDMA users experience an 800% increase in the salivary levels of cortisol after clubbing, compared with pre-drug baseline. Moreover, the same review reported that heavy MDMA users exhibited a 400% increase in hair cortisol levels 3 months after drug discontinuation, compared with both light MDMA users and non-users. In this regard, it is noteworthy that the HPA releases cortisol to cope with stressful situations, and that cortisol reactivity and homeostasis can be impaired when the body is subjected to repeated stressors. This impairment is reflected by deficits in core psychological functions, including memory, cognition, sleep, and well-being (Parrott et al., 2014). Therefore, these effects on cortisol may underlie some of the actions of MDMA as an acute metabolic stressor (Parrott, 2006, 2014).

MDMA consumed for recreational purposes produces transient and dose-dependent cardiovascular effects, such as tachycardia and moderate increases in both systolic and diastolic blood pressure (Vollenweider et al., 1998). It should also be mentioned that MDMA may exacerbate latent cardiovascular problems, such as labile hypertonia, and that high or repeated doses of MDMA, such as those that are often consumed during all-night dance sessions, may potentially lead to severe hypertensive reactions and arrhythmias (Vollenweider et al., 1998). Remarkably, hypertension, in combination with coagulopathy, might be potentially responsible for the cerebral insults induced by MDMA, as suggested by a single case report (Vollenweider et al., 1998).

#### **4. Neurotoxicity of MDMA**

A number of reports have demonstrated that MDMA binds to all three presynaptic monoamine transporters, although interspecies differences for this effect exist. In rats, MDMA has the highest affinity for the 5-HT transporter (SERT), and lower affinities for the NA transporter (NET) and DAT (Steele et al., 1987; Rudnick and Wall, 1992). Similar affinities were reported in mice, although in this species MDMA seems to act as a dopaminergic neurotoxin, rather than a serotonergic neurotoxin (Kindlundh-Högberg et al., 2007). In humans, and at variance with that observed in rodents, MDMA displays higher affinity for NET, and lower, but similar, affinities for SERT and DAT (Verrico et al., 2007). However, the ability to release intracellular monoamines is higher in SERT-expressing cells than in either DAT- or NET-expressing cells, and this may justify the toxic effects of MDMA on SERT density observed in the human brain (Reneman et al., 2001a, b). Once translocated to the cytoplasm, MDMA increases the extracellular levels of 5-HT, DA, and NA in multiple brain regions (Gudelsky and Yamamoto, 2008). MDMA causes the dissipation of the proton gradient between the vesicles and the cytosol that is necessary for the proper functioning

of the vesicular monoamine transporter (VMAT<sub>2</sub>), inhibiting VMAT<sub>2</sub>-mediated influx and proper storage of 5-HT, DA, and NA in the neuronal terminal (Rudnick and Wall, 1992; Cozzi et al., 1999). This event is boosted by the blockade of the reuptake by presynaptic terminals, and by the partial inhibition of the monoamine oxidase type B enzymes (MAO-B), located in the outer membrane of the mitochondria of serotonergic neurons (Leonardi and Azmitia, 1994).

MDMA has attracted the attention of several researchers since besides having abuse potential it may also elicit neurotoxic effects. The occurrence of neurotoxicity induced by MDMA has been investigated in both humans and experimental animals, although the issue appears to be complex. In fact the features of MDMA-induced neurotoxic damage seem to vary, depending on the gender and strain of animals, which may influence the response to different dosing regimens and administration routes of MDMA (Ricaurte et al., 1988b; Colado et al., 1995; Itzhak et al., 2003). The basis for the interspecies variations in MDMA neurotoxicity is still unknown, but it has been suggested that differences in MDMA disposition and metabolism by cytochrome P450 enzymes (CYP) may play a key role in these variations (Green et al., 2012).

### **5. *In vitro* studies: cell cultures**

Several studies have explored the effects of MDMA in cell cultures, primarily cortical neurons and human neuroblastoma-derived SH-SY5Y cells (table 1). Cortical neurons can be studied in serum-free cultures and contain virtually no microglia and few astroglial cells (less than 10%, in respect to the total population). These features allow for the evaluation of the specific effects of MDMA on neurodegeneration, and the exclusion of the influence of neuroinflammatory mechanisms (Capela et al., 2006a,b).

Using this model, Capela and coworkers (2006a,b) have established a relevant *in vitro* paradigm to study MDMA-induced neurotoxicity, in which they were able to reproduce the apoptotic cell death elicited by the drug *in vivo*. In that study, the cultures were treated with either MDMA or its metabolites, and were incubated at normal temperature (36.5°C) or at hyperthermic temperature (40°C) for 24 hours. The study revealed that MDMA-induced neurotoxicity in cortical neurons is not only concentration- and time-dependent, but also aggravated by hyperthermic conditions. Moreover, MDMA-induced neuronal death was found to follow an apoptotic pattern, which is at least partially mediated by the direct stimulation of the 5-HT<sub>2A</sub> receptor (Capela et al., 2006b).

Human SH-SY5Y cells are a tumor-derived cell line that has attracted the attention of scientists involved in the study of neurological diseases affecting dopaminergic neurons (Presgraves et al., 2004; Brown et al., 2006). When differentiated with retinoic acid, these cells express the features of a dopaminergic phenotype, making them a suitable tool for evaluating the effects induced by neurotoxins on dopaminergic neurons and the mechanisms involved (Presgraves et al., 2004; Brown et al., 2006). A study from Ferreira and colleagues has analyzed the neurotoxic effect of MDMA and its catechol metabolites  $\alpha$ -methyldopamine ( $\alpha$ -MeDA) and N-Me- $\alpha$ -MeDA on SH-SY5Y cells (Ferreira et al., 2013). This investigation found that these two metabolites of MDMA are neurotoxic in a concentration- and time-dependent manner, similar to that previously observed for MDMA in cortical neurons (Capela et al., 2006a). However, the same study also found that MDMA by itself failed to induce a concentration- and time-dependent cell death, and that the neurotoxic effects of MDMA metabolites in SH-SY5Y cells appeared to be completely independent of caspase, which is crucial for the activation of neuronal apoptotic pathways. In particular,  $\alpha$ -MeDA and N-Me- $\alpha$ -MeDA promoted cell rupture, a characteristic feature of necrosis. Additionally, these MDMA metabolites promoted a significant mitochondrial dysfunction, as early as 24 hours after their application. Finally, a recent study, has confirmed that the toxicity induced by the catechol metabolites of MDMA is potentiated by hyperthermia, in line with earlier evidence (Barbosa et al., 2014). Taken together, these *in vitro* experiments have contributed to the elucidation of the mechanistic interactions of MDMA and its metabolites with cellular components.

## 6. *In vivo* studies in experimental animals

### 6.1. Mice

When administered to mice, MDMA decreases the concentrations of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) in several brain regions. Moreover, and most importantly, MDMA produces long-term degeneration of dopaminergic nerve terminals (Brodkin et al., 1993; Colado et al., 2001; Izco et al., 2010; Costa et al., 2013) and a decrease in tyrosine hydroxylase (TH), the rate-limiting enzyme for DA synthesis, in the striatum (Green et al., 2003; Costa et al., 2013). A study by Granado and coworkers has provided significant insight into the elucidation of the effects of MDMA on the nigrostriatal system of mice (Granado et al., 2008b). The study demonstrated that MDMA induces a loss of TH and DAT fibers in the striatum, but not the nucleus accumbens, indicating that the dopaminergic neurotoxicity of MDMA targets the nigrostriatal system, while sparing the mesolimbic pathway (Fig. 1). Interestingly, the same group observed that MDMA administration induces a significant decrease in TH-positive neurons in the SN pars compacta (SNc), further supporting the toxic effect of MDMA on the

nigrostriatal system, and that MDMA does not reduce the synthesis of TH, but rather damages dopaminergic terminals, with an effect that appears more pronounced in the striosomal compartment than in the matrix (Granado et al., 2008c). Taken together, these results are in line with earlier evidence describing dopaminergic terminal loss in the mouse striatum following MDMA administration (Fornai et al., 2004). Furthermore, and most notably, a similar pattern of striosomal damage has been observed following the administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Iravani et al., 2005). Since MPTP is a toxin known to induce PD in humans and experimental animals, this would account for similarities between the effects of MDMA and those of a dopaminergic neurotoxin.

Recent studies have provided further preclinical evidence that early exposure to MDMA may render the dopaminergic neurons more vulnerable to the detrimental effects of later neurotoxic insults. The neuroinflammatory and neurotoxic effects elicited by MPTP in both motor (SNc and striatum), and non-motor (hippocampus and medial prefrontal cortex) brain areas have been shown to be more marked in adult mice treated with MDMA during adolescence, compared with mice never exposed to MDMA (Costa et al., 2013, 2014). Furthermore, these neurochemical changes were associated with cognitive deficits, which were demonstrated by reduced performance in the novel object recognition task, a behavioral test used to evaluate non-spatial short-term memory in rodents (Costa et al., 2014). By contrast, oleoylethanolamide, an endocannabinoid that reduce L-DOPA-induced dyskinesias (Gonzalez-Aparicio and Moratalla, 2014) also reduces cognitive deficits and TH-induced by MDMA (Plaza-Zabala et al., 2010). Interestingly, these results are in line with earlier evidence that demonstrates the existence of noxious interactions between MDMA and other substances that act on the CNS such as ethanol (Izco et al., 2010; Ros-Simó et al., 2012) or caffeine (Khairnar et al., 2010). A recent study revealed that the coadministration of DPCPX, an A<sub>1</sub> receptor antagonist, amplifies the effects of MDMA on both microglial and astroglial activation in the striatum, suggesting that the A<sub>1</sub> receptor could be the adenosine receptor subtype most involved in the exacerbation of MDMA-induced gliosis by caffeine (Khairnar et al., 2014).

An important point to consider when discussing the effects of MDMA on the mouse brain, is that the majority of studies on this issue have employed racemic (±)-MDMA. However, evidence exists that the effects of MDMA are stereospecific, as indicated by the finding that S(+)-MDMA induces motor activation more efficiently than R(-)-MDMA (Young and Glennon, 2008). Moreover, it has been reported that S(+)-MDMA elevates body temperature, while R(-)-MDMA does not (Fantegrossi et al., 2003). A recent study by Frau and coworkers has specifically investigated the



effects elicited by either MDMA enantiomer on body temperature and neuroinflammation in mice. The results obtained have shown that S(+)-MDMA stimulates the activation of both microglia and astroglia in the striatum, whereas R(-)-MDMA has no significant influence on glial activation (Frau et al., 2013b). Moreover, the same study observed a positive correlation between the neuroinflammatory effects and the elevation in body temperature stimulated by S(+)-MDMA. This could suggest that a link may exist between hyperthermia and glial activation observed in response to MDMA (Frau et al., 2013b). See table 1 for a summary of the protocols of the studies described above.

### 6.2. Rats

Earlier studies in adult rats have reported that MDMA may damage serotonergic axons, terminals and cell bodies in areas such as the hippocampus, hypothalamus, striatum, and neocortex (Commins et al., 1987; Scallet et al., 1988). These results were later confirmed by other investigations and extended to other brain areas such as the thalamus, septum, and amygdala (Adori et al., 2006; Kovács et al., 2007). Remarkably, the toxic effects of MDMA on markers of serotonergic viability lasted for weeks, months, or even years, after drug discontinuation (Battaglia et al., 1987; Fischer et al., 1995; Crawford et al., 2006). In addition to this, it has been shown that astrocyte hypertrophy, leading to enhanced expression of glial fibrillary acidic protein (GFAP), can parallel the damage to serotonergic fibers observed after single MDMA injection (Adori et al., 2006). Xie and coworkers studied the *in vivo* expression of SERT after acute MDMA administration, and compared the effects of MDMA with those of 5,7-dihydroxytryptamine (5,7-DHT), a well-known serotonergic neurotoxin (Xie et al., 2006). Interestingly, this investigation observed that MDMA produced lasting reductions in 5-HT, (5-hydroxyindoleacetic acid) 5-HIAA, and [<sup>3</sup>H]paroxetine-labeled SERT, similar to that observed after the administration of 5,7-DHT, further indicating the ability of MDMA to act as a serotonergic neurotoxin. However, it is noteworthy that studies that examined the effects of prenatal or neonatal exposure of rats to doses of MDMA known to be neurotoxic in adult animals, have demonstrated that these MDMA regimens produce little serotonergic neurotoxicity (Meyer et al., 2004; Crawford et al., 2006).

The serotonergic system is implicated in sensation seeking, inhibitory control, impulsivity, anxiety, regulation of mood, and aggression (Linnoila et al., 1983; Jacobs and Fornal, 1999; Winstanley et al., 2004). Furthermore, reduced serotonergic activity is associated with heightened impulsivity and enhanced sensation seeking (Linnoila et al., 1983), and lesions of the serotonergic neurons elicit anxiolytic effects and increased impulsivity in rats (Harrison et al., 1997; Soderpalm and Svensson,

1999). However, studies on the effects of MDMA-exposure in experimental models of anxiety have produced conflicting data (Morley and McGregor, 2000; Piper, 2007). With regard to the long-standing controversy on the role of 5-HT in the regulation of behavior in animal models of anxiety, Green and McGregor have suggested that MDMA-induced 5-HT depletion might produce either anxiolytic effects in rats with a high basal level of anxiety, or anxiogenic effects in rats with a low basal level of anxiety (Green and McGregor, 2002; Winstanley et al., 2004). However, a recent study by Cox and coworkers has refuted this speculation, by showing an anxiety-like behavior following repeated administration of MDMA and/or abstinence from MDMA (Cox et al., 2014).

Besides these emotional effects, MDMA has been reported to induce learning deficits (Piper and Meyer, 2004) and periadolescent MDMA exposure has been found to be associated with a behavioral pattern reminiscent of impaired working memory and behavioral disinhibition (Piper et al., 2005). At the same time, Meyer and coworkers observed that periadolescent exposure to MDMA is associated with a significant reduction of SERT-immunoreactive fiber density in the hippocampus (Meyer et al., 2008). See table 1 for a summary of the protocols of the studies described above.

### *6.3. Non-human primates*

Non-human primates appear to be very susceptible to the neurotoxic effects of MDMA. A dose-dependent reduction in the content of 5-HT in several brain regions has been observed in squirrel monkeys after the repeated administration of MDMA at moderate doses (Ricaurte et al., 1988a). Another study where MDMA was administered at moderate doses reported significant decreases in the levels of 5-HIAA in the cerebrospinal fluid (CSF), and of both 5-HT and 5-HIAA in the cerebral cortex and striatum (Insel et al., 1989). In addition, a study on the long-term effects of exposure to MDMA in squirrel monkeys has revealed altered patterns of 5-HT innervation in the brain that persisted for up to 7 years after drug discontinuation. Interestingly, the severity of the changes involving 5-HT transmission appeared to be region-dependent, as the decrease in 5-HT immunoreactivity only appeared to be long-lasting in the hippocampal CA1 and CA2 fields, dentate gyrus, and subiculum (Hatzidimitriou et al. 1999). Importantly, the route of administration seems to influence the degree of 5-HT depletion induced by MDMA in non-human primates, as oral administration has been reported to be less toxic than subcutaneous injection (Ricaurte et al., 1988b). In this regard, data have been obtained which show how repeated subcutaneous administration of MDMA at a dose of 5 mg/kg produces a 86% depletion of 5-HT in the frontal cortex, while the same dose of MDMA elicits a 42% depletion of cortical 5-HT when repeatedly

administered orally (Ricaurte et al., 1988b). Furthermore, since it has been calculated that a single 5 mg/kg oral dose administered to non-human primates is equivalent to a 1.4 mg/kg dose administered to a 70-kg human (McCann and Ricaurte, 2001), which corresponds to a medium dose, these data may indicate a possible risk of brain serotonergic nerve terminal injury in humans who consume MDMA (Ricaurte et al., 1988b; McCann and Ricaurte, 2001).

Although several studies clearly demonstrate the neurotoxic effects of MDMA in non-human primates, data on the behavioral and cognitive effects of MDMA in these animals are scarce. Some studies have suggested that no changes in a repeated acquisition tasks (Frederick et al., 1998; Winsauer et al., 2002) or in a battery of operant tasks (Frederick et al., 1995) occur in non-human primates exposed to varying dosing regimens of MDMA. See table 1 for a summary of the protocols of the studies described above.

## **7. Studies in humans**

The evaluation of the long-term effects of MDMA, including neurotoxicity, in humans is complex, since MDMA users frequently consume it in the context of poly-drug abuse, together with other psychoactive substances, such as ethanol, cannabis, and cocaine (Schifano et al., 1998).

The use of amphetamine-related drugs in humans has been linked to the emergence of deficits in cognitive and executive functions. However, in the case of MDMA users, it still remains to be elucidated whether a relationship exists between these effects and the possible serotonergic or dopaminergic toxicity induced by the drug.

Single photon emission computed tomography (SPECT), positron emission tomography (PET), and functional magnetic resonance imaging (fMRI) studies have generally found reductions in SERT binding in MDMA users (McCann et al., 2008; Kish et al., 2010; Urban et al., 2012) as well as in abstinent subjects (Semple et al., 1999; Reneman et al., 2001a,b). With regard to this, it is interesting to mention that some evidence has been collected that indicates SERT recovery in subcortical regions and neocortex after prolonged MDMA abstinence (Buchert et al., 2006; Benningfield and Cowan, 2013) suggesting that at least some MDMA-induced neurotoxicity could be reversible.

A SPECT study performed by Reneman and coworkers in a cohort of MDMA-naïve individuals, and in moderate, heavy, and abstinent MDMA users, reported a dose-related decrease of SERT

levels in heavy MDMA users, while the SERT levels in abstinent drug users was similar to that detected in MDMA-naïve individuals (Reneman et al., 2001b,c). Interestingly, a reduction in SERT levels was seen in heavy MDMA users of both genders, although it reached statistical significance in women, but not in men (Reneman et al., 2001b). These results indicate that heavy use of MDMA may be associated with toxic effects on serotonergic neurons, and that women might be more susceptible to these effects. Although these findings would suggest that at least some of the MDMA-induced effects on serotonergic system may be reversible (Reneman et al., 2001c), a study in former MDMA abusers has observed the presence of impairments in verbal memory even at 1 year after drug discontinuation (Reneman et al., 2001c). These latter data would suggest that, in contrast to the effects on cortical serotonergic neurons, memory deficits induced by MDMA may be long-lasting. In this regard, it is also noteworthy that earlier findings demonstrated how MDMA users exhibit significant deficits in a series of cognitive tasks and that these behavioral deficits are associated with decreased levels of 5-HIAA in the CSF (McCann et al., 1999). In addition, recent studies have observed that abstinent heavy MDMA users display hyperexcitability (Bauernfeind et al., 2011) and chronic alterations in cortical 5-HT signaling compared with drug-naïve individuals in both the primary and secondary visual cortex (Di Iorio et al., 2012). The hippocampus of heavy MDMA users might be particularly vulnerable to the neurotoxic effects of MDMA (Gouzoulis-Mayfrank et al., 2003; Daumann et al., 2005; Kish et al., 2010), and functional alteration in this area evaluated by fMRI may predict incipient cognitive decline several years before the manifestation of a measurable cognitive failure. Interestingly, Daumann and coworkers also found a heightened parietal activation during working memory tasks after prolonged periods of either continued use or abstinence from MDMA (Daumann et al., 2004). Based on these findings, it was suggested that altered cortical activation might appear before the manifestation of cognitive deficits and may reflect the early stage of neuronal injury induced by MDMA (Daumann et al., 2004).

Besides possibly promoting memory deficits, the long-term damage produced by MDMA has been suggested to be a potential causal factor in psychiatric disorders, such as anxiety, phobia, obsessive-compulsive behavior, and psychosis (Flaum and Schultz, 1996; Parrott et al., 2001). However, conflicting results have been obtained in clinical investigations on this issue. It is noteworthy that a recent PET study that compared 49 moderate MDMA users with 50 non-users has reported a significant reduction in SERT binding throughout all cerebral cortices and hippocampus (Kish et al., 2010), which are brain areas involved in the regulation of mood. Moreover, the same cohort of MDMA users, although not displaying overt behavioral abnormalities, reported subnormal mood and exhibited modest, non-significant, deficits in some tests of attention and memory, which might

potentially stem from the decrease in SERT binding sites (Kish et al., 2010). Although the occurrence of psychotic disorders in MDMA users has not been as thoroughly studied as METH-associated psychosis, alterations in hippocampal activity could potentially contribute to the documented ability of MDMA to induce psychotomimetic effects (Nifosi et al., 2009; Potash et al., 2009; Patel et al., 2011).

While several studies have suggested that MDMA may harm the serotonergic system in the human brain, it is less clear whether MDMA may be toxic to human dopaminergic neurons. Over the years, several clinical reports have found that patients diagnosed with neurodegenerative diseases, such as PD, had a higher rate of exposure to amphetamine-related drugs at a young age, compared with the general population (Parrott et al., 2004; Callaghan et al., 2010; Christine et al., 2010; Curtin et al., 2015). Since several clinical studies recognize that PD has multiple origins, one hypothesis is that amphetamine-related drugs may be part of the wide array of factors leading to the dopaminergic neuron degeneration that causes the disease (Obeso et al., 2010). In this regard, it is noteworthy that MDMA has significant affinity for DAT (Verrico et al., 2007) and promotes the release of DA in multiple brain regions. Therefore, it is conceivable that prolonged exposure to MDMA, similar to that proposed for other amphetamine-related drugs (Garwood et al., 2006), may damage the dopaminergic neurons in the human SNc. Hence, it is conceivable that these damaged neurons could die earlier, therefore depleting the reserve of neural cells necessary for normal neurological functions, eventually ending up in the manifestation of PD (Garwood et al., 2006; Todd et al., 2013).

## **8. Mechanisms of MDMA neurotoxicity**

### *8.1. Biochemical mechanisms: Oxidative stress and excitotoxicity*

Metabolism of MDMA results in the formation of reactive oxygen species (ROS), which ultimately induce long-term neurotoxic effects. Interestingly, several studies have reported that the intracerebroventricular (ICV) administration of MDMA does not induce neurotoxicity, even at doses much higher than those having neurotoxic effects when administered peripherally (Paris et al., 1992; Esteban et al., 2001; Escobedo et al., 2005). These latter findings indicate that MDMA has to be systemically metabolized to produce its neurotoxic effects, and suggest that MDMA metabolites are responsible for these effects, as suggested by studies in cell cultures (see above). When MDMA is administered at low doses to rats, it is chiefly N-demethylated to form 3,4-methylenedioxyamphetamine (MDA) (Easton et al., 2003), whereas in humans, O-demethylation to

3,4-dihydroxymethamphetamine (HHMA) predominates after MDMA administration, irrespective of the dose (De la Torre et al., 2004). MDA is metabolized to  $\alpha$ -MeDA that can react either with glutathione (GSH) to form 5-(GSH)- $\alpha$ -MeDA or with N-acetylcysteine (NAC) to form 5-(NAC)- $\alpha$ -MeDA, and these compounds might be the main metabolites responsible for the neurotoxic effects of MDMA observed in rats. Miller and colleagues reported that acute ICV administration of 5-(GSH)- $\alpha$ -MeDA and 5-(NAC)- $\alpha$ -MeDA to male rats produced neurobehavioral changes similar to those observed after systemic MDMA (Miller et al., 1996). In addition, 2,5-bis-(GSH)- $\alpha$ -MeDA decreased the levels of 5-HT in the striatum, hippocampus, and cortex 7 days after its ICV administration to rats (Miller et al., 1997). Further support for the involvement of metabolites in the neurotoxic effects of MDMA comes from evidence that pretreatment of rats with acivicin, a  $\gamma$ -glutamyltranspeptidase inhibitor, which increases the brain uptake of 5-(GSH)- $\alpha$ -MeDA, was associated with a more marked depletion of 5-HT and 5-HIAA by MDMA in the striatum, hippocampus, and cortex (Bai et al., 2001). Similar findings on the role of metabolites in MDMA-mediated neurotoxicity have also been observed by *in vitro* studies, where 5-(GSH)- $\alpha$ -MeDA, showed higher toxicity to cultured cortical neurons than its parent compound, MDMA (Capela et al., 2006a).

MDMA dose-dependently increases DA release and production of hydroxyl radicals in the mouse striatum (Górska et al., 2014a,b). ROS, together with reactive nitrogen species (RNS), and the formation of neurotoxic MDMA metabolites, may all contribute to neurotoxicity by MDMA (Puerta et al., 2010; Green et al., 2003). Moreover, DA and 5-HT are metabolized by MAO-B, inducing the formation of superoxide ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ) (Capela et al., 2009). This suggests that MAO-B is involved in the neurotoxic effects of MDMA, a hypothesis which is further substantiated by the finding that the MAO-B inhibitor selegiline prevents serotonergic neurotoxicity in MDMA-treated rats (Sprague and Nichols, 1995; Alves et al., 2007). Nitric oxide (NO) generated from neuronal NO synthase (nNOS) also seems to be involved in MDMA-induced neurotoxicity, as suggested by previous studies that have demonstrated how nNOS inhibitors provide significant neuroprotection against long-term DA depletion in mice treated with MDMA (Colado et al., 2001). Further support for the involvement of oxidative stress in MDMA-induced neurotoxicity comes from studies showing that this effect of MDMA is amplified by either reduced levels or inactivation of antioxidant enzymes, such as catalase, superoxide dismutase (SOD) and GSH peroxidase (Cadet et al., 2001; Sanchez et al., 2003). In line with this, MDMA produces a less marked oxidative stress and striatal depletion of both DA and 5-HT in transgenic mice overexpressing SOD than in wild-type mice (Jayanthi et al., 1999). Furthermore, it is worth mentioning that treatment with

antioxidant agents has been found to afford neuroprotection in MDMA-treated rats. In 1996, Gudelsky first found that pretreatment with sodium ascorbate and cysteine prevented the depletion of 5-HT observed after MDMA administration in rats (Gudelsky, 1996). Similar findings were obtained by subsequent studies that found beneficial effects of  $\alpha$ -lipoic acid (Aguirre et al., 1999) and ascorbic acid (Shankaran et al., 2001) on MDMA-induced neurotoxicity.

Excitotoxicity includes a series of events, such as excessive glutamate (GLU) release and activation of GLU receptors, culminating in the increase in intracellular calcium levels, generation of NO, and activation of apoptotic pathways, ultimately resulting in cellular damage (Bruno et al., 1993; Yamamoto et al., 2010). Importantly, glutamatergic dysfunction has been linked to the manifestation of neurodegenerative disorders such as Alzheimer's disease and PD. This, together with the ability of amphetamine-related drugs to stimulate the release of GLU (Anneken et al., 2013; Shoblock et al., 2003), suggests that this excitatory amino acid could play a role in the neurodegenerative processes induced by these drugs (Lipton et al., 1994; Quinton et al., 2006).

However, limited evidence has been collected so far that supports a role of GLU in MDMA-induced neurotoxicity. Earlier studies have reported that blockade of the N-methyl-D-aspartate (NMDA) GLU receptor produces hypothermia and attenuates serotonergic nerve ending damage induced by MDMA in rats (Finnegan et al., 1989; Farfel et al., 1992; Tao et al., 2014). Moreover, studies by Colado and coworkers demonstrated that NMDA receptor antagonists are neuroprotective against MDMA-induced neurotoxicity only at doses that produce hypothermia, and suggested that GLU is not crucial for MDMA-induced neurotoxicity in both rats and mice (Colado et al., 1998, 2001). In contrast, by performing *in vitro* studies with rat cortical neurons, Capela and coworkers found that the NMDA receptor antagonist MK-801 partially prevented the toxic effects of MDMA in both normothermic and hyperthermic conditions (Capela, 2006b). Taken together, these studies fail to provide clear evidence of an involvement of GLU in the neurotoxic effects of MDMA, and further studies on this issue appear warranted.

## 8.2. Neuroinflammation

Another relevant issue related to MDMA-induced neurotoxicity is that MDMA can trigger inflammatory processes in those brain areas that exhibit dopaminergic and/or serotonergic terminal degeneration, but not in brain areas where no modifications in either DA or 5-HT levels occur (Yamamoto et al., 2010). Recent studies have demonstrated that glial activation participates in the events that induce neuronal damage, since chronic neuroinflammation elevates the levels of glia-

derived cytokines that exert neurotoxic effects on vulnerable dopaminergic neurons (Barcia et al., 2011). This mechanism provides support for a causal relationship between MDMA-induced neurotoxicity and neuroinflammation.

Several preclinical studies in rats and mice have demonstrated that MDMA elicits astroglial and microglial activation in the mouse striatum (Granado et al., 2008b; Costa et al., 2013; Frau et al., 2013b), as well as in the cortex (Herndon et al., 2014; Costa et al., 2014), and hippocampus (Costa et al., 2014; Lopez-Rodriguez et al., 2014). Moreover, independent studies have shown that the toxic and inflammatory effects of MDMA are exacerbated by its concurrent administration with other psychoactive substances, such as caffeine or ethanol (Hernandez-Rabaza et al., 2010; Khairnar et al., 2010; Frau et al., 2013a) and, at the same time, MDMA worsens neuroinflammation produced by toxins inducing PD, such as MPTP (Costa et al., 2013).

### *8.3. Role of hyperthermia*

The mechanism of MDMA-induced hyperthermia is complex, and appears to not only involve serotonergic and dopaminergic systems, but also adrenergic transmission (Sprague et al., 1998). Moreover, MDMA-induced hyperthermia could depend, at least in part, on the enhancement of ROS production and release of cytokines, such as IL-1 $\beta$ , IL-6, and TNF (Green et al., 2004; Capela et al., 2009). Finally, as mentioned above, manifestation of hyperthermia in rats treated with MDMA is greatly influenced by ambient temperature and housing conditions. MDMA administration produces hypothermic response in rats housed at low ambient temperature, while the drug can cause hyperthermic responses in rats housed at high ambient temperature (Green et al., 2005). This can be explained by considering that MDMA interferes with heat-loss mechanisms, and consequently higher ambient temperatures are more likely to impair the ability of the body to dissipate the excessive heat.

Previous studies have demonstrated that several compounds that elicit protective effects on MDMA neurotoxicity in rats, such as MK-801 (Farfel et al., 1992), the 5-HT<sub>2A</sub> receptor antagonist ketanserin, and the DA synthesis inhibitor  $\alpha$ -methyl-p-tyrosine (Malberg et al., 1996), are also able to lower body temperature. Interestingly, studies of these compounds found that when the core body temperature of rats was kept elevated, the neuroprotective effect was lost. Furthermore, pharmacological agents that amplify the hyperthermic effects of MDMA, such as caffeine, have been reported to potentiate MDMA-induced serotonergic deficits (McNamara et al., 2006). However, experiments with the 5-HT reuptake blocker fluoxetine have demonstrated that this drug



can provide protection against MDMA-induced neurotoxicity without affecting the increase in body temperature (Capela et al., 2009). Taken together, these findings indicate that while possibly playing an important modulatory role, hyperthermia could not be an essential factor in MDMA-induced neurotoxicity (Fig. 2).

#### 8.4. Role of dopamine and of D<sub>1</sub> and D<sub>2</sub> dopamine receptors

As mentioned above, MDMA promotes the release of DA, therefore, the possibility may exist that DA receptors play a role in MDMA-mediated neurotoxicity (Capela et al., 2009, Bisagno and Cadet, 2014). In this regard, it is noteworthy that previous studies have demonstrated how antagonists of DA receptors may be neuroprotective against neurotoxicity induced by amphetamine-related drugs (Albers and Sonsalla, 1995). Granado and coworkers (Granado et al., 2014) studied MDMA-induced neurotoxicity in DA D<sub>1</sub> or D<sub>4</sub> receptor knock-out mice, and found that inactivation of D<sub>1</sub> receptors attenuated MDMA-induced hyperthermia and prevented the striatal loss of DA and its metabolites, DOPAC and HVA (Granado et al., 2014). In addition, D<sub>1</sub> receptor knockout mice showed a reduction in the expression of GFAP, a marker for reactive astrogliosis (Aguirre et al., 1999; Adori et al., 2006) in the striatum and a concomitant attenuation of dopaminergic terminal loss induced by MDMA. With regard to D<sub>1</sub> receptors, it is also worth mentioning that activation of these receptors induces hyperthermia in mice (Zarrindast and Tabatabai, 1992). In contrast, inactivation of D<sub>4</sub> receptors was not able to counteract the neurotoxic effects induced by MDMA.

Earlier evidence has demonstrated that the inactivation of D<sub>2</sub> receptors decreases the DAT activity in the striatum (Dickinson et al., 1999), and that D<sub>2</sub> receptor knock-out mice are protected from MDMA-induced hyperthermia, striatal DA loss, and gliosis (Granado et al., 2011a). Antagonism or inactivation of D<sub>2</sub> receptors provides neuroprotection, possibly by either reducing the cytosolic DA content through DAT inhibition or attenuating hyperthermia, which is one of the mechanisms implicated in MDMA neurotoxicity (Green, 2005, Capela et al., 2009).

Further support for a possible involvement of DA in the neurotoxic effects of MDMA comes from pharmacological studies. In 1988, Stone and coworkers proposed that endogenous DA is important for the development of long-term MDMA-induced neurotoxicity in rats (Stone et al., 1988). In line with an important role of DA in MDMA neurotoxicity, bilateral lesions of the dopaminergic terminals with 6-hydroxydopamine (6-OHDA) were reported to block MDMA-induced serotonergic neurotoxicity in rats, while administration of the DA precursor 3,4-dihydroxy-l-phenylalanine (L-DOPA) exacerbated this effect of MDMA (Schmidt et al., 1990). Later, in 1995,

Yamamoto and colleagues demonstrated that MDMA indirectly elicits DA release by the activation of 5-HT<sub>2A</sub> receptors subsequent to the release of 5-HT in rats.

Furthermore, DAT inhibitors, such as GBR12909 and mazindol, which prevent the increase in DA levels and ultimately the generation of ROS, have been reported to attenuate serotonergic toxicity by MDMA in rat (Stone et al., 1988). In addition, Benamar and coworkers performed combined microdialysis and biotelemetry studies to determine the occurrence of parallel changes in the extracellular levels of DA and body temperature after the administration of MDMA (Benamar et al., 2008). They found that the hyperthermic response produced by MDMA is accompanied by an increase in the levels of DA in the preoptic anterior hypothalamus, which was attenuated by the D<sub>1</sub> antagonist SCH-23390 (Benamar et al., 2008). Finally, it is interesting to observe that previous studies indicate that, at low ambient temperature, MDMA induces hypothermia (Gordon et al., 1991; Dafters 1998), which is mediated by stimulation of the D<sub>2</sub> receptor (Faunt and Crocker, 1987; Mechan et al., 2002). Conversely, at high ambient temperature, MDMA induces hyperthermia, which stems from the stimulation of D<sub>1</sub> receptors (Mechan et al., 2002). Taken together, these data indicate that both D<sub>1</sub> and D<sub>2</sub> DA receptors could play an important role in MDMA-induced neurotoxicity.

## **Methamphetamine (METH)**

### **9. History of METH**

METH, a variant of amphetamine, was first synthesized by Nagayoshi Nagai in Japan in 1893 from the precursor chemical ephedrine (Nagai and Kamiyama, 1988; Meredith et al., 2005). METH use became prominent during World War II (1940s), when various governments began giving their military personnel the drug to ward off fatigue and enhance endurance and alertness (Meredith et al., 2005; McGuinness, 2006). Amphetamine itself was first synthesized by Lazar Edeleanu, a Romanian chemist at the University of Berlin in 1887. However, it was not used clinically until 1920s, when Gordon Alles re-synthesized it for use in asthma, hay fever and colds (Alles, 1993; Wolkoff, 1997; Sulzer et al., 2005). In 1932 an amphetamine-based inhaler (Benedrine) was marketed for the treatment of nasal congestion (McGuinness, 2006), and later amphetamines were promoted as treatments for various ailments such as rhinitis and asthma (Derlet and Heischouer, 1990; Alles, 1993; Wolkoff, 1997; Sulzer et al., 2005; McGuinness, 2006).

The first METH epidemic occurred in Japan where large quantities of over-the-counter METH pills

were available after World War II (Wolkoff, 1997). The onset of epidemic in the USA was slower due to prescription requirement. Nevertheless, the prevalence of amphetamine use in the 1950s was high among civilians including groups such as college students, truck drivers, athletes, housewives, and individuals performing monotonous jobs (Wolkoff, 1997; Donaldson and Goodchild, 2006). Although amphetamine-based inhalers were banned by the Food and Drug Administration (FDA) in late 1950s due to increases in their abuse (Roll et al., 2006), amphetamine itself and some of its derivatives were promoted as therapeutic agents for hyperactivity, obesity, narcolepsy, and depression. Abuse of METH gained popularity in the 1960s especially among individuals already using other illicit drugs (Wolkoff, 1997). In the 1970s, the Controlled Substance Act and classification of amphetamines as Schedule II drugs (i.e., drugs that have an accepted medical use but also have a high potential for abuse and are available only by prescription) reduced the diversion of pharmaceutical amphetamines in the USA (Wolkoff, 1997). However, despite these efforts, the use of METH increased in the 1980s, due in large part to production of the drug from the precursor chemical ephedrine in clandestine labs (Cunningham and Thielemeir, 1996). To counter this problem, the federal government regulated the sale of pseudoephedrine commonly used as cold medicine (Cunningham and Liu, 2003). Although this act temporarily reduced METH-related problems (Cunningham and Liu, 2003; Cunningham et al., 2008), the void in supplies was filled by foreign producers (Cunningham et al., 2008; Cunningham and Liu, 2008), where attempts to bring that production under control have been problematic (NDIC, 2007). Nowadays, METH abuse is a worldwide problem and concerted efforts by governments and enforcing agencies are conducted to combat this epidemic (UNODC, 2014). In the USA, street-names for METH include crystal, crank, ice, glass, go, meth, speed, and zoom.

## **10. General toxicity of METH**

At low therapeutic doses, METH can cause an elevated mood and increase alertness, concentration, and energy in fatigued individuals, while at higher doses it can induce psychosis, rhabdomyolysis (a condition in which damaged skeletal muscle tissue breaks down rapidly and may lead to kidney failure) and cerebral hemorrhage.

The side effects of METH can include anorexia, hyperactivity, dilated pupils, flushed skin, excessive sweating, headache, irregular heartbeat, rapid breathing, high or low blood pressure, high body temperature, diarrhea, constipation, blurred vision, dizziness, twitching, numbness, tremors, dry skin, acne, pallor, dry mouth and "meth mouth", a condition where the addicts may lose their teeth abnormally quickly (Winslow et al., 2007; National Geographic Channel, 2013; United

StatesFDA, 2014). It is also important to note that METH may cross the placental barrier or may appear in breast milk, as a consequence of which exposed infants can have a significantly smaller head circumference and birth weight, as well as manifest neonatal withdrawal symptoms such as agitation, vomiting and tachypnea (Winslow et al., 2007; Chomchai et al., 2010). METH may also lead to complex psychological effects including euphoria, dysphoria, changes in libido, insomnia or wakefulness, self-confidence, sociability, irritability, restlessness, grandiosity and repetitive and obsessive behaviors as well as anxiety, depression, psychosis, suicide, and violent behaviors (Westfall and Westfall, 2010; O'Connor, 2012; United StatesFDA, 2014). At extremely large overdose METH may produce symptoms such as adrenergic storm, cardiogenic shock, brain bleed, circulatory collapse, dangerously high body temperature, pulmonary hypertension and kidney failure (Schep et al., 2010; Westfall and Westfall, 2010; O'Connor, 2012; United StatesFDA, 2014). Death from METH poisoning is preceded by convulsions and coma (United StatesFDA, 2014). METH withdrawal is also associated with a variety of conditions including anxiety, drug craving, dysphoric mood, fatigue, increased appetite, increased or decreased movement, lack of motivation, sleeplessness or sleepiness, and vivid or lucid dreams that may persist for 3-4 weeks (Shoptaw et al., 2009). Curiously, the mental depression following METH withdrawal is more severe and lasts longer than that of cocaine (Winslow et al., 2007).

### **11. Neurotoxicity of METH: selectivity for the dopaminergic system**

METH is directly neurotoxic to dopaminergic neurons and hence its abuse may increase the risk of developing PD by 2 to 3 fold (Callaghan 2010, 2012; Curtin et al., 2015). METH can also result in neurotoxicity to other neurons including serotonergic (Krasnova and Cadet, 2009; Silva et al., 2014), glutamatergic (Simões et al., 2007; Miyazaki et al., 2013; Zhang et al., 2014), GABAergic (Zhang et al., 2006; Mizoguchi and Yamada, 2011; Shen et al., 2013), as well as cholinergic neurons (Lim et al., 2014). Serotonergic interactions of METH may be more related to its mood altering effects (Silva et al., 2014), glutamatergic interactions of METH may be most relevant to its addictive, memory impairments and psychotic-like effects related to glutamate receptors distribution (Simões et al., 2007; Grant et al., 2012; Miyazaki et al., 2013; Henley et al., 1989) whereas METH GABAergic interactions may be most relevant to its cognitive dysfunctions and anxiety-like symptoms observed during withdrawal (Mizoguchi and Yamada, 2011; Shen et al., 2013). Striatal cholinergic effects of METH are believed to play a role in the motor impairments induced by the drug (Lim et al., 2014). In addition, interactions of these neurotransmitter systems with dopaminergic systems can also influence the final behavioral and neuronal effects of METH (Miyazaki et al., 2013). However, in this review the primary focus will be on METH interaction

with the dopaminergic system and its particular implications for PD.

### **12. *In vitro* studies: cell cultures**

A number of studies have employed various dopaminergic cell lines including the neuroblastoma-derived SH-SY5Y cells and pheochromocytoma-derived PC12 cells to investigate the mechanism of METH-induced neurotoxicity and evaluate novel neuroprotectants. Thus, earlier experiments using PC12 cells showed that METH significantly increased formation of 3-nitrotyrosine (3-NT), a biomarker of peroxynitrite, a reactive nitrogen species (RNS), and cellular toxicity. In the same study, the authors reported that pretreatment with antioxidants such as selenium and melatonin could completely protect against the formation of 3-NT. Moreover, application of a selective neuronal nitric oxide synthase (nNOS) inhibitor, 7-nitroindazole (7-NI), significantly protected against the formation of 3-NT (Imam et al., 2001), also, in PD models (Solís et al., 2015). Parallel to these *in-vitro* studies, it was shown that METH caused striatal dopamine depletion and that same agents were able to restore the dopamine level back to normal, suggesting that peroxynitrite plays a major role in METH-induced dopaminergic neurotoxicity and that selective antioxidants and peroxynitrite decomposition catalysts can protect against METH-induced neurotoxicity (Imam et al., 2001). Later, using SH-SY5Y cells, it was shown that METH enhances oxidative stress and aberrant mitochondrial biogenesis in these cells and that vitamin E pretreatment can attenuate these effects (Wu et al., 2007). Moreover, using the same cell lines, it was observed that HIV protein, Tat, dramatically increases the toxicity of METH (Cai and Cadet, 2008). See table 2 for a summary of the protocols of the studies described above.

More recently, it has been reported that melatonin can attenuate METH-induced disturbances in mitochondrial dynamics and degeneration in neuroblastoma SH-SY5Y cells (Parameyong et al., 2013). Interestingly, caffeine inhibition of autophagy may increase METH-induced toxicity in these cell lines (Pitaksalee et al., 2015). Moreover, it was shown that acutely, METH may inhibit voltage-gated  $\text{Ca}^{2+}$  channels, but chronically upregulates L-type channels, thus  $\text{Ca}^{2+}$  channel blockers may be of therapeutic potential in METH-induced toxicity and dependence (Andres et al., 2015).

### **13. *In vivo* studies in experimental animals**

As mentioned above, METH is a neurotoxic drug that causes deficits and alterations in central dopaminergic pathways. Repeated administration of METH in rodents has been shown to cause neurodegeneration of dopaminergic axon terminals in the striatum, reducing TH and DAT,

accompanied by a reduction in TH activity as well as reductions in the levels of DA, DOPAC and HVA, and decreased levels of VMAT<sub>2</sub>. These effects occur primarily in the striatum, but are also seen in the cortex, thalamus, hypothalamus, and hippocampus (Ares-Santos et al., 2012; Granado et al., 2010, 2011a; Guilarte et al., 2003; Krasnova and Cadet, 2009; Ricaurte et al., 1980). METH induces neurotoxicity in a dose-dependent manner (Seiden and Sabol, 1996), as do other amphetamine-derivatives like MDMA (Granado et al., 2008a 2008b). The loss of axonal DA can be detected as early as 24 hours after exposure to a relatively high concentration of METH. Although some partial recovery of striatal TH and DAT immunoreactivity may occur few days post-treatment, the recovery is not complete and some of the losses persist for long periods (Granado et al., 2011a; 2011b; Ares-Santos et al., 2012; 2014). Neurotoxic effects persist for more than seven days and up to a month after METH exposure (Ares-Santos et al., 2012; O'Callaghan and Miller, 1994; Granado, et al., 2011b; Ares-Santos et al., 2014). In addition, there is partial recovery of dopamine levels in the striatum (Ares-Santos et al., 2012; Granado et al., 2008a; Granado, 2011a), strongly suggesting that the regrown terminals are functional. The mechanisms responsible for the partial recovery are not known, but might involve compensatory sprouting and branching as has been reported for regrowth following MPTP-induced damage (Bezard et al, 2006). DA terminal recovery has also been described in rhesus monkeys and velvet monkeys, although in these species, it appears to occur on a slower timescale than in mice. METH-induced dopaminergic damage persists for more than 12 weeks in velvet monkeys and more than 3 years in rhesus monkeys (Melega et al., 1997; Seiden and Sabol, 1996), demonstrating the persistence of METH-induced brain damage.

The persistent loss of DA axons in the striatum has been correlated with DA cell body loss in the SNc, demonstrated by rigorous stereological measurement of cell numbers with both TH and Nissl staining. METH administration produces DA cell body loss in the SNpc, as indicated by stereological counts in TH-stained SN sections from treated mice (Granado et al, 2011b, Ares-Santos et al, 2012). The observed pattern of TH-stained neuron loss is very similar to the pattern of Nissl-stained neuron loss, indicating that neuronal loss is specific to dopaminergic neurons. DA cell body loss was confirmed with Fluoro-Jade, a general marker of neuronal degeneration that fluoresces after administration of known dopaminergic toxins such as 6-OHDA and MPTP (Schmued and Bowyer, 1997). Dopaminergic cell loss is further supported by measurements of the markers of apoptosis and neuronal death (Sonsalla et al., 1996; Hirata and Cadet, 1997; Granado et al., 2011a; 2011b; Ares-Santos et al., 2012).

Interestingly, METH primarily affects the nigrostriatal pathway and spares the mesolimbic dopaminergic pathway as TH levels in the nucleus accumbens, the terminal field of VTA, are not affected by METH (Fig. 3) (Granado et al., 2010), paralleling the situation in PD (Hurtig et al., 2000). It may be possible as well, that the strong fiber loss produced at early times brought dendritic spine loss as seen in PD models (Suarez et al., 2014). Furthermore, within the striatum, it is the striosomes rather than the matrix that are more vulnerable to the damaging effects of METH (Fig. 4) (Granado et al., 2010). Curiously, this pattern of degeneration is also observed following administration of other neurotoxins such as MPTP, MDMA, quinolinic acid and other NMDA receptor agonists, as well as in other neurodegenerative diseases including early Huntington's disease (Hedreen and Folstein, 1995) and ischemia/reperfusion injury (Burke and Baimbridge, 1993). The differential vulnerability of the striosomes may at least be partially due to the low levels of superoxide dismutase(SOD), a known antioxidant enzyme, in this area compared to the matrix (Medina et al., 1996).

In addition, it is now well recognized that stress can alter the neurotoxic effects of various substances including those of METH. Surprisingly though, an original study by Miller and O'Callaghan reported that restraint stress actually protected against neurotoxic effects of d-amphetamines (d-AMP) (Miller and O'Callaghan, 1996). This effect of restraint stress, as suggested by the authors is likely due to hypothermic actions of such a stressor, as adrenalectomy also provided protection against d-AMP-induced striatal damage (Miller and O'Callaghan, 1996). In general, however, stress is likely to exacerbate the damaging effects of toxic substances via activation of the HPA axis (Jonhson et al., 2005). Indeed, recent studies have verified the stimulating effects of METH on the HPA axis and have provided evidence of neuronal c-Fos activation in specific brain regions by METH (Tomita et al., 2013). Interestingly, in the same study in mice it was demonstrated that HPA activation by METH is correlated with METH-induced hyperthermia (Tomita et al., 2013).

METH interaction with the HPA axis is likely to contribute to the psychological and psychiatric symptoms, particularly depression, that is observed in adolescent and adult METH abusers (King et al., 2010; Li et al., 2013). Thus, it is hypothesized that dysregulation of the HPA axis reflected specifically in cortisol inability to suppress ACTH secretion is a major contributor to METH-associated psychiatric symptoms (Li et al., 2013). It would be of significant interest to further investigate the relationship between motor and psychiatric effects of METH in both genders as

adolescent females appear to be more susceptible to the effects of METH than adolescent males (King et al., 2010).

#### 14. Studies in humans

METH has high popularity, attributed to its wide availability, relative low cost and long duration of psychoactive effects. The neurotoxic effects of METH in humans are similar to those observed in experimental animals. Neuroimaging (PET) studies in METH abusers have revealed reductions in striatal DAT levels that are associated with motor slowing and memory impairment, this is observed with brain PET studies in human abusers (Volkow et al., 2001a). Other reports of neurotoxic effects of METH in human abusers indicate poor motor performance associated with DAT loss in the caudate nucleus and putamen (Volkow et al., 2001a,b; McCann et al., 1998). Partial recovery of dopaminergic markers in the striatum has also been reported in human METH-abusers after periods of abstinence (Volkow et al., 2001a). Persistent DA terminal losses have been documented after 11 months (Volkow et al., 2001a) or 3 years of abstinence (McCann et al., 1998). Other studies with magnetic resonance imaging (MRI) demonstrated enlarged striatal volumes in METH abusers who recently abstained, while greater cumulative METH use or longer duration, was associated with smaller striatal structures (Chang et al 2007).

There are evidences of neurodegenerative changes in SN of METH abusers. For example, a specific decrease in pigmented neurons in SN of human METH abusers, similar to that seen in PD patients, has been reported (Büttner and Weis, 2006; Büttner, 2011). Moreover, the morphology of the SN (as measured by transcranial sonography) in individuals with a history of stimulant abuse, including METH, is abnormal, and is associated with reduced DA uptake in the striatum and increased risk for development of PD (Todd et al., 2013). It is therefore not unreasonable to expect that METH consumers may be more susceptible to neurodegenerative disorders such as PD (Granado et al., 2013).

Recent epidemiological studies provided evidence that the risk for developing PD is almost doubled in individuals with a history of METH use (Callaghan et al., 2010). These results have been reproduced using a larger and more age-diverse group of patients (Callaghan et al., 2012), and another recent study suggests the risk may be somewhat higher than previous estimates and indicates differences in risk estimates between women and men (Curtin et al., 2015). Use of METH has also been associated with impaired executive and psychomotor function, as well as learning and attention, underscored by alterations in basal ganglia and cortical circuits (Paulus et al., 2002;



Chang et al., 2007). Although motor deficits have been reported in chronic METH abusers, these deficits do not typically involve gross movements, but rather affect fine motor dexterity, e.g. placing pegs in a pegboard (Caligiuri and Buitenhuis, 2005; Rusyniak et al., 2011).

## 15. Mechanisms of METH neurotoxicity

### 15.1. Biochemical mechanisms: Oxidative stress and excitotoxicity

The mechanisms responsible for the damage following METH administration are complex and may involve various processes. In this regard, oxidative stress is believed to be a prominent factor as METH induces ROS such as hydroxyl radicals and superoxides that lead to cellular toxicity (Cubells et al., 1994; Toborek et al., 2013). METH, in addition to increasing DA release, impairs VMAT<sub>2</sub> function, hence increasing cytosolic DA oxidation (Hansen et al., 2002) and DA metabolism by MAO-B, producing DOPAC and hydrogen peroxide (Cadet and Brannock, 1998; Olanow and Tatton, 1999). Within the terminals, H<sub>2</sub>O<sub>2</sub> reacts with transition metal ions producing highly toxic hydroxyl radicals (OH<sup>•</sup>) through the Fenton reaction. Moreover, cytosolic DA can produce superoxide anion (O<sub>2</sub><sup>•-</sup>) that in turn can also generate hydroxyl radicals, leading to lipid peroxidation and activation of proteases that finally trigger the cell death cascade. Anion superoxide reacts with NO producing highly toxic peroxynitrites (ONOO<sup>-</sup>) causing cellular damage to proteins, nucleic acids and phospholipids by overwhelming the antioxidative enzymes (Cadet and Brannock, 1998). ROS and RNS can also damage DNA structures causing loss of genetic information leading to accelerated mitochondrial dysfunction by inhibition of complex II electron transport chain. This mitochondrial dysfunction, in particular, mediates the long-term deficits in markers of striatal dopaminergic terminals (Brown and Yamamoto, 2003; Brown et al., 2005). The DA-induced oxidative stress after METH is consistent with the selective degeneration of DA terminals. Thus, reducing cytosolic DA with alpha-methyl-para-tyrosine (α-MPT), that inhibits DA synthesis, one can significantly reduce METH-induced damage (Ares-Santos et al., 2012; De Vito and Wagner, 1989; Fumagalli et al., 1998; Granado et al., 2011; Wagner et al., 1986). Conversely, increasing cytosolic DA with L-DOPA, reserpine or in VMAT<sub>2</sub> heterozygous mice, one can increase METH-induced damage (Albers and Sonsalla, 1995; Ares-Santos et al., 2012; Fumagalli et al., 1999; Granado et al., 2011). Noteworthy, these oxidative stress mechanisms induced by METH are also shared by other amphetamine derivatives.

It is now well accepted that excess GLU, the most abundant excitatory neurotransmitter in the brain, is responsible for excitotoxic damage which is primarily mediated through increase in Ca<sup>2+</sup> influx.

METH has been shown to be a potent releaser of GLU, resulting in activation of GLU receptors and eventual neuronal damage (Yamamoto and Bankson, 2005). Indeed, blockade of NMDA receptors, the major mediators of GLU excitotoxicity, by selective antagonists (e.g. MK-801) can attenuate METH-induced neurotoxicity in cell cultures (e.g. in PC12 cells) (Uemura et al., 2003) as well as in various brain regions (Battaglia et al., 2002; Bowyer et al., 2001; Chipana et al., 2008; Farfel et al., 1992; Fuller et al., 1992; Golembiowska et al., 2003; Green et al., 1992; Ohmori et al., 1993; Sonsalla et al., 1989, 1991; Weihmuller et al., 1992).

Interestingly chronic stress, suspected of enhancing METH-induced toxicity is believed to be acting via further increase in GLU concentrations and hence exacerbating damage to striatal DA terminals (Quinton and Yamamoto, 2007; Tata and Yamamoto, 2008). This contention is supported by the findings that corticosterone synthesis inhibitor (e.g. metyrapone), can attenuate stress-induced enhanced GLU release and result in reduced METH neurotoxicity (Tata and Yamamoto, 2008).

METH interaction with astrocytes may also contribute to its excitotoxicity as astrocytes on their own exhibit a form of excitability through increase in  $Ca^{2+}$  influx. Thus, it is hypothesized that activation of GLU receptors, coupled to various intracellular signaling cascades, including activation of PKC, Akt, cAMP/PKA/cAMP responsive element binding protein (CREB), and mitogen-activated protein kinase (MAPK) and Janus kinase (JAK) pathways, can lead to eventual METH-induced toxicity (Narita et al, 2006, Cisneros and Ghorpade, 2012).

Over-expression of  $\alpha$ -synuclein may also play an important role in METH-induced death of dopaminergic neurons (Jiang et al., 2014; Wang and Witt, 2014). Interestingly, oxidative stress, a process implicated in neuronal toxicity may also result in generation of  $\alpha$ -synuclein oligomers (Norris et al., 2003).  $\alpha$ -Synuclein is a protein that is intimately involved in maintaining the supply of synaptic vesicles in presynaptic terminals and may also help regulate the release of DA (Ronzitti et al., 2014).  $\alpha$ -Synuclein is localized in the cytosol as well as in the inner membrane of neuronal mitochondria where it may exert an inhibitory effect on complex I activity of mitochondrial respiratory chain, predisposing some neurons to degeneration (Liu et al., 2009; Lam et al., 2011; Gaugler et al., 2012). Indeed,  $\alpha$ -synuclein knock-down in SH-SY5Y cells or in rats can protect against METH-induced toxicity (Chen et al., 2013; Tai et al., 2014). It is also of interest to note that S-nitrosylating protein disulphide isomerase may mediate METH-induced  $\alpha$ -synuclein aggregation and that inhibition of this enzyme may offer a therapeutic potential (Wu et al., 2014). Recently a role for insulin-like growth factor binding protein 5 in METH-induced apoptosis of dopaminergic neurons was proposed (Qiao et al., 2014). In addition, altered energy metabolism and

inflammatory processes (see below) have also been linked to METH-induced toxicity (Downey and Loftis, 2014).

### 15.2. Neuroinflammation

Microglial activation can occur rapidly after METH administration. Thus, METH can trigger the release of a number of pro-inflammatory cytokines that can lead to glial dysfunction as well as neuronal death. Indeed, it has been shown that inhibition of microglial activation by minocycline protects against the neurotoxic effects of amphetamine derivatives, including METH. METH increases reactive microglia in the striatum, hippocampus, cortex, and SN, peaking one day after administration. METH-induced microglial activation was also recently observed in *indusium griseum*, considered to be an extension of the hippocampus (Carmena et al. 2015). Interestingly, microglial activation occurs exclusively in DA-innervated areas (Ares-Santos et al, 2014).

METH also increases GFAP immunoreactivity in the striatum (Ares-Santos et al., 2013) and in *indusium griseum* (Carmena et al. 2015). Reactive gliosis is considered a universal reaction to injury in the CNS and is used as a sensitive marker of neuronal damage. Studies with other amphetamine-related drugs, such as MDMA, and the neurotoxin 6-OHDA have found a similar pattern of increase in GFAP (Ares-Santos et al., 2013). Of relevance is that astrocytes, in contrast to microglia, can play a protective role by increasing the levels of GSH, and facilitating sprouting. Thus, providing growth factors, guidance molecules and scaffolding for axonal regeneration (see Ares-Santos et al., 2013 for more detail).

Given that suppression of neuroinflammation mediated by microglia activation may help prevent, or reverse, neurodegenerative diseases, a number of drugs have been tested over the years that act on different parts of the inflammatory pathways. Some drugs including nicotine, resveratrol, curcumin, and ketamine as potential novel therapeutic agents in neurodegenerative diseases have been suggested (Hurley and Tizabi, 2013).

### 15.3. Role of DA receptors: $D_1$ receptors in METH-induced neurotoxicity

The first studies to implicate striatal  $D_1$ -like receptors in the neurotoxic effects of METH were pharmacological studies using SCH-23390. This compound, an antagonist of  $D_1$ -like receptors (Bourne, 2001), also has agonist effects at 5-HT<sub>1C</sub> and 5-HT<sub>2C</sub> receptors (Millan et al., 2001). When SCH-23390 was administered with METH, TH loss and METH-induced toxicity decreased (Bronstein and Hong, 1995). Sonsalla et al. first reported in 1986 that SCH-23390 effectively

inhibited METH-induced depression of striatal TH activity in a dose-dependent manner and also prevented the decrease in concentration of DA, DOPAC and HVA, in this area (Sonsalla et al., 1986). These findings were confirmed by others showing prevention of METH-induced DA depletion by SCH-23390 (O'Dell et al., 1993; Broening et al., 2005). Moreover, it was further demonstrated that this compound also attenuated TH and DAT depletion in the striatum (Metzger et al., 2000; Angulo et al., 2004; Xu et al., 2005) and inhibited the increases in GFAP, caspases 3 and 8, and mediators of the calcineurin/NFAT/FasL-dependent apoptotic cell death pathway (Jayanthi et al., 2005; Krasnova and Cadet, 2009). However all these protective effects could be due to the inespecific affects of SCH-23390 on 5-HT receptors as these receptors have been shown to protect against MPTP intoxication (Bezard et al, 1996). At the molecular level, D<sub>1</sub>-like receptors blockade decreases DA-induced oxidation and cytotoxicity mediated by the activation of extracellular-signal-regulated kinase (ERK) and c-Jun N-terminal kinases (JNK) (Chen et al., 2004). In addition, SCH-23390 completely blocked METH-induced expression of endoplasmic reticulum stress-related proteins (ATF3, ATF4, CHOP/Gadd153, HSPs and caspase 12), inhibited METH-induced increase in pro-survival genes in response to endoplasmic reticulum stress (including BIP/GRP-78 and P58IPK) and increased expression of the mitochondrial anti-apoptotic protein Bcl-2 in the striatum (Beauvais et al., 2011). However, as SCH-23390 does not distinguish between D<sub>1</sub> and D<sub>5</sub> receptors (Moratalla et al., 1996b) and, as mentioned above, interacts with 5-HT receptors (Kvernmo et al., 2006), it was not clear exactly which receptor subtypes were involved in METH-induced neurotoxicity. A study in D<sub>1</sub> receptor knockout mice specifically implicated the D<sub>1</sub> receptor in METH-induced toxicity (Ares-Santos et al., 2012). Thus, D<sub>1</sub> receptor knockout mice were protected against reductions in striatal TH and DAT expression (Fig. 5) and against loss of dopaminergic neurons in the SNc following METH administration. In addition, D<sub>1</sub> receptor knockout mice did not exhibit an increase in GFAP or in macrophage-1 antigen (Mac-1) expression in these areas. However, under these conditions, the D<sub>1</sub> receptor knockout mice treated with METH not only did not develop hyperthermia, but had a hypothermic response instead (Ares-Santos et al., 2012). Other studies with intrastriatal SCH-23390 injections supported the results obtained with the D<sub>1</sub> receptor knockout mice, implicating hypothermia as an important mechanism in prevention of METH toxicity in such cases (Friend and Keefe, 2013).

In summary, D<sub>1</sub>-like receptors involvement in METH-induced toxicity is supported by the findings that inactivation of these receptors provides neuroprotection against METH-induced toxicity. Several mechanisms are postulated for this protection, including blockade of the hyperthermic response, decreased DA content and turnover and redistribution of DA inside the terminal.

Moreover, animals lacking D<sub>1</sub> receptors can store more DA in vesicles (Fig. 6). Because cytosolic DA levels determine the severity of METH-induced toxicity (Guillot et al., 2008; Thomas et al., 2008), the reduction in cytosolic DA in mice lacking D<sub>1</sub> receptor is a plausible mechanism for the decrease in METH-induced dopaminergic damage (Fig. 6) (Ares-Santos et al., 2012).

#### *15.4. Role of DA receptors: D<sub>2</sub> receptors in METH-induced neurotoxicity*

The ability of D<sub>2</sub>-like receptors antagonists to prevent the long-term effects of METH has been evaluated by several groups. Pharmacological agents that block D<sub>2</sub>-like receptors, can partially or completely protect against METH-induced toxicity and can also prevent the hyperthermic response (Albers and Sonsalla, 1995; Eisch and Marshall, 1998; Metzger et al., 2000). Thus, sulpiride, a D<sub>2</sub>-like receptor antagonist, dose-dependently blocked METH-induced toxicity in mice, and eticlopride and raclopride (other D<sub>2</sub>-like receptor antagonists) prevented METH-induced depletion of the striatal DA content (O'Dell et al., 1993; Metzger et al., 2000). These drugs also prevented reductions in DAT immunoreactivity (Albers and Sonsalla, 1995; Xu et al., 2005) and DAT activity following METH administration (Metzger et al., 2000). In addition, eticlopride attenuated the decrease in VMAT<sub>2</sub> caused by METH treatment (Chu et al., 2010), while raclopride attenuated METH-induced increases in GFAP in the striatum (Xu et al., 2005). Hence, pharmacological studies using D<sub>2</sub>-like receptor antagonists indicate a role for D<sub>2</sub>-like receptors in METH-induced apoptosis of striatal neurons (Xu et al., 2005) and other METH-induced toxicities. Moreover, unilateral striatal infusion of sulpiride protects against DAT loss, implicating striatal D<sub>2</sub>-like receptors in METH-induced neurotoxicity (Gross et al., 2011). However, D<sub>2</sub>-like receptors antagonists are not specific for the D<sub>2</sub> receptor subtype as they may also block other D<sub>2</sub>-like receptors (e.g. D<sub>3</sub> and D<sub>4</sub> receptors). Nevertheless, due to its presynaptic location and its role in regulating DA release, the D<sub>2</sub> receptor subtype appears to be the most likely mediator of the neuroprotective effects of these antagonists. Confirmation of this contention was provided with D<sub>2</sub> receptor knock-out animals. It was demonstrated that genetic inactivation of D<sub>2</sub> receptors prevents: METH-induced reductions in striatal DA levels, fiber loss as seen with TH and DAT immunoreactivity as well as micro- and astrogliosis (Fig. 5). Moreover, inactivation of D<sub>2</sub> receptor prevented dopaminergic neuronal loss in the SNc (Granado et al., 2011a). However, the absence of the D<sub>2</sub> receptor also blocked the hyperthermic response to METH. Indeed, D<sub>2</sub> receptor knock-out mice exhibited a hypothermic response, even at higher ambient temperature (29–30 °C) that is known to potentiate METH-induced hyperthermic response (Granado et al., 2011a). The inability to separate neuroprotective effects from attenuation of hyperthermia after inactivation of D<sub>2</sub> receptor does not completely rule out a specific role for D<sub>2</sub> receptor in METH-induced neurotoxicity. Recent findings suggest that D<sub>2</sub>

receptor inactivation does lead to neuroprotection that depends on factors other than an effect on body temperature. For example, reserpine, which blocks hyperthermia, strongly potentiated METH-induced neurotoxicity in wild-type mice indicating that hyperthermia is not essential for neurotoxicity (Granado et al., 2011a). However, in D<sub>2</sub> receptor knock-out mice, not even pretreatment with reserpine was able to abolish the neuroprotective effect of D<sub>2</sub> receptor inactivation against the toxicity caused by METH, strongly suggesting that neuroprotection conferred by D<sub>2</sub> receptor inactivation is not completely dependent on its effect on body temperature (Granado et al., 2011a). This contention is further supported by the finding that the rectal temperatures were very similar in reserpine-treated wild-type mice and in D<sub>2</sub> receptor knockout mice after METH injection, whereas the neurotoxic effects clearly differed between these two experimental groups (Granado et al., 2011a). Other plausible hyperthermia-independent mechanisms underlying the neuroprotection observed after D<sub>2</sub> receptor inactivation are provided by the evidence that the activity of striatal DAT, which is crucial for METH-induced neurotoxicity (Fumagalli et al., 1998; Manning-Bog et al., 2007; Afonso-Oramas et al., 2009), is reduced in the absence of D<sub>2</sub> receptor (Dickinson et al. 1999). Thus, it is proposed that the decrease in DAT activity produced by inactivation of the D<sub>2</sub> receptor blocks DA reuptake, resulting in lower intracytosolic DA levels and hence, neuroprotection (Fig. 6) (Ares-Santos et al., 2013).

The above suggests that DA release and activation of DA D<sub>2</sub> receptor are critical for METH-induced neurotoxicity. Furthermore, the neuroprotection against METH observed in the absence of the D<sub>2</sub> receptor might depend, at least in part, on inhibition of the hyperthermic response produced by METH as well as a decrease in vesicular DA levels.

Thus, inactivation of either the D<sub>1</sub> or D<sub>2</sub> receptor subtype may provide protection against the neurotoxicity induced by METH, albeit by slightly different mechanisms. Although the protection provided by D<sub>1</sub> or D<sub>2</sub> receptor inactivation is due primarily to decrease in cytosolic DA levels, the mechanisms by which cytosolic DA are lowered in D<sub>1</sub> and D<sub>2</sub> receptor knockout mice may differ (Fig. 6). In D<sub>1</sub> knockout mice, the decrease in cytosolic DA can be mostly attributed to a high proportion of vesicular DA (Fig. 6) (Ares-Santos et al. 2012). In contrast, in D<sub>2</sub> receptor knockout mice, cytosolic DA is decreased by blocking DA reuptake and decreasing DAT activity (Fig. 6) (Granado et al., 2011a). In addition, blockade of hyperthermia is also likely to play a role in the neuroprotection observed in both genotypes.

## 16. Conclusions and future directions

In summary, MDMA and METH are synthetic drugs whose consumption started at the beginning of the twentieth century with therapeutic purposes for their sympathomimetic properties, but are now popular illegal recreational drugs in many countries due to their psychostimulant effects. Their augmented popularity is a cause of concern for health professionals and policy makers, as MDMA and METH users can become highly addicted to these drugs and suffer undesirable consequences including increased risk of developing PD. MDMA and METH effects are related to their structural analogy to DA, and to their ability to release this neurotransmitter. The mechanisms responsible for their neurotoxic actions are complex and may involve inflammation, oxidative stress, GLU, NO, hyperthermia, mitochondrial dysfunction, gliosis, etc . The fact that DA receptors, in particular, D<sub>1</sub> and D<sub>2</sub> subtypes, play an important role in initiation of the toxic effects of these drugs, offers a window for therapeutic developments. Moreover, because of observations that the neurotoxicity produced by MDMA and METH is similar in humans and rodents, animal models and in particular knockout mice represent extremely useful tools for further elucidation of the molecular mechanisms of these toxicities. New genetic technologies make it possible to identify new targets that may lead to novel therapeutic strategies in prevention of the devastating neurotoxic effects of MDMA and METH.

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## Figure Legends

**Figure 1. MDMA affects the nigrostriatal pathway but has no effect in the mesolimbic pathway.** Photomicrographs of TH immunoreactivity in the caudate putamen (CPu) and nucleus accumbens shell and core (AcbSh, AcbC) respectively of mice treated with saline (Sal) or MDMA. Bar indicates: 500  $\mu\text{m}$ .

**Figure 2. Proposed mechanisms underlying MDMA- and METH-induced neurotoxicity.** The figure shows the potential interactions among the several factors, such as hyperthermia, ROS and RNS, toxic metabolites, excitotoxicity, neuroinflammation, and high cortisol levels that may contribute to MDMA- and METH- induced neurotoxicity. Reports in experimental animals indicate that high ambient temperatures can influence the neurotoxic effect of MDMA and METH. Moreover, MDMA and METH stimulate the release of DA and NA, while MDMA may also promote the release of 5-HT. Catabolism of DA and 5-HT mediated by MAO-B enzymes lead to the generation of reactive aldehyde species, and  $\text{H}_2\text{O}_2$ . This latter species promotes formation of ROS, which contribute to MDMA and METH neurotoxic effects. Both MDMA and METH may stimulate the release of GLU, either directly or indirectly via activation of serotonergic 5-HT<sub>2A</sub> receptors. By acting on NMDA receptors, GLU activates NOS, leading to the overproduction of NO and further stimulating the formation of damaging ROS. Neuroinflammation appears also to play an important role in neurotoxicity by MDMA and METH, since both these substances induce microglial activation that can lead to the production of many reactive species (e.g., NO,  $\text{O}_2^-$ , cytokines) resulting in neurodegeneration. Finally, both, MDMA and METH affect neuroendocrine functioning by stimulating the activity of the HPA axis, and increasing the levels of cortisol that can also participate in the neurotoxic effects of these drugs.  $\uparrow$  = elevation; + = potentiation of effect, as observed by preclinical studies in experimental animals. \* = the importance of this mechanism has been clearly described for METH-mediated neurotoxicity only. See the text for further details on each mechanism and its relevance.

**Figure 3. The nigrostriatal pathway is more vulnerable than the mesolimbic pathway to METH-induced neurotoxicity.** Photomicrographs of TH immunoreactivity in the caudate putamen (CPu) and nucleus accumbens shell and core (AcbSh, AcbC) respectively of mice treated with saline (Sal) or METH. Bar indicates: 500  $\mu\text{m}$ .

**Figure 4. Striosomes are more vulnerable than the matrix to neurotoxicity induced by METH.** TH-ir loss occurs predominantly in striosomes after METH treatment. Striatal adjacent sections from a mouse treated with METH were stained for TH or  $\mu$  opioid receptor (MOR). Striatal weak

TH patches corresponded with striosomes as demonstrated by MOR-1 immunostaining. Bar indicates 500 $\mu$ m.

**Figure 5. Inactivation of D<sub>1</sub> or D<sub>2</sub> receptors protects against METH-induced decreases in TH expression in the striatum.** Photomicrographs of striatal sections of mice 1 day after treatment with saline or METH, stained for TH. D<sub>1</sub> and D<sub>2</sub> receptors knockout mice were protected against TH loss induced by METH. Bar indicates 500 $\mu$ m.

**Figure 6. Diagram of the different intracellular DA distribution in striatal dopaminergic terminals in WT, D<sub>1</sub> and D<sub>2</sub> receptor knockout mice.** D<sub>1</sub> receptor knockout mice (D<sub>1</sub>R<sup>-/-</sup>) have lower DA levels than WT animals. D<sub>1</sub>R<sup>-/-</sup> mice have higher vesicular DA and lower cytosolic DA than WT mice, as demonstrated by fast scan cyclic voltammetry and by HPLC (Ares-Santos et al, 2012). D<sub>2</sub> receptor knockout mice (D<sub>2</sub>R<sup>-/-</sup>) have lower vesicular and cytosolic DA and higher extracellular DA levels than WT mice, as demonstrated by fast scan cyclic voltammetry and by HPLC (Granado et al, 2011). The reduced intracellular DA content in D<sub>2</sub>R<sup>-/-</sup> mice may be due to a lower DA re-uptake activity as a consequence of decreased DAT function. Modified from Ares-Santos et al, 2014.



Figure 1  
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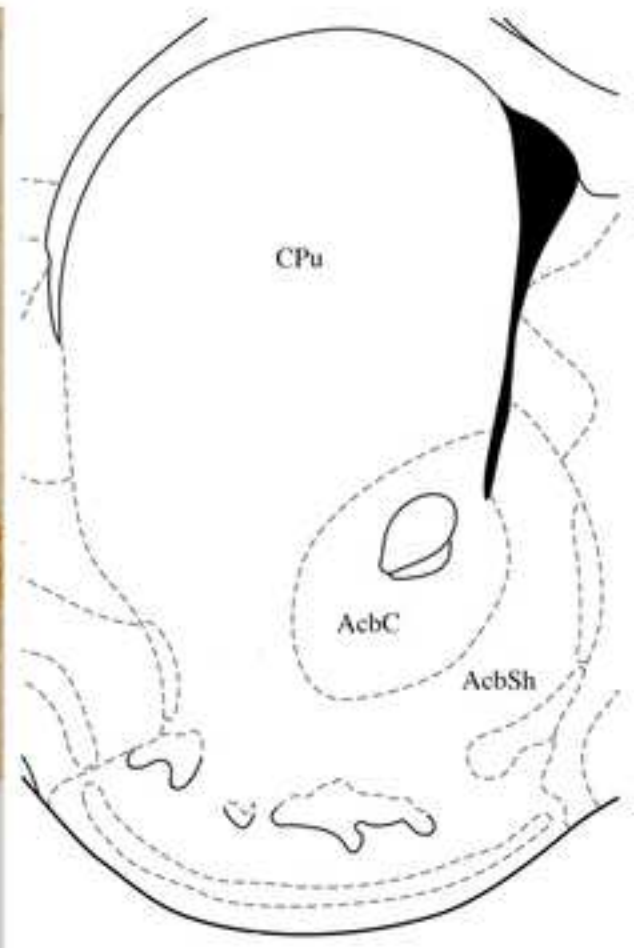
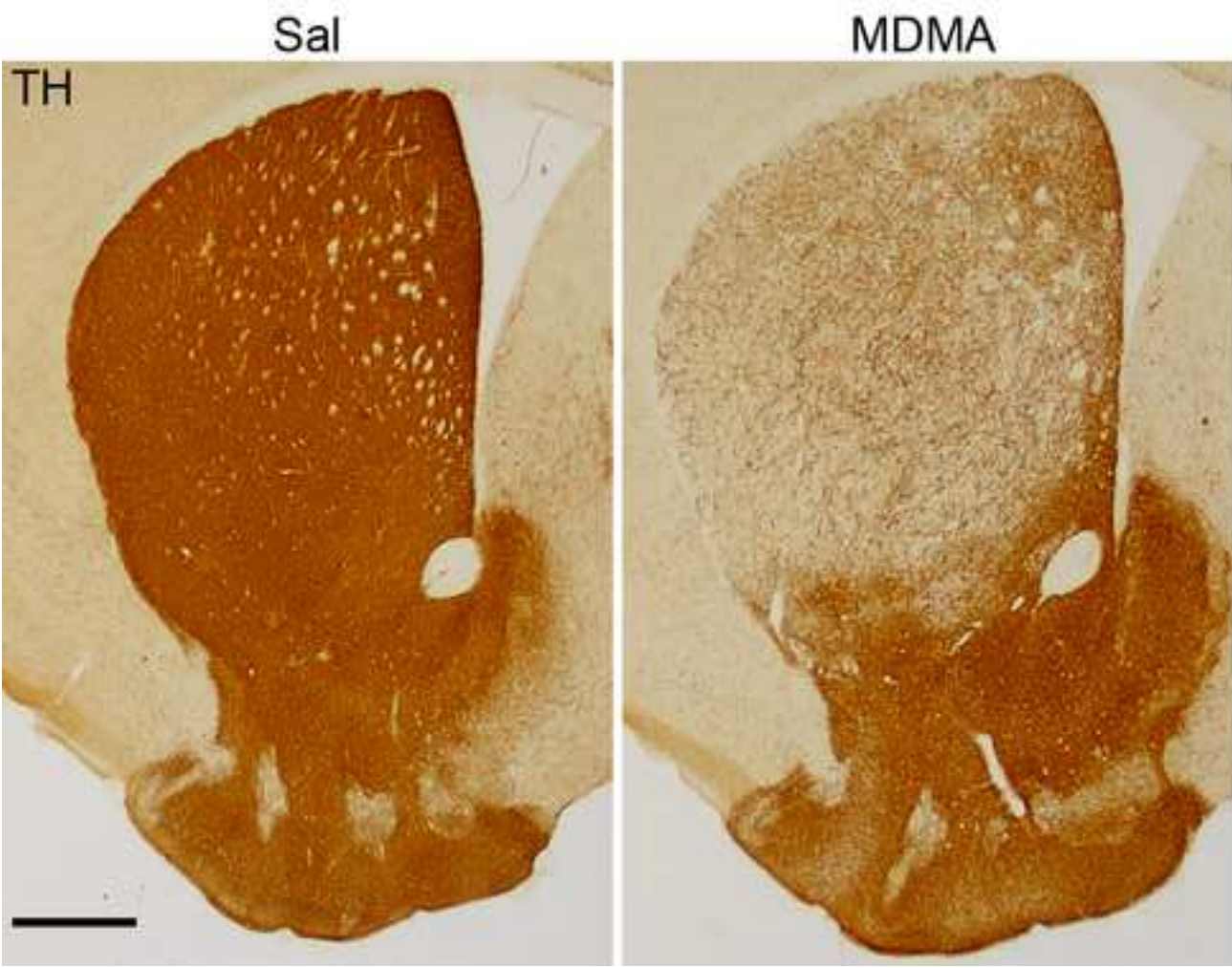


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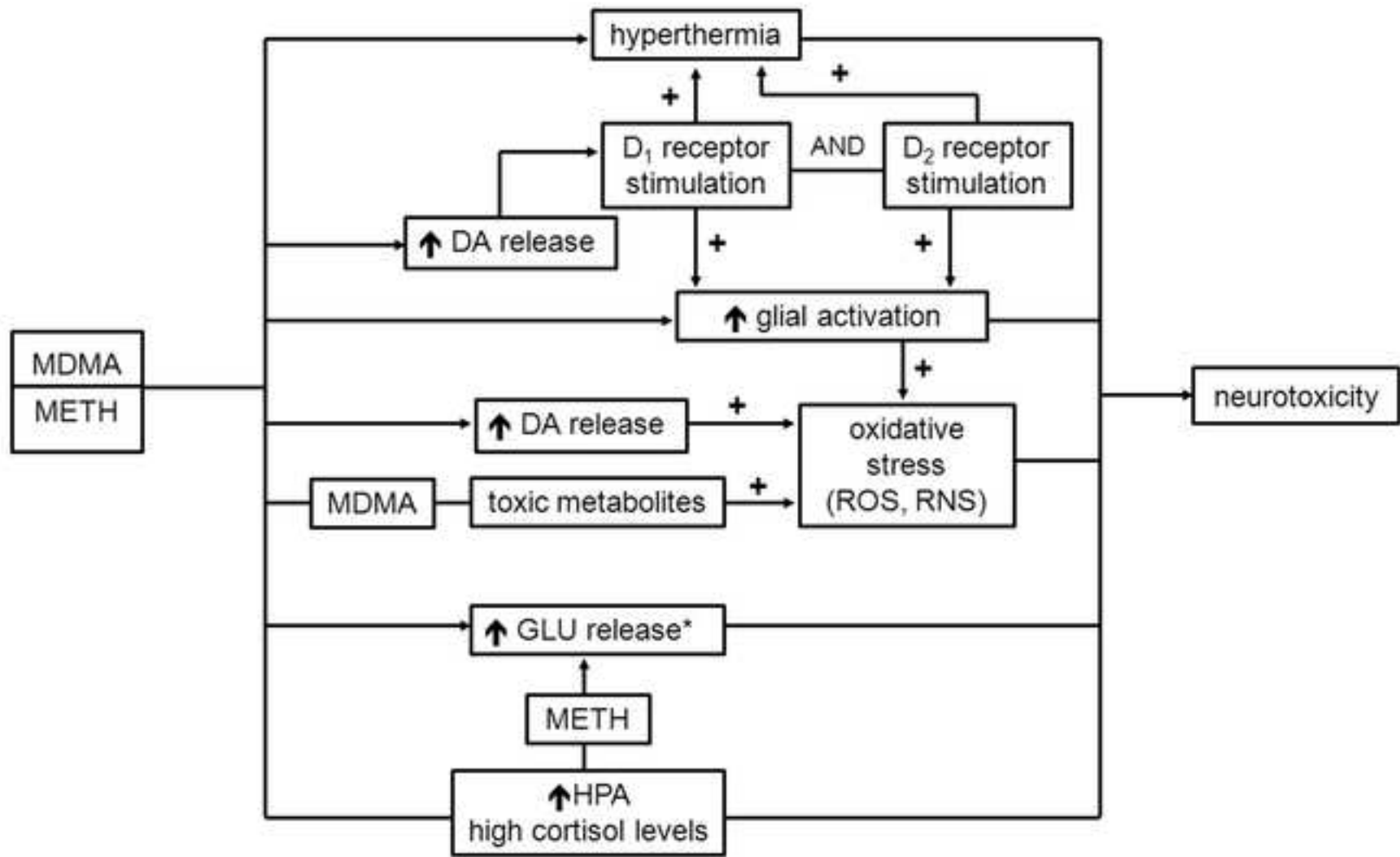


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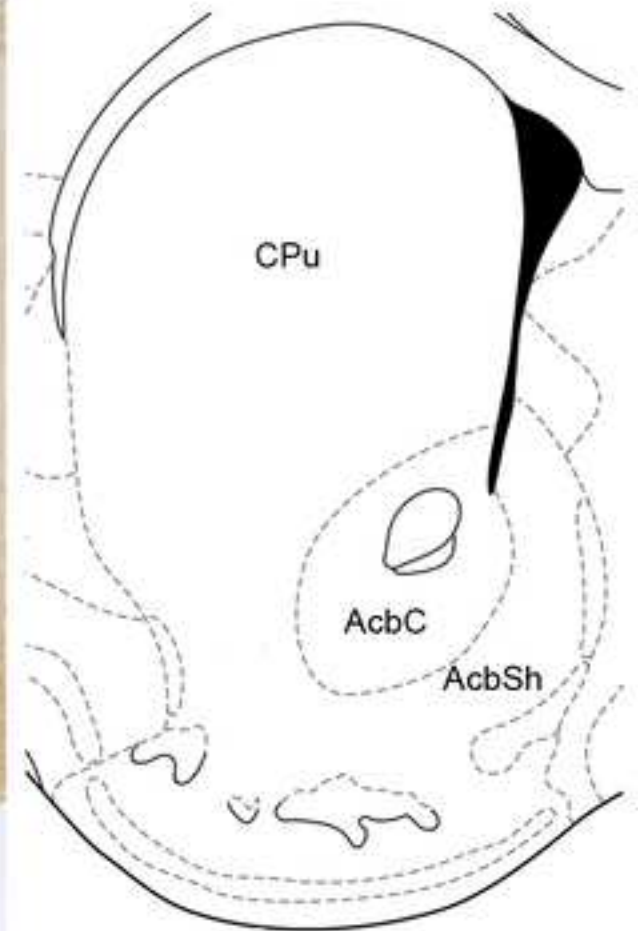
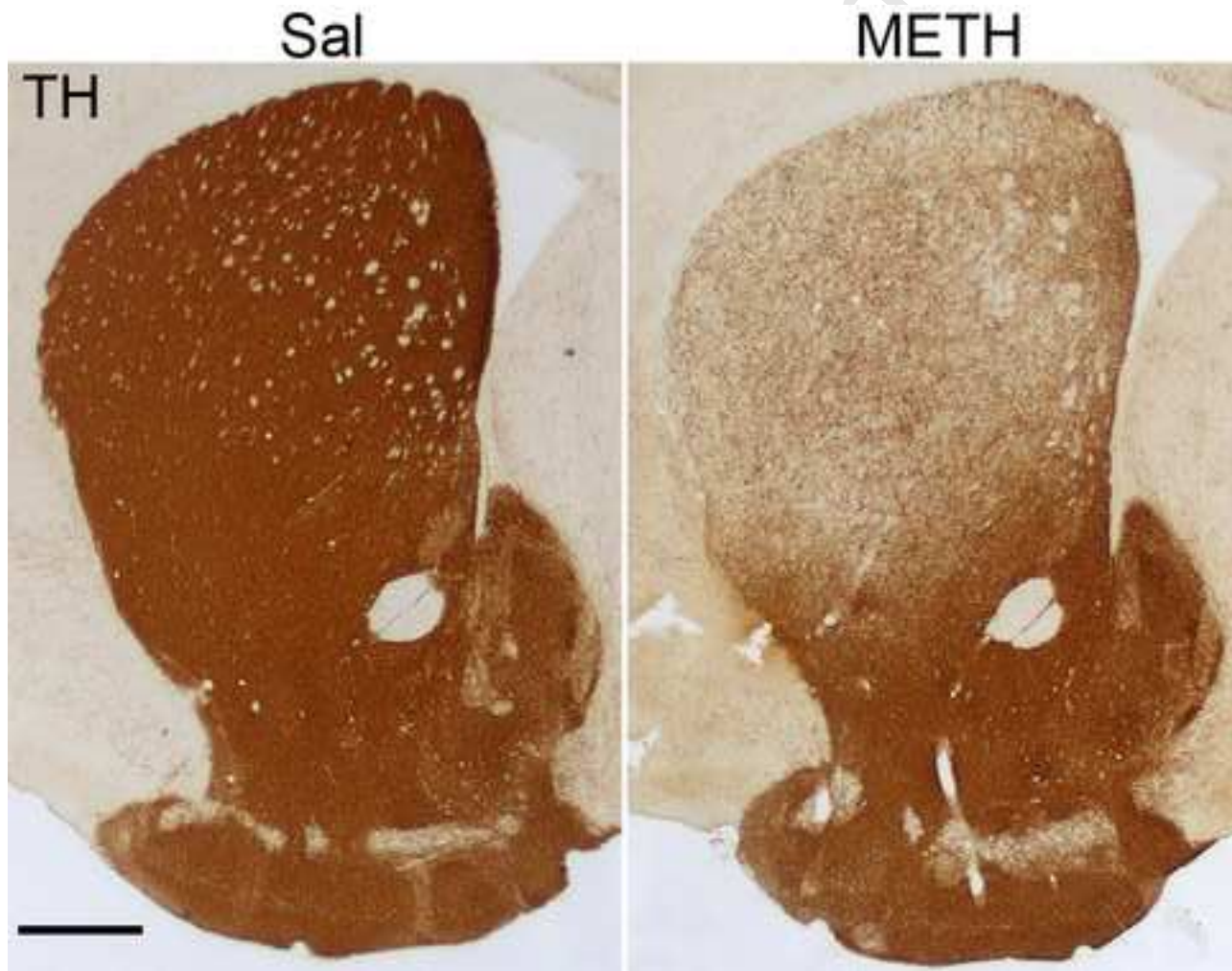


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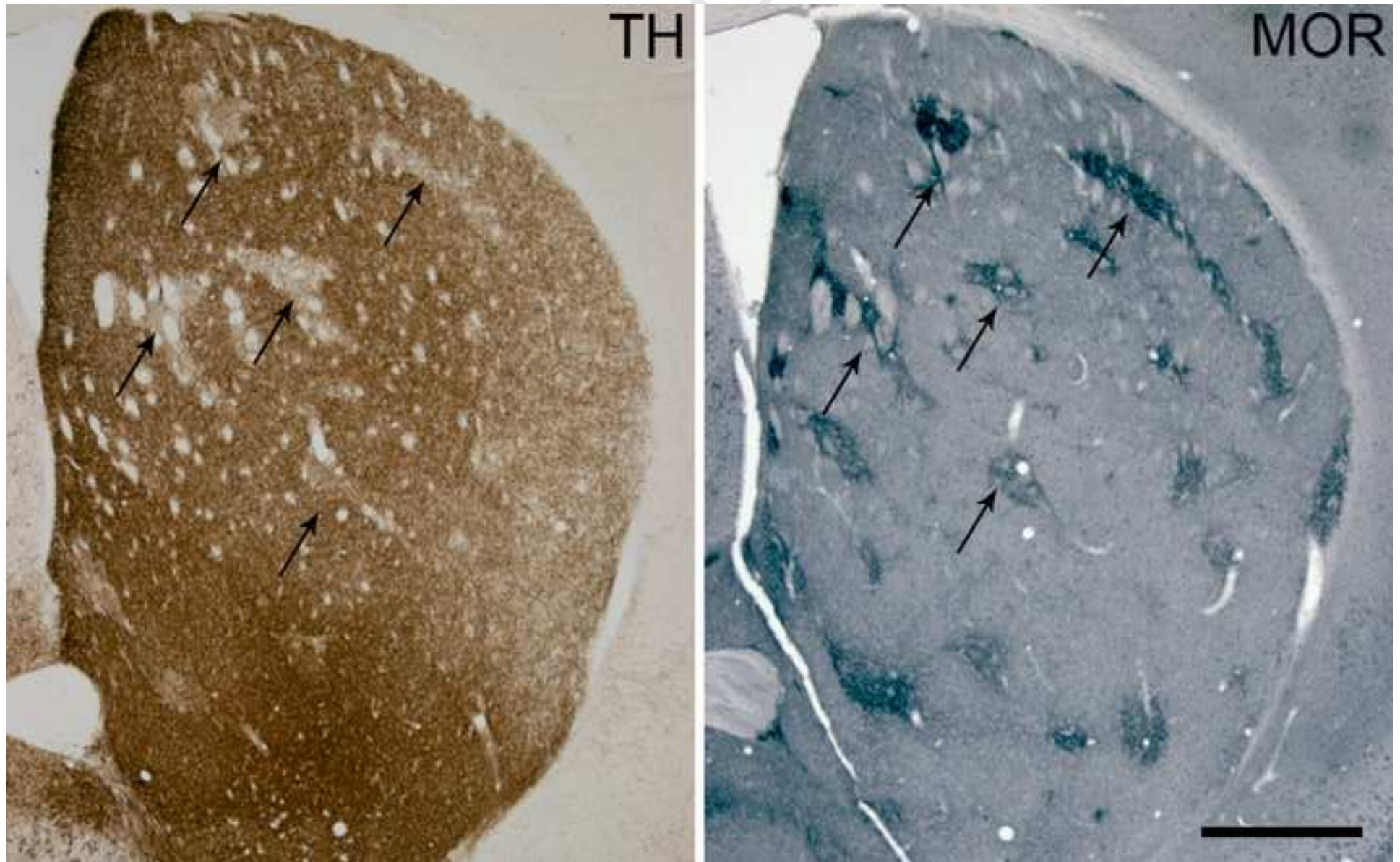


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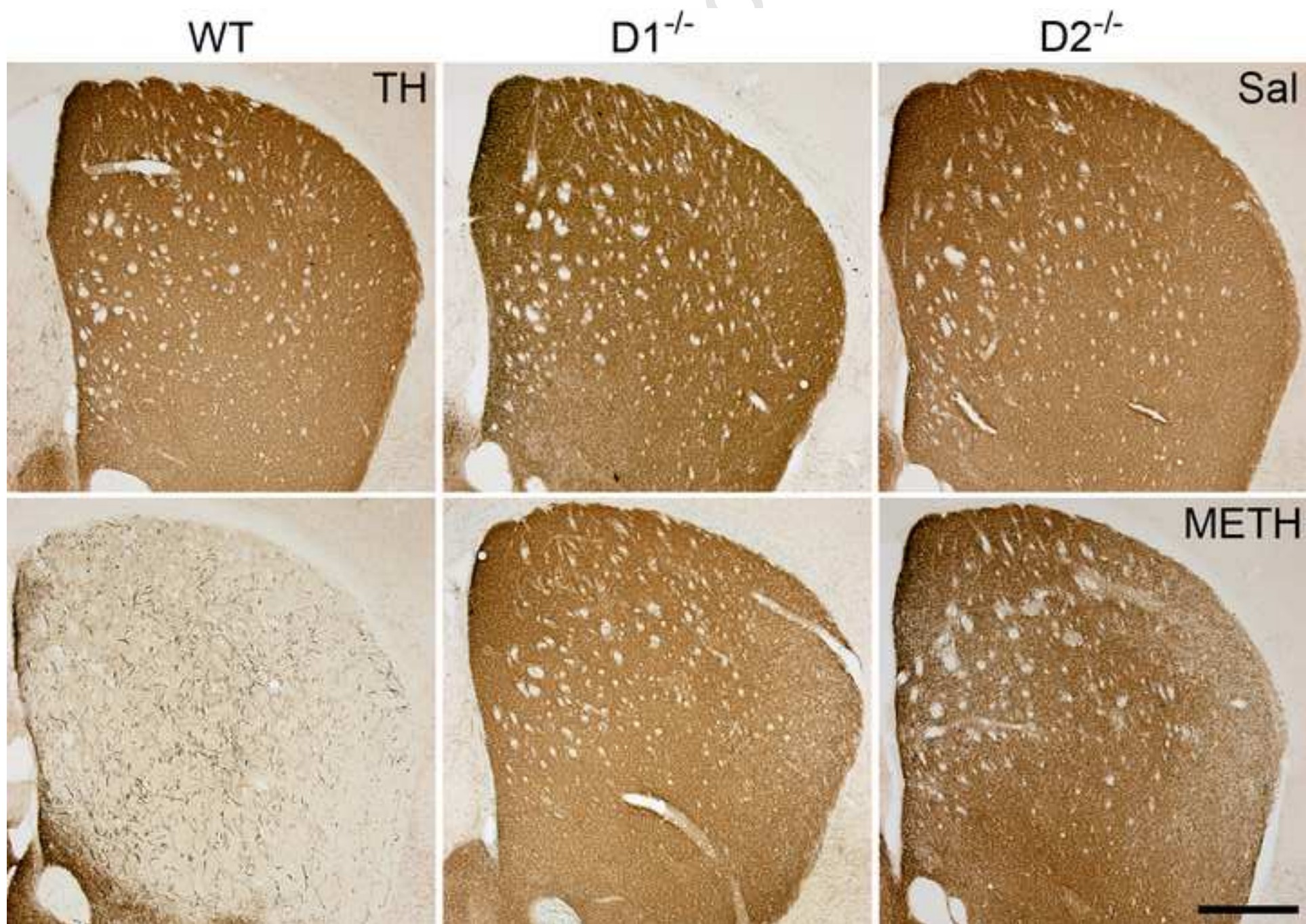


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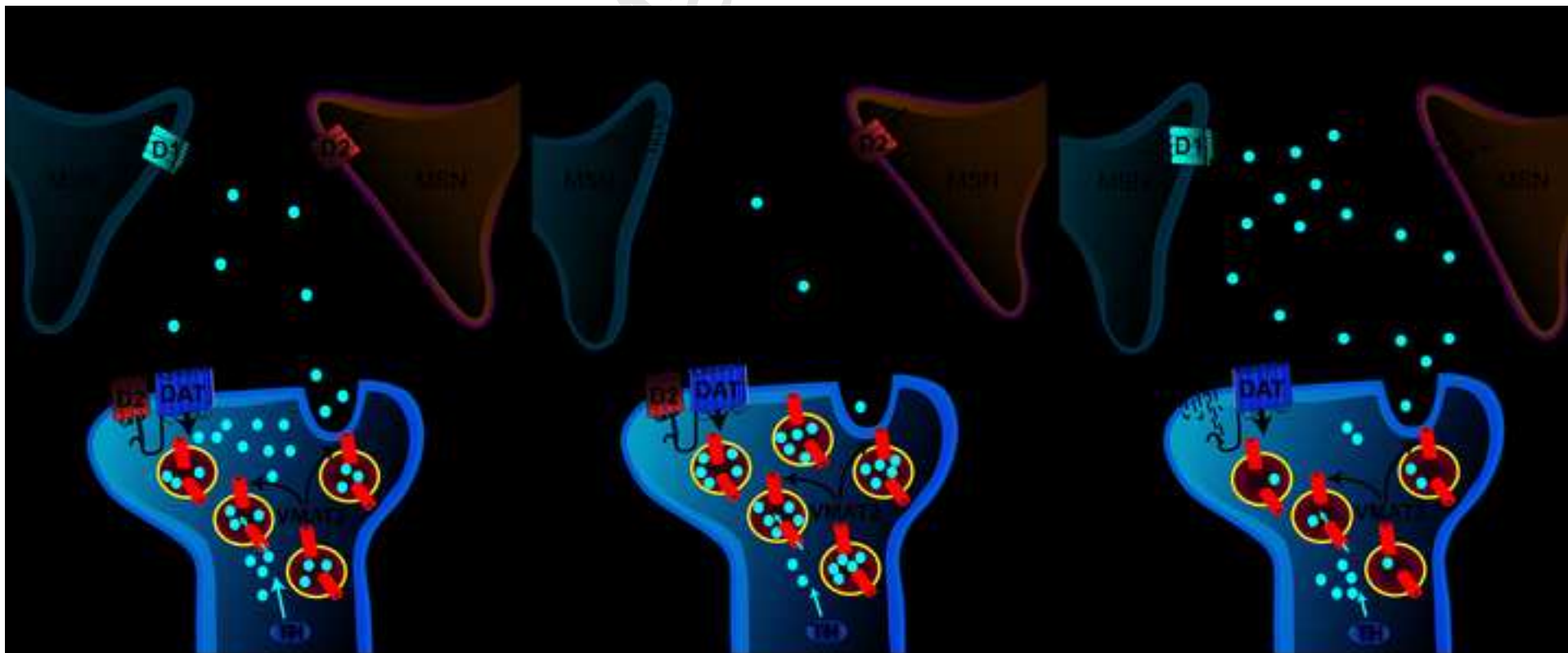


Table 1

## Experimental studies on neurotoxicity of MDMA

Species	Protocol of MDMA administration	Reference
Primary neuronal cultures of cerebral cortex	concentration range 100–800 $\mu$ M; single application without feeding for the following 24 or 48 h	Capela et al., 2006a, b
Human SH-SY5Y neuroblastoma cells	concentration range: 100, 200, 400 and 800 $\mu$ M, for 24 or 48 h	Ferreira et al., 2013
Mice	20 or 30 mg/kg i.p. x3, at 3 h intervals	Granado et al., 2008a, b
Mice	5 mg/kg i.p. x4, at 2 h intervals	Fornai et al., 2004
Mice	10 mg/kg i.p. x2, at 4-6 h intervals, twice weekly on the 2nd and 5th days of the week, according to a 9-week administration schedule	Costa et al., 2013, 2014
Mice	20 mg/kg i.p x4, at 2 h intervals	Frau et al., 2013
Rats	20 mg/kg s.c. x2, at 8 h intervals, post-natal day (PND) 11–20	Crawford et al., 2006
Rats	10 mg/kg i.p. X2, at 4 h intervals, PND 35–60	Shen et al., 2011
Rats	15 mg/kg o.s. x3, at 1.5 h intervals	Xie et al., 2006
Rats	10 mg/kg i.p. x2, at 4 h intervals every 5 days, PND 35-60	Piper and Meyer, 2004 Meyer et al., 2008
Rats	5 mg/kg i.p. every 5 days, PND 35-60	Piper et al., 2004 and 2005
Non-human primates	2.50 mg/kg , 3.75 mg/kg , or 5.00 mg/kg s.c. X2, at 9 h intervals, for 4 days	Ricaurte et al., 1988a
Non-human primates	2.5 or 10 mg/kg i.m. x2, for 4 days	Insel et al., 1989
Non-human primates	5 mg/kg s.c. x2, for 4 days	Hatzidimitriou et al. 1999
Non-human primates	10 mg/kg i.m. x2, for 4 days	Frederick et al., 1998
Non-human primates	2.5 mg/kg i.m. x2, at 12 h intervals, for 4 days. Eighteen days after , a higher dose of MDMA (5 mg/kg i.m.) was administered x2, at 12 h intervals, for 4 days	Winsauer et al., 2002

Table 1

Table 2

## Experimental studies on neurotoxicity of METH

Species	Protocol of METH administration	Reference
Primary microglial cell cultures	100 $\mu$ M for 24 h	Wang et al, 2014
PC12 cells and SH-SY5Y cells	2.0 -3.0mM for 24h	Huang et al, 2015
Mice	20 mg/kg, s.c	Sriram et al, 2006, Kelly et al, 2012
Mice	3x4mg/kg, i.p., at 2 h intervals	Thomas et al, 2010, Angoa et al, 2013
Mice	3x5mg/kg or 3x10mg/kg, i.p, at 2-3 h intervals	Granado et al 2010, 2011a, 2011b, Ares-Santos et al, 2012, 2014, Urrutia et al, 2014, Carmena et al, 2014
Mice	30 or 40mg/kg, single injection, i.p.	Deng et al, 2007, Bowyer et al 2008, Fantegrossi et al, 2008, Ares-Santos et al, 2014
Rats	Self-administer for 9 h per day for 14 days	Krasnova et al, 2014
	Self-administer for 3 h per day for 14 days	Mata et al, 2015
Rats	4x10mg/kg, i.p, at 2 h intervals	Beauvais et al, 2011
Rats	40mg/kg, single injection, s.c. or i.p.	Cappon et al, 2000, Jayanthi et al, 2005
Monkeys	Scaling-dose treatment for 4 weeks	Jiang and Capitanio, 2014
Monkeys	Scaling-dose treatment for 3 weeks	Melega et al, 2008

**Table 2**



**List of abbreviations:**

AC, adenylyl cyclase  
AcbSh, nucleus accumbens shell  
AcbC, nucleus accumbens core  
cAMP, cyclic adenosine monophosphate  
CNS, central nervous system  
CPu, caudate putamen  
CREB, cAMP responsive element binding protein  
CSF, cerebrospinal fluid  
CYP, cytochrome P450 enzymes  
DA, dopamine  
DAT, DA transporter  
5,7-DHT, 5,7-dihydroxytryptamine  
L-DOPA, 3,4-dihydroxy-l-phenylalanine  
DOPAC, 3,4-dihydroxyphenylacetic acid  
DPCPX, dipropylcyclopentylxanthine, adenosine A<sub>1</sub> receptor antagonist  
ERK, extracellular-signal-regulated kinase  
FDA, Food and Drug Administration  
fMRI, functional magnetic resonance imaging  
GBR12909, vanoxerine, antagonist of DAT  
GFAP, glial fibrillary acidic protein  
GLU, glutamate  
GSH, glutathione  
HHMA, 3,4-dihydroxymethamphetamine  
5-HIAA, 5-hydroxyindoleacetic acid  
HPA, hypothalamus–pituitary–adrenal axis  
5-HT, serotonin  
HVA, homovanillic acid  
HPLC, high-performance liquid chromatography  
JAK, Janus kinase  
JNK, c-Jun N-terminal kinases  
ICV, intracerebroventricular  
IL, interleukin  
Mac-1, Macrophage-1 antigen

MAO-B, monoamine oxidase type B  
MAPK, mitogen-activated protein kinase  
 $\alpha$ -MeDA,  $\alpha$ -methyldopamine  
MDA, 3,4-methylenedioxyamphetamine  
MDMA, 3,4-methylenedioxymethamphetamine  
METH, methamphetamine  
MK-801, dizocilpine, NMDA receptor antagonist  
MOR-1,  $\mu$  opioid receptor  
 $\alpha$ -MPT, alpha-methyl-para-tyrosine  
MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine  
MRI, magnetic resonance imaging  
NA, noradrenaline  
NAC, N-acetylcysteine  
NET, NA transporter  
7-NI, 7-nitroindazole  
NMDA, N-methyl-D-aspartate  
NO, nitricoxide  
3-NT, 3-nitrotyrosine  
nNOS, neuronal NO synthase  
6-OHDA, 6-hydroxydopamine  
PD, Parkinson's disease  
PET, positron emission tomography  
PKC, protein kinase C  
ROS, reactive oxygen species  
RNS, reactive nitrogen species  
SCH23390, dopamine D1 receptor antagonist  
SERT, 5-HT transporter  
SN, substantia nigra  
SNc, SN pars compacta  
SOD, superoxide dismutase  
SPECT, single photon emission computed tomography  
TH, tyrosine hydroxylase  
TNF, tumor necrosis factor  
VMAT<sub>2</sub>, vesicular monoamine transporter

VTA, ventral tegmental area

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