Neuropharmacology and toxicology of novel amphetamine-type stimulants

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Academic Dissertation

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"…I feel absolutely clean inside, and there is nothing but pure euphoria. I have never felt so great, or believed this to be possible. The cleanliness, clarity, and marvelous feeling of solid inner strength continued throughout the rest of the day, and evening, and through the next day. I am overcome by the profundity of the experience…"

- Alexander Shulgin, commenting on the subjective effects of 120 mg MDMA

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Original Publications

This thesis is based on the following publications:

I Klomp A, den Hollander B, de Bruin K, Booij J, Reneman L (2012). The effects of ecstasy (MDMA) on brain serotonin transporters are dependent on age-of-first exposure in recreational users and animals. *PLoS One, doi: 10.1371/journal.pone.0047524*.

II den Hollander B, Rozov S, Linden AM, Uusi-Oukari M, Ojanperä I, Korpi ER (2013). Long-term cognitive and neurochemical effects of "bath salt" designer drugs methylone and mephedrone. *Pharmacol Biochem Behav, 103, 501-9*.

III den Hollander B, Dudek M, Ojanperä I, Kankuri E, Hyytia P, Korpi ER (2015). Manganese-enhanced Magnetic Resonance Imaging Reveals Differential Long-term Neuroadaptation after Methamphetamine and the Substituted Cathinone 4-Methylmethcathinone (Mephedrone). *Int J Neuropsychopharmacol, doi: 10.1093/ijnp/pyu106*.

IV den Hollander B, Sundström M, Pelander A, Ojanperä I, Mervaala E, Korpi ER, Kankuri E (2014). Keto Amphetamine Toxicity-Focus on the Redox Reactivity of the Cathinone Designer Drug Mephedrone. *Toxicol Sci, doi: 10.1093/toxsci/kfu108*.

These papers are referred to in the text by their roman numerals.

Abbreviations

4-MMC	4-methylmethcathinone, mephedrone
5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
AMPH	Amphetamine
ATP	Adenosine triphosphate
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase
DA	Dopamine
DAT	Dopamine transporter
DOPAC	3,4-dihydroxyphenylacetic acid
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ETC	Electron transport chain
HVA	Homovanillic acid
MAO	Monoamine oxidase
MDMA	3,4-methylenedioxymethamphetamine, ecstasy
MDMC	3,4-methylenedioxymethcathinone, methylone
MEMRI	Manganese-enhanced MRI
METH	Methamphetamine
MRI	Magnetic resonance imaging
NE	Norepinephrine
NET	Norepinephrine transporter
NMDA	N-methyl-D-aspartate
PFC	Prefrontal cortex
PKC	Protein kinase C
ROS	Reactive oxygen species
SERT	Serotonin transporter
SPECT	Single-photon emission computed tomography
TH	Tyrosine hydroxylase
TPH	Tryptophan hydroxylase
VMAT-2	Vesicular monoamine transporter-2
WST-1	Water soluble 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt
[^{123}I]β-CIT	^{123}iodine-labeled 2β-carbomethoxy-3β(4-iodophenyl)tropane

Abstract

INTRODUCTION

In recent years there has been a large increase in the use of a new kind of amphetamine-type stimulants known as substituted cathinones. These compounds have a short history of human use, and little is known about their potential neurotoxicity. Two of the most popular substituted cathinones, 4-methylmethcathinone (4-MMC, mephedrone) and 3,4-methylenedioxymethcathinone (MDMC, methylone) are, aside from their β-ketone group, close structural analogues of potentially neurotoxic amphetamines such as methamphetamine (METH) and 3,4-methylenedioxymethamphetamine (MDMA, ecstasy). This has led to concern about the potential neurotoxicity of these novel compounds, and warrants a closer investigation into their possible long-term neurotoxic effects.

METHODS

The long-term effects of METH and MDMA as well as 4-MMC and MDMC were assessed using a range of biochemical assays, including assessment of monoamine levels and their transporters. The effects on brain activity were investigated using manganese-enhanced magnetic resonance imaging. Furthermore, behavioral experiments assessing cognition and neuropsychiatric function were performed. Finally, *in vitro* experiments in a neuroblastoma cell line were performed to identify mechanisms responsible for the observed differences in toxicity between the amphetamines and cathinones.

RESULTS

Unlike METH and MDMA, which produced strong reductions in dopamine and serotonin levels or brain activation, 4-MMC produced few notable effects on monoamine levels and had only minor effects on brain activation, although MDMC produced a reduction in 5-HT levels similar to MDMA. No clear effects on behavioral tests of memory function were observed as both increases and decreases in test performance were seen following 4-MMC and MDMC. *In vitro* experiments revealed that cathinones differ from amphetamines in their redox properties, and 4-MMC produced different effects than METH on the mitochondrial electron transport chain.

CONCLUSIONS

The substituted cathinones 4-MMC and MDMC do not appear to be more neurotoxic than METH and MDMA. If anything, they show a more favorable safety profile. Therefore, these substances do not appear to present an imminent and severe threat to public health. From a harm reduction perspective, these compounds may be good alternatives to METH and MDMA. However, future work is needed to assess with certainty the long-term effects of amphetamine-type stimulants in humans.

1. Introduction

Amphetamine-type stimulants comprise a wide range of substances including amphetamine, methamphetamine and ecstasy which have been used medically and recreationally for decades. They belong to a class of drugs known as psychostimulants that, when consumed, produce sensations of euphoria, decreased need for sleep, and increased levels of energy and mental alertness. The fact that these drugs exert such a powerful stimulatory effect on the brain has led to questions and concern about their potential neurotoxicity. Just as researchers were attempting to answer these questions, a game-changing development occurred on the worldwide illicit drug market.

Between 2000 and 2010 the use of Internet increased rapidly, becoming an integral part of everyday life and the preferred method of communication in many parts of the world. A revolution in commerce ensued, and companies such as Amazon introduced the world to the concept of e-commerce: the possibility of buying goods on the Internet with the click of a button. It was around this time that some Internet vendors began selling a wide range of novel psychoactive substances for recreational use that had little or no history of human consumption. Amongst the most popular new drugs were the ones that belong to a particular kind of amphetamine-type stimulants known as cathinones.

Cathinones are very, but not entirely, similar to normal amphetamines: although they produce almost identical euphoric and wakefulness-promoting effects, the slight changes in their molecular structure meant they were not covered by existing drug laws, and could therefore legally be distributed and sold. The term “designer drug” which is often used for these drugs, actually refers to the notion that the molecules are specifically designed to circumvent drug laws. More recently, they have also been known as “bath salts” or “plant food”, labels initially applied to these substances by Internet vendors in order to circumvent laws banning their sale for human consumption.

The explosive increase in popularity of these new, legal and widely available drugs sparked a panic in media and politics about the possible dangers of these novel compounds. Sensationalized tabloid reports of violence, self-mutilation and even cannibalism occurred frequently. Although these reports were mostly false or exaggerated, they did point out some important questions. What risks are people taking by consuming these new substances? Are they neurotoxic, or do they produce any serious long-term effects on the brain? If so, are these cathinones more or less harmful than the well-known, normal amphetamines?

It is the goal of this thesis to provide answers to some of these questions, as they will provide urgently needed information that is important for the development of an effective and evidence-based public health policy and harm reduction approach with regards to these new substances.

2. Literature review

2.1 Overview

The literature review is organized as follows. First, a brief description of a number of important terms, such as amphetamines, cathinones, and the differences between them will be explained. After this, a section is devoted to the history of amphetamines and their impact on society. Subsequently, a short review of the 5-HT, DA and NE neurotransmitter systems will be given, as these systems are the primary targets of amphetamines. The next section extends on this, and discusses in more detail the pharmacology and mechanisms of action of amphetamines in the nervous system.

After this general overview, the focus will shift towards the primary topic of this thesis, namely the alleged long-term neurotoxic effects of amphetamine-type stimulants. The existing evidence of neurotoxicity in both humans and animal models will be reviewed broadly, and include a discussion of studies ranging from neuroimaging and neurocognitive studies in humans to preclinical studies employing biochemical as well as behavioral methods. Finally, the last section of the review deals with the proposed mechanisms underlying the development of amphetamine neurotoxicity, and focuses primarily on preclinical and *in vitro* mechanistic studies that provide clues about the mechanisms involved in producing amphetamine neurotoxicity.

2.2 Defining amphetamine-type stimulants

The pharmacology and toxicology of amphetamine-type stimulants is the primary topic of this thesis. Understanding the terms used to refer to amphetamine-type stimulants can be can be somewhat complicated, particularly in the case of this thesis, which deals with two different kinds of amphetamine-type stimulants, namely amphetamines and cathinones: two closely related, but not entirely similar substances. For clarity, a brief description of these terms is provided here.

Amphetamine-type stimulants – amphetamine-type stimulants is a catch-all phrase for all amphetamines, cathinones as well as other drugs that resemble amphetamines in their structure or pharmacological action, such as methylphenidate.

Amphetamine and amphetamines – the word amphetamine is a contraction of α-methyl-phenethylamine. In the strictest sense of the word, amphetamine refers to the corresponding molecule shown in Fig 2.1. The phenethylamine part of the word refers to the most basic "backbone" of the molecule consisting of a 6-carbon phenyl ring connected to an amino (NH_2) group by a two-carbon side chain. The carbons in the side chain are referred to as α and β carbons. The α-carbon, located closest to the amino

group, has a methyl (-CH_3) group attached to it, hence the α-methyl part of the name. When referring specifically to this molecule, the abbreviation AMPH will be used in this thesis. As Fig. 2.1 shows, the basic amphetamine structure is not unique to AMPH. The amino group can be methylated and functional groups can be added to numerous locations on the ring and side chain of the amphetamine molecule, producing a vast number of different substances, for instance METH and MDMA. This entire group of compounds is known as substituted amphetamines or just amphetamines for short. In this thesis, the terms amphetamine and amphetamines refer to any specific amphetamine or to the entire group of amphetamines, rather than AMPH specifically.

Cathinone and cathinones – In the strictest form, cathinone is an AMPH molecule with a specific substitution: namely a ketone group on the side chain β carbon (Fig. 2.1). It derives its name from the fact that it is the primary psychoactive compound of the *Catha edulis* plant. Similar to AMPH, cathinone can also have functional groups added to it, producing a large possible variety of so-called substituted cathinones, or cathinones for short. In this thesis, the term cathinone or cathinones will refer to any specific cathinone or to the entire group of cathinones. The substances 4-MMC and MDMC, also known as mephedrone and methylone (Fig. 2.1) are examples of such cathinones, and will play a key role in this thesis.

B-keto amphetamines and non-keto amphetamines - due to the ketone group on the β carbon, cathinones are sometimes also referred to as β-keto amphetamines or simply keto amphetamines. In contrast, normal amphetamines can be referred to as non-keto amphetamines. Cathinones can best be viewed as a subtype of amphetamines. Importantly, this implies that all cathinones, by definition, are amphetamines, but only amphetamines with the β-ketone group are cathinones. For this reason, the terms β-keto amphetamine, keto amphetamine and non-keto amphetamine will also be used in this thesis when appropriate to avoid any ambiguity.

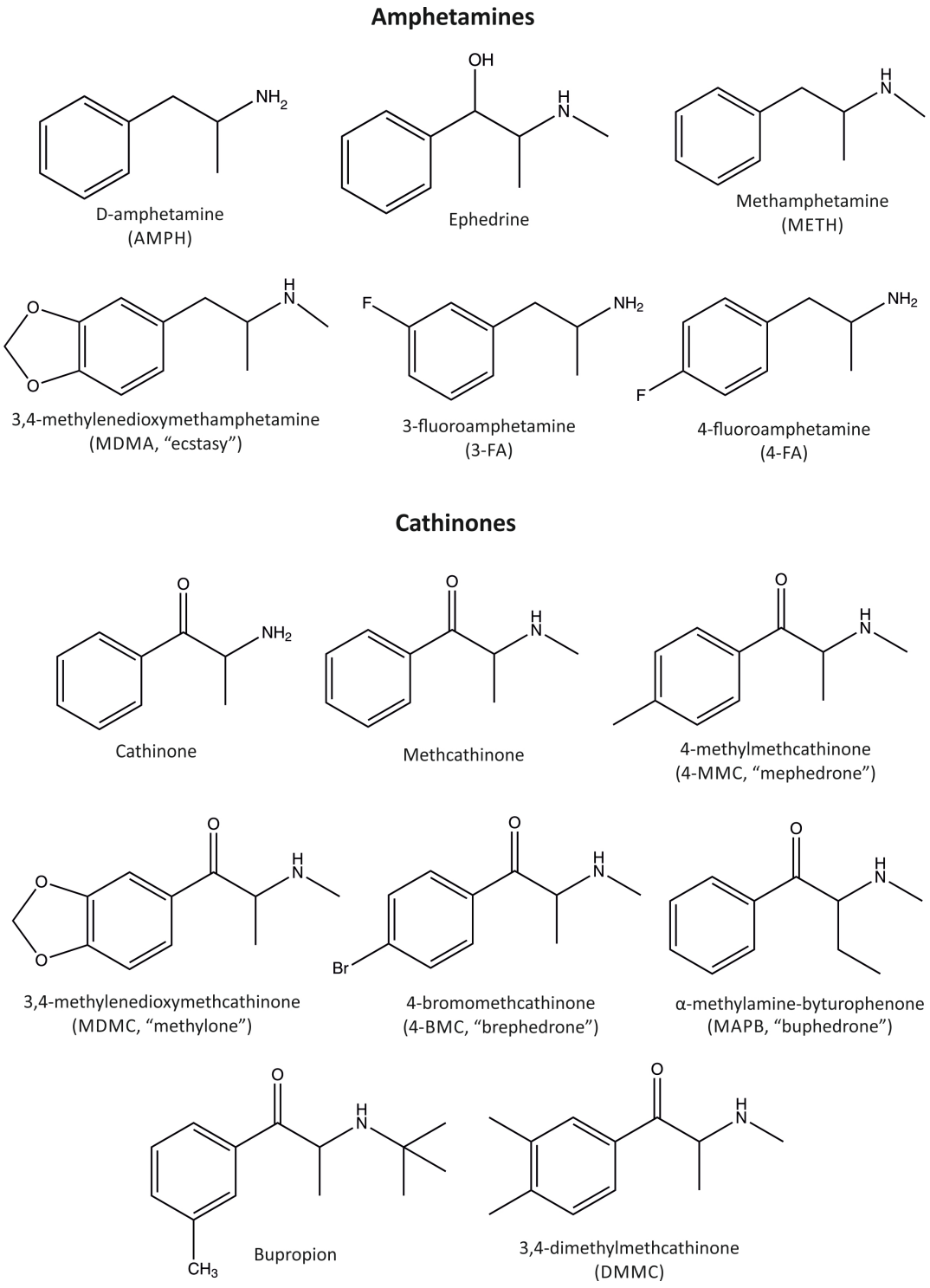


Figure 2.1 Molecular structures of amphetamine-type stimulants mentioned in this thesis.

2.3 History of amphetamines

2.3.1 Amphetamines in pre-industrial societies

There is evidence that the psychoactive and stimulating effects of amphetamines have been known to humans for millennia. The *Ephedra sinica* plant, which contains ephedrine (Fig. 2.1), a stimulant closely related to AMPH, has been found in Neolithic archeological sites in the Middle East and India. It has been suggested that Soma, the Vedic ritual drink mentioned in the Rigveda, may have been an *Ephedra*-containing concoction, although other psychoactive constituents, such as *Amanita muscaria,* have also been mentioned as possible candidates (Furst, 1976; Mahdihassan and Mehdi, 1989). Clearer evidence of its use comes somewhat later, from a first century AD Chinese text that details the use of ephedra for treating asthma and upper respiratory infections, suggesting that also its medical applications have also been known for a long time (Sulzer et al., 2005).

A second example of the lengthy history of human amphetamine use is the consumption of the *Catha edulis* plant, which is commonly consumed by chewing fresh leaves of the plant for an extended period. The compound responsible for the psychoactive effect of the khat plant is cathinone. Khat is frequently used in the Arabian Peninsula and parts of Africa. Exactly when the chewing of khat was introduced in these societies is not known, with estimates ranging between 525 AD and the 13th century. However, it is clear that since the 13th century, khat has been widely enjoyed for its psychoactive and stimulating effects and as a social ritual. At present, it is estimated that between 80-90% of adult males and 10-60% of adult females in these regions consume khat on a daily basis (Elmi, 1983; Pantelis et al., 1989; Al-Motarreb et al., 2002; Warfa et al., 2007)

2.3.2 Synthetic amphetamine and methamphetamine

Although amphetamines have been used for thousands of years, the chemical synthesis of AMPH is a more recent event that was first described by Rumanian chemist Lazar Edeleanu (Edeleano, 1887). However, the drug received relatively little attention until its psychostimulant properties were discovered several decades later (Alles, 1933). It was first introduced commercially in 1932 by Smith Kline and French, under the trade name Benzedrine, as an inhaler for treating asthma and allergies, as it effectively enlarged nasal and bronchial passages. After its introduction in tablet form in 1936 it quickly became one of the most popular and well-known drugs of all times. Its effects were welcomed by people across different social strata and professional groups, ranging from stay-at-home moms and truck drivers to artists, musicians and scientists. The surge in popularity led to AMPH being scheduled as a prescription-only medicine in 1939, in an attempt to decrease its use. However, due to aggressive marketing by the pharmaceutical industry – AMPH was marketed for treating anything from hay fever, fatigue and obesity to depression,

schizophrenia and even opiate addiction - and high demand for the drug, its use only increased. By 1970 the pharmaceutical industry produced 10 billion tablets and it is estimated that 50-90% of this amount was re-sold or otherwise diverted to the black market (Sulzer et al., 2005; Rasmussen, 2008b).

Shortly after AMPH, the synthesis of METH was first described in 1893 by Japanese chemist Nagai Nagayoshi (Grobler et al., 2011). Just like AMPH, METH was embraced by pharmaceutical companies for similar indications. For instance Obetrol, a mixture of AMPH and METH, became a highly popular weight loss drug in the 50's and 60's (Rasmussen, 2008a). The use of METH by the German armed forces during World War II has been well-documented. Although Nazi officials formally discouraged the use of METH, it is clear that some commanding officers would give the drug to their soldiers to combat fatigue during long missions. The METH, which was sold in Germany under the trade name Pervitin was referred to by the soldiers as tank chocolates ("*Panzerschokolade*") (Hurst, 2013). Notably, amphetamines are currently still in use by armed forces up until this day (Estrada et al., 2012).

Today, AMPH remains a widely used drug. Although the number of indications for which AMPH is commonly prescribed has decreased significantly, it still remains one of the primary treatments for ADHD, a disease affecting up to 5% of the adult population, with an even higher prevalence in children (Wigal, 2009; Willcutt, 2012). Furthermore, AMPH is commonly prescribed off-label for a number of other disorders (Bazzano et al., 2009). Importantly, aside from its medical uses, amphetamines are also widely used recreationally. According to the United Nations Office on Drugs and Crime, amphetamines are the second most widely used illicit drug in the world, after cannabis (UNODC, 2011). Therapeutic doses of AMPH and METH are in the range of 10-60 mg/day, corresponding to approximately 0.1 – 0.8 mg/kg/day for an 80 kg individual, while recreational doses are commonly north of this number.

2.3.3 Rise of MDMA

Aside from AMPH and METH, another drug known as MDMA or ecstasy, has also had a significant influence on society, albeit primarily as an illegal drug. MDMA was originally developed by Merck in 1912, but did not become widely known until after it was first resynthesized by American medicinal chemist Alexander Shulgin in 1965. In an effort to determine the psychoactive properties of the drug, Shulgin (1995) tested it on himself and discovered that MDMA, in addition to possessing the known wakefulness promoting effect of amphetamines, also produced a number of very different effects, such as an increased willingness to communicate and feelings of love, empathy and closeness to others. Later the term empathogen or entactogen was coined to refer to substances producing these types of effects. Shulgin realized that these effects might make the drug an effective tool in aiding psychotherapy sessions by improving communication between

patient and therapist. He introduced the drug to psychotherapists, and the drug was used fora period of time for this purpose. However, as more people became familiar with its effects, its recreational use increased rapidly, and in 1985 the US Drug Enforcement Agency scheduled MDMA as a controlled substance (Benzenhofer and Passie, 2010). Despite its legal status, the drug is still one of the most widely used drugs in the world and has exerted tremendous influence on music, nightlife and festival culture (Reynolds, 2013). Interestingly, there has recently been renewed interest in the possible clinical applications of MDMA as initially envisioned by Shulgin (Mithoefer et al., 2011; Oehen et al., 2013). A common dose of MDMA is around 100 mg, which corresponds to 1.3 mg/kg for an 80 kg person, and in the abovementioned clinical study the initial dose was 125 mg. However, in recreational settings, MDMA is commonly (but not always) consumed in the form of ecstasy pills containing variable amounts of MDMA, making it difficult for users to accurately dose the drug (Morefield et al., 2011).

2.3.4 Substituted cathinones and the new drug market

Prior to the turn of the century, the amphetamines in widespread recreational use were limited to AMPH, METH and MDMA. All three of these substances are subject to tight legal control measures. Sale and possession of these substances is illegal and they are covertly distributed to users via the black market. However, this was all about to change. Between 2000 and 2010, advances in information technology and infrastructure drastically increased the percentage of households with an Internet connection. This caused revolutionary changes in communication patterns and increased the ability of people all over the globe to exchange information with each other. These developments eventually led to a second revolution, namely in commerce, as companies such as Amazon introduced the world to the concept of electronic commerce – the possibility of buying goods at the click of a button without having to leave your house.

The Internet revolution did not go unnoticed to the drug market either. Towards the end of the first decade of this century, the number of Internet vendors selling a variety of novel, hitherto practically unknown, psychoactive substances increased exponentially. Figure 2.2 shows the number of novel psychoactive substances reported through the EMCDDA early warning system from 2005 – 2013, and shows the large increase of novel psychoactive sbustances starting around 2009.

Amongst the most popular substances are the substituted cathinones. As described previously (see Fig. 2.1), the chemical structure of cathinones is very similar to that of amphetamines. It is therefore not entirely surprising that they also produce almost identical euphoric and wakefulness-promoting effects. In fact, the primary reason that these cathinones quickly became so popular was because they caused similar pleasant subjective effects as amphetamines, but due to the ketone group on the β carbon, were

not covered by existing drug laws. They could therefore be legally distributed and sold in this vast, newly developed, online e-commerce-based grey market (Power, 2013).

The term “designer drug” which is often applied to these types of drugs, actually refers to the idea that the molecules are specifically designed to circumvent drug laws. More recently, however, the cathinones have also been known as “research chemicals”, “bath salts” or “plant food”, referring to labels that vendors would place on the products in order to circumvent laws, specifically the Federal Analogue Act, banning the sale of these substances specifically for human consumption.

A wide array of substituted cathinones has been reported to be sold and used. However, there are two specific ones that deserve some more attention, namely 4-MMC and MDMC. They are particularly interesting for several reasons. For one, they were amongst the first cathinones to be sold in this newly developed drug market infrastructure. Second, they are the ones which are the most favored by users, producing the best subjective effects (Sogawa et al., 2011; Winstock et al., 2011). Third, they are very close

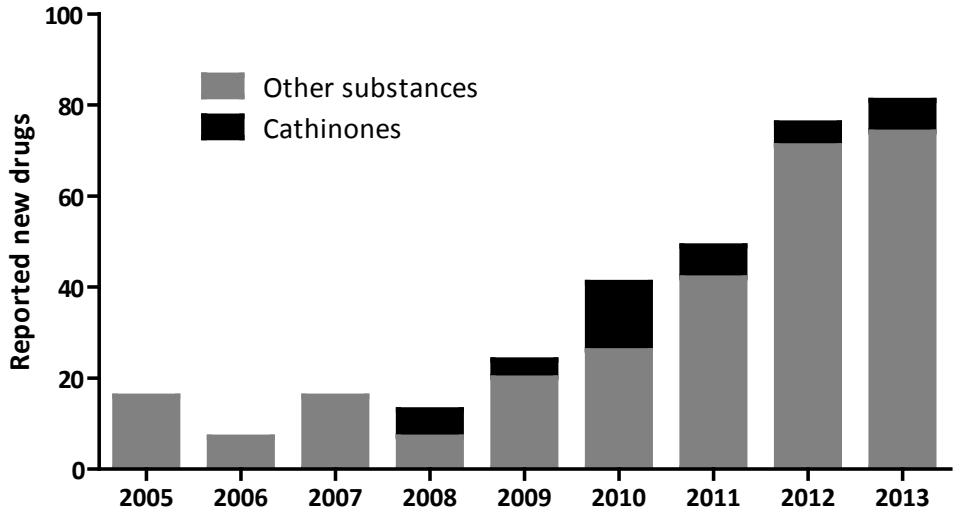


Figure 2.2 The number of new psychoactive substances notified for the first time in the EMCDDA Early Warning System since May 2005. A distinction has been made between cathinones specifically and all other reported substances, including cannabinoids, phenethylamines, tryptamines, opioids and others. Adapted from the EMCDDA–Europol 2013 Annual Report on the implementation of Council Decision 2005/387/JHA(EMCDDA, 2014a).

structural analogues of METH and MDMA, respectively. Aside from the β-ketone group, 4-MMC differs from METH only by the addition of a methyl group on the 4-position on the phenyl ring, while MDMC is direct β-keto analogue of MDMA.

Although 4-MMC and MDMC are now banned in many countries due to the rapid surge in popularity they enjoyed, the amphetamine ring and side chain offer countless possibilities for substitutions. Chemists quickly came up with a large number of different substituted cathinones with names such as buphedrone, brephedrone, DMMC (Fig 2.1) and countless others, in order to replace the substances which had been banned. The cat-and-mouse game between governments and grey market chemists, where one drug is banned while a new one is already being developed and brought to the market, continues until the present day.

2.4 DA, 5-HT and NE systems

The primary targets of most amphetamines are the DA, 5-HT and NE systems. This is perhaps not surprising, as all three of these systems belong to the monoamine system, meaning they employ transmitter molecules consisting of an aromatic ring connected to an amino group by a two-carbon side chain, and thus bear strong resemblance to the basic phenethylamine structure of amphetamines (compare Fig. 2.1 and 2.4/2.5). What follows is a brief overview of the anatomy and function of these three monoamine systems, as well as a summary of the biogenesis and metabolism of their respective neurotransmitter molecules.

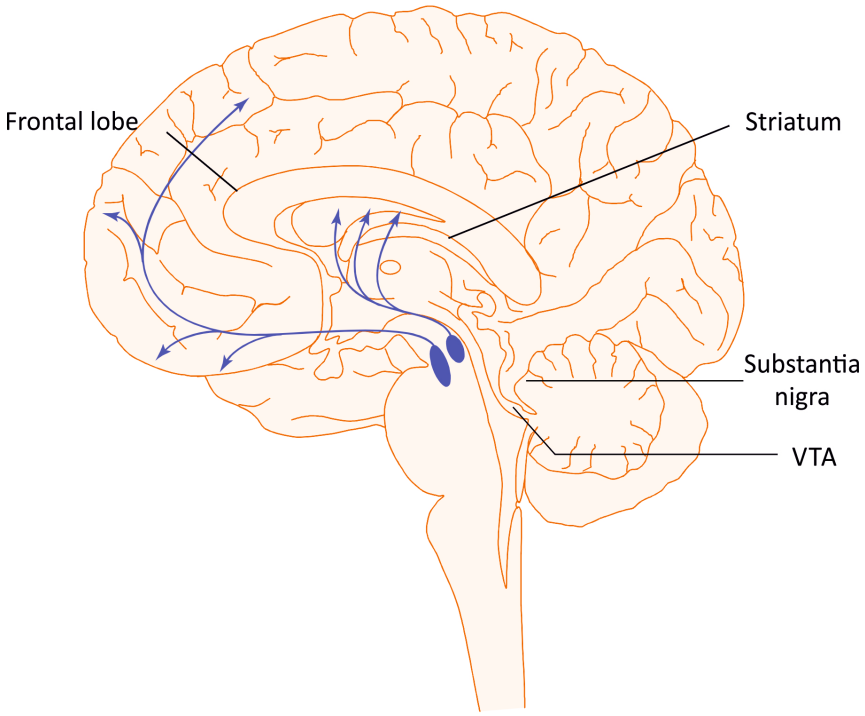
2.4.1 Neuroanatomy

The primary projections of the NE, DA and 5-HT system are shown in Fig. 2.3.

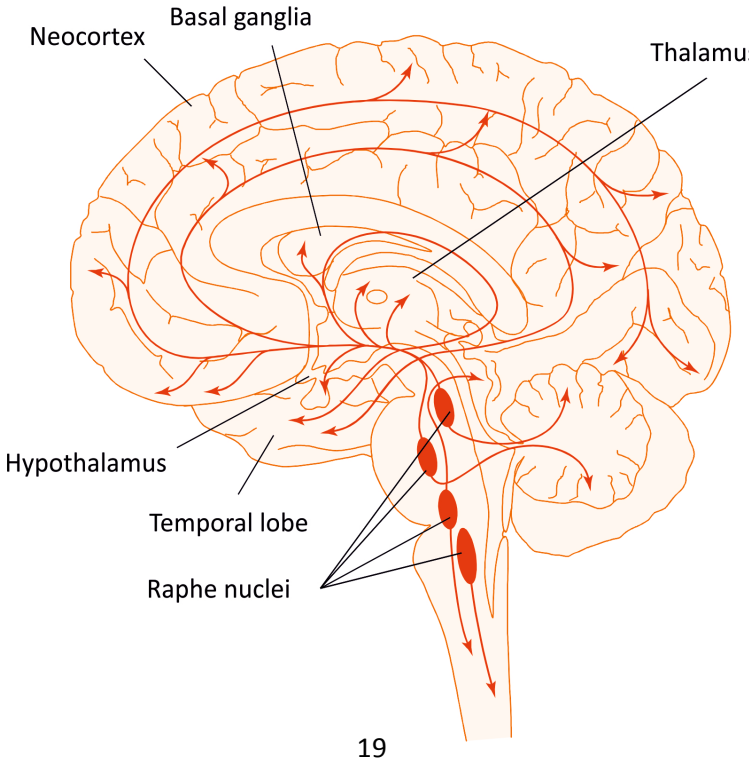
Dopamine – DA neuronal cell bodies are located in various parts of the brain and give rise to several projections: the nigrostriatal pathway, consisting of cell bodies located in the substantia nigra that send projections to the striatum; the mesocorticolimbic pathway, consisting of cell bodies in the VTA projecting to the limbic system and cortex and a smaller tuberoinfundibular pathway from the hypothalamus to the pituitary (Björklund and Dunnett, 2007). The different DA pathways are involved in regulating separate brain functions. The nigrostriatal projection plays an important role in regulating movement while the mesocorticolimbic system controls many different aspects of motivation and cognition. The tuberoinfundibular pathway plays a role in prolactin secretion (DeLong, 1990; Porter et al., 1990; Di Chiara, 1998; Floresco and Magyar, 2006)

Serotonin – 5-HT neurons have cell bodies located in the raphe nuclei from which axonal projections travel upwards, similarly to NE axons, also via the medial forebrain bundle to form widespread synaptic contacts with targets in the cortex, basal ganglia, limbic system and hypothalamus (Sullivan, 1995). Serotonin plays an important role in several brain

Dopamine system



Serotonin system



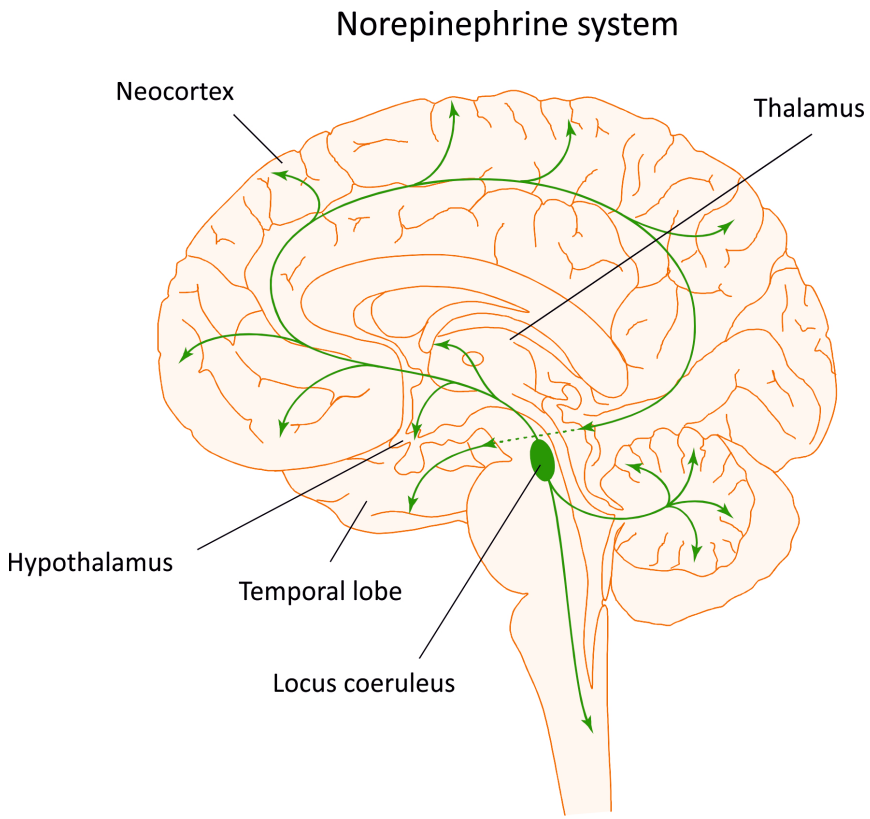


Figure 2.3 Schematic drawing of the DA, 5-HT and NE systems in the human brain. The primary DA projections are the mesocorticolimbic and the nigrostriatal pathways. A smaller projection known as the tuberoinfundibular pathway (not shown) exists between the hypothalamus and pituitary gland and is involved in prolactin secretion. Projections of the 5-HT and NE systems originate in the raphe nucleus and locus coeruleus, respectively, and proceed upward via the medial forebrain bundle to numerous targets including the cortex, hippocampus and basal ganglia or downward into the spinal cord.

functions such as sleep, and the regulation of mood and appetite (Ursin, 2002; Halford et al., 2005; Martinowich and Lu, 2008)

Norepinephrine – NE neuronal cell bodies are primarily located in the locus coeruleus. From here, axons travel upward in the medial forebrain bundle, forming connections throughout the cortex, hippocampus, (hypo)thalamus and cerebellum (Jones and Moore, 1977). The locus coeruleus also sends projections downward to the spinal cord (Westlund and Coulter, 1980). The brain NE system plays an important role in regulating a number of brain functions including arousal, attention, memory and anxiety (Tanaka et al., 2000; Marzo et al., 2009).

2.4.2 Neurotransmitter biogenesis and metabolism

Catecholamines – The neurotransmitters DA and NE are both catecholamines, and their biosynthesis occurs in the same pathway (Fig 2.4). TH converts L-tyrosine to levodopa, which is subsequently converted to DA by aromatic amino acid decarboxylase. NE is produced from DA by dopamine-β-hydroxylase. DA is metabolized by MAO and catechol-*O*-methyltransferase to produce HVA. NE is similarly metabolized by MOA and catechol-*O*-methyltransferase to produce 3-methoxy-4-hydroxyphenylglycol and vanillylmandelic acid (Brady et al., 2011)

Serotonin – The biosynthesis of 5-HT begins with tryptophan, which is converted by TPH to 5-hydroxytroptophan and subsequently converted by aromatic amino acid decarboxylase to 5-HT. The metabolism of 5-HT by MAO and aldehyde dehydrogenase yields the metabolite 5-HIAA (Fig. 2.5). Furthermore, a separate circadian rhythm-dependent metabolic shunt exists in the pineal gland, through which 5-HT is converted to melatonin (Brady et al., 2011).

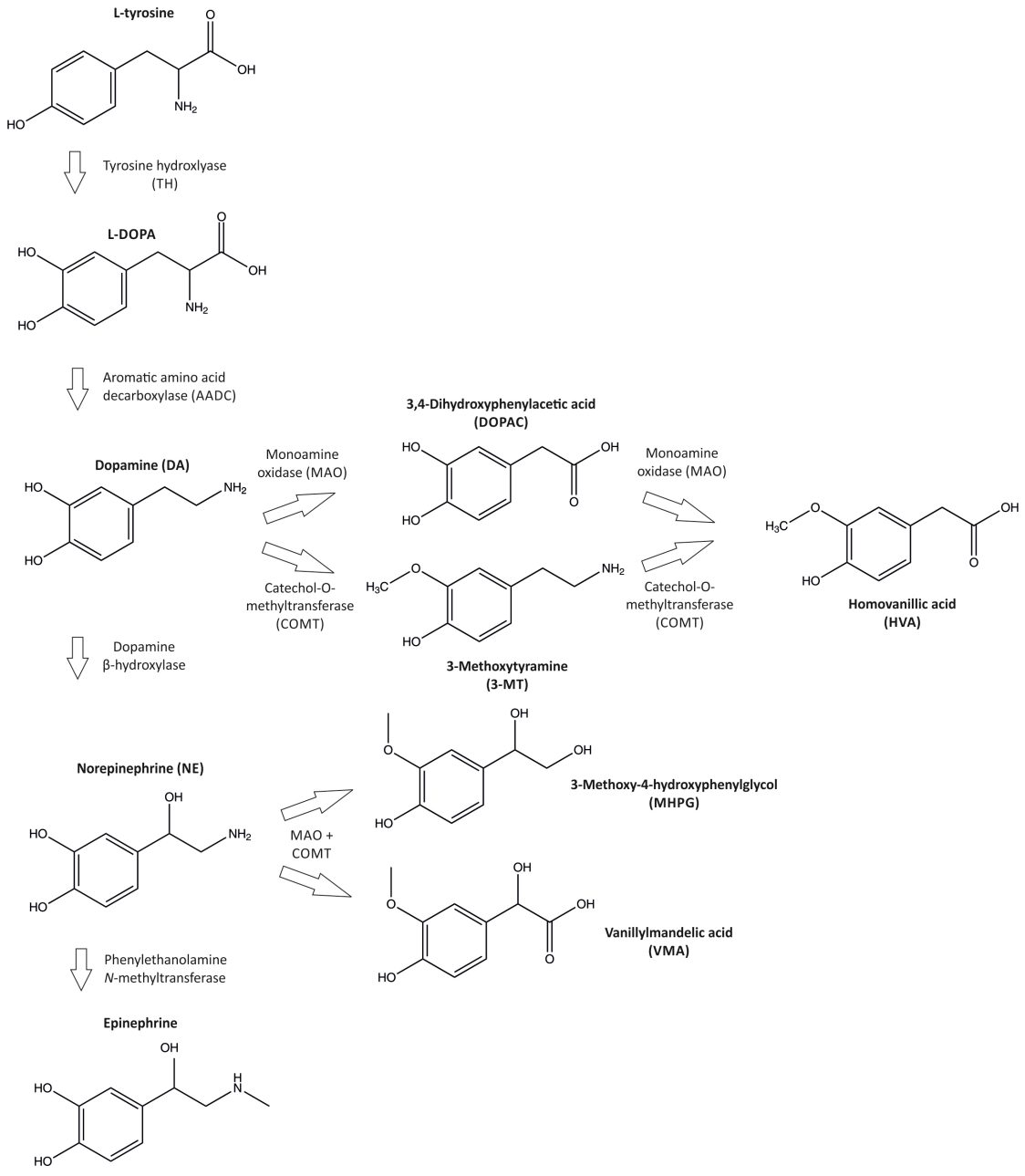


Figure 2.4 Overview of catecholamine biosynthesis and metabolism. The left row shows the synthesis of DA and NE from L-tyrosine. The rows to the right show the metabolism into their respective breakdown products. Arrows indicate enzymatic reactions and the name of the enzymes are given near the arrows.

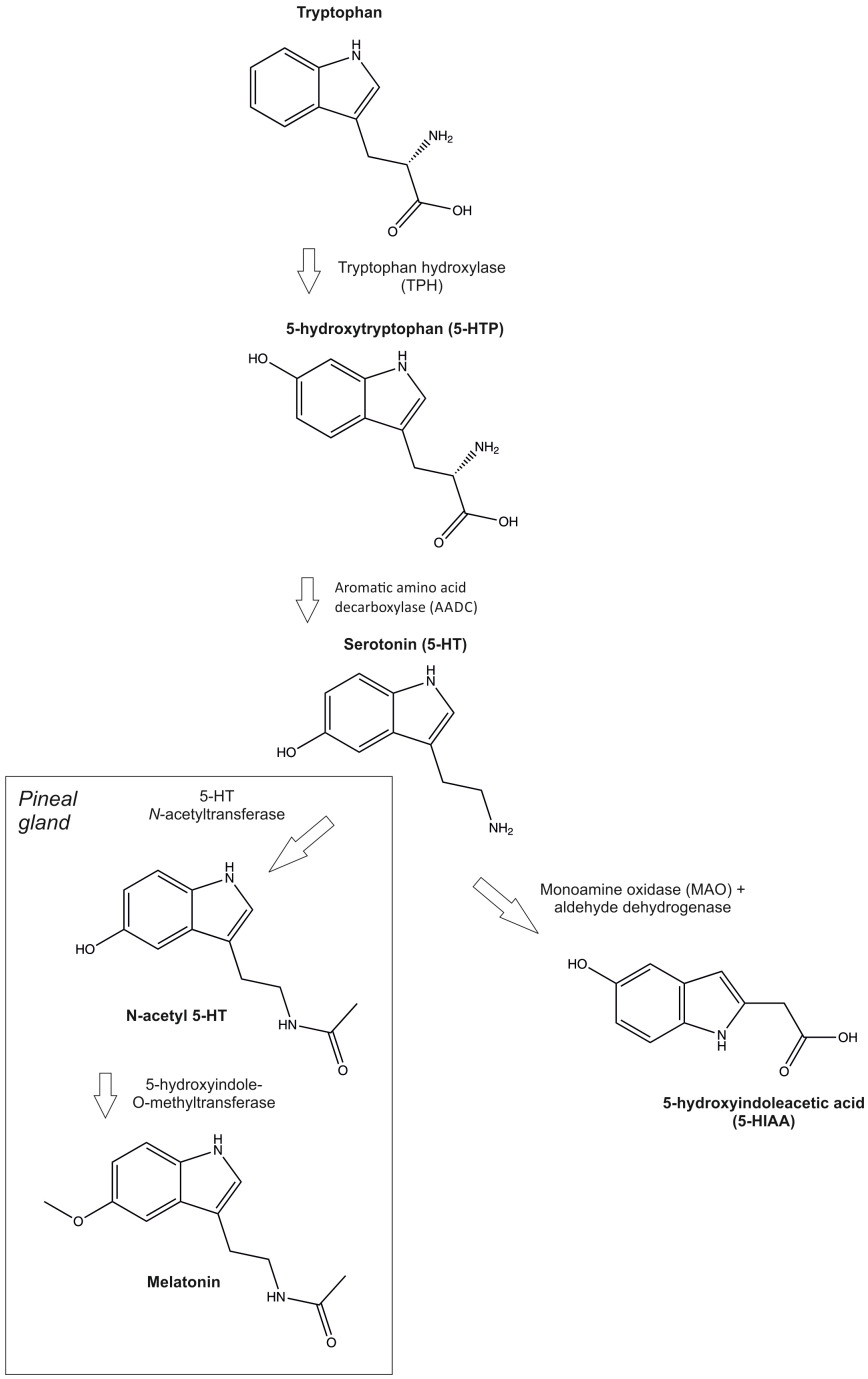


Figure 2.5 Overview of 5-HT biosynthesis and metabolism. The top half of the figure shows the biosynthesis of 5-HT from its precursor tryptophan. The bottom half of the figure shows the metabolism of 5-HT into 5-HIAA or melatonin (in the pineal gland). The arrows indicate enzymatic reactions performed by the enzyme listed next to the arrow.

2.5 Neuropharmacology of amphetamines

Several decades of research have revealed many details about the neuropharmacology of amphetamines. Historically, the mechanisms that have received the most attention are the ability of AMPH to inhibit the reuptake and enhance the release of DA via the DAT, as well as its ability to promote the release of DA from secretory vesicles (Sulzer et al., 2005; Fleckenstein et al., 2007). The primary mechanisms of AMPH action in the nerve terminal are shown in Fig. 2.6. An overview of the main properties and neuropharmacological effects of amphetamines is provided in Table 2.1.

Unraveling the pharmacological action of AMPH has been a century-long endeavor and is in many ways an extension of even earlier work on the effects of epinephrine. In early work, British physician George Oliver discovered that adrenal gland extract would increase blood pressure when he injected it into his son's radial artery. The discovery that the vassopressive action was due to the presence of epinephrine gave rise to the theory of secretory transmission: the idea that nerves communicate via the excretion of chemical messengers. Subsequently, it was discovered that also other substances, such as AMPH, could produce a similar effects as epinephrine, and these substances were termed sympathomimetics, due to their ability to mimic the effects of the sympathetic nervous system. In the 1950's, experiments with reserpine demonstrated that some sympathomimetics, such as epinephrine, were capable of contracting blood vessel even after reserpine treatment, whereas other sympathomimetics, such as AMPH, where not. Thereby the discovery of the distinction between directly and indirectly acting drugs - the latter being dependent on the presence of an intact sympathomimetic system acting by releasing epinephrine-like substances - was made (Burn and Rand, 1958; Sulzer et al., 2005).

Recently it has become clear that, aside from inhibiting reuptake and enhancing release of neurotransmitter via reuptake transporters, AMPH is also capable of modulating action potential-dependent neurotransmitter release (Branch and Beckstead, 2012; Daberkow et al., 2013). Furthermore, AMPH regulates DA neurotransmission via a number of mechanisms and also has an array of targets outside the DA system (Fung and Uretsky, 1982; Robinson, 1985; Ritz and Kuhar, 1989; Matsumoto et al., 2014; Reese et al., 2014). Many of these targets have been identified only recently, and their importance in mediating the physiological, behavioral, therapeutic, reinforcing and toxic effects of AMPH is just beginning to be understood. Nonetheless, it is now clear that amphetamine action is much more complex and extends far beyond the mechanisms which have traditionally received the most attention.

Drug property or effect	Section	Brief description
AMPH vs. METH	2.5.1	- The subjective effects of the drug are similar and neither drug appears more addictive than the other. - Subtle differences exist between AMPH and METH as AMPH releases more DA in the PFC. - Due to their similar pharmacological action the drugs will be considered interchangeable here.
D- and l-isomers	2.5.1	D-AMPH appears to be a more potent DA releaser than L-AMPH and produce stronger behavioral effects such as locomotor activity.
Action at the plasmalemmal DAT	2.5.2	- AMPH prevents reuptake of DA by competitive inhibition of DAT. - AMPH releases DA by reversing the direction of DAT and inducing transient channel-like states during which DA is transported outwards
Regulation of DAT surface expression	2.5.2.2	- The effects of AMPH are determined by DAT surface expression and function, which is regulated by PKC/CaMKII phosphorylation and ROS signaling. - AMPH can target these mechanisms to regulate DAT surface expression.
Modulation of exocytotic DA release	2.5.3	- Recently it has become clear that AMPH also enhances exocytotic DA release. - The exact mechanism is unknown but could be related to the ability of AMPH to induce persistent transporter-mediated ion leakage and excitatory conductance.
Effects on secretory vesicles	2.5.4	- AMPH promotes the release of neurotransmitter from secretory vesicles. - Mechanisms include disruption of the vesicle membrane proton gradient as well as inhibition and internalization of VMAT-2.
Regulation of TH and MAO	2.5.5.1	- AMPH reduces MAO function and can both increase and decrease TH activity. - Mechanisms include direct interaction with the feedback inhibition site or induction of phosphorylation at other regulatory sites.
Outside the DA system	2.5.5.2	- In addition to DAT, AMPH also inhibits reuptake and enhances release of neurotransmitters via NET and SERT. - Recently, novel AMPH targets such as the σ receptor and trace amine associated receptor-1 have been identified that appear to play an important role in mediating the effects of AMPH.
Substituted amphetamines	2.5.6	- Substituted amphetamines and cathinones also inhibit reuptake and increase release of monoamines via transporters, but differ from AMPH in their relative preference for SERT, DAT and NET

Table 2.1 The table lists the main neuropharmacological effects of AMPH together with a brief description and a reference to the sections in which the respective effects are discussed in further detail.

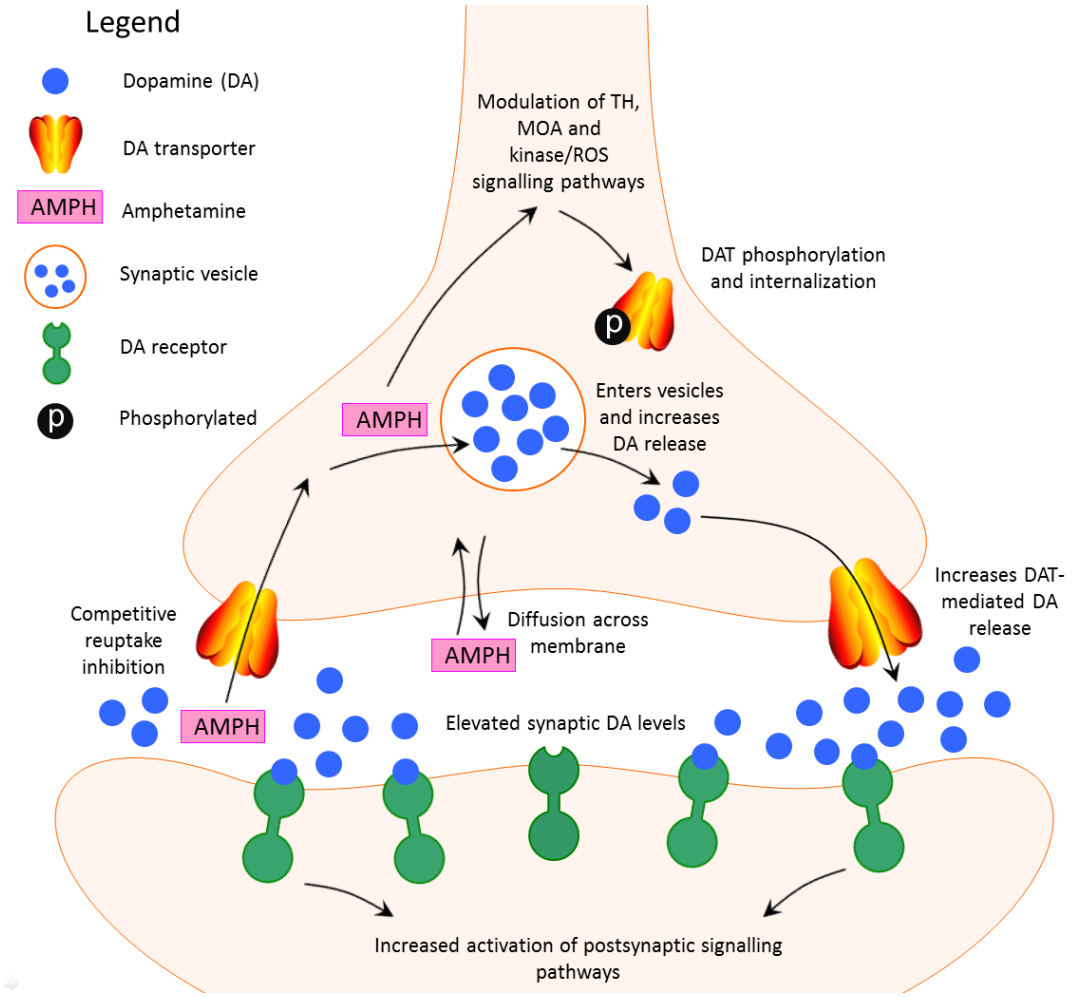


Figure 2.6 A graphical representation of some of the important actions of AMPH in the nerve terminal. AMPH is taken up by the DAT but also diffuses across the membrane. Inside the nerve terminal, it may enter synaptic vesicles, resulting in release of DA from the vesicles into the cytosol. Competitive reuptake inhibition and increased DA release lead to elevated synaptic DA levels and subsequent increases in the postsynaptic response. AMPH also activates numerous signalling pathways and enzymes, leading to cellular responses such as phosphorylation and internalization of DAT.

In this section, the current state of the art of amphetamine neuropharmacology is reviewed. First, the effects of AMPH at the plasmalemmal DAT and the complex mechanisms by which AMPH regulates DAT function will be discussed. Second, the effects on secretory vesicles will be discussed and third, other AMPH targets outside the DA system will be briefly mentioned. The section ends with a discussion about the neuropharmacology of substituted amphetamines and cathinones, and how their mechanism of action differs from that of AMPH.

2.5.1 AMPH, METH and their isomers

Prior to discussing the mechanism of amphetamine action in the nervous system, it is important to distinguish two slightly different drugs which both have been used for studying the pharmacological and neurochemical effects of amphetamines, namely AMPH and METH. These two substances, as well as their isomers, display subtle differences in terms of their pharmacology. Nonetheless, the notion that one of them is more powerful and addictive than the other has been hard to back up empirically.

One study showed that equimolar concentrations of AMPH and METH evoke a similar amount of DA release in the striatum and show similar elimination rates (Melega et al., 1995). Another study confirmed that the two substnaces produce similar increases in nucleus accumbens DA levels and actually found that AMPH increases PFC DA levels more than METH, while also producing a higher peak locomotor activity (Shoblock et al., 2003b). In line with the effect on PFC DA levels, it was discovered that AMPH has a stronger effect than METH on working memory function. The effects of AMPH and METH on working memory are bimodal, with a small dose increasing performance while higher doses produce a decrease. For AMPH, a dose of 0.5 mg/kg (doses calculated based on free base weight) increased working memory performance whereas a 2 mg/kg dose decreased it. METH, on the other hand, had little effect at 0.5 mg/kg, produced the highest increase at 2 mg/kg and a decreased performance only at 4 mg/kg (Shoblock et al., 2003a).

In terms of subjective effects, AMPH fully substitutes for METH in animals trained to discriminate METH from placebo (Desai and Bergman, 2010) and also causes dose-dependent increases in METH-appropriate responding in humans taught to discriminate 10 mg METH from placebo (Sevak et al., 2009), suggesting that also the subjective effects are very similar, if not indistinguishable. Moreover, it was shown that varying doses (between 12 and 50 mg intranasally) of AMPH or METH produce similar subjective effects and did not differ from each other with regards to the rate at which participants would opt for a cash reward instead of a dose of the drug (Kirkpatrick et al., 2012), thereby not corroborating the notion that METH is more addictive or reinforcing than AMPH.

Both AMPH and METH exist as isomers with subtle differences in their neurochemical and behavioral properties. For AMPH, the d-isomer appears to be a more potent DA releaser, whereas the l-isomer is an equally or more potent releaser of NE (Heikkila et al., 1975; Holmes and Rutledge, 1976; Mendelson et al., 2006). Furthermore, the d-isomers of AMPH and METH appear more potent than the l-isomers in producing behavioral effects such as locomotor activity, self-administration and taste aversion (Balster and Schuster, 1973; Yokel and Pickens, 1973; Carey and Goodall, 1974; Segal, 1975). Illicit METH is usually distributed as either a racemic mixture of d- and l-isomers or as pure d-isomer, depending on the method of synthesis most prevalent at the time (Logan, 2002; Mendelson et al., 2006)

In scientific literature, by tradition, work on the mechanism of action of amphetamines is primarily done with AMPH, whereas studies on neurodegeneration and toxicity preferentially employ METH (Sulzer et al., 2005). However, since these substances seem to show more similarities than differences, the assumption will be made here that results and conclusions from experiments employing AMPH can be generalized to METH, and vice versa, in order to better integrate literature from these two overlapping fields.

2.5.2 Action of AMPH at the plasmalemmal DAT

The DAT, together with the NET and SERT, belong to the solute carrier-6 gene family of secondary-active transporters consisting of 12 transmembrane domains as well as intracellular domains with phosphorylation and binding sites that are vital for its regulation. Normally, the transporters depend on a pre-existing concentration gradient of Na^+ and Cl^- to co-transport one molecule of Na^+ together with one neurotransmitter molecule from the extracellular space into the cytoplasm (Robertson et al., 2009).

The ability of AMPH to increase extracellular DA levels in regions such as the nucleus accumbens is well-established (Carboni et al., 1989; Pierce and Kalivas, 1995; Pontieri et al., 1995). Early studies already demonstrated that AMPH inhibits the reuptake of DA via the DAT (Parker and Cubeddu, 1988). However, intracytoplasmic injections of AMPH also increase extracellular DA, and this effect is blocked by the competitive DAT inhibitor nomifensine, indicating that AMPH, aside from blocking the reuptake, also enhances the release of intracellular DA (Sulzer et al., 1995; Tatsumi et al., 1997). It was also shown that the uptake of radiolabelled AMPH into striatal synaptosomes is a saturable, high-affinity, ouabain-sensitive (ouabain blocks the plasmalemmal Na^+/K^+ ATPase) and temperature-dependent process, suggesting that AMPH is itself transported into the cell via the DAT as a substrate (Zaczek et al., 1991). This notion was supported by a study employing cells transfected with human DAT, which confirmed that AMPH elicited voltage-dependent inward currents that were Na^+ dependent, ouabain-sensitive and inhibited by the DAT blocker cocaine (Sitte et al., 1998). Although it is clear that AMPH blocks the reuptake of DA and is taken up via the DAT as a substrate, it is important to note that AMPH is a

lipophilic weak base (Mack and Bonisch, 1979; Gulaboski et al., 2007) which will readily diffuse through the plasma membrane and enter the cytoplasm independently of the DAT. Thus, entry of AMPH into the cytoplasm is not dependent on the presence of DAT.

2.5.2.1 Mechanisms of DAT-mediated DA release

Exchange-diffusion

Although it is clear that AMPH releases DA via the DAT, it raises the question by which mechanism this occurs. The early exchange-diffusion model (Fischer and Cho, 1979) stated that AMPH, like DA, would bind the extracellular binding site, producing a conformational change in the DAT protein resulting in the binding site traversing the membrane, releasing AMPH into the cytoplasm and simultaneously binding a DA molecule which would then be transported outwards. This model implied that the exchange was limited to one molecule of AMPH exchanging for one molecule of DA. Although it is possible that this type of exchange does take place it has become clear that exchange-diffusion is not limited to 1:1 ratio of AMPH exchange for DA. This is evidenced by the fact that intracytoplasmic injections of AMPH also induce DAT-mediated DA efflux without AMPH first being taken up by the DAT, as mentioned above. Moreover, the DAT can be regulated, as discussed below, via intracellular second messengers to switch between "reluctant" and willing" states for AMPH-mediated DA efflux without affecting inward transport (Khoshbouei et al., 2004).

Channel-like transport

Next to the exchange-diffusion mechanism of DA release, AMPH is also capable of producing rapid bursts of DA release via the DAT. This is indicative of AMPH causing a conformational change to the DAT that result in a channel-like state, in which DA is rapidly transported outward via a pore in the transporter protein. The channel phenomenon is transient, consisting of millisecond bursts, and is inhibited by the presence of DA on both sides of the plasma membrane, and therefore not capable of transporting DA against its concentration gradient (Kahlig et al., 2005).

2.5.2.2 Regulation of DAT function and surface expression

The ability of AMPH to induce DAT-mediated DA release is considered one of its most important properties. However, this ability is highly dependent on the degree of DAT function and surface expression on the plasma membrane. These parameters are controlled by several intracellular messenger systems which are described below.

PKC phosphorylation

PKC is a serine/threonine kinase that plays an important role in regulation of DAT function and surface expression. It exists in a number of different isotypes which can be divided into the classical isotypes that are activated by Ca^{2+} and diacylglycerol and includes the α, βI/II and ɣ isotypes. A second group known as the novel PKC isotypes is not activated by Ca^{2+}, but requires only diacylglycerol. This group includes the isotypes, ε, and δ. Finally, a third group referred to as the atypical PKC isotypes are insensitive to both Ca^{2+} and diacylglycerol, and includes the isotypes ζ and λ (Mellor and Parker, 1998; Olive and Messing, 2004). Unfortunately, selective PKC modulators have not been used in many studies(Mochly-Rosen et al., 2012), and therefore not much can be concluded about the influence of specific isotypes of PKC on DAT regulation.

PKC-activating and phosphatase-inhibiting drugs both decrease the uptake of DA into striatal synaptosomes. The effect is inhibited by the PKC inhibitor staurosporine and does not affect total DAT-binding (Copeland et al., 1996; Zhang et al., 1997). Studies in striatal synaptosomes employing [^{32}P]orthophosphatase to assess the amount of DAT phosphorylation confirmed that phosphorylation of intracellular DAT-domains is regulated by PKC, and that the degree of DAT phosphorylation is negatively correlated to the efficacy of DA uptake (Vaughan et al., 1997). These results may be explained by the fact that PKC activation removes DAT from the membrane to endosomes, whereas PKC inhibition promotes the insertion of DAT into the membrane, something which has been confirmed using immunofluorescent confocal microscopy (Pristupa et al., 1998; Sorkina et al., 2003).

In addition to affecting DA uptake, PKC-mediated DAT phosphorylation also affects AMPH-induced DA release. One study found that PKC-inhibiting drugs almost completely inhibited AMPH-induced DA efflux, while increasing baseline DA uptake without the presence of AMPH (Kantor and Gnegy, 1998). DAT proteins with mutations of serine to alanine on N-terminus, the main target of PKC phosphorylation, show a strong reduction of AMPH-induced DA release (Khoshbouei et al., 2004; Foster et al., 2012). The effects of PKC-mediated DAT phosphorylation thus appear to affect both baseline DA uptake as well as AMPH-induced DA efflux. However, whereas increases in phosphorylation appear to decrease the efficacy of baseline DA uptake, the opposite appears to be the case for AMPH-mediated DA efflux, which is enhanced by phosphorylation.

Exposure to METH induces DAT phosphorylation both *in vivo* and *in vitro* (Cervinski et al., 2005) and, furthermore, even a single injection of METH *in vivo* rapidly (within 1 h) and reversibly decreased plasmalemmal DA uptake, without affecting total binding of the membrane permeable DAT ligand [^{3}H]WIN35428, indicative of METH-induced transient receptor internalization or loss of function (Fleckenstein et al., 1997c; Kokoshka et al., 1998). The loss of surface DAT following AMPH was also directly confirmed using microscopy in human DAT-expressing cells (Saunders et al., 2000). These data suggest

that AMPH produces transporter internalization in a similar way as PKC activation does. However, there appear to be subtle differences between PKC-mediated and AMPH-mediated DAT internalization. First, although AMPH-induced DAT internalization is clearly observed *in vitro* , it has been difficult to show *in vivo*, as even repeated AMPH or METH treatment do not appear to cause DAT internalization in the same way as the PKC-activating drugs do, but still produce a decrease in synaptosomal DA transport, suggesting the DAT is close to the membrane, but not functioning normally (German et al., 2012). Recent work may offer an explanation for these results as it was shown that both AMPH and PKC activation result in loss of cell surface DAT *in vitro*. However, contrary to the PKC-induced internalization, which was dependent on ubiquitination of the transporter and subsequent sorting to lysosomes for degradation, the AMPH-induced internalization sorted DAT to recycling endosomes (Hong and Amara, 2013), likely destined for rapid re-insertion into the cell membrane.

CaMKII phosphorylation

AMPH-mediated DA efflux appears to be influenced also by CaMKII. This kinase binds the DAT at the distant C terminus and, similarly to PKC, phosphorylates N-terminal serine residues, leading to enhanced AMPH-induced DA efflux. Mutations of DAT N-terminal serines to alanines which inhibit phosphorylation block this enhancement, demonstrating that both CaMKII and PKC increase AMPH-induced DA efflux via phosphorylation of the same serine residues present in the first 22 DAT N-terminal amino acids (Granas et al., 2003; Fog et al., 2006). CaMKII appears to influence AMPH-induced DA efflux via the DAT through Syntaxin A1, a SNARE protein which, aside from being involved in synaptic vesicle release, also interacts with and regulates transmembrane proteins, including DAT. Cells overexpressing Syntaxin A1 show an increased AMPH-mediated DA efflux and this effect is blocked by CaMKII inhibition, suggesting a model were binding of CaMKII at the C-terminal leads to subsequent phosphorylation of N-terminal residues which in turn promote binding of Syntaxin A1 to DAT and leading to the facilitation of AMPH-mediated DA efflux (Binda et al., 2008; Robertson et al., 2009). Interestingly, association of Syntaxin A1 with the DAT is also subject to regulation by AMPH itself (Binda et al., 2008), pointing to another target by which AMPH regulated its own effects at the DAT.

ROS signaling

Finally, aside from regulation of DAT directly via kinase-mediated phosphorylation there is also evidence suggesting that ROS signaling may be involved in regulating cell surface DAT expression (Fleckenstein et al., 2007). The oxygen radical generating enzyme xanthine oxidase reduces DA uptake into striatal synaptosomes, an effect inhibited by co-application of the free radical scavenger superoxide dismutase (Berman et al., 1996;

Fleckenstein et al., 1997b). As discussed in more detail below, AMPH is also capable of generating ROS, so also this regulatory pathway is open to modulation by AMPH itself.

Regulation of DAT function via phosphorylation and ROS are some mechanisms by which DAT surface expression and function is regulated. As these mechanisms are affected by the presence of AMPH itself, it appears that the final amount of DA released into the synapse in response to AMPH depends on a complex interplay between AMPH and intracellular second messenger systems. To add to this complexity, it has more recently become clear that AMPH can induce DA release via mechanisms independent of the DAT as well.

2.5.3 AMPH-mediated modulation of exocytotic DA release

Although non-exocytotic release via DAT-mediated reverse transport was long considered the primary mechanism by which AMPH increases extracellular DA levels, this claim has been challenged recently, as evidence mounts that AMPH also affects action potential-dependent DA release. One study which examined this demonstrated that AMPH, at 10 mg/kg, enhanced electrically evoked DA release measured within 30 minutes after the injection. Additionally, a low dose (1 mg/kg) of AMPH facilitated the DA release associated with a rewarding stimulus cue, suggesting that AMPH acts, at least in part, by altering the characteristics of action potential-dependent exocytotic DA release (Daberkow et al., 2013). The exact mechanism by which AMPH enhances the Ca^{2+}-dependent release of DA is unknown, but it may be related to the ability of AMPH to induce persistent transporter-mediated ion-leakage and excitatory conductance (Ingram et al., 2002; Branch and Beckstead, 2012; Rodriguez-Menchaca et al., 2012).

Thus, in addition to inhibiting the reuptake and enhancing the release of DA via the DAT, AMPH also enhances action potential-dependent vesicular DA release. The interplay between these mechanisms is responsible for the final behavioral and neurochemical effects of AMPH, and the relative importance of these mechanisms can be affected by several factors. It has been suggested that reuptake inhibition is primarily associated with lower drug concentrations corresponding to those in the therapeutic range, whereas doses in the recreational range shift the primary mechanism to DA release (Calipari and Ferris, 2013). Furthermore, there appear to be region-specific changes in the relative importance of DAT-dependent and action potential-dependent DA release, with enhancement of vesicular release being more important in the dorsal striatum compared to the ventral striatum (Avelar et al., 2013).

2.5.4 Effects of AMPH on secretory vesicles

In addition to releasing DA from the cytoplasm to the extracellular space, AMPH also promotes the release of DA from secretory vesicles into the cytosol. Several mechanisms by which AMPH can induce release of DA from secretory have been suggested, including AMPH weak base effects and direct interactions with the vesicular monoamine transporter 2 (VMAT-2).

AMPH is a lipophilic weak base (Mack and Bonisch, 1979; Gulaboski et al., 2007), and as such, is capable of traversing the membranes of synaptic vesicles. The lumen of secretory vesicles is acidic, with a pH of around 5.5 (Mellman et al., 1986; Njus et al., 1986). Thus, once inside the vesicle lumen, AMPH is protonated, which decreases its membrane permeability and leads to accumulation of AMPH inside the vesicles. The protonation of AMPH also results in loss of the proton gradient established by the H^+-ATPase across the vesicular membrane, which is required by the secondary active VMAT-2 transporter to sequester DA into the vesicles, and eventually leads to the release of DA into the cytoplasm (Sulzer and Rayport, 1990; Sulzer et al., 2005). The loss of DA from secretory vesicles is evidenced by a decrease in the quantal size due to the lower amount of DA in individual vesicles. However, after prolonged (6-48 hour) exposure to METH, a rebound hyperacidification and subsequent increase in quantal size has been reported (Markov et al., 2008), indicating the presence of homeostatic mechanisms capable of preventing long-term loss of DA from secretory vesicles.

In addition to promoting release by interfering with the membrane proton gradient, AMPH has affinity for VMAT-2 itself, suggesting it may also interfere with the uptake of DA into vesicles. The competitive inhibition of vesicular DA uptake together with the ongoing leakage of transmitter from the vesicles would eventually also result in a net loss of vesicular DA levels (Sulzer et al., 2005). Finally, it has been shown that repeated high-dose METH treatment decreases vesicular DA uptake without affecting total binding of the VMAT-2 ligand dihydrotetrabenazine, consistent with removal of VMAT-2 from the vesicular membrane (Brown et al., 2000; Hogan et al., 2000). Recent work with N-terminally mutated VMAT-2 indicated the presence of phosphorylation sites involved in regulating, in similarity to the DAT N-terminus, METH-mediated DA efflux (Torres and Ruoho, 2014) and provides a possible mechanism involved in the regulation of VMAT-2 membrane surface expression (Gonzalez et al., 1994; Fleckenstein et al., 2007).

2.5.5 Other AMPH targets

2.5.5.1 Regulation of MAO and TH function

In addition to its actions at the plasmalemmal DAT and secretory vesicles, AMPH also exerts influence on DA neurotransmission by other means. The ability of AMPH to block MAO has been repeatedly demonstrated both *in vitro* and *in vivo* (Mantle et al., 1976; Green and el Hait, 1978; Clarke et al., 1979; Miller et al., 1980; Robinson, 1985). Furthermore, AMPH also increases DA synthesis by enhancing TH function (Costa et al., 1972; Kuczenski, 1975), although higher concentrations appear to decrease TH function (Kogan et al., 1976; Hotchkiss and Gibb, 1980). This highlights that AMPH, at least to a certain extent, compensates for its enhancement of DA efflux by increasing cellular DA levels through inhibiting DA breakdown and increasing DA biosynthesis. As part of a normal regulatory mechanism, TH is subject to feedback inhibition by catecholamines. Although the exact mechanism by which AMPH alters TH function is not known, it is possible that AMPH competes with catecholamines for this binding site without producing the same inhibitory effect. Additionally, several kinases, including CaMKII and protein kinase A, extracellular signal-related kinase and mitogen activated protein kinase can phosphorylate a range of TH serine residues and produce effects such as activation or alleviation of feedback inhibition (Daubner et al., 2011). Since it has been shown that AMPH exposure indeed results in phosphorylation of regulatory TH serine sites (Klongpanichapak et al., 2008), this mechanism is most likely involved in the regulation of TH function by AMPH.

2.5.5.2 Outside the DA system

Aside from increasing DA levels, AMPH also increases extracellular levels of 5-HT and NE (Kuczenski and Segal, 1997) in a similar fashion, namely by inhibiting the reuptake and enhancing the release of these neurotransmitters via their respective plasmalemmal transporters (Parada et al., 1988; Rothman et al., 2001; Hilber et al., 2005). The effects of AMPH in these other monoamine systems play an important role in mediating its behavioral effects (Sloviter et al., 1978).

Several other AMPH targets are also known. Recently the trace amine associated receptor-1 (Reese et al., 2014) and σ receptor (Matsumoto et al., 2014) have received attention. The trace amine associated receptor-1 is a G-protein coupled receptor and co-localizes with DAT in certain regions. It exerts a modulatory effect on monoamine neurotransmission (Xie and Miller, 2009), and therefore also plays an important role in mediating the action of amphetamines. The importance of this receptor is demonstrated by the fact that trace amine associated receptor-1 knock-out mice show clear behavioral differences and respond much more strongly to the locomotor activating and rewarding

effects of AMPH (Achat-Mendes et al., 2012). The σ receptor is another interesting target as recent data shows this receptor to play a key role in mediating METH neurotoxicity, with blockade of the σ receptor attenuating the striatal DA depletions, cytokine activation and cognitive impairments induced by high-dose METH treatments in rodent models (Robson et al., 2013a; Robson et al., 2013b; Seminerio et al., 2013).

For AMPH and METH, the DA system is clearly important in mediating its neurochemical and behavioral effects, although it is clear that some of its effects are also mediated by different neurotransmitter systems, some of which only recently have been identified. Furthermore, once the amphetamine molecule is subjected to substitutions, its affinity for the different monoamine system changes in distinctive ways.

2.5.6 Action of substituted amphetamines and cathinones

The substituted amphetamines MDMA, 4-MMC and MDMC have a mechanism of action which is reminiscent of AMPH and METH, but differ in the ratio of their effects at various monoamine systems. As mentioned above, AMPH and METH, in addition to its action at the DAT, also display affinity for NET and SERT. However, both METH and AMPH show a strong preference for the catecholamine transporters, and have much lower affinity for the SERT (Nichols, 1994; Simmler et al., 2013). Subsequently, their main effect *in vivo* is to increase extracellular DA levels in the nucleus accumbens and frontal cortex without very pronounced effects on 5-HT release (Carboni et al., 1989; Melega et al., 1995; Shoblock et al., 2003b; Kehr et al., 2011)

2.5.6.1 MDMA

Substitutions on the phenyl ring on the amphetamine molecule produces compounds with much higher affinity for SERT, while in many cases maintaining some DA releasing properties as well (Nichols, 1994). Thus, the ring substituted amphetamine MDMA is a potent 5-HT releasing agent with relatively weak effects at the DAT (Cozzi et al., 1999; Verrico et al., 2007; Simmler et al., 2013). Thus, *in vivo*, it produces increases in both 5-HT and DA levels, but with 5-HT levels increasing more than those of DA (Kehr et al., 2011; Baumann et al., 2012). Furthermore, like AMPH and METH, MDMA also induces neurotransmitter efflux from secretory vesicles (Rudnick and Wall, 1992; Mlinar and Corradetti, 2003).

2.5.6.2 4-MMC and MDMC

The substituted cathinones 4-MMC and MDMC have similar affinity for plasmalemmal monoamine transporters as their non-keto analogues, but display a more than 10-fold lower affinity for the VMAT-2 transporter (Cozzi et al., 1999; Baumann et al., 2012;

Martinez-Clemente et al., 2012). The exact importance of the lower VMAT-2 affinity is currently unknown, however, since 4-MMC is a weak base (Santali et al., 2011) it is not unlikely that it is also capable of inducing neurotransmitter efflux from secretory vesicles in a similar way as non-keto amphetamines. *In vivo*, 4-MMC produces a neurochemical effect best described as somewhere in between MDMA and METH, producing large increases in both DA and 5-HT levels. Meanwhile, MDMC produces a neurotransmitter release pattern similar to but slightly weaker than MDMA, increasing primarily 5-HT levels (Kehr et al., 2011; Baumann et al., 2012; Wright et al., 2012b), but not as much as MDMA.

In summary, the primary effect of amphetamines is to increase monoamine neurotransmission by inhibiting the reuptake and enhancing the release of the monoamines DA, NE and 5-HT. The monoamine release is dependent on a range of interactions between AMPH and cellular constituents such as second messenger systems, secretory vesicles and enzymes. Moreover, substitutions of the amphetamine molecules produce important changes in the relative affinity of the drugs for different monoamine systems, producing a wider possible range of effects. Amphetamines have been used for a long time, both for medical as well as recreational purposes. This raises the next question, namely whether amphetamines have any long-term neurotoxic effects on the brain that persist for long after the initial effects wear off.

2.6 Evidence of amphetamine neurotoxicity

2.6.1 Preclinical studies

2.6.1.1 Studies suggesting structural toxicity

The work on METH and MDMA neurotoxicity gained pace in the 1980s as the drugs gained popularity amongst recreational users. One study (Ricaurte et al., 1982) demonstrated that high doses of METH (3 injections of 50 mg/kg spaced over 8 hours) caused destruction of nerve terminals in the rat striatum as observed 4 days after the treatments using a silver staining method, considered the gold standard of neurotoxicity testing (Switzer, 2000). Furthermore, it was reported that similar regimens also reduced levels of DA and 5-HT in the striatum and other brain regions, such as the frontal cortex and amygdala (Ricaurte et al., 1980; Ricaurte et al., 1982). The observation that high doses of METH produce neurotoxicity, visible as swollen fibers and nerve terminals indicative of axonal damage has been confirmed by other investigators (Escalante and Ellinwood, 1970; Lorez, 1981). Recently, evidence of toxicity measured using silver staining was observed for METH even at lower doses (3x 5 mg/kg) (Ares-Santos et al., 2014).

There is also evidence of structural damage to neurons after high doses of MDMA. For instance, one study reported axonal degeneration following 80 mg/kg MDMA twice daily for two days (Commins et al., 1987b). Another study where MDMA was administered twice daily for two days at doses between 25 and 150 mg/kg appears to show significant degeneration of axons at doses of 50 mg/kg and higher based on the presented graphs, although statistics are not presented (Jensen et al., 1993). Similar results were reported by Molliver et al. (1990) who administered MDMA (20 mg/kg twice daily for 4 days) and found evidence of structurally damaged axons which appeared as swollen stumps in cortical regions using silver staining. The axonal damage coincided with loss of fine axons 5-HT immunoreactivity in cortical regions 2 weeks after treatment.

2.6.1.2 Immunological and neurochemical studies

Thus, it appears that high doses of both METH and AMPH can produce structural damage to neurons, which often coincides with decreases in levels of DA or 5-HT, or the levels of their respective transporters and synthesizing enzymes. A large body of evidence suggests that high-dose amphetamines produce alterations in the DA and 5-HT systems, such as decreases in DA and 5-HT levels, DAT and SERT binding and decreases in the levels and activity of the synthesizing enzymes TH and TPH, after various dosing regimens and survival intervals.

METH

In rodents, numerous studies demonstrate that METH can affect 5-HT and DA axons. For instance, METH produces axons that appear swollen and varicose, reminiscent of what is seen with silver staining, and display a decrease in 5-HT immunoreactive boutons (Fukui et al., 1989). Similarly, both METH and AMPH were reported to produce reductions in TH-positive neurons in the caudate nucleus and frontal cortex, with AMPH also producing swollen and enlarged axons (Kadota and Kadota, 2004; Bowyer and Schmued, 2006).

Quantitative ligand-binding and neurochemical methods also suggest alterations in monoamine systems following METH. Numerous studies have reported decreases in striatal DA and 5-HT levels following treatment with METH, with at least partial recovery occurring over time (Wagner et al., 1980; Seiden et al., 1988; O'Dell et al., 1991; Cass, 1996; Callahan et al., 1998; Cass and Manning, 1999). Also DAT and SERT levels, as measured by ligand-binding or uptake measurements, are decreased following METH (Hirata and Cadet, 1997; Brown et al., 2000; Ladenheim et al., 2000; Schroder et al., 2003; Armstrong and Noguchi, 2004). Finally, decreases in the levels and function of the synthesizing enzymes TH and TPH have been reported, also with recovery occurring as the survival interval increases (Hotchkiss and Gibb, 1980; Bakhit et al., 1981; Haughey et al., 1999; Schroder et al., 2003).

MDMA

MDMA appears to have an effect on 5-HT axons, but not on DA axons, in rats. For instance, decreased 5-HT positive neurons were observed in the parietal cortex and forebrain regions following treatment with high doses of MDMA, and similar to METH, the remaining axons were ablated or thick and varicose. However, no damage was observed to the cell bodies in the raphe nucleus or to catecholaminergic neurons. Furthermore, the loss of 5-HT axons recovered to a large extent after one year (O'hearn et al., 1988; Molliver et al., 1990; Scanzello et al., 1993).

The loss of 5-HT immunoreactivity, as expected, coincides with loss of 5-HT and SERT-binding in brain regions including the cortex, striatum, hippocampus and hypothalamus. Also decreases in TPH function have been reported. The alteration of these markers commonly recovers with time. Further, the alterations are limited to the 5-HT system, and no studies report any alterations in the catecholaminergic systems in rats (Stone et al., 1986; Battaglia et al., 1987; Battaglia et al., 1988; Souza et al., 1990; Sprague et al., 1994; Armstrong and Noguchi, 2004).

Notably, MDMA appears to produce pronounced differences in its toxicity profile depending on what species is used for testing. In mice, even very high doses of MDMA (60 mg/kg) do not affect 5-HT system markers such as brain 5-HT or TPH activity levels (Stone et al., 1987), but instead decrease DA levels, particularly in the striatum (Logan et al., 1988; O'Callaghan and Miller, 1994).

Studies in non-human primates

Studies in rhesus monkeys, vervet monkeys and baboons support the plasticity profile observed in rats, with METH decreasing DA as well as DAT and VMAT-2 binding in various brain regions such as the striatum and cortex, but with recovery occurring over time (Woolverton et al., 1989; Melega et al., 1997; Villemagne et al., 1998; Hashimoto et al., 2004). Meanwhile, MDMA affects only the 5-HT system, as observed by decreased levels of 5-HT and SERT binding as well as loss of 5-HT positive axons in brain regions including cortical regions and the hippocampus, again with at least partial recovery occurring as the time between the drug treatments and the measurements increases (Insel et al., 1989; Souza et al., 1990; Hatzidimitriou et al., 1999).

It seems clear that high doses of METH and MDMA can induce consistent and long-term alterations to the brain DA and 5-HT systems in several species. This raises the question if these alterations have any significance in terms of their effect on important brain functions, such as those related to cognition, anxiety and mood.

2.6.1.3 Long-lasting cognitive and behavioral effects of amphetamines

In rodents, cognition and neurobehavioral function can be assessed using a range of animal behavioral tests. Examples include memory tests such as the novel object recognition test and the Morris water maze, tests of anxiety-like behavior in emergence tests, elevated plus maze tests and social interaction tests as well as the forced swim test, said to measure aspects of depression (McGregor et al., 2003a; Clemens et al., 2004; Clemens et al., 2007).

METH

METH was shown to decrease recognition memory in the novel object recognition test for up to several weeks after drug treatments (Schroder et al., 2003; Marshall et al., 2007; Herring et al., 2008). Also decreases in spatial memory have been observed using tests such as the radial arm maze and Morris water maze (Nagai et al., 2007; Camarasa et al., 2010). However, other studies report no effect on memory using the novel object recognition test (Clemens et al., 2007). Further, inconsistent results are obtained for anxiety-related behavior depending on the type of test used, and no effect was found on the forced swim test (Clemens et al., 2004; Clemens et al., 2007).

MDMA

With MDMA, the evidence of persistent long-lasting effects on rodent behavioral tests is also difficult to interpret. Evidence of decreased memory performance has been reported using a variety of behavioral paradigms (Marston et al., 1999; Mechan et al., 2002; Piper and Meyer, 2004), as have increases in anxiety and depressive-like behavior using such tests as the elevated plus maze, emergence test, social interaction test and forced swim test (McGregor et al., 2003a; McGregor et al., 2003b). However, decreases in anxiety after MDMA has been observed using these same tests (Mechan et al., 2002; Piper and Meyer, 2004), and also memory deficits following MDMA are not always observed (McGregor et al., 2003b). In fact, some studies found no evidence of impairment in MDMA-treated animals tested on a wide range of behavioral tests, including memory tests such as the radial arm maze and the Morris water maze, despite the fact that large decreases in cortical 5-HT levels were present (Seiden et al., 1993; Marston et al., 1999), indicating that the relationship between observed monoamine system deficits and functional outcome in terms of behavior and cognitive function is not straightforward.

In summary, the effects of amphetamines on outcomes related to behavioral measures of cognition and neuropsychiatric function are inconsistent. Although alterations in tests measuring aspects of memory are sometimes reported, this is also by far the most studied outcome measure, where type I errors and publication bias may well produce a

skewed picture. The inconsistency may also suggest that the measured effects are not causally related to neurotoxicity but rather reflect changes in behavior due to stress associated with non-contingent high-dose drug treatments.

2.6.2 Human studies

A number of different types of studies and study designs have been used to assess the effects of amphetamines in humans. Most commonly, these studies employ neuroimaging, cognitive testing or, ideally, a combination of these. Certain studies have even examined post-mortem brain tissue of amphetamine users. Unfortunately, the cross-sectional design employed in most of these studies do not allow for firm conclusions to be drawn about causality of the observed effects (see also 6.6.1).

2.6.2.1 Neuroimaging

Positron emission tomography and SPECT

Positron emission tomography and SPECT have been used to assess brain monoamine ligand binding and metabolism in human METH users. In analogy to what is seen in animals, these studies also show evidence of monoaminergic deficits, such as decreased DAT and VMAT-2 availability, as evidenced by decreases in [^{11}C]WIN-35,428 and [^{11}C]dihydrotetrabenazine binding in brain regions including the striatum and frontal cortex (McCann et al., 1998b; Sekine et al., 2001; Johanson et al., 2006). Metabolic positron emission tomography studies with [^{18}F]fluorodeoxyglucose suggest that METH users show altered resting state or cognitive task-dependent metabolic activity in numerous cortical and subcortical brain regions which sometimes, but not always, is associated with lower cognitive performance or increases in neuropsychiatric symptoms such as anxiety and depression (Volkow et al., 2001a; London et al., 2004; Kim et al., 2005; London et al., 2005; Kim et al., 2009)

In abstinent MDMA users, the findings from positron emission tomography and SPECT studies also generally appear to support what is seen in preclinical studies. Abstinent MDMA users show decreases in binding of the SERT ligands [^{11}C]-McN5652 and [^{11}C]DASB in brain regions including the cortex, thalamus, hypothalamus and caudate nucleus. Additionally, abstinent MDMA users perform worse on tests of memory function, and their performance on these tests is correlated with the SERT-binding observed in the insula and hippocampus (McCann et al., 1998a; Kish et al., 2010). However, a study employing SPECT to assess [^{123}I]beta-CIT binding to SERT found a decrease only in recently abstinent female, but not male, MDMA users. Furthermore, in this study, the decreases in binding appeared to recover with protracted abstinence and in fact reached a level higher than control subjects in the group of subjects with prolonged abstinence

from MDMA (Reneman et al., 2001). In addition to changes in SERT-binding, decreased metabolism has also been reported with [^{18}F]fluorodeoxyglucose positron emission tomography in regions such as the cingulate, hippocampus and amygdala and striatum, however, no correlation was found between metabolism and the amount of MDMA consumed (Obrocki et al., 1999; Buchert et al., 2001).

Structural MRI

Both structural and functional MRI studies have reported differences between METH users and non-users. It has been reported that abstinent METH users have lower gray matter density in the frontal cortex as well as decreased cognitive task performance compared to drug-free control subjects (Kim et al., 2006). Another study found decreased cingulate and hippocampal volumes that correlated with performance on a verbal memory test (Thompson et al., 2004). Correlations between increased METH exposure and reductions in the volume of the prefrontal cortex have also been described (Daumann et al., 2011). Interestingly, striatal volume is reportedly increased in abstinent METH users in a study which, however, did not find any evidence of decreased cognitive performance (Chang et al., 2005). Another study found increased putamen volume in METH users that correlated negatively with impulsivity scores (Jan et al., 2012). Decreased cortical and hippocampal volumes have also been reported in MDMA users (Cowan et al., 2003; den Hollander et al., 2012)

Functional MRI

Functional MRI studies also have reported differences in amphetamine users, such as decreased activity in the cingulate and prefrontal cortex of METH users that correlated with higher impulsivity scores (Monterosso et al., 2007; Hoffman et al., 2008). Decreased activation was also observed in frontal and insular cortex of METH users while performing a Stroop task, although actual task performance was not consistently decreased (Salo et al., 2009; Nestor et al., 2011). In MDMA users a weaker signal while performing a working memory task was noted in the frontal and temporal cortex in heavy compared to light users. However, when comparing users to non-users, there was no clear effect. Furthermore, task performance was also similar between MDMA users and controls (Daumann et al., 2003). Conversely, another study reported an increased signal in the frontal cortex in response to a memory task, on which MDMA users also were not impaired (Moeller et al., 2004). Similarly, hippocampal signal intensity in response to episodic or associative memory tasks revealed alterations in MDMA users versus controls, and in continuing versus abstinent MDMA users. However, also here no differences were observed on the actual task performance (Daumann et al., 2005; Becker et al., 2013).

2.6.2.2 Effects on cognition, mood and anxiety

Some studies suggest that abstinent METH users show decreases in measures of cognitive function. Deficits in abstinent METH users compared to control subjects have been observed on tests such as immediate and delayed recall and digit span tests, which measure performance on memory-related domains, such as verbal memory, long-term declarative memory and working memory. Furthermore, deficits are also observed on tests such as the Wisconsin card sorting test, Stroop test and trail-making test, indicating deficits also in the cognitive domains of executive function and attention (Simon et al., 2000; Kalechstein et al., 2003; Han et al., 2008; Rendell et al., 2009). Furthermore, METH users appear to experience higher levels of anxiety and depression than non-users (Zweben et al., 2004; Vik, 2007).
Similar to METH, abstinent MDMA users also show deficits in certain cognitive domains. Also here, the most common observations include deficits in short term and long term memory, verbal reasoning, attention and executive functions as well as decreased performance in tests reflecting aspects of general intelligence (Parrott et al., 1998; McCann et al., 1999; Gouzoulis-Mayfrank et al., 2000; Heffernan et al., 2001; von Geusau et al., 2004). Other studies of abstinent MDMA users have also reported higher ratings of depression and anxiety in addition to decreases in memory performance (McCardle et al., 2004; Thomasius et al., 2006).

The main issue with these studies is that drug users and non-users are often not properly matched in terms of age, education, IQ and other relevant variables. In an attempt to minimize these confounding factors one study recruited all participants from the same demographic group (rave attendees). Furthermore, drug testing at the time of the neuropsychological evaluation was used to exclude subjects acutely under the influence of any substances. Here, no evidence was found of any consistent deficits in MDMA users. MDMA users showed lower vocabulary scores, but since these are usually not affected by neurological insults they were interpreted as a pre-existing difference (Halpern et al., 2011). The lack of consistent results highlights the difficulty in interpreting cross-sectional studies and has led to a number of attempts at gaining more insight into actual drug-dependent effects through a prospective study design.

2.6.2.3 Prospective studies

The first large prospective cohort study of MDMA users was done in The Netherlands. Subjects who were considered to be at high risk of commencing MDMA were recruited for an in-depth set of neuroimaging experiments at the time of recruitment, prior to using MDMA, and during a follow-up within 3 years. Individuals who started using MDMA showed decreased cerebral blood flow in the globus pallidus and putamen and decreased fractional anisotropy in thalamic and cortical white matter, compared to subjects who did not use MDMA. Importantly, however, no difference was observed between groups on

the most important outcome measure, namely SERT binding. A dose-response relationship between SERT binding and cumulative MDMA use was also not present (de Win et al., 2008). Cognitive function was also assessed. Those who started using MDMA scored significantly lower on immediate and delayed verbal recall compared to subjects who did not. Nonetheless, scores were within the normal range, and no effects were observed on any other neurocognitive tests. Most importantly, the verbal memory scores of MDMA users did not decrease, as the difference between groups was in fact due to an increase in performance of the non-users during follow-up (Schilt et al., 2007; Krebs and Johansen, 2008).

Another prospective study also assessed MDMA-naïve subjects at baseline and during a follow-up after 12 months. Subjects were tested on a wide range of neurocognitive tests. Like in the Dutch study, no effects were found on verbal memory or other measures of neurocognitive function, with significant differences between groups being reported only for immediate and delayed visual memory. Here, the difference was indeed due to a decreased performance in the MDMA users, whereas the performance of individuals who stayed MDMA-naïve remained stable (Wagner et al., 2013).

2.6.2.4 Studies on post-mortem tissue

Aside from neuroimaging and cognitive studies in amphetamine users, analysis have also been made of post-mortem brain material and a decrease in striatal DA, DAT and TH levels have been reported in the brains of subjects who had used METH (Wilson et al., 1996). Also a decrease in D_1 receptor-mediated stimulation of adenylyl cyclase in response to DA has been reported (Tong et al., 2003), suggesting alterations both in the level and function of DA-related proteins in the brains of METH users. However, since the brains in these studies are likely sourced from individuals who died as a result of a METH overdose, their relevance for normal recreational users must be questioned.

In summary, studies examining amphetamine users and non-users appear to show certain differences in brain function, such as decreases in monoamine transporter binding, metabolism and MRI signal intensities. Other studies also suggest decreased memory performance, but it remains difficult to interpret the results from these studies, due to inherent limitations of the study designs. Nonetheless, a large body of literature suggests that amphetamines have some long-term effects. A number of subsequent studies have attempted to outline what mechanisms may be involved in producing these effects.

2.7 Mechanisms of amphetamine neurotoxicity

A number of mechanisms are thought to play a role in amphetamine neurotoxicity. The four mechanisms which have received the most are oxidative stress, mitochondrial dysfunction, excitotoxicity and hyperthermia. Additionally, a number of other processes, including inflammation and blood-brain barrier disruption have been implied to play a role. Also other neurotransmitter systems, such as the cannabinoid system and σ receptors, are now thought to play a role in mediating amphetamine neurotoxicity (Yamamoto et al., 2010; Achat-Mendes et al., 2012; Goncalves et al., 2014; Halpin et al., 2014; Matsumoto et al., 2014). The proposed mechanisms are summarized in Table 2.2 and discussed in more detail below.

Mechanism	Section	Brief description
ROS and oxidative stress	2.7.1	- Oxidative stress is a major contributor to amphetamine neurotoxicity. - Sources of ROS include (semi-)quinones from DA as well as reactive drug metabolites
Mitochondrial dysfunction	2.7.2	- Amphetamines can inhibit specific complexes of the mitochondrial ETC. - The subsequent decrease in ATP production may produce energy deficits. - Inhibition of mitochondrial complexes results in electron leakage and production of the superoxide radical.
Excitotoxicity	2.7.3	- A number of excitotoxic processes mediate amphetamine toxicity. - Activation of Ca2+-dependent enzymes such as xanthine oxidase, nitric oxide synthase and calpain causes production of superoxide and peroxynitrite and can initiate proteolysis of cytoskeletal protein.
Hyperthermia	2.7.4	- Amphetamines increase core body temperature. Further increasing body temperature by administering the drug in high ambient temperature exacerbates toxicity while preventing hyperthermia attenuates toxicity. - Hyperthermia may catalyze the rate at which other toxic processes proceed.
Other mechanisms	2.7.5	- A number of other mechanisms have been suggested to be involved in amphetamine toxicity. - Examples include inflammation, defects in the ubiquitin proteasomal system, blood-brain-barrier disruption and altered neurogenesis.

Table 2.2 The table lists the primary mechanisms proposed to be involved in amphetamine neurotoxicity as well as a brief description of each mechanism and a reference to the section where they are discussed in more detail.

2.7.1 ROS and oxidative stress

ROS are highly reactive molecules that can easily react with numerous biomolecules, including cellular lipids, proteins and DNA. This reaction leads to alteration of their composition or conformational structure, thereby rendering the affected molecules incapable of performing their normal functions. The damaging reactions between ROS and cellular constituents are known as oxidative stress. (Balasubramanian et al., 1998; Du and Gebicki, 2004; Catala, 2009).

There are several types of oxidative stress. Protein oxidation refers to ROS forming covalent bonds with amino acids, for instance at the sulfur atom present on cysteine, thereby posing a threat to a vast number of proteins (LaVoie and Hastings, 1999). Lipid peroxidation is the process by which radicals oxidize cellular lipids. Particularly polyunsaturated fatty acids, such as those in cellular membranes, are vulnerable to oxidation. Aside from damaging membranes and other cellular constituents, the products of lipid peroxidation, such as hydroxy-2-nonenal, resulting from the oxidation of fatty acids, are themselves reactive, and can initiate adduct formation with other biomolecules such as proteins and ribonucleic acids (Sprague and Nichols, 1995; Yamamoto and Zhu, 1998; Gluck et al., 2001; Catala, 2009), resulting in further damage.

2.7.1.1 Sources of amphetamine-induced ROS

Some of the major pathways of reactive species generation as a result of amphetamines are summarized in Fig. 2.7. One of the major sources of increased oxidative stress following amphetamines is the large increase in cytoplasmic and extracellular DA levels, and the subsequent metabolism of DA into toxic metabolites (Yamamoto and Raudensky, 2008). DA can be both enzymatically and non-enzymatically metabolized, and both routes can result in the production of reactive products. DA can be by MAO, resulting in the production of 3,4-dihydroxyphenylacetaldehyde, the transient precursor of 3,4-dihydroxyphenylacetic acid (DOPAC), and hydrogen peroxide. When glutathione is incapable of detoxifying all the hydrogen peroxide, it may react with Fe^{2+} in a Fenton reaction, resulting in the production of the hydroxyl radical OH•. Moreover, both DA and 3,4-dihydroxyphenylacetaldehyde can undergo auto-oxidation to form DA- or 3,4-dihydroxyphenylacetaldehyde-(semi)quinones (Sulzer and Zecca, 2000; Anderson et al., 2011). Both OH• and DA-(semi)quinones are highly reactive species, capable of reacting with a plethora of different biomolecules (LaVoie and Hastings, 1999; Munoz et al., 2012)

Other potential sources of ROS are amphetamine metabolites themselves. Particularly MDMA has been implied as a source of reactive metabolites. The catechol-like hepatic metabolites of MDMA, 3,4-dihydroxymethamphetamine (HMMA) and 3,4-dihydroxyamphetamine (HHA) are thought to be capable of auto-oxidizing, much like DA itself. The resulting quinones are highly reactive and could easily form thioether

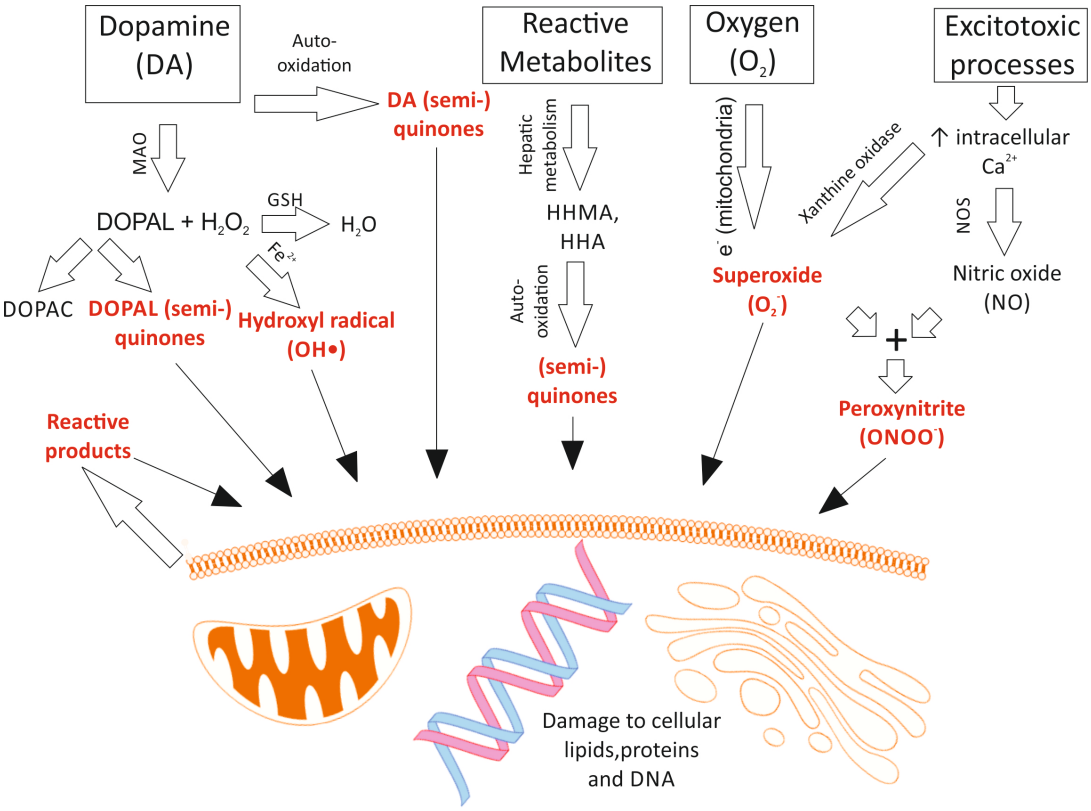


Figure 2.7 Overview of important pathways for generation of reactive species. Major sources of reactive species are DA, reactive metabolites of amphetamines, oxygen in the mitochondria and excitotoxic processes (in black boxes). The reactions leading to reactive species (marked in red) are indicated by thick arrows. DA metabolism produces reactive DA-quinones and hydrogen peroxide which may lead to production of hydroxyl radicals. The transient DA metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL) can form 3,4-dihydroxyphenylacetaldehyde -quinones. In mitochondria, electrons escaping the electron transport chain may reduce oxygen, producing superoxide while excitotoxic processes, which increase Ca^{2+} levels, may produce additional superoxide via activation of enzymes such as xanthine oxidase. Increased nitric oxide synthase (NOS) activity elevates nitric oxide, which can react with superoxide to produce peroxynitrite. These reactive species may damage cellular lipids, proteins and DNA. Furthermore, lipid peroxidation may itself produce reactive products which cause further damage.

conjugates with sulfur-containing compounds such as glutathione and N-acetyl-cysteine. These thioether compounds retain the ability to redox cycle and are toxic to 5-HT neurons. In fact, they are more toxic than MDMA itself (Monks et al., 2004; Ferreira et al., 2013). Evidence supporting this pathway comes from detection of the abovementioned adducts *in vivo* (de la Torre and Farre, 2004; Perfetti et al., 2009).

Finally, another very important reactive species is superoxide (O_2^-). Superoxide is produced, for instance, during DA oxidation. Moreover, a number of other enzymes produce superoxide during their normal functioning. However, another important source of amphetamine-induced superoxide production is the mitochondrion (see section 2.7.2).

2.7.1.2 Role of oxidative stress in amphetamine toxicity

Support for the involvement of oxidative stress as a causal mechanism in amphetamine toxicity comes from a number of studies demonstrating attenuation of METH-induced monoamine loss with antioxidants such as N-acetyl-cysteine, ascorbic acid, vitamin E and selenium (Wagner et al., 1985; De Vito and Wagner, 1989; Fukami et al., 2004; Barayuga et al., 2013). Further, both METH and MDMA were found to increase the production of OH•, and pre-treatment with spin trapping agents or antioxidants attenuated the long-term monoamine depletions normally observed after these amphetamines (Colado and Green, 1995; Yamamoto and Zhu, 1998; Shankaran et al., 1999). Accordingly, depletion of glutathione, inhibition of superoxide dismutase and the feeding of a selenium-deficient diet all increase the toxicity observed after MDMA and METH (De Vito and Wagner, 1989; Sanchez et al., 2003; Chandramani Shivalingappa et al., 2012).

2.7.1.3 Factors mediating ROS production

DA

As mentioned in the beginning of this section, DA is an important factor in amphetamine-induced ROS production. Further evidence for this comes from the fact that blockade of DAT and inhibition of DA synthesis both reduce the METH-induced loss of DA, 5-HT or TPH activity (Schmidt et al., 1985; Marek et al., 1990; Yamamoto and Zhu, 1998). Additionally, the potent electron transport chain inhibitor 6-hydroxydopamine has been observed in the brain following METH treatment and are thought to also be a result of oxidation of DA by OH• (Seiden and Vosmer, 1984; Commins et al., 1987a; Glinka et al., 1997; Wrona et al., 1997). Oxidative stress following METH, as measured by lipid peroxidation and protein oxidation, was significantly decreased in PKCδ knockout mice or mice treated with the PKCδ-inhibitor rottlerin. These mice also did not show any METH-induced alterations in TH, DAT and VMAT-2 function. Since inhibitors of other PKC isoforms had no effect, these results suggest a specific role of the PKCδ isoform in mediating METH-induced oxidative

stress (Shin et al., 2012). These data are particularly interesting considering the role of PKC-mediated phosphorylation in enhancing AMPH-induced, DAT-mediated DA efflux (see section 4.3.2.2).

DA in MDMA toxicity

Less obvious may be that DA also plays a role in mediating MDMA toxicity, as this drug exerts less effect on DA neurotransmission than METH. Nonetheless, MDMA-induced decreases in 5-HT were potentiated by levodopa administration, while inhibition of DA synthesis protected against decreases in 5-HT and TPH function (Stone et al., 1988; Schmidt et al., 1990). Moreover, the DAT blocker mazindol decreased the amount of MDMA-induced production of OH• and loss of 5-HT and TPH function, while knockdown of MAO-B using by antisense oligonucleotides attenuated the loss of 5-HT levels in the striatum (Shankaran et al., 1999; Falk et al., 2002), indicating an important role of DA oxidation in mediating the toxic effects of MDMA. It has been even shown that MDMA increased tyrosine levels in the hippocampus and that administration of extra tyrosine enhanced, while administration of an aromatic amino acid decarboxylase-inhibitor decreased, MDMA-induced 5-HT depletion. These results suggest a mechanism whereby levodopa, following enzyme-independent hydroxylation from tyrosine, is converted to DA by aromatic amino acid decarboxylase (Breier et al., 2006), and demonstrate that DA may play a role in MDMA toxicity even in brain regions very sparsely innervated by DA, such as the hippocampus.

Excitotoxicity

A number of additional sources of amphetamine-induced oxidative stress are related to excitotoxicity, and are discussed in more detail in section 2.7.3.

2.7.2 Mitochondrial dysfunction

Amphetamines can inhibit specific complexes of the mitochondrial ETC, resulting in increased levels of ROS and deficits in energy production (Fig. 2.8). METH and MDMA are known to decrease the levels and activity of mitochondrial complexes I, II and IV in the striatum (Burrows et al., 2000a; Brown et al., 2005; Klongpanichapak et al., 2006; Puerta et al., 2010). MDMA was also shown to cause deletions in mitochondrial deoxyribonuncleic acid coding for subunits of complexes I and IV following a two week survival interval (Alves et al., 2007). More recently it was shown that even relatively low doses of both AMPH and METH (2mg /kg or less) decrease complex I, II or IV activity in various brain regions, including the striatum, frontal cortex and amygdala in tissue obtained from animals sacrificed while still under the influence of the drug (Feier et al., 2012).

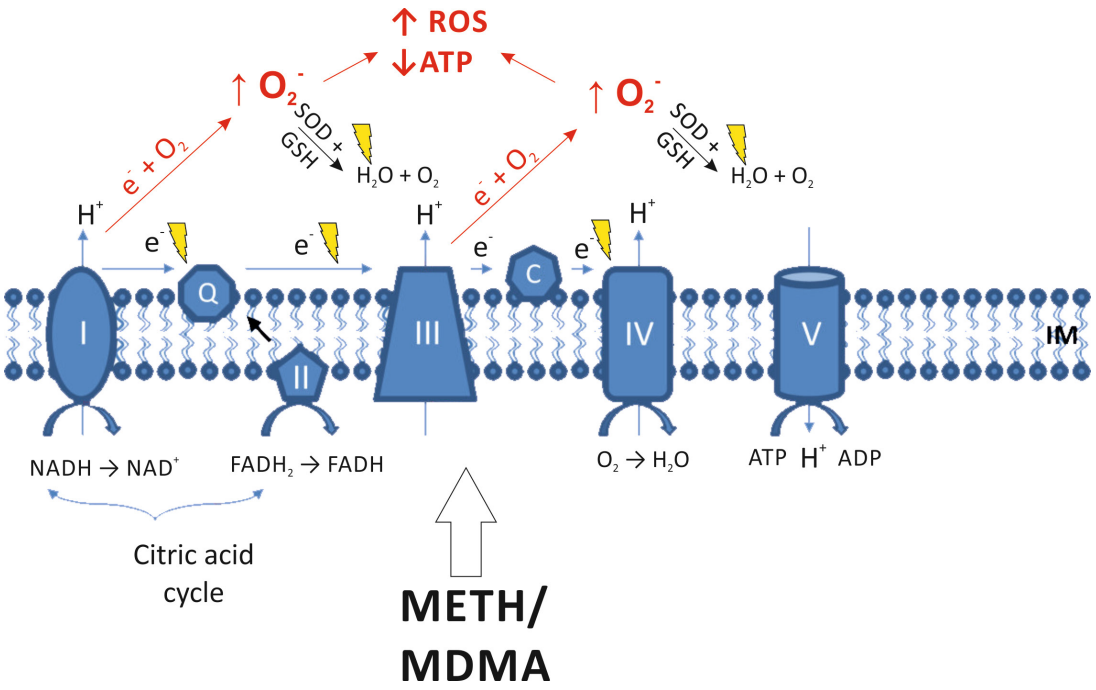


Figure 2.8 The figure shows the mitochondrial ETC which consists of complex I (NADH dehydrogenase), complex II, (succinate dehydrogenase), complex III (ubiquinone-cytochrome c reductase), complex IV (cytochrome c oxidase) and complex V (ATP synthase). METH and MDMA are known to inhibit several mitochondrial complexes, including I, II and IV. The resulting breakdown of the normal productive electron flow through the mitochondrial complexes (marked by yellow lightning strikes next to the electrons), necessary to produce the electrochemical H^+ gradient needed for ATP production, results in increased leakage of electrons from the ETC (red arrows from complexes I and III), where they readily react with molecular oxygen. The subsequent superoxide production can exceed the capacity of superoxide dismutase (SOD) and glutathione (GSH) (again marked with yellow lightning strikes), resulting in increased production of superoxide and ROS, as well as a cellular energy deficit due to the inhibited ATP production. Figure adapted from Li et al. (2013).

Inhibition of the mitochondrial ETC by amphetamines can result in reduction of ATP levels following impairment in oxidative phosphorylation, as well as increases in oxidative stress due to excessive leakage of superoxide from the collapsed ETC. It has even been suggested that amphetamine-induced mitochondrial dysfunction may cause apoptosis.

2.7.2.1 Failure of ATP production and cellular energy deficit

It is known that METH decreases ATP and oxygen levels in the striatum and cortex while increasing AMP levels (Shiba et al., 2011). The role of decreased ATP levels in amphetamine toxicity is demonstrated by an experiment where neural glucose uptake was prevented by administration of 2-deoxyglucose, a manipulation that enhanced both the decrease in ATP levels observed directly after METH treatment as well as the loss of

striatal DA one week later (Chan et al., 1994). Similarly, inhibition of metabolism by directly interfering with the ETC also increases toxicity. For instance, the complex II inhibitor malonate exacerbated METH-induced depletions of striatal DA levels (Burrows et al., 2000b). Similarly, malonate decreased both 5-HT and DA levels after a 5 day survival interval at a dose of MDMA which did not produce any reductions in the levels of these transmitters on its own (Darvesh et al., 2005). One cannot conclude with certainty that the effects on monoamine levels are due to decreased ATP, as the toxicity may also have been exacerbated due to oxidative stress resulting from ETC inhibition (see below).

However, since application of the energy substrates decylubiquinone or nicotinamide attenuated the METH-induced loss of striatal DA after 7 days, it is likely that it does play a role, particularly as this effect was only present when the substrates were infused 6 hours following METH administration, but had no effect when they were administered together with METH (Stephans et al., 1998), suggesting that the protective effects were mediated by increasing ATP levels in the time following METH treatment. Deficient energy metabolism may have important consequences. Aside from producing monoaminergic toxicity, it has even been suggested that METH-induced neurotoxicity occurring from bioenergetics failure in brainstem regions may be directly responsible for cardiovascular collapse occurring with lethal METH doses, as it was shown that co-treatment with coenzyme-Q10 in the medulla prevented both superoxide production and death (Li et al., 2012).

2.7.2.2 Superoxide leakage and increased oxidative stress

Aside from reducing ATP levels, ETC inhibition can also exacerbate amphetamine toxicity by increasing oxidative stress. During normal mitochondrial functioning, large amounts of electrons are transported through the ETC, eventually leading some electrons to escape and react with molecular oxygen to form superoxide, the primary reactive species in mitochondria. During normal oxidative phosphorylation it is estimated that about 1-4% of mitochondrial oxygen will be reduced to superoxide. However, when mitochondrial

complexes are inhibited and the existing electron flow through the complexes is blocked, the electron leak and subsequent superoxide production will increase dramatically. Normally, the amount of superoxide dismutase, glutathione and catalases present in mitochondria is sufficient to detoxify the produced superoxide, but the increased electron leakage following amphetamine-mediated ETC inhibition may outrun the capacity of detoxification enzymes, leading to the generation of other reactive species such as OH• and subsequent damage to cellular components. The exact source of electron leakage from within the ETC following inhibition from METH and MDMA is hard to predict, as it depends on several factors, including mitochondrial substrate utilization status and which specific complexes are inhibited, however, it is clear that any inhibition of mitochondrial complexes will increase ROS production (Adam-Vizi, 2005). The result is clear, as the ROS

production, which is already increased following amphetamines, will be further enhanced due to increased mitochondrial ROS (Brown and Yamamoto, 2003), thereby further increasing the likelihood of oxidative stress-mediated toxicity.

2.7.2.3 Apoptosis

Finally, the mitochondrial-dependent apoptotic pathway has also been suggested to play a role in amphetamine toxicity (Cadet et al., 2005). METH increases the expression of pro-apoptotic genes such as BAD, BAX and BID, while decreasing the expression of anti-apoptotic genes Bcl-2 and Bcl-XL (Jayanthi et al., 2001; Krasnova et al., 2005; Beauvais et al., 2011). Indeed, METH has been shown to trigger the release of apoptosis inducing factor, cytochrome c and smac/DIABLO from the mitochondria to the cytoplasm, leading to the recruitment of caspases and initiation of apoptotic cell death (Jayanthi et al., 2004). Both overexpression of Bcl-2 and inhibition of caspases, protects against METH toxicity (Cadet et al., 1997; Uemura et al., 2003). It is important to note that this effect has primarily been observed following very high doses of METH or *in vitro*, and the *in vivo* relevance remains unclear (Cadet et al., 2003).

2.7.3 Excitotoxicity

Excitotoxicity is the process by which excessive stimulation of glutamate receptors leads to influx of toxic levels of Ca^{2+} into neurons. All types of glutamate receptors, including the metabotropic glutamate receptors, have been implicated in the process of excitotoxicity, but the N-methyl-D-aspartate (NMDA) receptor is considered to be the primary mediator, due to the high capacity of its ion channel for Ca^{2+} transport. The high levels of intracellular Ca^{2+} lead to number of downstream processes such as depolarization of the mitochondrial membrane potential, activation of Ca^{2+}-dependent enzymes including nitric oxide synthase and PKC (primarily the classical Ca^{2+}-dependent isoforms α, βI/II and ɣ have been associated with excitotoxicity), as well as the activation of various phospholipases, phosphatases and proteases such as calpain and caspases which can have potentially detrimental effects on cellular components including mitochondria, endoplasmatic reticulum, cytoskeletal proteins and DNA, and may eventually cause cell death via apoptosis or necrosis (Buchner et al., 1999; Mark et al., 2001; Arundine and Tymianski, 2003; Dong et al., 2009; Lee et al., 2014).

2.7.3.1 Glutamate receptor involvement

Studies employing in vivo microdialysis have shown that METH increases glutamate release in the striatum (Nash and Yamamoto, 1992; Stephans and Yamamoto, 1994; Mark et al., 2004), and that blockade of metabotropic glutamate receptors attenuates METH-induced depletion of striatal DA and other markers of toxicity (Battaglia et al., 2002; Shah

et al., 2012), indicating that aspects of excitotoxicity play a role in METH toxicity. NMDA receptor blockade using dizocilpine also attenuates METH toxicity (Farfel et al., 1992; Bowyer, 1995). However, since NMDA receptor antagonism also decreases METH-induced hyperthermia, it is impossible to determine whether the protective effects are due to attenuation of excitotoxicity-mediated aspects of toxicity or if it is a general protective effect mediated by the attenuation of hyperthermia (see below). Furthermore, the NMDA antagonist amantadine apparently increases, rather than attenuates, the toxicity of METH and this demonstrates that NMDA receptor antagonism is not always sufficient to prevent METH toxicity. Importantly, amantadine also produces actions besides NMDA receptor antagonism that may influence its effects of amphetamine toxicity (Ossola et al., 2011; Thrash-Williams et al., 2013).

2.7.3.2 Intracellular excitotoxic processes

The increases in intracellular Ca^{2+} activates downstream processes that lead to further increases in ROS production and to the activation of Ca^{2+}-dependent proteases, both of which can damage cellular components.

Excitotoxicity-mediated ROS production

The METH-induced glutamate release and subsequent increases in intracellular Ca^{2+} may lead to increased ROS production in several ways. Increased Ca^{2+} leads to activation of enzymes such as calpain and xanthine oxidase. Xanthine oxidase catalyzes the conversion of xanthine to uric acid in a process that also produces superoxide. Also Ca^{2+}-dependent activation of phospholipase A_2, which cleaves membrane phospholipids and releases arachidonic acid, may result in production of superoxide, following lipoxygenase- and cyclooxygenase-mediated metabolism of arachidonic acid (Yamamoto and Raudensky, 2008).

Another enzyme dependent on Ca^{2+} that is elevated during METH treatments is nitric oxide synthase, which catalyzes the conversion of arginine to nitric oxide. NO can readily react with superoxide to produce peroxynitrite ($ONOO^-$), a highly reactive anion of the reactive nitrogen species class. Like ROS, it readily reacts with a range of biomolecules and its targets include lipids, proteins as well as DNA (Pacher et al., 2007). Peroxynitrite produces toxicity through nitrosylation, the addition of nitric oxide to proteins, in particular to tyrosine and cysteine residues. Direct evidence for its involved in METH toxicity comes from data showing that METH-induced depletion of DA is accompanied by increased levels of striatal nitrate and nitrotyrosine, indicative of nitrosative toxicity (Anderson and Itzhak, 2006; Wang et al., 2008). Furthermore, blockade of nitric oxide synthase provides full protection against METH-induced loss of DA (Itzhak and Ali, 1996) while co-administration of a peroxynitrite decomposition catalyst together with METH

partially prevents toxicity (Imam et al., 1999). Even specific target proteins have been identified, as METH-induced nitrosylation coupled with loss of function has been observed on the VMAT-2 protein (Eyerman and Yamamoto, 2007).

Activation of proteases

Another event associated with excitotoxicity and increased intracellular Ca^{2+} levels is proteolysis of the cytoskeletal proteins, such as spectrin, by the Ca^{2+}-activated enzyme calpain (Lee et al., 1991). Spectrin proteolysis has been observed in the rat striatum following METH treatments, while METH- and MDMA-induced increases in calpain activation and spectrin proteolysis were blocked by the calpain inhibitor calpastatin *in vitro* (Staszewski and Yamamoto, 2006; Warren et al., 2007; Suwanjang et al., 2012).

The involvement of glutamate receptors as well as downstream mechanisms such as spectrin-mediated proteolysis of cytoskeletal proteins and increased nitric oxide synthesis and nitrosative stress clearly indicate that excitotoxic, Ca^{2+}-dependent mechanisms play an important role in mediating METH toxicity. Nonetheless, the fact that the NMDA receptor antagonist amantadine exacerbated rather than attenuated METH toxicity indicates that there are still some unresolved questions regarding the exact role of excitotoxicity within the whole picture of amphetamine toxicity.

2.7.4 Hyperthermia

Hyperthermia plays a well-established and important role in mediating the toxicity of amphetamines. For instance, when METH is administered in a cold environment, the depletion of striatal DA levels is strongly attenuated. Moreover, the body temperature of individual animals is correlated to striatal DA levels and other measures of neurotoxicity (Ali et al., 1994; Bowyer et al., 1994). Ambient temperatures have a similar effect on the 5-HT deficits observed after MDMA administration (Goni-Allo et al., 2008). In general, it seems that low ambient temperatures reduce, whereas high ambient temperatures increase, the neurotoxic effects of amphetamines (Broening et al., 1995; Malberg and Seiden, 1998).

Alteration of body temperatures by means other than modulating ambient temperature similarly influences the effect of amphetamines on monoamine levels. For instance hypophysectomized or thyroparathyroidectomized rats, which do not develop hyperthermia in response to MDMA, are more resistant to the 5-HT neurotoxicity (Sprague et al., 2003). Pharmacological induction of hypothermia with drugs including diazepam and haloperidol also protect against depletion of brain monoamine levels (Bowyer et al., 1994).

Several neurotransmitter systems are involved in mediating the hyperthermic effects of METH. As discussed above, NMDA receptor antagonism reduces METH-induced hyperthermia and toxicity (Sonsalla et al., 1991; Farfel et al., 1992). However, a host of other receptors are also involved in modulating METH-induced hyperthermia. For instance, blockade of receptors including the DA D_1, D_2 and D_3 receptors; the α1-adrenoreceptor; the 5-HT_2 receptor as well as the σ and orexin-1 receptor, all block or reduce METH-induced hyperthermia. Furthermore, the suppression of hyperthermia is associated with significant protection against METH toxicity (Doyle and Yamamoto, 2010; Seminerio et al., 2011; Ares-Santos et al., 2012; Rusyniak et al., 2012; Seminerio et al., 2012; Kikuchi-Utsumi et al., 2013; Robson et al., 2013a; Sabol et al., 2013; Baladi et al., 2014). Conversely, trace amine associated receptor 1-knockout mice exhibit slightly quicker response to the hyperthermic effects of METH and MDMA (Panas et al., 2010).

The effect of body temperature on METH toxicity appears to depend on a specific interaction, as hyperthermia on its own does not deplete striatal DA levels (Bowyer, 1995). It has been suggested to be due to a general effect on the enzymatic and non-enzymatic reactions that are involved in amphetamine toxicity. For instance, it has been observed that hypothermia prevents METH-induced ROS production and oxidative stress (Fleckenstein et al., 1997a; Yamamoto et al., 2010) while hyperthermia, as expected, significantly increases ROS production and other measures of toxicity (Silva et al., 2013).

2.7.5 Other factors

In addition to oxidative stress, mitochondrial dysfunction, excitotoxicity and hyperthermia, a number of other mechanisms have been suggested to be involved in amphetamine toxicity as well. There is evidence of METH and MDMA-induced activation of microglia and subsequent increases in proinflammatory cytokines which can under some circumstances contribute to toxicity. Defects in the ubiquitin proteasomal system have also been reported and form an additional pathway affected by amphetamines which could have detrimental cellular effects. Furthermore, blood-brain-barrier dysfunction as well as interaction of amphetamine toxicity with factors such as stress and HIV-infections has been reported (Yamamoto et al., 2010).

Alterations in neurogenesis in neurons derived from the subventricular zone stem/progenitor cells and the dentate gyrus have also been reported (Goncalves et al., 2014). Further, toxic products such as ammonia, resulting from METH-induced damage to peripheral organs have also been suggested to play a role in mediating neurotoxic effects (Halpin et al., 2014).

It is also becoming clear that amphetamine neurotoxicity is subject to regulation by a plethora of other systems and receptors including the CB_1 and CB_2 receptors, the trace-amine associated receptor-1 and σ receptor which all play a modulator role in

monoamine neurotransmission and immune system function and microglial proliferation (Xie and Miller, 2009; Seminerio et al., 2013; Halpin et al., 2014). In many instances, these effects can be seen as downstream of, and dependent upon, the primary mechanisms of amphetamine toxicity described above. More details are available in recent reviews (Yamamoto et al., 2010; Goncalves et al., 2014; Halpin et al., 2014).

The large body of evidence suggesting amphetamine-induced neurotoxicity due to processes related to ROS production, mitochondrial dysfunction, excitotoxicity and hyperthermia raise important questions regarding the potential toxicity of novel substituted amphetamines, such as the cathinones.

2.8 Summary and rationale for the present study

Although amphetamines have a long history of human use, it is only recently that there has been a strong increase in the use of completely new amphetamines, such as the substituted cathinones (Fig 2.2). The large increase in the prevalence of these substances is concerning because their lack of history of human use means virtually nothing is known about their safety or toxicity. This is particularly concerning in the case of the substituted cathinones, such as 4-MMC and MDMC, which are amongst the most popular new psychoactive substances and, furthermore, show a close similarity to METH and MDMA, drugs which are potentially neurotoxic to recreational users.

The close similarity of the new substituted cathinones to well-known and potentially neurotoxic drugs, suggests that also the cathinones may produce similar neurotoxicity. Furthermore, the fact that the lifetime prevalence of illicit amphetamine use is over 10% in some countries (EMCDDA, 2014b), means that there are a large number of potential users for these new compounds, particularly as their apparent legality and relatively overt availability may lower the threshold for some people to try them. Therefore, a closer investigation of the potential toxicity of substituted cathinones, with a focus on 4-MMC and MDMC, is clearly warranted.

3. Aims of the study

The aim of this thesis is to investigate whether β-keto amphetamines differ from their non-keto amphetamine analogues in terms of their long-term neuropharmacological effects and possible neurotoxicity, and to elucidate possible mechanisms responsible for any observed differences.

The lifetime prevalence of recreational use of amphetamine-type stimulants is high, but most people use them infrequently and do not develop an addiction. In order to maximize the relevance of the obtained data, the studies were designed to answer the question of whether occasional binge-use of certain specific amphetamines or cathinones produces neurotoxicity or any other evidence of long-term neuropharmacological or neurophysiological effects. This information will provide valuable information for the development of an effective, evidence-based public health policy and harm reduction strategy.

The aims of the specific studies were:

I. To investigate the possible differential susceptibility to MDMA-induced long-term alterations of the 5-HT system in human subjects and rat models between adolescence and adulthood.

II. To investigate the long-term effects of 4-MMC and MDMC on the monoamine system, cognitive function and other neurobehavioral tests using rat and mouse models.

III. To explore how 4-MMC and METH differ in terms of their long-term effects on brain activity and memory function in rats.

IV. To investigate possible mechanisms responsible for the observed similarities and differences between the effects of keto amphetamines and non-keto amphetamines *in vitro*.

4. Materials and methods

This section briefly describes materials and methods used in more than one of the studies described below. Sections 4.2 – 4.6 provide an outline of materials and methods specific to the respective studies.

Experimental animals – Male Wistar rats and C57BL/6 mice were used in these experiments. All animal experiments were performed according to relevant national and international guidelines and approved by the Animal Care Committee of The Academic Medical Center in Amsterdam or The Southern Finland Provincial Government. Drugs were dissolved in saline and administered intraperitoneally at a volume of 10 ml/kg for mice or 1 ml/kg for rats. Control groups were administered equal amounts of saline.

Drugs and reagents – The cathinones used in these experiments were all obtained from the Hjelt Institute and, using gas chromatography and Fourier transform infrared spectroscopy, determined to be hydrochloride salts of ≥95% purity. Analytical grade METH hydrochloride was obtained from Sigma-Aldrich (St. Louis, MO, USA), D-AMPH sulfate from GlaxoSmithKline (Brentford, UK) and MDMA from THC Pharm GmbH (Frankfurt, Germany) and the Netherlands Forensic Institute (Rijswijk, The Netherlands). All other reagents used were analytical grade and obtained from Sigma-Aldrich, unless otherwise specified.

Dosing regimen and dose selection – Drugs were administered twice daily for four consecutive days in order to mimic a long weekend of binge-use of a certain substance. Doses of AMPH and METH were selected to be in a similar range as those used in other studies (Bowyer et al., 1993; LaVoie and Hastings, 1999; Ossola et al., 2011). The substituted cathinones 4-MMC and MDMC were injected at a dose of 30 mg/kg. This dose was established in pilot experiments to produce a consistent 2°C increase in body temperature, similar to AMPH and METH, without producing lethality.

Statistics – In all experiments statistical significance is assumed at $p < 0.05$ (2-tailed).

4.1 Study I: Effect of age on MDMA neurotoxicity

The effect of age on the susceptibility to MDMA neurotoxicity was assessed in both rats and humans. In a group of human MDMA users, the effect of age of first exposure to MDMA on SERT density was investigated using the radioactive ligand [^{123}I]β-CIT in combination with SPECT neuroimaging. In parallel, the effect of age on MDMA neurotoxicity was assessed in rats by measuring the binding of this same ligand one week after treatment with MDMA in both adolescent and adult rats. This ligand has previously been validated and found to be well-suited for detecting alterations in SERT (Reneman et al., 1999).

4.1.1 SPECT imaging in humans

4.1.1.1 Human subjects

The population used in this study has been described previously (Reneman et al., 2001b; Reneman et al., 2001a). Subjects were recruited by means of flyers posted at venues associated with the "rave scene" in Amsterdam and with the help from Unity, an organization that provides information about drugs and harm-reduction at festivals and events. Data from 33 was reanalyzed and employed for this study. Exclusion criteria included presence of one or more lifetime psychiatric disorders (assessed by telephone interview prior to inclusion in the study) or a positive urine drug test at the time of the scan. The experiments were approved by the institutional Medical Ethics Committee of The Academic Medical Center in Amsterdam, and written informed consent was obtained from all participants. The study was conducted according to the principles expressed in the Declaration of Helsinki.

4.1.1.2 SPECT imaging and data analysis

For this study, subjects were divided into two groups based on the age at which they had first used MDMA. The first group consisted of subjects who had started using MDMA prior to age 18 (the early-exposure group, N=8), while the second group (the late-exposure group, N=25) comprised subjects who had consumed MDMA for the first time after the age of 18. Subjects were administered approximately 140 millibecquerel [^{123}I]β-CIT and imaged 4 h later, at the time of maximum SERT binding. The brain-dedicated SPECT scanner (Strichman Medical Equipment 810X, Strichman Medical Equipment Inc., Medfield, Mass., USA) with a resolution of approximately 7.5 mm was used to acquire transversal slices in 5mm steps from the orbito-meatal line to the uppermost part of the head, producing acquisitions comprising approximately 15 slices each. The scanning time per slice was 3 min and the energy window was set to 135 – 190 KeV.

An investigator blind to the experimental conditions performed a region of interest analysis for the frontal cortex, midbrain and the cerebellum using a standard template developed from co-registered MRI images. The binding in the cerebellum was used as a reference region as it is presumed to be low in SERT. The ratios of specific to non-specific binding were subsequently calculated by dividing the frontal cortical and midbrain binding by the binding observed in the cerebellum.

4.1.2 SERT binding studies in rats

Rats (N=5-8 per group) were treated with MDMA (10 mg/kg, twice daily for four consecutive days) starting at either postnatal day 27 (adolescent) or 63 (early adulthood). Following a one week survival interval the animals were injected with [^{123}I]β-CIT . Three hours later the animals were sacrificed by means of bleeding via heart puncture under carbon dioxide anesthesia and the brains were dissected into the following regions: PFC, temporal cortex, occipital cortex, hypothalamus, amygdala, hippocampus, striatum, cerebellum and superior colliculus. Subsequently, the radioactivity in each region was measured using a gamma counter. Binding of [^{123}I]β-CIT to SERT was determined using the ratio of specific to non-specific binding using the cerebellum as a reference region (Reneman et al., 1999; van Dyck et al., 2000).

4.1.3 Statistics

In the SPECT experiment, differences in binding between the two groups were analyzed using the Student *t*-test while the association between age-of-first-exposure and SERT-binding was analyzed using Spearman's correlation coefficient and regression analysis.

For the rat binding studies, group differences in SERT binding were analyzed using multivariate analysis of variance with age and treatment as independent variables, followed by Bonferroni post-hoc tests were appropriate.

4.2 Study II: Long-term effects of 4-MMC and MDMC

In order to investigate the potential neurotoxic effects of 4-MMC and MDMC, rats and mice were treated twice daily for four consecutive days with 4-MMC (30 mg/kg), MDMC (30 mg/kg) or AMPH (12.5 mg/kg) as a positive control. Two weeks after the final drug treatments, levels of monoamine neurotransmitters as well as DAT and SERT density was assessed using high-performance liquid chromatography and high-affinity radioligands. Behavioral tests were performed to assess the effects of these substances on memory function, anxiety and depressive behavior. One group of mice was used to measure brain monoamine levels while another group was used for behavioral experiments. Rat brains were used to measure monoamine levels as well as SERT and DAT binding.

4.2.1 Measurement of monoamine levels

Tissue levels of 5-HT, DA, NE, 5-HIAA, HVA and DOPAC in the PFC, striatum and hippocampus were measured using high-performance liquid chromatography with electrochemical detection. Brain samples from the frontal cortex, striatum and

hippocampus were prepared by sonication in 2% perchloric acid and centrifuged for 30 min at 15,000×g after which 10 µl of filtered supernatant was injected into the high-performance liquid chromatography system (Waters Concorde electrochemical detector, potential + 0.80 V, column oven and a 5 µm 150 × 4.60 mm column). The mobile phase was comprised of purified water with 8% methanol, 50 mM citric acid, 1.5 mM 1-octanesulfonic acid, 0.05 mM ethylenediaminetetraacetic acid and 50 mM phosphoric acid. Column temperature was 37 °C with a flow rate of 1 ml/min. The retention times for NE, DOPAC, DA, 5-HIAA, HVA and 5-HT were 3.65, 5.47, 7.45, 9.16, 12.11 and 19.04 min, respectively. Concentrations of the neurotransmitters and metabolites were calculated from standard curves (linear from 10 nM to 1 µM).The lower limit of quantification was 10 nM or 0.1 pmol/g of wet tissue. Samples were standardized based on wet weight.

4.2.2 Measurement of DAT and SERT levels

SERT binding in the hippocampus and DAT binding in the striatum were assessed using a filter-assay of [^{3}H]paroxetine and [^{3}H]mazindol binding to brain homogenates according to well-established methods (Battaglia et al., 1987; Hrdina et al., 1990; Robledo et al., 2004; Chiu et al., 2007). Here, tissue samples were placed in a tube containing 10 ml incubation buffer (50mM Tris–HCl buffer with 120 mM NaCl and 5 mM KCl), and homogenized for 30 s with a Polytron. Tubes were centrifuged (48,000×g, 10 min) and then washed and resuspended to a volume of 15 mg wet weight/ml. Total [^{3}H]paroxetine binding was assessed by incubation of the samples in tubes containing 4 nM [^{3}H]paroxetine for 2 h at 22 °C. Non-specific binding was assessed in separate tubes by the addition of 20 µM citalopram to the incubation buffer in order to prevent[^{3}H]paroxetine binding to SERT. Total [^{3}H]mazindol binding was assessed by incubating the samples for 1 h at 4 °C in tubes containing 6 nM [^{3}H]mazindol in the presence of 0.3 µM desipramine to block [^{3}H]mazindol binding to NET. Non-specific binding was assessed in separate tubes by the addition of 100 µM nomifensine to the incubation buffer to block [^{3}H]mazindol DAT binding. Following incubation, the tube contents were filtered through Whatman GF/B filters pre-soaked with 0.05% polyethylenimine and washed with 15 ml of buffer. Finally, the filters were placed in tubes and 3 ml Optiphase HiSafe 3 scintillation fluid was added (PerkinElmer, Turku, Finland). These were counted at 47% efficiency in a scintillation spectrometer.

4.2.3 Behavioral experiments

Memory function, anxiety, depressive behavior and locomotor activity were assessed using the T-maze and Morris water maze, the elevated plus maze, the tail suspension test and exposure to novel cages. Behavioral metrics were either recorded manually (at the time of the test or later using video recordings) or automatically using Ethovision software (Noldus Information Technology, Wageningen, Netherlands).

Working memory – the T-maze spontaneous alternation test was used to assess working memory (Gerlai, 1998; Linden et al., 2007). The maze consists of three arms with a door separating a 12 cm compartment of the start arm, and two other doors separating 24 cm compartments of the other arms. Spontaneous alternation was assessed by placing the mouse in the start arm and opening the door after 5 s to allow the mouse to visit either the left or right arm. After entering either arm, the opposite arm was closed. Every time the mouse returned to the start arm after entering either arm, it was confined for 5 s in the start arm, after which the doors were opened again. The total test time was 15 min. A higher rate of spontaneous alternation of arm visits is assumed to reflect better working memory.

Spatial memory and reversal learning – spatial memory and reversal learning was assessed with the Morris water maze. Here, the rats were trained for 4 days (6 trials per day with a 10 min interval) to find a hidden platform (14 × 14 cm) submerged in a circular tub (120 cm diameter) filled with opaque water. On the 5^{th} day, a probe trial was performed to assess the time taken to find the platform. The time the rat spent in each of the quadrants was also assessed. Lower latency to the platform and preference for the platform quadrant were interpreted as evidence of spatial memory. On day 6, reversal learning (5 trials with a 10 min interval) was performed by placing the platform in the opposite quadrant of the tub. Finally, on day 7, a second probe trial was performed to assess reversal learning.

Anxiety – anxiety was measured using the elevated plus maze (Lister, 1987; Leppä et al., 2011), in which the mouse is placed in a plus-shaped maze with 2 closed and 2 open arms (each arm 5 × 40 cm). A preference for closed arms over open arms was interpreted as evidence of anxiety.

Depressive behavior – depressive behavior was assessed using the tail suspension test (Steru et al., 1985; Procaccini et al., 2011). In this test, increased immobility during a 6 min tail suspension session is used as a measure of depressive behavior.

Locomotor activity/habituation – Locomotor activity and habituation was assessed for 2 h after introduction of the animals to novel, empty cages.

4.2.4 Statistics

Data from transporter-binding, high-performance liquid chromatography and most behavioral experiments were analyzed using one-way analysis of variance with Fisher's least significant difference post-hoc tests or Kruskal–Wallis one-way analysis of variance and post-hoc pairwise comparisons. Data from the Morris water maze and locomotor

activity tests were analyzed by means of repeated measures analysis of variance with Greenhouse–Geisser correction.

4.3 Study III: Effect of 4-MMC and METH on brain activity

Since no clear long-term neurochemical effects of 4-MMC were observed in the previous study, an attempt was made to investigate the possible long-term neuropharmacological or neurophysiological effects of 4-MMC using MEMRI, a highly sensitive MRI method (Bangasser et al., 2013; Hoch et al., 2013). Following 4-MMC (30 mg/kg) or METH (5 mg/kg) twice daily for four consecutive days, a MEMRI scan was performed on rats two weeks after the final drug treatments. Furthermore, to determine if any observed neurophysiological alterations were accompanied by behavioral changes, experiments measuring locomotor activity, sensitization and memory performance (novel object recognition) were performed.

4.3.1 MEMRI procedure and data analysis

The MEMRI method is based on the fact that the manganese ion Mn^{2+} functions as a Ca^{2+} analogue *in vivo* and is primarily taken up by activated neurons. Since Mn^{2+} is paramagnetic, it functions as an MRI contrast enhancing agent, and brain regions showing higher signal intensity are assumed to reflect regions of increased brain activity (Koretsky and Silva, 2004; Silva and Bock, 2008).

One week prior to the scan, after the completion of all behavioral experiments, animals were surgically implanted with osmotic minipumps which delivered 200 µL of $MnCl_2$ at a rate of 1 µL/h to a total dose of 120 mg/kg over the course of one week. One week after pump implantation and two weeks after the final drug treatment the MRI scan was performed in a 4.7 T scanner.

MRI data processing was performed according to previously described methods (Friston et al., 1990; Jenkinson et al., 2002; Schwarz et al., 2006). For the region-of-interest analysis, 22 regions were selected on the basis of involvement in circuits mediating goal-directed behavior as well as regions known to be affected by psychostimulant drugs (Sesack and Grace, 2010; Klomp et al., 2012; Noori et al., 2012). Region of interest analysis was also performed according to previously described methods (Maldjian et al., 2003; Duff et al., 2007).

4.3.2 Behavioral experiments

Locomotor activity and sensitization – locomotor activity was measured for up to 6 hours in novel cages after the first of two daily drug administrations during the first and last day of the drug treatments. Locomotor activity at both days was analyzed, and sensitization was assessed by comparing the locomotor activity on the first and last day of treatment.

Recognition memory – memory function was assessed using the novel object recognition test (Motbey et al., 2012) in which the preference for exploring a novel object is used as a measure of recognition memory. The test was performed one week after the last drug treatment in a rectangular opaque blue box (50 x 80 x 30 cm). The test consisted of one 3-min sample trial where the rat was exposed to two identical objects, followed by a 3-min test trial 15 min later where the rat was exposed to one novel object and one object which was previously used in the sample trial. The objects were a coffee mug (8 x 8 x 12 cm, brown color) and two white translucent, rectangular plastic bottles taped together at the lids (3 x 3 x 18 cm). Objects were mounted securely to the bottom of the box using putty to prevent the rats from moving the objects. Novel object preference was assessed by recording the test trials with a video camera and subsequent manual analysis by an operator blind to the experimental conditions using Ethograph v. 2.06 (St. Petersburg, Russia). Active exploration of objects such as touching and sniffing was counted as investigation. Merely being in the vicinity of or perching over the objects was not.

4.3.3 Statistics

Novel object recognition test performance and individual region of interest comparisons were analyzed using one-way or two-way analysis of variance followed by Fisher's least significant difference post-hoc tests. Development of sensitization, or increased locomotor activity between the first and the last treatment day was analyzed by means of repeated-measures analysis of variance of average locomotor activity during the first two hours following drug administration on the first and last day.

4.4 Study IV: *In vitro* toxicity and redox reactivity

In order to identify possible mechanisms responsible for the differing effects of keto and non-keto amphetamines, we investigated the effects of these drugs on cytotoxicity and cellular proliferation in a neuroblastoma cell line *in vitro* and investigated differences in redox reactivity between these two classes of compounds. Finally, the effects of METH and 4-MMC on mitochondrial respiration were explored.

4.4.1 Cell culture

SH-SY5Y neuroblastoma cells between passage 6 – 20 were maintained at 37 °C/5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM/F12) containing 10% FBS and antimycin-A. This cell line was selected because it displays a catecholaminergic neuronal phenotype, as witnessed by the presence of markers such as TH, dopamine-β-hydroxylase and marked neurite outgrowth (Presgraves et al., 2004; Xie et al., 2010).

4.4.2 Cytotoxicity assay

Cytotoxicity was assessed by measuring the amount of lactate dehydrogenase released into the medium using the "Cytotoxicity Detection Kit" from Roche (Basel, Switzerland) according to the manufacturer's instructions. In experiments employing the DAT-blocker GBR12909, the cells were pre-incubated for 1 h with GBR12909 prior to applying other drugs. The validity of the assay was confirmed using 5mM nicotinamide adenine dinucleotide or 2mM hydrogen peroxide as positive and negative controls.

4.4.3 Cell proliferation and redox sensitivity assay

The "Roche Cell Proliferation Reagent WST-1" containing WST-1 together with the intermediate electron acceptor 1-methoxy-5-methyl-phenazinium methyl sulfate was used for measuring cellular proliferation as well as redox sensitivity of keto and non-keto amphetamines. For the proliferation assays, the resulting formazan dye production after 60 min incubation was measured spectrophotometrically at 440 nm. Similar to the cytotoxicity assay, the validity of the assay was confirmed using 5mM nicotinamide adenine dinucleotide or 2mM hydrogen peroxide as positive and negative controls.

For the drug redox sensitivity assays, WST-1 was applied together with the various keto and non-keto amphetamines in PBS or DMEM/F12 medium without cells present. A 60 min incubation time was also used for most of these experiments except the experiment measuring the reaction kinetics, were reactivity was measured every 10 min.

4.4.4 Analysis of reaction products

Detection and analysis of reaction products was performed using ultra-high performance liquid chromatography/high-resolution time-of-flight mass spectrometry under similar conditions as described previously (Sundström et al., 2013).

4.4.5 Mitochondrial respiration

Cells were seeded at 10.000 cells per 80 µL well on XF cell culture microplates and grown normally. One hour prior the assay the normal culture medium was replaced with XF base medium containing glucose (10mM), L-glutamine (1mM) and pyruvic acid (10mM) and the microplate was placed in a CO_2 free incubator until the time of the assay. Baseline and maximum mitochondrial respiration was measured using the XF^e96 Analyzer (Seahorse Bioscience, MA, USA). Oxygen consumption rate was measured prior to, during, and after injections with the uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, and complex I+II inhibitors antimycin A + rotenone (final concentration 0.5µMand 1µM+1µM, respectively). Basal respiration was calculated by subtracting non-mitochondrial respiration (respiration following antimycin A + rotenone injection) from baseline respiration measured before any drugs were injected. Maximum respiration was calculated by subtracting non-mitochondrial respiration from the maximum respiration measured after injection of the uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone.

4.4.6 Mitochondrial complex I/II assay

The complex I/II assay was performed using the Oxygraph-2k (Oroboros Instruments GmbH, Innsbruck, Austria). For this assay, 8 mg of mice frontal cortex tissue was dissected and placed in shredder tubes containing MiRO6 respiration buffer (0.5mM ethylene glycol tetraacetic acid, 3mM MgCl2·6H20, 60mM K-lactobionate, 20mM taurine, 10mM KH_2PO_4, 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 110mM sucrose, 1 g/l BSA, and 280 u/ml catalase) after which they were shredded using a SG3 shredder (Pressure Biosciences Inc., MA). The tube contents were diluted in 4.5 ml of MiRO6 and approximately 2.5 ml was transferred to the two Oxygraph-2k chambers for analysis.

For the analysis, 4-MMC, METH (20mM final concentrations) or water were injected into the chambers and allowed to incubate for 20 min. After this, a substrate-uncoupler-inhibitor titration protocol (Pesta and Gnaiger, 2012) was performed were substances were injected in the following order: L-malic acid (2mM), pyruvic acid (5mM), L-glutamic acid (10mM), adenosine diphosphate + Mg^{2+} (1mM), succinate (10mM), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, rotenone (0.2µM), malonate (5µM) and antimycin A (2.5µM). Oxygen flux control ratio was determined for complex I+II (after succinate injection) and complex II only (after rotenone).

4.4.7 Statistics

Lactate dehydrogenase release, WST-1 reduction and oxygen flux control ratios data were analyzed using analysis of variance and subsequent Dunnett's or Fisher's least significant difference post-hoc tests.

5. Results

5.1 Study I: Effect of age on MDMA neurotoxicity

5.1.1 Human SPECT neuroimaging

SPECT imaging revealed no differences in midbrain [^{123}I]ß-CIT binding between the early-exposure group (1.30 ± 0.30) and the late-exposure group (1.24 ± 0.17). There were also no differences in frontal cortex [^{123}I]ß-CIT binding between the early (0.77 ± 0.06) versus the late-exposure group (0.77 ± 0.08). Correlation and regression analysis indicated a strong inverse relationship between age of first ecstasy use and midbrain [^{123}I]ß-CIT binding in the early, but not the late-exposure group. Specifically, the correlation between age of first exposure and SERT binding in the early-exposure group was r^2 = 0.789 and r = -0.888 ($p < 0.01$) while it was r^2 = 0.032 and r = 0.179 ($p > 0.05$) in the late-exposure group. In the frontal cortex, no relationship between age of first exposure and [^{123}I]ß-CIT was found. Furthermore, other factors including age at the time of scanning, gender or duration and amount of ecstasy use also did not affect [^{123}I]ß-CIT binding.

5.1.2 Rat SERT binding

In the control group, PFC [^{123}I]ß-CIT binding was 21% higher in adult rats compared to adolescent rats (4.47 versus 3.55, $p < 0.01$). In other brain regions, including the midbrain, no differences in [^{123}I]ß-CIT binding were found between rats at with MDMA during adolescence or adulthood. MDMA treatment reduced [^{123}I]ß-CIT binding in all investigated brain regions except the striatum in both adolescent and adult rats (see Table 2 in **I**). Furthermore, an age-by-treatment interaction effect was observed in the PFC ($p < 0.01$) as adolescent rats appeared to show less reduction of [^{123}I]ß-CIT binding in this brain region following MDMA treatment than the adult rats (a decrease of 35% and 49%, respectively).

5.2 Study II: Long-term effects of 4-MMC and MDMC

5.2.1 Monoamine levels

All significant effects of AMPH, 4-MMC or MDMC on levels of monoamine neurotransmitters and their metabolites two weeks after the final treatments are shown in Table 5.1 Exhaustive data from these experiments, including all measured transmitter and metabolite levels for rats and mice in all investigated brain regions can be found in **II** (Tables 2 and 3).

Drug	Effects in rats	Effects in mice
AMPH	DA: ↓ 45% in striatum 5-HT: ↓ 33% in frontal cortex, ↓45% in striatum and ↓29% in hippocampus 5-HIAA: ↓ 24% in striatum	DA: ↓ 66% in striatum
4-MMC	No observed significant effects	HVA: ↓ 22 in striatum
MDMC	5-HT: ↓ 43% in frontal cortex, ↓ 35% in striatum and ↓ 38% in hippocampus 5-HIAA: ↓ 21% in striatum and ↓ 22% in hippocampus	No observed significant effects

Table 5.1 Summary of the significant effect of AMPH, 4-MMC and MDMC treatments on levels of monoamine neurotransmitters and their metabolites two weeks after the final drug treatments in study II.

Test	Main results
Elevated plus-maze	No significant treatment effects
T-maze	25% ↓ in alternation ratio following 4-MMC
Locomotor activity	No significant treatment effects
Morris water maze	Indications of increased reversal learning performance following 4-MMC and MDMC: 4-MMC: 25% ↓ in time spent in old quadrant during the reversal learning probe trial. MDMC: 39% ↓ in time spent in the old quadrant and a 57% ↑ in the time spent in the new quadrant during the reversal learning probe trial.
Tail suspension test	No significant treatment effect

Table 5.2 Summary of all results from behavioral tests performed 2 weeks or longer after the final treatment with 4-MMC or MDMC in study II.

Test	Main result
Locomotor activity/sensitization	↑ Acute locomotor activity following METH and 4-MMC on both the first and last day of drug treatments. 35% ↑ in total locomotor activity following METH on the last day of drug treatments. No evidence of locomotor sensitization at the last day of drug treatments.
Novel object recognition	No significant treatment effects.

Table 5.3 Summary of the results obtained from the behavioral experiments in study III.

5.2.2 SERT and DAT binding

MDMC produced a 24% decrease in PFC [^{3}H]paroxetine binding ($p < 0.05$) and a 65% decrease in hippocampal [^{3}H]paroxetine binding, but had no effect on [^{3}H]mazindol binding in the striatum. On the other hand, 4-MMC had no effect on [^{3}H]paroxetine or [^{3}H]mazindol in any of the investigated brain regions.

5.2.3 Behavioral experiments

A summary of the results obtained from the behavioral experiments are shown in Table 5.2. For complete results, please refer to **II** (Table 2 and results section).

As seen in Table 5.2, the results indicate that only behavior related to memory, but not to anxiety or depression, was affected by 4-MMC and MDMC. However, the observed effects of 4-MMC and MDMC on memory are ambiguous. Although 4-MMC appears to decrease working memory performance, it actually appears to improve the performance on aspects of spatial memory. Specifically, both MDMC and 4-MMC appeared to improve spatial reversal learning.

5.3 Study III: Effect of METH and 4-MMC on brain activity

5.3.1 Assessment of brain activity with MEMRI

Functional brain activity – METH and 4-MMC produced surprisingly different long-term effects on brain activity. METH produced a widespread reduction in brain activity in regions including the primary motor cortex, cingulate, insular cortex, striatum, thalamus, hippocampus, amygdala, superior colliculus and raphe nucleus. On the other hand, 4-MMC produced an *increase* in brain activity. The increases in activity were much less widespread and were limited primarily to certain cortical regions and the hypothalamus. Detailed maps of altered brain activity following METH and 4-MMC as are shown in **III** (Fig. 4 and 5)

Region of interest analysis – Out of the 22 regions of interest that were investigated, METH treatment produced significant decreases in brain activity in the nucleus accumbens core, caudate/putamen, lateral globus pallidus, cingulate cortex, raphe nucleus and superior colliculus. In all regions, the decreases were between 6% and 7% (all $p < 0.05$). Conversely, 4-MMC produced a 10% increase in brain activity. This effect was limited to only one region of interest, namely the hypothalamus ($p < 0.01$).

5.3.2 Behavioral experiments

The significant results obtained from the locomotor activity and sensitization experiment as well as the novel object recognition experiment is shown in Table 5.3. The data is also summarized graphically in Fig. 2 (locomotor activity) and Fig. 3 (novel object recognition) of **III**.

The most important result from the behavioral experiment was that no effect was observed on memory performance. This was contrary to expectation, as a decrease in memory performance had previously been reported using the same test and 4-MMC doses, although with a 25% higher cumulative 4-MMC dose (Motbey et al., 2012).

In this experiment we also did not find any evidence of locomotor sensitization. This was not unexpected, as sensitization develops preferentially following treatments with low doses of psychostimulants, and not high doses, such as employed here (Frey et al., 1997). Furthermore, a previous study employing the same 4-MMC doses as here also reported that no sensitization had taken place (Motbey et al., 2012). The development of locomotor sensitization is associated with drug addiction (Robinson and Berridge, 1993), and the fact that no sensitization was observed in this experiment strengthens the hypothesis that the observed changes in brain activity are due to neurophysiological processes unrelated to addiction.

5.4 Study IV: *In vitro* toxicity and redox reactivity

5.4.1 Cytotoxicity and cellular proliferation

Cytotoxicity – The cytotoxic effects of a 48 h treatment with 4-MMC became apparent at concentrations of 500µM and higher. At this concentration a 16% increase in lactate dehydrogenase release was observed ($p < 0.001$) compared to untreated cells. The observed lactate dehydrogenase release increased to 75% ($p < 0.001$) at 2mM, the highest concentration tested. MDMC and METH produced significant increases in lactate dehydrogenase release of 24% ($p < 0.001$) and 5% ($p < 0.05$), respectively. However, this effect was only observed at the highest concentration tested (2mM). Notably, all drugs produced a decrease in lactate dehydrogenase release of between 18% and 34% (all $p < 0.001$) at concentrations between 2 – 100µM. Furthermore, pre-treatment with the DAT blocker GBR12909 reduced 2mM 4-MMC-induced lactate dehydrogenase release by 38% ($p < 0.01$).

Cellular proliferation – As expected, METH produced a decrease in cell viability. At 1mM, a 19% decrease in viability was observed ($p < 0.01$). At the 2mM concentration the decrease had reached 20% ($p < 0.001$). Notably, both 4-MMC and MDMC did not produce

a decrease in WST-1 reduction indicative of decreased cellular proliferation. Instead they produced an exceptionally large and dose-dependent increase in WST-1 reduction of up to 280% for 4-MMC and 187% for MDMC (both p <0.001). Examination of the cells under a light microscope, however, did not reveal any apparent increase in cellular proliferation in the drug-treated cells (**IV**, Fig. 2).

5.4.2 Redox reactivity of β-keto amphetamines

The experiments using WST-1 as a redox sensitivity assay produced the following main results:

1. The observed increase in WST-1 reduction occurred with all keto amphetamines 4-MMC, MDMC, methcathinone, 3,4-dimethylmethcathinone, 4-bromomethcathinone/brephedrone, α-methylamine-byturophenone/buphedrone and bupropion, but not with any of the non-keto amphetamines AMPH, METH, MDMA, 3-fluoroamphetamine (3-FA) and 4-fluoroamphetamine (4-FA).
2. The kinetics of the observed reaction between 4-MMC and WST-1 was linear between 0 and 60 min.
3. Although the reaction occurred in phosphate buffered saline, the presence of DMEM/F12 culture medium increased the reactivity.
4. The reactivity was increased as a function of increasing pH (range 6.4 – 8.4).
5. The reactivity was blocked or attenuated by the antioxidants and superoxide scavengers including L-Ascorbic acid-2-phosphate, 4-Hydroxy-TEMPO and Tiron.

The results are also summarized graphically in **IV**, Fig. 4 and 5.

5.4.3 Effects on mitochondria

Respiration – Figure 3.1 shows the effect of METH or 4-MMC either alone or in combination with GBR12909 on basal and maximum respiration. Both METH and 4-MMC produced dose-dependent decreases in baseline and maximum respiration. The decrease in respiration following 4-MMC was significantly larger than the decrease observed after METH. Furthermore, GBR12909 blocked the 4-MMC-induced decreases in respiration.

Complex I/II assay – Following a 20 min incubation with METH, a 69% decrease in complex II-mediated flux control ratio was observed ($p < 0.01$). No such effect was found following incubation with 4-MMC.

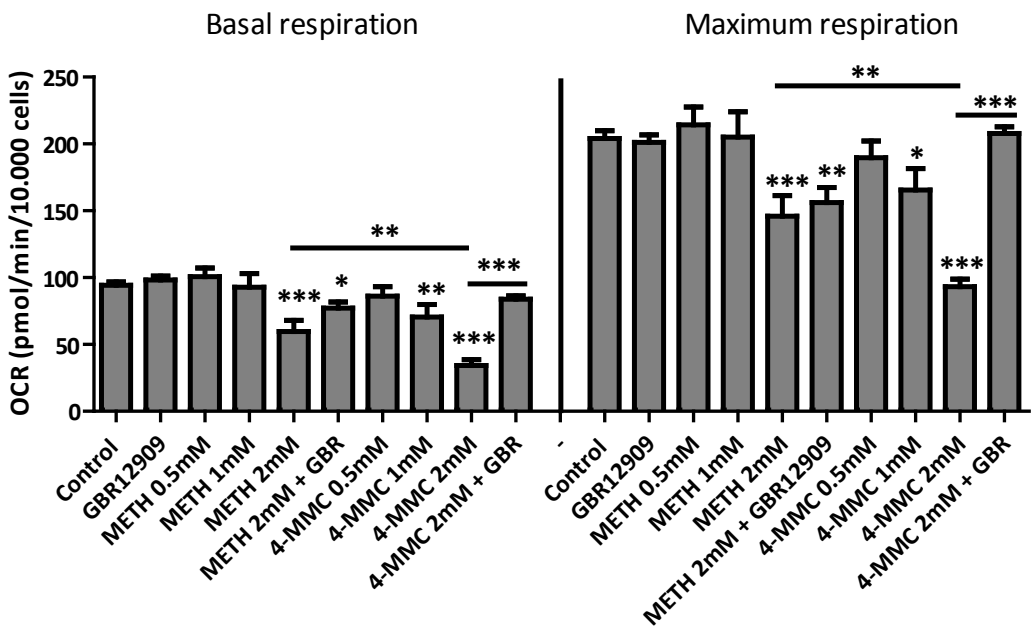


Figure 5.1 The effects of METH or 4-MMC alone or in combination with DAT-blocker GBR12909 on basal respiration (left) maximum respiration (right). The basal and maximum metrics were calculated by subtracting oxygen consumption after rotenone + antimycin A injection from the baseline pre-drug respiration or the maximum carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone-induced respiration. Data presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. N ≥ 5 per group.

5.4.4 Reaction products

Analysis of the reaction products following the reaction of 4-MMC or MDMC with WST-1 yielded the two sets of compounds shown in Fig. 5.1 (see **IV**, Fig. 6 for the corresponding chromatograms). Reaction of WST-1 with 4-MMC resulted in the production of *N*,4-dimethylbenzamide and *N*-Ac-4-MMC. The reaction with MDMC produced 3,4-methylenedioxy-*N*-methylbenzamide and *N*-Acetyl-MDMC. Additionally, reduced WST-1 was detected in both samples.

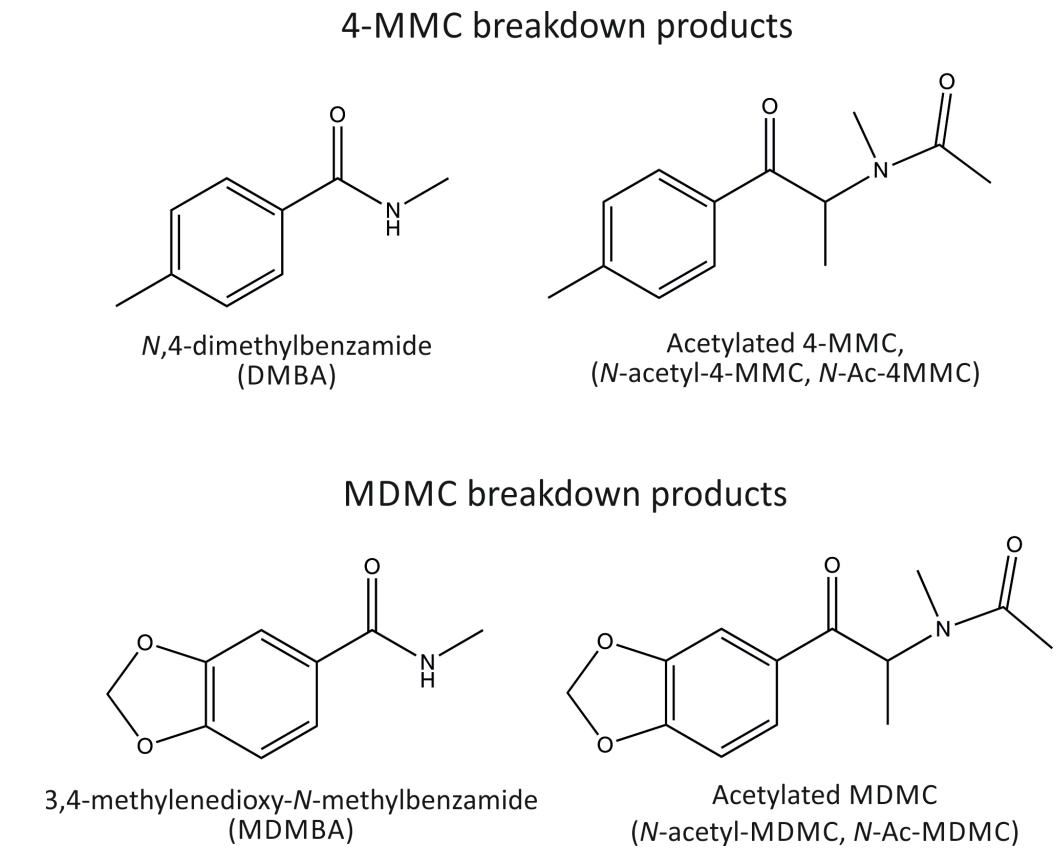


Figure 5.1 Incubation of 4-MMC and MDMC with the electron acceptor WST-1 produced *N*,4-dimethylbenzamine and 3,4-methylenedioxy-*N*-methylbenzamine, respectively.

6. Discussion

The first three studies demonstrate that the non-keto amphetamines AMPH, METH and MDMA produce long-term reductions in brain levels of monoamines, monoamine transporter binding or brain activity. However, alterations in these physiological parameters are not associated with any consistent effect on cognitive function or other neurobehavioral measurements. Conversely, the keto-amphetamine 4-MMC does not produce any long-term alterations in brain levels of monoamines, transporter binding or widespread decreases in brain activity that would be suggestive of neurotoxicity or detrimental long-term neuropharmacological or neurophysiological adaptations. MDMC produces long-term effects on the 5-HT system that are similar to MDMA. Neither keto amphetamines produce any consistent effect on cognition, mood or anxiety. Finally, in the last study it is shown that keto and non-keto amphetamines possess different redox properties and have different effects at the level of the mitochondrial ETC, something which may play a role in their differing biological and toxicological effects.

What follows is a brief discussions of specific results obtained in the individual studies, followed by a more general discussion on cathinone toxicity, the limitations associated with the models used in the present studies, as well as future directions for the study of substituted amphetamines and other novel psychoactive substances in general.

6.1 Effect of age on MDMA neurotoxicity

6.1.1 No clear age-of-first exposure effect observed in humans

The hypothesis that the neurotoxic effects of MDMA are dependent upon the initial integrity 5-HT system is not supported by the obtained data. In humans, midbrain SERT density decreases with increasing age in individuals 18 and older, whereas no age-associated changes in SERT binding are observed prior to the age of 18 (Dahlstrom et al. 2000, van Dyck et al. 2000). For this reason, it was expected that, due to the natural age-related loss of SERT, decreases in midbrain SERT density would be less pronounced in individuals exposed to MDMA at a later age in the late-exposure group. However, no correlation between age of first exposure and SERT density was found in adult subjects. Furthermore, in the early-exposure group, where no age-related changes were hypothesized to take place, a negative correlation between age of first MDMA exposure and midbrain SERT density was observed.

One explanation for the observed effect in the early-exposure group is that 5-HT exerts neurotrophic effects during development (Lauder and Krebs 1978, Lauder et al. 1983), which suggests that increases in 5-HT levels due to MDMA may produce neurotrophic effects. It has previously been shown that MDMA exerts neurotrophic effects on midbrain

primary cultures *in vitro* (Azmitia et al., 1990). However, it is difficult to predict whether a similar effect would occur *in vivo*. There are important differences between short-term application of MDMA in an *in vitro* model and human MDMA use. Importantly, MDMA produces a quick peak, followed by a more long-term decrease in 5-HT levels (McCann et al., 1994). Therefore, it is perhaps unlikely that, on average, 5-HT levels would be much higher as a result of MDMA in human recreational users.

A number of limitations apply to this experiment. The sample size of the early-exposure group was relatively small. Furthermore, the age of first exposure to MDMA, as well as the total consumption of MDMA and other drugs were based solely on self-reports and may thus not be entirely accurate. Finally, it is possible that the results are primarily due to pre-existing differences between individuals exposed to MDMA at different ages. With these limitations in mind, it is difficult to draw definitive conclusions based on this neuroimaging data.

6.1.2 Age-related increase in SERT density increases MDMA neurotoxicity in rats

Unlike what was seen in the human study, the results from the rat experiment do support the hypothesis that increased SERT density conveys vulnerability to the neurotoxic effects of MDMA. In rats, SERT densities in the midbrain reach a plateau during weaning, whereas in later maturing brain regions, such as the PFC, there is a steady increase in SERT density from weaning till old age (Brunello et al. 1985, Moll et al. 2000, Slotkin et al. 1997). Accordingly, the results indicated higher baseline PFC SERT and increased MDMA-induced PFC SERT loss in adult compared to adolescent rats. Furthermore, no age-related differences in SERT loss were observed in the midbrain or any other brain regions where no differences in baseline SERT levels were observed. Thus, the results from this experiment suggest that, compared to adult rats, MDMA is less toxic to the 5-HT system in the PFC of adolescent rats.

Unfortunately it is difficult to compare the results obtained from the rat brain SERT binding with the human SPECT neuroimaging due to ontogenetic differences in SERT expression across species, but it is clear that there is no neurotrophic effect of MDMA in rats, like human data may have suggested. This may have been because the employed dose of 10 mg/kg administered in a binge-like fashion, is very high (Baumann et al., 2007), and may have reached a level were the toxic effects of MDMA outweigh its neurotrophic effects. Another general limitation is related to the fact that the [^{123}I]β-CIT shows affinity for both DAT and SERT, with DAT and SERT binding occurring preferentially at different times after administration. Due to the presence of DAT in the midbrain, thalamus and cortex, the observed changes cannot with certainty be attributed to altered SERT availability. Nonetheless, displacement studies suggest that binding in these regions is primarily associated with SERT (Scheffel et al., 1992; Farde et al., 1994). Furthermore, the

analysis time was selected based on the time period of maximum SERT binding, suggesting that the observed changes in brain regions besides the striatum primarily reflect SERT-specific binding.

6.2 Neurotoxicity of 4-MMC and MDMC

As expected, AMPH produced a strong reduction in DA levels in both mice and rats. Surprisingly, however, 4-MMC did not produce, aside from a minor reduction of striatal HVA levels in mice, any significant effects in mice or rats. MDMC, conversely, produced a decrease in 5-HT levels in all brain regions investigated. A similar picture emerged with regards to monoamine transporters, as MDMC reduced SERT binding, while no effects on SERT or DAT transporters were observed after 4-MMC. Memory function was also affected, and this is discussed in section 6.3.2.

6.2.1. 4-MMC does not affect monoamine and transporter levels

The most important and unexpected result was that 4-MMC did not produce any long-term decreases in the levels of DA, 5-HT or their respective transporters. If one uses these measurements as evidence of neurotoxicity, it would suggest that no neurotoxicity took place following 4-MMC treatments. This is interesting, considering the similarity of this drug, in terms of both structure and pharmacology, to METH. Similar experiments have recently been done by several other groups, and it has now repeatedly been shown that similar binge-treatments with 4-MMC produce no effect on levels of DA, 5-HT, TH, microglial activation or glial fibrillary acidic protein between 2 days and several weeks after treatment (Angoa-Perez et al., 2012; Baumann et al., 2012; Motbey et al., 2012). The apparent lack of neurotoxic effects of 4-MMC is striking and provides information that is important for both public health policy and harm reduction strategies related to this substance.

6.2.2 MDMC reduces 5-HT and SERT levels

The effects of MDMC have been less studied, thus making it harder to draw definitive conclusions regarding the long-term effects of this drug. One study reported no long-term effects on DA or 5-HT levels at doses up to 3 times 10 mg/kg (Baumann et al., 2012). This suggests that MDMC can produce a long-term reduction in 5-HT at very high, but not at lower doses. It also shows that MDMC is similar to MDMA, in that they both affect the 5-HT system, although higher doses of MDMC than MDMA are required to produce this effect. Nonetheless, relative effects on 5-HT levels at equimolar doses may not be entirely representative of real-life differences in toxicity, due to differences in the potency of the

subjective effects and commonly consumed recreational doses between the two substances.

Another interesting similarity between MDMC and MDMA is related to the observed species differences in their effects. In this study, the long-term effect of MDMC on 5-HT levels was observed only in rats, but not in mice. This is reminiscent of the species difference of MDMA between mice and rats, where MDMA reduces 5-HT levels in rats, while reducing DA levels in mice (Logan et al., 1988). With MDMA it has been suggested that this is because of metabolic differences between mice and rats, with hepatic metabolites toxic to 5-HT neurons being more important in rats, while DA release and subsequent ROS toxicity to DA neurons being the primary mechanism in mice (Stone et al., 1987; de la Torre and Farre, 2004).

6.3 Effect of METH and 4-MMC on brain activity and memory

6.3.1 Contrasting effects of 4-MMC and METH on brain activity

The brain regions showing decreased activity after METH, such as the striatum, correspond closely to regions known to be vulnerable to METH-induced neurotoxicity (Seiden et al., 1988; Cass and Manning, 1999; Haughey et al., 2000; Schroder et al., 2003). They also overlap closely with regions showing altered metabolism in abstinent human METH users (Volkow et al., 2001b; London et al., 2004; Wang et al., 2004). This speaks for the construct validity of the method in terms of detecting psychostimulant-induced alterations in brain function and, furthermore, suggests MEMRI may be an interesting translational method that may help bridge the gap between human neuroimaging and preclinical studies on amphetamine neurotoxicity.

The differences between METH and 4-MMC in terms of brain activity are striking. The MEMRI method has previously been employed to detect changes in brain activity following various behavioral paradigms (Bangasser et al., 2013; Hoch et al., 2013). The fact that this method is sensitive enough to detect neurophysiological alterations following only behavioral paradigms, suggests it may also detect long-term alterations following 4-MMC that are not detectable using neurochemical and immunological methods. Although regions of altered activity were observed, they were much more limited than what was observed with METH. Furthermore, unlike METH, 4-MMC produced only increased brain activity. This would appear to confirm the results from previous experiments, namely that the long-term effects of 4-MMC are very limited when compared to METH.

6.3.2 Behavioral tests of cognition provide ambiguous results

No effect was found of 4-MMC or MDMC on tests aside from some of the tests of memory function. The only long-term after-effect of MDMC was an improvement in spatial reversal learning. Improvement in reversal learning was also observed after 4-MMC, but this drug in turn reduced working memory performance in the T-maze while having no effect on object recognition (studies **II** and **III**). The observed effect on working memory is interesting as decreased working memory performance has also been reported in human 4-MMC users, although this effect was measured while users were acutely under the influence of the drug (Freeman et al., 2012). On the other hand, a study in rhesus macaques, also performed while animals were under the influence of 4-MMC, found no evidence of detrimental effects on working memory, and actually reported a pronounced improvement in visuospatial learning and memory (Wright et al., 2012a). However, assessments made during acute intoxication are not a good reference when one is interested in the long-term residual effects.

Thus, the implications of the results obtained from the behavioral tests in these studies depend largely on how they are interpreted. Based on the decrease in working memory performance one could argue that 4-MMC has a detrimental effect on memory, but this would not do justice to the fact that other tests produced no measurable effect or in fact produced an improvement in certain test metrics. One thing that can be said is that the behavioral results are very much in accord with previous literature on the long-term effects of amphetamines on cognition, which is equally ambiguous and inconsistent (see 2.6.1.3). They highlight that behavioral paradigms, as they are presently used, are not a very good method for investigating the long-term effects of amphetamines on cognition as interfering factors, such as stress from high-dose drug treatments, could easily affect the results.

6.3.2.1 A role for stress?

The inconsistency of the results obtained from behavioral tests measuring memory performance raises the question if they really measure any drug toxicity-related alterations in these parameters. It is likely that performance is affected also by other parameters likely to be affected by drug treatments, such as stress. For instance, it is likely that non-contingent, high-dose amphetamine administration induces a much higher level of stress than corresponding saline injections in experimental animals.

It is therefore notable that one of the few brain regions affected by 4-MMC on the MEMRI scan was the hypothalamus, which showed a robust 10% increase in activity compared to saline-treated animals. Moreover, when looking at novel object recognition performance in the same experiment, the percentage of time spent exploring the novel object was significantly correlated to signal intensity in two brain regions across all

subjects (unpublished data). The first region was the ventral hippocampus ($r = 0.43$, $N = 22$, $p < 0.05$), which was expected, considering the importance of the hippocampus in learning and memory (Felix and Levin, 1997; Lipska et al., 2002; Broadbent et al., 2004; Winters et al., 2004). The second region showing significant correlation was the lateral hypothalamus ($r = 0.48$, $N = 22$, $p < 0.05$), a region known for its pivotal role in mediating the stress response (Herman and Cullinan, 1997; Ida et al., 2000). This could indicate that stress may be an important factor in determining the performance in rodent tests of memory after amphetamines. The effect of stress on memory performance is complex, and both improvement and impairment of memory function as a result of stress has been reported (Park et al., 2001; Duncko et al., 2009; Sunada et al., 2010), which could explain the largely inconsistent results obtained from animal models of cognition following treatment with 4-MMC, but also with other amphetamines.

Further work is needed to establish the exact role of stress in mediating the performance of rodents in behavioral tests of memory function following exposure 4-MMC and amphetamines in general. Meanwhile, conclusions based on these results should be drawn with great caution.

6.4 Keto amphetamine redox reactivity and toxicity

6.4.1 β-keto amphetamines produce cytotoxicity *in vitro*

Previously it has been shown that METH is toxic and causes cell death in SH-SY5Y cells at concentrations in a similar range as those used here (Wu et al., 2007; Wisessmith et al., 2009; Nara et al., 2010; Suwanjang et al., 2010). Here, we show that 4-MMC and MDMC produce a higher degree of cytotoxicity than METH. Furthermore, 4-MMC toxicity was partially blocked by DAT inhibitor GBR12909.

A recent study reported on the *in vitro* toxicity of a range of β-keto amphetamines in rat hepatocytes (Araujo et al., 2014). Additionally, there is one report of MDMC toxicity in Chinese hamster ovary cells transfected with SERT DAT and NET. Here, only the SERT transfected cells displayed increased cytotoxicity after MDMC incubation at 100 µM. However, DAT SERT and NET transfection all increased the toxicity of METH when the two drugs were administered together (Sogawa et al., 2011). This is reminiscent of the *in vivo* findings of 4-MMC not being toxic on its own, but increasing the toxicity of METH when administered together (Angoa-Perez et al., 2013). Aside from the studies mentioned above, this appears to be the first report specifically on the *in vitro* toxicology of substituted cathinones in neuron-like cells.

6.4.2 β-keto amphetamines possess specific redox reactivity

The increase in WST-1 reduction observed in the experiments set out to measure cellular proliferation after 4-MMC and MDMC treatment was striking due to the magnitude of the reactivity, and the fact that no evidence of increased proliferation was visible when examining the cells visually. It suggested that another factor was responsible for the increased reactivity. WST-1 is a water soluble tetrazolium salt, and the WST-1 assay is a colorimetric assay based upon the reduction of the pale yellow WST-1, via an intermediate electron acceptor, to the darker colored formazan (Berridge et al., 2005). The next step was therefore to investigate the possibility that β-keto amphetamines can function as reducing agents in the presence of this electron acceptor.

6.4.3 β-keto amphetamine as reducing agents: biological significance

The experiments using the WST-1 reagents as a redox sensitivity assay clearly indicate that β-keto amphetamines are powerful and selective reducing agents in the presence of an electron acceptor, compared to their non-keto counterparts. Furthermore, the reducing properties appear to be enhanced as a function of increasing pH in the physiological range and are blocked by antioxidants. This raises the question whether or not this property has any biological or toxicological significance. One possibility is that the reducing properties of β-keto amphetamines could interfere with cellular redox buffering systems. Such is the case, for instance, with redox-active iron, which has been shown to be a source of oxidative stress associated with apoptosis (Smith et al., 1997; Savory et al., 1999).

To study the possible biological significance of the redox properties of 4-MMC it was chosen to study the effects on mitochondria, as this organelle is dependent upon a high volume of redox reactions for normal functioning and also has a slightly alkaline pH. The results showed that METH, but not 4-MMC, produced an inhibition of mitochondrial complex II. The observed effect of METH on complex II is consistent with previous findings demonstrating inhibition of this complex by METH (see section 2.7.2). The finding that 4-MMC produced no apparent inhibition of specific mitochondrial complexes is surprising, considering the fact that it produced a general decrease in both baseline and maximum mitochondrial respiration that was much higher than what was observed after METH.

One reason for the observed discrepancy may be that different types of assays were used for the respiration assay and complex I/II assay. Whereas respiration was measured after one day incubation, the complex I/II assay was performed after only 20 min incubation with the drugs, albeit in a higher concentration. Thus, it is possible that more time may have been needed to induce inhibition of mitochondrial complexes, or that the longer incubation led to production of metabolites with more potent ETC-inhibitory effects.

Furthermore, the complex I/II assay was performed on mouse cortical tissue homogenates, whereas the respiration experiments were performed in SH-SY5Y cells.

The redox properties of β-keto amphetamines may play an important role in their interaction with cellular targets. Further study of the exact biological significance of β-keto amphetamine redox properties in terms of their interaction with mitochondria and other cellular targets is clearly warranted.

6.4.4 The cathinones 4-MMC and MDMC produce breakdown products

The analysis of 4-MMC and MDMC reaction products was initially performed in order to test the hypothesis that these drugs were capable of forming adducts with amino acids, something which has been implicated in the neurotoxicity of ketone-containing substances such as acrylamide (Lopachin and Decaprio, 2005). We did not find any evidence of adduct formation, but instead observed the breakdown products shown in Fig. 5.1.

In the 4-MMC sample *N*,4-dimethylbenzamide and *N*-Acetyl-4-MMC was detected. Accordingly, the MDMC sample contained the dioxol-ring substituted analogues of these compounds, namely 3,4-methylenedioxy-*N*-methylbenzamide and N-Acetyl-MDMC. Interestingly, these products were previously detected after incubation of 4-MMC in alkaline pH (Tsujikawa et al., 2012), and suggest a specific degradation pathway whereby 4-MMC is converted via two intermediate substances to *N*,4-dimethylbenzamide, in the process releasing acetic acid, which reacts with remaining 4-MMC to produce *N*-Acetyl-4-MMC (see **IV**, Fig. 9). Here, we demonstrate that this reaction occurs also in the presence of an electron acceptor.

Currently, the biological significance of these breakdown products is unknown. A further investigation into breakdown products of keto amphetamines is needed in order to draw conclusions about their toxicological relevance.

6.5 Neurotoxicity of cathinones and amphetamines

6.5.1 Cathinones are less neurotoxic than amphetamines

It is interesting to note that the occurrence of novel psychoactive substances that started gaining pace in 2009 is not the first time there has been interest in the toxicology of cathinones. A handful of studies investigated the neurotoxicity of cathinone and methcathinone in the 1980s and 90s, as it enjoyed brief popularity as a recreational drug and due to khat use (Wagner et al., 1982; Roper, 1986; Gygi et al., 1996; Sparago et al.,

1996). Sparago et al. (1996) treated animals with methcathinone and reported reductions in DA levels after a two week survival interval, but only at doses of 80 mg/kg or higher administered repeatedly. Gygi et al. (1996) reported no effects at doses of 10-20 mg/kg but found that doses of 30 mg/kg were associated with decreases in DA, TH, 5-HT and TPH levels. However, the 30 mg/kg dose also produced high lethality and, furthermore, all measurements were performed no later than 3 days after the final drug treatments, and thus did not strictly measure long-term effects.

The previous interest in cathinones, however, was brief. Interest soon shifted towards the, at that time, more prevalent MDMA and METH. But the enormous increase in novel psychoactive substance use in recent years has led to renewed research interest in cathinones. The data presented in this thesis have been published simultaneously with a number of other studies initiated in response to the recent increase in substituted cathinone use (see section 5.2.3). Taking into consideration the previous and current data on cathinone neurotoxicity, together with the results presented in this thesis, it appears that cathinones have a more favourable safety profile than amphetamines. From a harm-reduction point of view this is important information. However, it should be stressed that the present data focuses on neurotoxicity. Amphetamines can, particularly at high doses, exert powerful and potentially detrimental effects on the hepatic, renal and cardiovascular systems (Carvalho et al., 2012), and it is still unclear how cathinones compare to amphetamines in this respect.

6.5.2 Added stress factors may induce 4-MMC neurotoxicity

The results presented above suggest that 4-MMC produces little, if any, toxicity when administered on its own under normal conditions. However, a few circumstances have been identified under which 4-MMC does appear to produce or enhance neurotoxicity in a fashion reminiscent of what is seen with non-keto amphetamines.

One such condition is administration of 4-MMC at high (> 27°C) ambient temperatures. For instance, decreased 5-HT uptake has been demonstrated in synaptosomes prepared from rats treated with 4-MMC at high ambient temperatures one week earlier (Hadlock et al. 2011). Similarly, it was reported that binge 4-MMC treatments produced a reduction in DAT and SERT binding in mice when measured 3 days after the final drug treatment. However most of these values restored to control levels when measured at the 7 day time point. Furthermore, there were no clear effects in terms of TH, TPH and glial fibrillary acidic protein activity levels at 7 days either (Martinez-Clemente et al., 2014).

In a set of interesting experiments, one group investigated the interaction between 4-MMC and non-keto amphetamine neurotoxicity in mice. Specifically, they investigated if concurrent treatment with 4-MMC would affect the depletion of striatal and hippocampal

DA and 5-HT caused by METH and MDMA. The results confirmed that 4-MMC on its own did not affect monoamine levels, but showed that 4-MMC did exacerbate METH- and MDMA-induced depletions of striatal DA when the two drugs were administered together. On the other hand, 4-MMC did not appear to exacerbate the effects of these drugs on hippocampal 5-HT levels (Angoa-Perez et al., 2013; Angoa-Perez et al., 2014).

The experiments on the effect of ambient temperatures and co-treatment with non-keto amphetamines described above are particularly valuable as they provide specific and useful harm reduction information.

6.6. Limitations

The results presented in this thesis provide important new information about the potential neurotoxicity and long-term neuropharmacological effects of new amphetamine-type stimulants obtained using a wide range of methods, ranging from studies in human recreational users to preclinical and *in vitro* experiments. All these methods are subject to certain limitations, which are addressed in this section.

6.6.1 Human studies

The results from the human neuroimaging study presented in this thesis (**I**) did not support the hypothesis or the data obtained from the analogous animal experiment. Unfortunately, the inconsistency of data appears to be a general trend in studies of human amphetamine users. For instance, although DAT binding was decreased in abstinent METH users (McCann et al., 2008), there was no correlation between DAT binding and the time of abstinence or between DAT binding and measures of cognitive function (Johanson et al., 2006). With regards to MDMA, one study (McCann et al., 1998a) reported decreased SERT binding in MDMA users but no correlation between decreases in SERT binding and time of abstinence. Investigations of cerebral metabolism and blood flow also demonstrate alterations in amphetamine users compared to control groups that do not show any correlation between cognitive test performance (Daumann et al., 2003; Daumann et al., 2005; Becker et al., 2013).

With regards to effects on cognition, the results are not clear either. Although groups of abstinent amphetamine users sometimes display statistically significant lower scores on memory tests compared to control groups (see section 2.6), they still perform well within the normal range, making it unclear if it really can be considered a deficiency, due to the lack of any clinically significant impairment (Hart et al., 2012). The issue is particularly salient as the cross-sectional design of most studies makes it impossible to draw conclusions with regards to causality. Thus, the question remains whether the observed effects are actually due to drug consumption or if amphetamine users exhibit pre-existing

differences in parameters such as monoamine transporter binding and memory function, compared to non-users.

This question was one important reason for performing the MEMRI study presented in this thesis. This study demonstrated changes in brain activity following METH that showed a large degree of overlap with those shown to be affected in abstinent METH users (see **III** and section 5.3.2). Translating the results of this study would suggest that changes in brain chemistry could indeed be related to amphetamine use. Further MEMRI studies are clearly warranted to gain more insight into the dose-dependency and time course of the observed effects. However, no effect was found on memory function, meaning any observed effect on memory in human studies may just reflect pre-existing differences. The exact functional ramification, if any, of amphetamine-induced long-term alterations in neurophysiological parameters remain to be determined

6.6.2 Preclinical studies

The animal studies presented in this thesis (**I**, **II**, **III**) have all employed relatively high drug doses between 5 and 30 mg/kg administered in a binge-like fashion over several days. One of the major controversies in the preclinical literature on amphetamine toxicity is the issue of interspecies scaling (McCann and Ricaurte, 2001; Vollenweider et al., 2001). Rodents are typically administered doses of 10-20 mg/kg or more, often repeatedly over the course of one to several days, whereas a human recreational dose of MDMA is around 1.3 mg/kg (based on a 100 mg pill consumed by an 80 kg individual). Even with several tablets consumed in one night, the cumulative dose usually does not come close to the doses regularly administered to rodents in a laboratory setting. The rationale for these high doses comes from the concept of allometric interspecies scaling, a technique commonly used in medicine development to calculate human doses based on animal data. This method is based on compensating for faster basal metabolic rate, heart rate and circulation times that are expected to result in more rapid drug metabolism in smaller animals. However, there are a number of objections against using this formula for certain substances, particularly amphetamines, which are extensively metabolized, thereby complicating simple scaling based on basal metabolic rate. MDMA also displays nonlinear kinetics, with increasing doses producing unexpectedly high plasma concentrations, and, furthermore, possible species-dependent differences in metabolism, tissue uptake or other parameters are also not taken into consideration (Baumann et al., 2007).

Due to the problems associated with allometric scaling, Baumann et al. (2007) has suggested using an alternative method, effects scaling, to compensate for differences between species. This method is based on establishing the lowest dose which produces a specific pharmacological effect, such as measurable neurotransmitter or hormone release, or the dose needed to produce specific behavioral effects such as reinforcement and drug discrimination. This method appears more sensible in this context as it is based

purely on the effects of the drug at a certain dose, without having to predetermine numerous factors as would be required in order to correctly employ allometric scaling. When using effects scaling, the doses required to achieve similar amounts of 5-HT release, prolactin secretion, reinforcement or drug discrimination in rodents and humans are very similar, and all in the 1-3 mg/kg range (Baumann et al., 2007), closely corresponding to the doses used recreationally by humans. More importantly, when administered at these doses (< 5 mg/kg) there is no evidence of long-lasting monoamine depletions (O'Shea et al., 1998; Baumann et al., 2007).

Another important issue which limits the validity of preclinical studies is related to the dosing regimens commonly employed. Most commonly, the drug is administered as multiple, equal injections of the compounds. Aside from the issues associated with allometric scaling, on which these high doses are based, they are a poor reflection of how humans commonly use psychostimulants, as human use is generally characterized by initial light use, which gradually escalates to the intake of larger amounts. This is particularly relevant as several studies show that preconditioning animals with low doses of METH or MDMA is protective, and drastically reduces the effect of subsequent high drug doses on monoamine levels and ROS production (Graham et al., 2008; Cadet et al., 2009; El Ayadi and Zigmond, 2011; Hodges et al., 2011; Mayado et al., 2011). Other factors, such as voluntary exercise (Toborek et al., 2013) also offer protection against METH toxicity and indicate that administration of high-doses of amphetamines in relatively low-enriched caging environment may not correctly model the effects in humans.

Importantly, effects scaling or naturalistic dosing regimens were not employed in the present study. The primary reason for this was to be able to directly compare the effects of keto and non-keto amphetamines, and since the majority of the literature employs the aforementioned high, non-progressive doses, using similar doses provides the best way of directly comparing the two types of drugs. Conclusions about the relative toxicity of cathinones and amphetamines are therefore defendable. However, whether, or to what extent, the toxicity that is seen in animals also occurs in humans at common recreational doses in the first place is still unclear.

6.6.3 *In vitro* studies

The results from the *in vitro* study presented in this thesis (**IV**) demonstrated cytotoxic effects of 4-MMC and MDMC at millimolar concentrations. It is important to note that these results should not be translated in a way to suggest that these drugs are more neurotoxic *in vivo*, primarily because the actual *in vivo* data does not support this conclusion. Furthermore, these millimolar concentrations are around a thousand times higher than the low micromolar concentrations observed in human users (Logan et al., 1998). At the more physiologically relevant concentrations, a decrease in toxicity was in

fact observed. Nonetheless, a number of factors known to mediate neurotoxicity *in vivo*, such as ROS generation and mitochondrial damage, are replicated in the SH-SY5Y cell line at these high concentrations (Wu et al., 2007; Wang et al., 2008), suggesting that the SH-SY5Y cell line is a valid model for comparing and contrasting the effects of keto and non-keto amphetamines and for investigating mechanisms potentially involved in producing toxic effects of these substances *in vivo.*

6.7 Future directions

The phenomenon of new and obscure psychoactive drugs occurring on the drug market via online distribution, which started in the early 2000's but gained pace rapidly around 2009, does not show any sign of slowing down. In fact, the number of new psychoactive substances which have been officially reported has been increasing every year since 2008, with over 80 new substances reported in 2013 alone (Fig. 2.2). The actual number of new substances may be even higher, considering the likelihood that not all new substances are rapidly detected by authorities.

6.7.1 Ongoing screening of new drugs

As new drugs continue to appear, it is important to continuously keep an eye on this new online drug market, and to identify new trends and substances in order to subject them to scientific scrutiny. With the number of new substances being reported every year, it is currently impossible to study each compound in-depth. Nonetheless, preclinical and *in vitro* studies of the most popular compounds is clearly warranted in order to make a reasonable risk assessment, to identify mechanisms involved in producing potential neurotoxic effects, and to identify possible strategies for harm reduction.

6.7.2 Answers in human studies

In light of the occurrence of new amphetamines and other psychedelic drugs, it is important to note the recently renewed interest in these substances as potential treatments for a wide range of mental disorders, including depression, anxiety disorders and addiction. MDMA has received the most interest so far in terms of completed studies (Mithoefer et al., 2011; Chabrol, 2013; Mithoefer et al., 2013; Oehen et al., 2013) and ongoing clinical trials (NCT01211405, NCT01793610 and NCT01689740) for treatment of post-traumatic stress disorder. The antidepressant effect of ketamine has been known for some time, but is now again receiving more attention (Berman et al., 2000; Salvadore and Singh, 2013; Moaddel et al., 2014 and NCT01558063). Also 5-HT hallucinogens such as lysergic acid diethylamide and psilocybin are being tested for applications ranging from anxiolysis to smoking cessation (Grob et al., 2011; Gasser et al., 2014 and NCT01943994).

Interestingly, the results from the published studies have so far been quite favorable. The studies are also important for another reason, as they can provide clear answers regarding the effects of drugs such as MDMA at relevant clinical and recreational doses in humans. After around three decades of research on MDMA there are still a number of unanswered questions about its long-term effects on humans. It is therefore advisable that the ongoing and future clinical studies of these substances include carefully planned and meticulously designed cognitive tests and, when possible, neuroimaging experiments, in order to provide more definitive answers regarding the effects of these drugs in humans.

6.7.3 A toolbox of novel psychoactive substances

The rise of the novel psychoactive substance phenomenon is often considered mostly in terms of its potential public health threat. Although this is not unwarranted - it is always best to approach unknown compounds with caution – it is perhaps not right to only see it in this light. The results presented in this thesis emphasize that novel does not necessarily imply more dangerous. In fact, new substances may even have more favorable safety profiles, compared to their more well-known analogues and counterparts. Aside from empathogenic phenethylamines such as 4-MMC and MDMC discussed in this thesis, other novel psychoactive substances include tryptamines, cannabinoids and ketamine analogues. These are exactly the classes of drugs which have been showing very favorable results in recent clinical trials. Therefore, novel psychoactive substances represent not only a threat to public health but also, when approached systematically and studied meticulously, a toolbox filled with an almost unlimited amount of new tools, with potential applications in psychiatry, neurology and possibly other branches of medicine.

7. Conclusions

Based on the results of specific studies described in this thesis, it can be concluded that:
1. In rats, treatment with MDMA during adolescence is less toxic to 5-HT neurons in the PFC compared to treatment in early adulthood. However, no such age-related effect on 5-HT neurotoxicity was observed in humans.

2. The substituted cathinone 4-MMC produces no evidence of neurotoxicity in mice or rats. MDMC produces 5-HT toxicity similar to MDMA in rats, although the effect may be less severe. Furthermore, neither drug produces any consistent long-term effects on cognition or other neurobehavioral measurements in mice.

3. The long-term effects of 4-MMC on rat brain activity are much less pronounced than those of METH.

4. Specific differences in chemical properties between β-keto amphetamines and non-keto amphetamines, such as altered redox reactivity, could play a role in their differing long-term effects.

When translating these results, the following more general conclusions about the long-term neuropharmacological and neurotoxic effects of cathinones can be drawn:

1. Occasional recreational use of the substituted cathinones 4-MMC and MDMC does not appear to be more harmful than the use of amphetamines such as METH and MDMA. In fact, they may be less harmful.

2. At present, there is no indication that these cathinones constitute an imminent and severe public health threat, as was initially feared. However, further work is needed before definitive conclusions about its safety in humans can be drawn.

3. From a harm reduction perspective, the cathinones may be interesting because they produce similar subjective effects as amphetamines, but may have a more favourable safety profile.

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