

**Comparative neuropharmacology of the substituted amphetamines,
p-methoxyamphetamine (PMA) &
3,4-methylenedioxymethamphetamine (MDMA)**

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Abstract

Dramatic growth in substituted amphetamines ('Ecstasy') use since the 1980's has correlated with increased incidence of acute toxicity and residual neuropsychological deficits. This thesis aimed to characterise the acute neurochemical mechanisms and residual neurochemical alterations produced by p-methoxyamphetamine (PMA), which is usually sold as 'ecstasy' and is associated with greater acute toxicity than 3,4-methylenedioxymethamphetamine (MDMA). While both PMA and MDMA primarily modulate dopaminergic and serotonergic neurotransmission, little is known of the differences in the neurochemical effects of PMA within the central nervous system, *in vivo*. This thesis used *in vivo* chronoamperometry to elucidate the acute neurochemical alterations in monoaminergic pharmacology *in vivo* after local application of PMA or MDMA within discrete brain nuclei in anaesthetised rats. Measurement of evoked release of monoamines including serotonin (5-HT), and inhibition of neurotransmitter uptake via membrane transporters were assessed.

Initial studies compared pharmacodynamic responses of PMA and MDMA, showing PMA to have greater efficacy and potency for alteration of core body temperature in rats, a primary cause of acute toxicity, within minimal alteration in locomotion. Dose-response studies indicated local PMA application within striatum resulted in significantly greater 5-HT evoked release than MDMA, yet lesser dopaminergic release, as predicted by the pharmacodynamic data. Only PMA-evoked release could be partially blocked by pre-treatment with a 5-HT reuptake inhibitor (SERT). Differences in both the qualitative and quantitative nature of striatal evoked-release of 5HT and dopamine were noted for both drugs, which had not been previously seen. Both PMA and MDMA inhibited 5-HT clearance, but only MDMA inhibited dopamine clearance in striatum. Dose-response studies in the CA3 region of hippocampus indicated PMA was also more efficacious than MDMA in the inhibition of 5-HT clearance *in vivo*.

While the question of whether long term MDMA use induces selective neurodegeneration (reductions in serotonergic *in vitro* biomarkers) is still unclear, it was not known for PMA prior to this work. Repeated PMA administration was shown to result in reductions in cortical SERT (indicative of potential loss of 5-HT terminal axons), cortical 5-HT content was unaltered. A subsequent comprehensive study followed, comparing the residual effects of PMA or MDMA

administration on *in vitro* serotonergic biomarkers (markers of selective neurodegeneration) and SERT function *in vivo*. PMA administration resulted in reductions in hippocampal SERT binding and [³H]-5HT synaptosomal uptake, correlating with *in vitro* biomarkers previously used. SERT function *in vivo* using chronoamperometric techniques was reduced, as would be predicted. However, hippocampal 5-HT content was again not reduced, indicating that selective neurodegeneration of 5-HT fibres may not in fact be occurring. MDMA administration reduced all measured *in vitro* serotonergic biomarkers, however SERT function *in vivo* was completely unaltered. These data indicate that reductions of *in vitro* biomarkers of 5-HT axonal degeneration do not necessarily predict the potential compensatory mechanisms that maintain SERT function *in vivo*. Compensatory mechanisms appear to exist *in vivo* to maintain clearance of extracellular 5-HT that may be disrupted or eliminated during tissue preparation for *in vitro* assays.

In summary, while PMA produced significantly greater alterations, compared to MDMA, in processes intrinsic to 5-HT neurotransmission in both striatum and hippocampus, the magnitude of these responses did not explain the significantly higher risk of acute toxicity seen clinically with PMA use. The second component of the thesis extended beyond prior work, investigating the potential neurodegenerative effects of PMA and MDMA through the assessment of changes in *key functional processes* in 5-HT neurotransmission. It is hoped this will contribute to the subsequent characterisation of the mechanism(s) of functional compensation in 5-HT neurotransmission which may lead to more targeted treatments to modulate potential psychological/psychiatric deficits that occur in regular 'ecstasy' users.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

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Daws L. C., Irvine R. J., **Callaghan P. D.**, Toop N. P., White J. M. and Bochner F. (2000) Differential behavioural and neurochemical effects of para-methoxyamphetamine and 3,4-methylenedioxyamphetamine in the rat. *Prog Neuropsychopharmacol Biol Psychiatry* 24, 955-977.

Callaghan P. D., Irvine R. J. and Daws L. C. (2005) Differences in the *in vivo* dynamics of neurotransmitter release and serotonin uptake after acute para-methoxyamphetamine and 3,4-methylenedioxyamphetamine revealed by chronoamperometry. *Neurochem Int* 47, 350-361.

Callaghan P. D., Farrand K., Salem A., Hughes P., Daws L. C. and Irvine R. J. (2006) Repeated administration of the substituted amphetamine p-methoxyamphetamine produces reductions in cortical 5-HT transporter binding but not 5-HT content, unlike 3,4-methylenedioxyamphetamine. *Eur J Pharmacol* 546, 74-81.

Callaghan P. D., Owens W. A., Javors M. A., Sanchez T. A., Jones D. J., Irvine R. J. and Daws L. C. (2007) *In vivo* analysis of serotonin clearance in rat hippocampus reveals that repeated administration of p-methoxyamphetamine (PMA), but not 3,4-methylenedioxyamphetamine (MDMA), leads to long-lasting deficits in serotonin transporter function. *J Neurochem* 100, 617-627.

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Mr Callaghan had a major input in the experimental design, conducted all *in vivo* electrochemical experimental procedures, statistical analysis and graphical presentation of the data collected, and was involved in preparation of the manuscript for submission.

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Professor Bochner was involved in the experimental design, and contributed to the interpretation of the data collected and preparation of the manuscript.

Professor White was involved in the experimental design, and contributed to the interpretation of the data collected and preparation of the manuscript.

Associate Professor Irvine was involved in the experimental design, conducted telemetric studies and contributed to the interpretation of the data collected and preparation of the manuscript.

Associate Professor Daws was involved in the experimental design, contributed to the interpretation of the data collected and preparation of the manuscript

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Mr. Hughes was involved in the experimental design and conducted animal procedures for experiment 2.

Dr. Salem was involved in the experimental design, contributed to the experimental procedures, interpretation of the data collected and preparation of the manuscript.

Associate Professor Irvine was involved in the experimental design, contributed to the experimental procedures, interpretation of the data collected and preparation of the manuscript.

Associate Professor Daws was involved in the experimental design, and contributed to the interpretation of the data collected and preparation of the manuscript.

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Mr. Owens was involved in the autoradiographic procedures, and interpretation of the data collected.

Ms. Sanchez conducted *in vitro* hippocampal 5-HT and noradrenaline uptake procedures, and interpretation of the data collected.

Professor Jones was involved in the experimental design of *in vitro* hippocampal 5-HT and noradrenaline uptake procedures, contributed to the experimental procedures and interpretation of the data collected.

Professor Javors was involved in conducting HPLC analysis of extracted 5-HT samples, and interpretation of the data collected.

Associate Professor Irvine was involved in the experimental design, and contributed to the interpretation of the data collected and preparation of the manuscript.

Associate Professor Daws was involved in the experimental design, and contributed to the interpretation of the data collected and preparation of the manuscript.

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Auxiliary publications not for assessment for this thesis

The following publications would be presented as auxiliary publications, indicating my current research contribution to the field of the pharmacology of stimulants and drug abuse. These publications will not be presented for assessment for the degree of Doctor of Philosophy.

Kaminskas L.M., Irvine R.J., Callaghan P.D., White J.M. and Kirkbride P. 2002. The contribution of the metabolite p-hydroxyamphetamine to the central actions of p-methoxyamphetamine. *Psychopharmacology (Berl)* 160, 155-160. Impact factor: 3.625 (2006)

Daws L.C., Callaghan P.D., Moron J.A., Kahlig K.M., Shippenberg T.S., Javitch J.A. and Galli A. 2002. Cocaine increases dopamine uptake and cell surface expression of dopamine transporters. *Biochem Biophys Res Commun* 290, 1545-1550. Impact factor: 2.855 (2006)

Irvine, R.J., Keane, M., Felgate. P., McCann, U.D., Callaghan, P.D., White, J.M. 2005. Plasma Drug Concentrations and Physiological Measures in 'Dance Party' Participants. *Neuropsychopharmacology*. 31(2), 424-30. Impact factor: 5.889 (2006)

Abbreviations, prefixes and symbols

PMA	p-methoxyamphetamine
MDMA	3,4-methylenedioxyamphetamine
CNS	Central nervous system
POHA	p-hydroxyamphetamine
5-HT	5-hydroxytryptamine
5-HIAA	5-hydroxy-indolacetic acid
MAO	Monoamine oxidase
SERT	5-HT transport protein
DAT	Dopamine transport protein
IC ₅₀	Concentration of 50% inhibitory response
EC ₅₀	Concentration of 50% response
E _{max}	Maximal response
MTA	Myoclonic twitch activity
PKC	Protein kinase C
NET	Noradrenaline transporter protein
KO	Knockout
GABA	gamma-hydroxybutyric acid
LD ₅₀	Dose resulting in 50% lethality
CYP	Cytochrome P450 isozyme
MDA	Methylenedioxyamphetamine
HHMA	Dihydroxymethamphetamine
HHA	Dihydroxyamphetamine
OHMDMA	6-hydroxymethylenedioxyamphetamine
THMA	2,4,5-trihoxymethamphetamine
COMT	catechol-O-methyl transferase

MMAI	5-methoxy-6-methyl-2-aminoindan
BID	Twice daily
GFAP	Glial acidic fibrillary protein
PBR	Peripheral benzodiazepine receptor
kDa	Kilodalton
NO	Nitric oxide
nNOS	neural nitric oxide synthase
L-DOPA	L-3,4-dihydroxyphenylalanine
m-CPP	m-chlorophenylpiperazine
CSF	Cerebrospinal fluid
PET	Positron emission tomography
SPECT	Single photon emission computed tomography
OCT	Organic cation transporter
mRNA	Messenger ribonucleic acid

*And the world's concerns with its rights and wrongs
Shall seem but small things
Poet or painter, a singer of songs,
Thine art is all things.*

*For the wine of life is a woman's love
To keep beside thee
But the love of Art is a thing above
A star to guide thee.*

*As the years go by with thy love of Art
All undiminished
Thou shalt end thy days with a quiet heart
Thy work is finished.*

Passage from *'Ambition and Art'*, by A.B. 'Banjo' Patterson

1. Research background

The substituted amphetamines, 3,4-methylenedioxymethamphetamine (MDMA) and p-methoxyamphetamine (PMA) are members of a relatively new class of drugs that have become immensely popular amongst the “Rave” and dance music scene since the 1980’s. Their positive mood and stimulant effects and supposed lack of prominent acute side effects (in comparison to so-called ‘hard’ drugs, like heroin and cocaine) have led to the perception that they are relatively benign (Solowij et al. 1992). Media reports have drawn attention to the ‘dark’ side of these drugs, in particular the severe acute adverse responses that can lead to the need for urgent medical care. Both MDMA and PMA have been attributed to causing fatalities worldwide. Death results primarily from an acute hyperthermic response, which in turn leads to rhabdomyolysis, kidney failure and other cardiovascular disturbances. Also, most fatalities occur in young, healthy people and unlike most illicit drugs, acute adverse effects do not always appear to be related to the dose of drug taken (Dowling et al. 1987; Screamon et al. 1992; Felgate et al. 1998).

Due to the ever increasing prevalence of use of ‘ecstasy’ worldwide, there has been a considerable need for further investigation of the clinical pharmacology and neurochemical mechanisms that underlie the acute adverse responses of these drugs, in addition to investigation of what the ramifications of long term use will be.

As such, there has been extensive characterisation of the pharmacological and physiological effects of the compound sold as ‘ecstasy’, MDMA. Illicit PMA use, again under the name ‘ecstasy’, has a more restricted market and geographical distribution, yet appears to be associated with a much greater incidence of acute adverse responses, compared to MDMA (White et al. 1997; Felgate et al. 1998; Byard et al. 1999a, b; Ling et al. 2001; Kaminskis et al. 2002; Caldicott et al. 2003; Irvine et al. 2006). PMA use only became more widespread in relatively recent times (since 1994 in Australia, and the late 1990’s worldwide) and relatively little is still known about its clinical and basic

pharmacology and toxicology (Kraner et al. 2001; Chodorowski et al. 2002; Galloway and Forrest 2002; Voorspoels et al. 2002; Becker et al. 2003; Dams et al. 2003; Johansen et al. 2003; Refstad 2003; Lora-Tamayo et al. 2004). Reports of increased toxicity seen with PMA in comparison to MDMA have come from retrospective case studies investigating fatal and non-fatal acute adverse effects (Felgate et al. 1998; Byard et al. 1999a, b; Ling et al. 2001; Caldicott et al. 2003; Irvine et al. 2006). These studies firmly suggest that PMA used recreationally has considerably greater potential for acute toxic events compared to MDMA.

The primary aim of this project is to investigate the mechanisms of action of PMA and MDMA *in vivo*, in order to understand the potential differences in the neurochemical mechanism of action that may underlie the increased propensity for toxicity seen clinically with PMA. This will involve *in vivo* investigation of PMA and MDMA effects on the neurochemistry within the brain pathways involved in their primary psychoactive properties, namely their ability to modulate monoamine pharmacology. In addition, these studies will be extended to examine the longer term effects of repeated use on *in vivo* neurochemical function in these same pathways.

1.1. Prevalence of use (Australia and worldwide)

The growth in ecstasy and substituted amphetamine use and manufacture worldwide has reached alarming proportions. Recent data from the United Nations indicate 40 million people worldwide (1% of world population) use amphetamine type substances, such as amphetamine, methamphetamine and ecstasy (UNODC 2003). Of these, 8 million people have used ecstasy in the 12 months prior to the completion of this study. World averages indicate that 18-20 year old people are the greatest users of ecstasy, with 6 times greater use than the population average.

Use of ecstasy was highest in east and south-east Asia, Europe, Australia and the United States. Figure 1 outlines the prevalence of ecstasy and methamphetamine use on a country by country basis (UNODC 2003). This indicates Australia to have the highest use of ecstasy per capita in the world, followed by Ireland, UK, Spain, Belgium and the USA respectively. This study also highlighted the worrying fact that unlike many other illicit drugs, younger ecstasy users are not giving up as they age, and in fact older people are commencing use. As previously mentioned, most ecstasy users perceive the drug as safe, in spite of the significant amount of conflicting information available in the public domain over the internet and in the media. Figure 2, from the UN report on global amphetamine use indicates this to be the case, with the perception of the drug harmfulness of ecstasy decreasing over time despite the increased occurrence of acute adverse events, as measured by emergency room admissions (UNODC 2003).

In light of the fact that Australia has the highest prevalence of ecstasy use (per capita), and the second highest prevalence of methamphetamine use (per capita) in the world, it is not surprising that novel substituted amphetamines such as PMA would come to prominence in this location (figure 3). Current data indicate 7.5% of the Australian population aged 14 or over have tried ecstasy, a trend that has been increasing each year since 1988, when data were first collected, as shown in figure 4 (AIWH 2005). Prevalence of PMA use appears to be much lower, but is only detected by its presence in drug seizures, or through clinical admissions to medical institutions (Cimbura 1974; Felgate et al. 1998; James and Dinan 1998; Byard et al. 1999a, b; Kraner et al. 2001; Ling et al. 2001; Martin 2001; Becker et al. 2003; Caldicott et al. 2003; Dams et al. 2003; Johansen et al. 2003; Refstad 2003; Lora-Tamayo et al. 2004; Irvine et al. 2006). PMA is not currently included in any of the epidemiological tools used for assessment of drug use trends in Australia, probably due to apparent sporadic appearance and disappearance within the illicit drug market.

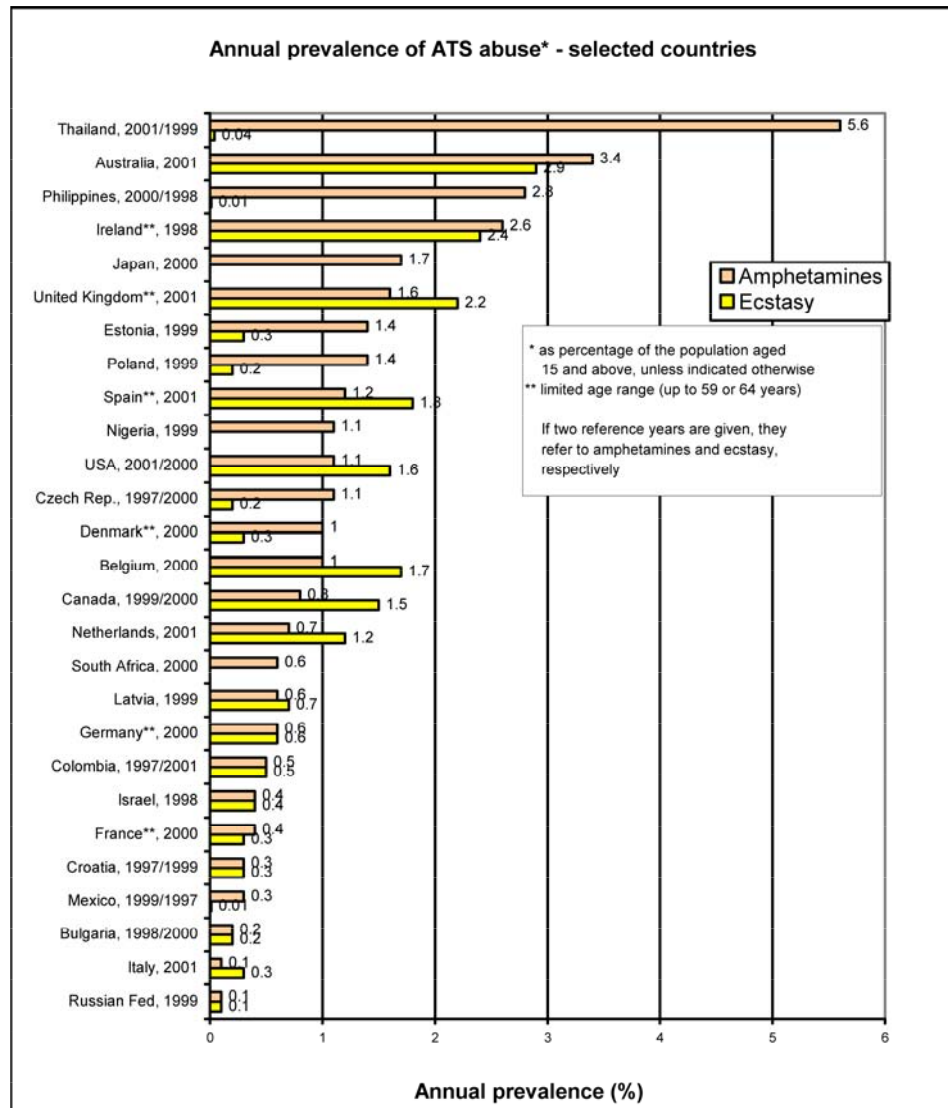


Figure 1: Prevalence of Amphetamine type substances on a country by country basis. Taken from UNODC (2003). Sources: Annual Reports Questionnaires Data; Government Reports; US Department of State; EMCDDA; UNODC estimates.

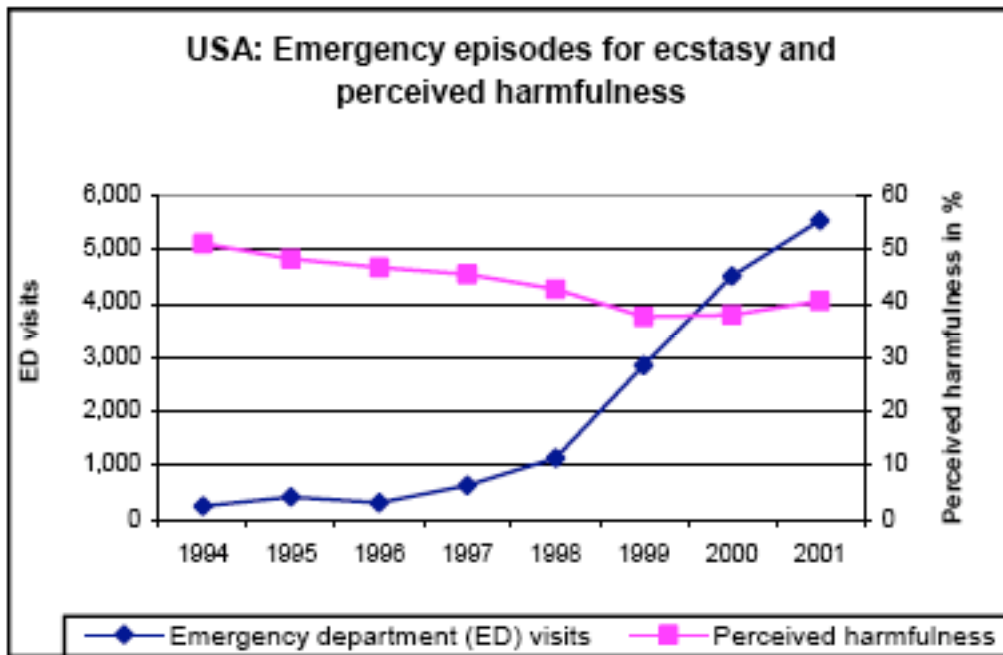


Figure 2: Perceived harmfulness of ecstasy does not correlate with occurrence of acute adverse event, as measured by emergency room admissions. Taken from UNODC (2003). Source: Drug Abuse Warning Network (DAWN) 2002. NIDA, Monitoring the future Volume 2. 2002.



Figure 3: Clandestine manufacture of Ecstasy. Taken from UNODC (2003). Source: UNODC, Annual reports Questionnaire data

NOTE:

This figure is included on page 6 of the print copy of the thesis held in the University of Adelaide Library.

Figure 4: Prevalence of ecstasy use in Australia, 1988-2004. (Figure taken from AIWH, 2005).

The USA and Australia do not appear to be major sites for manufacture of ecstasy (UNODC 2003), which differs from the situation for methamphetamine manufacture in these countries. The main exporters of ecstasy to Australia are Europe and Asia. Figure 3 indicates main sites of ecstasy manufacture worldwide (UNODC 2003). How and why PMA manufacture has come to prominence in Australia is still not well understood. What is clear is that PMA must be synthesised on purpose, and is not a by-product of failed MDMA synthesis (Kirkbride et al. 2001; Blachut et al. 2002; Waumans et al. 2003). In Australia, the growth in the illicit ecstasy market has correlated with increases in seizures by Australian Customs, but in the last 2004-5 period the rate of increase has more than doubled (AIWH 2005). The much smaller scale of the PMA market appears to be supplied by local manufacture.

1.2. Historical origins of PMA and MDMA

MDMA was originally patented in 1914 for use as an appetite suppressant, but never marketed (Dowling et al. 1987). Some psychotherapists during the 1960-1980's sanctioned it as a useful adjunct in therapy, especially in marriage guidance counselling, but this was controversial (Green et al. 2003). Small numbers of psychiatrists began to use MDMA as an adjunct to psychotherapy, due to the drug's ability to facilitate communication and increase self-esteem. Despite this rather controversial medicinal use, MDMA has been primarily used as a recreational drug. Its use as an illicit drug grew during the late 1970's and particularly within the 1980's, leading to it being declared an illegal substance in 1977 in the UK, and in 1985 in the USA (Green et al. 2003).

PMA was not synthesised as a therapeutic compound like MDMA, but was discovered in the seminal work of Alexander Shulgin, investigating the structure-function relationships of substituted amphetamines on CNS function (Smythies et al. 1967; Shulgin et al. 1969). It was originally postulated that PMA may be involved in amphetamine-induced psychosis,

being formed by methylation of p-hydroxyamphetamine, the primary metabolite of amphetamine (Angrist et al. 1970; Schweitzer et al. 1971). This theory was later discounted, but by this stage the first illicit use of PMA had been noted in Canada (Cimbura 1973). Early studies investigating the potential psychiatric properties of various substituted amphetamines indicated PMA to have potent hallucinogenic properties, suggesting an involvement with the 5-HT neurotransmitter system (Smythies et al. 1967; Shulgin et al. 1969). PMA illicit use did not reappear until the early 1990's, in Adelaide, Australia (Byard et al. 1998; Felgate et al. 1998; James and Dinan 1998). Since this point in time, PMA has been responsible for the overwhelming majority of Australian 'ecstasy' related deaths (Byard et al. 1998; Felgate et al. 1998; James and Dinan 1998; Byard et al. 1999b; Ling et al. 2001; Caldicott et al. 2003). Within ten years, PMA-related deaths had been noted in Europe and North American, indicating a significant dispersion in drug use (Kraner et al. 2001; Martin 2001; Becker et al. 2003; Dams et al. 2003; Johansen et al. 2003; Refstad 2003; Lora-Tamayo et al. 2004).

1.3. Acute clinical pharmacology

Only in recent years have controlled clinical studies been conducted to characterise the acute pharmacodynamic actions of MDMA. No such studies exist for PMA. The primary reason for this is related to the potential for these drugs to induce 'neurotoxic damage', as shown in animal models, and indirectly in clinical studies on regular ecstasy users. Due to this major ethical dilemma, reports of clinical effects have primarily come from case notes and forensic reports from subjects who use the drug illicitly.

1.3.1. Mood state

'Ecstasy' induces a positive mood state, with users reporting feelings of euphoria, closeness to others and increased sensuality (Solowij et al. 1992). While these positive

effects predominate in recreational users, increased doses and increased frequency of use leads to a decrease in the pleasurable effects and an increase in the negative effects. The negative effects include palpitations, increased blood pressure, chills, sweating, muscle aches, tremor, loss of appetite, jaw clenching, teeth grinding, increased thirst, impaired balance, tremor and insomnia (Solowij et al. 1992; McCann et al. 1996). Many of these negative effects are a component of ‘serotonin syndrome’, and are induced by drugs that increase synaptic concentrations of 5-HT (Gillman 1997; Green et al. 2003). In a controlled clinical study with low dose MDMA administration, some subjects noted an inability to concentrate, and altered thought processes, in addition to serotonin syndrome behaviours (Vollenweider et al. 1998). The negative emotional effects are known to persist for up to 24 hours after drug taking (Solowij et al. 1992; Solowij 1993; Vollenweider et al. 1998; Topp et al. 1999). Repeated use of MDMA during this refractory period can lead to an exacerbation of this ‘hangover’ effect, leading to feelings of depression, irritability and social withdrawal (Solowij et al. 1992; Parrott 2001). Additionally, acute psychoses, panic attacks and anxiety are noted.

Anecdotal reports suggest PMA to have similar mood altering effects to that of MDMA, with one significant difference being a purported lesser ‘stimulant effect’ (Ling et al. 2001; Caldicott et al. 2003). Similar “negative” effects have been noted (James and Dinan 1998; Ling et al. 2001; Caldicott et al. 2003). However, due to the sporadic occurrence of PMA in the illicit drug marketplace and its usual covert sale as ‘ecstasy’, retrospective studies of the subjective effects of the drug are very difficult if not impossible at this point.

1.3.2. Body temperature

The effects of MDMA and other substituted amphetamines on thermoregulatory dysfunction are primarily responsible for most acute adverse effects of these drugs. The drug-induced hyperthermia can lead to rhabdomyolysis if untreated, which in turn leads to

disseminated vascular coagulation, and renal failure and coma in the most severe cases. Cases of extreme hyperthermia, resultant rhabdomyolysis, coma and death are thought to be related to serotonin syndrome (Gillman 1997; White et al. 1997). This is further supported by the increased acute morbidity when users take drugs such as monoamine uptake inhibitors or selective serotonin uptake inhibitors in addition to MDMA (Vuori et al. 2003). Users report a greater ‘high’ with lower doses of MDMA required, but other unwanted effects of serotonin syndrome are also reported.

A similar presentation of serotonin syndrome and sympathomimetic effects such as hyperthermia, anxiety, agitation, nausea and palpitations have also been noted in retrospective case note studies for PMA in Australia (Ling et al. 2001; Caldicott et al. 2003). Hyperpyrexia and resultant rhabdomyolysis and coma have been noted in cases of fatality. As previously noted, within Australia in particular, in spite of the highest use of MDMA per capita in the world, all but two ‘ecstasy’-linked deaths have been related to PMA, rather than MDMA (Byard et al. 1998; Felgate et al. 1998; Byard et al. 1999a, b; Ling et al. 2001; Caldicott et al. 2003). It is this forensic data that indicates PMA’s high level of toxicity.

Currently, no approved targeted pharmacotherapies exist to reverse this resultant hyperthermia. Treatments rely on lowering body temperature by conventional means. Most deaths from acute ‘ecstasy’ use result from complications of, or directly from the acute hyperthermic response. Further understanding of the underlying neurochemical mechanism of MDMA and other substituted amphetamine-induced hyperthermia will allow better targeted pharmacotherapies.

1.3.3. Stimulant effects (activity/cardiovascular)

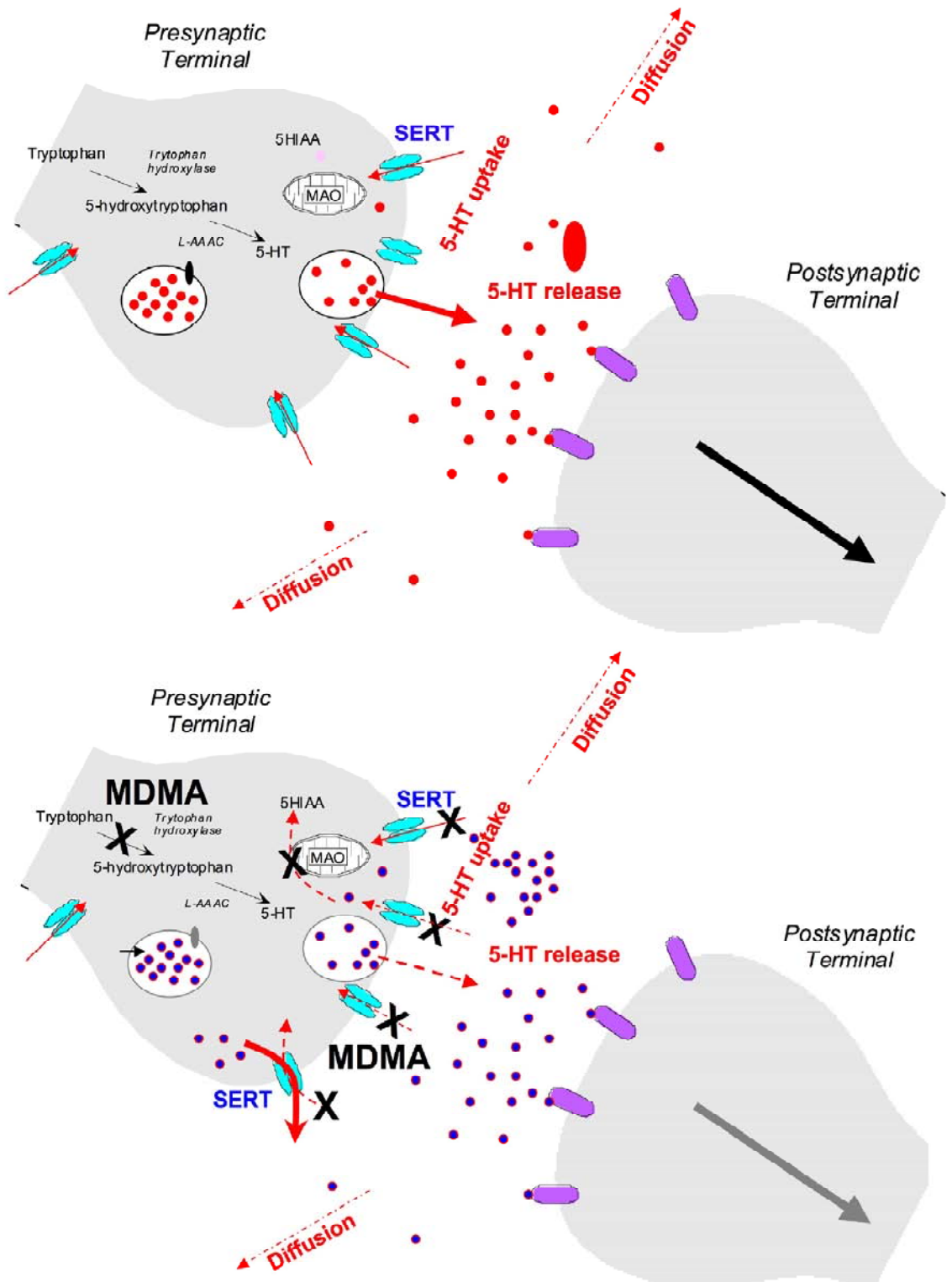
In addition to the above mentioned responses, MDMA is also a sympathomimetic drug. MDMA use is known to be associated with cerebrovascular “accidents”, in addition to

cardiac effects (Reneman et al. 2000). This is consistent with data shown in animal models where MDMA mediates cardiac stimulant effects, namely tachycardia and arrhythmia (Gordon et al. 1991). MDMA was also shown to have vasoconstrictor properties in rodent aortic rings, further fitting with its profile as a sympathomimetic (Fitzgerald and Reid 1994). MDMA dose dependently increases mean arterial blood pressure (O'Cain et al. 2000), in addition to increasing heart rate (Bexis et al. 2004). This is consistent with the increases in mean arterial blood pressure seen in humans given low doses of MDMA within a controlled clinical setting (Vollenweider et al. 1998; Lester et al. 2000)

1.4. Mechanism of action

As their primary mode of action, substituted amphetamines modulate key processes in monoaminergic (dopamine, serotonin and noradrenaline) synaptic transmission, as illustrated in figures 5A & 5B. With normal monoaminergic neurotransmission, after depolarisation of the presynaptic terminal, vesicles containing neurotransmitter are sequestered to the presynaptic membrane after calcium ion influx from calcium channels, and neurotransmitter is released into the synaptic cleft. Termination of the subsequent pre- and/or post-synaptic receptor signalling by the neurotransmitter is controlled by three major processes: diffusion of neurotransmitter from the synaptic cleft; metabolism of the neurotransmitter in situ, and active transport of neurotransmitter by specific membrane-bound transporter proteins back into the presynaptic terminal. Substituted amphetamines modulate these processes to induce their physiological and psychedelic effects.

Figure 5: Sketch of the neurochemical effects of MDMA within a typical 5-HT synapse. **A.** Normal mechanisms involved in synthesis, release and re-uptake of 5-HT within the synapse. **B.** The initial action of MDMA involves binding to the SERT (see **X**) and a resulting reversal of transporter function to increase 5-HT efflux (see **red solid arrow**) into the synaptic cleft. Normal active uptake back into the presynaptic terminal via the SERT is inhibited (see **red dashed arrow**), resulting in further increases in synaptic 5-HT concentration. In addition, metabolism of 5-HT via monoamine oxidase (MAO) is inhibited (see **X**), leading to further increases in synaptic 5-HT. After the initial 5-HT release events, there is a depletion of pre-synaptic 5-HT stores, which is accentuated by blockage of the rate-limiting enzyme controlling 5-HT synthesis, tryptophan hydroxylase (see **X**), by MDMA. This leads to reduction in serotonergic pre- and post-synaptic activation, which correlates with the ‘come-down’ phase after MDMA use in humans.



The acute actions of PMA and MDMA are mediated primarily by their interactions with either the 5-hydroxytryptamine (5-HT, serotonin) transporter (SERT) and/or the dopamine transporter (DAT) to evoke release of neurotransmitter, and inhibit neurotransmitter uptake from presynaptic and extrasynaptic sites within the CNS (Menon et al. 1976a; McKenna and Peroutka 1990; Daws et al. 2000b; Gough et al. 2002a; Kaminskas et al. 2002; Green et al. 2003; Callaghan et al. 2005; Freezer et al. 2005). *In vivo* and *in vitro* evidence for these mechanisms, and how they relate to physiological effects are discussed below.

1.4.1. Serotonergic neurotransmission

1.4.1.1. MDMA effects on 5-HT release and reuptake processes

Acute MDMA administration has been demonstrated to produce a rapid and dose dependent release of 5-HT in terminal regions in the CNS (Gough et al. 1991; Yamamoto et al. 1995; Gudelsky and Nash 1996; Sabol and Seiden 1998; Shankaran and Gudelsky 1999; Nixdorf et al. 2001; Rothman et al. 2001; Gough et al. 2002a; Mechan et al. 2002). This results in a subsequent depletion of 5-HT content in terminal field regions, such as striatum, hippocampus, hypothalamus and cortex (Schmidt et al. 1986; Stone et al. 1987a; Battaglia et al. 1988a; Finnegan et al. 1988; Logan et al. 1988; Colado and Green 1994; Callaghan et al. 2006). This has been shown to be a carrier-mediated process both *in vivo* and *in vitro* (Gudelsky and Nash 1996; Crespi et al. 1997; Esteban et al. 2001). Microdialysis studies show that MDMA evoked 5-HT release is attenuated by administration of the SERT inhibitor fluoxetine, in striatum and hippocampus, suggesting a pivotal role of SERT in the actions of MDMA to modulate serotonergic neurotransmission (Gudelsky and Nash 1996; Mechan et al. 2002). Additionally, MDMA produces a dose-dependent release of 5-HT from brain slice preparations, which was again blocked by SERT inhibitors (Schmidt 1987b; Schmidt et al. 1987; Berger et al. 1992; Crespi et al.

1997; Koch and Galloway 1997). MDMA also evoked dose dependent release of [H^3] 5-HT release from cortical, striatal and hippocampal synaptosomes (Berger et al. 1992; O'Loinsigh et al. 2001; Rothman et al. 2001).

While MDMA binds to all three monoamine transporters, it exhibits the highest affinity for the SERT (Rothman and Baumann 2002; Verrico et al. 2007). Data from homogenate binding studies show MDMA to have a submicromolar affinity for SERT (Battaglia et al. 1988b). In addition, binding is stereoselective, with the *S*-(+) isomer more potent (Steele et al. 1987). MDMA has potent effects on 5-HT release (again with a submicromolar affinity), inducing release at 10 fold lower concentrations than those required to induce DA release (Steele et al. 1987; Rothman et al. 2001).

1.4.1.2. PMA effects on 5-HT release and reuptake processes

In contrast, much less is known about the actions of PMA to modulate serotonergic neurotransmission. *In vitro*, PMA has been shown, like MDMA, to be a potent 5-HT releasing agent in rat brain synaptosomes (Nichols et al. 1982). Initial studies showed a ten fold greater efficacy in elevating brain 5-HT release and reducing 5-HIAA content, compared to amphetamine (Hitzemann et al. 1971). Additionally PMA (sub micromolar affinity) was a far more potent 5-HT releasing agent than amphetamine after local application in cortical and striatal brain slices (Tseng et al. 1976). Sub-micromolar concentrations of PMA also inhibited [H^3]-5-HT uptake into cortical and striatal brain slices (Tseng et al. 1976). More recent studies have shown PMA to have a low micromolar affinity (IC_{50}) for inhibition of [H^3]-5-HT uptake into striatal homogenate preparations (Hegadoren et al. 1994). Administration of PMA also produced a significant, acute increase in cortical 5-HT content, as expected (Hegadoren et al. 1995).

Initial investigations of the role in 5-HT in PMA actions *in vivo* relied on an indirect measure of brain 5-HT release, the myoclonic twitch activity (MTA) of the suprahyoidal

muscle (Menon et al. 1976a; Tseng 1978b, 1979). Dose dependent effects of PMA on MTA were blocked by administration of SERT inhibitors, fluoxetine and chlorimipramine (Menon et al. 1976a; Clineschmidt and McGuffin 1978; Tseng 1979). Additionally, this effect was shown to be mediated indirectly through 5-HT receptors as the antagonist methysergide blocked PMA induced MTA. Administration of the tryptophan hydroxylase inhibitor, p-chlorophenylalanine also reduced PMA-induced MTA, indicating that PMA was not directly interacting with postsynaptic 5-HT receptors, but required an intracellular store of 5-HT to be present in order to increase extracellular 5-HT concentration (Tseng 1978b). MTA induced by PMA were not blocked by xylamidine, a 5-HT receptor antagonist that supposedly doesn't pass through the blood-brain barrier, indicating MTA is mediated through changes in CNS 5-HT levels (Clineschmidt and McGuffin 1978). More recent microdialysis studies have shown PMA dose dependently increased extracellular 5-HT concentrations in striatum and substantia nigra (Gough et al. 2002b; Freezer et al. 2005; Hewton et al. 2007; Stanley et al. 2007). Interestingly, the comparison of maximal 5-HT efflux indicated MDMA to have either equivalent or slightly greater efficacy compared to PMA. This was also noted in the recent study of Romero and coworkers, where perfusion of PMA and MDMA yielded similar efficacy in striatal 5-HT release (Romero et al. 2006). As such, it is difficult to understand how acute differences in thermogenic responses between MDMA and PMA could be related to 5-HT release. As such, further comparison of the potency and efficacy of PMA and MDMA is needed to further understand this key issue. In addition, the monoamine transporter dependency of PMA evoked neurotransmitter release had not previously been investigated. This was investigated within the scope of this thesis.

1.4.1.3. MDMA and PMA effects on 5-HT synthesis

In addition to interactions involving 5-HT release and reuptake, MDMA has been shown to significantly inhibit the synthesis of 5-HT through inactivation of the rate-limiting enzyme of production, tryptophan hydroxylase (Schmidt and Taylor 1987; Stone et al. 1987a; Stone et al. 1987b; Schmidt and Taylor 1988; Che et al. 1995a). MDMA inactivates tryptophan hydroxylase within 15 minutes in striatum, frontal cortex, hippocampus and hypothalamus (Stone et al. 1987b). Activity remains attenuated more than 2 weeks after a single dose of MDMA (Schmidt and Taylor 1987). Interestingly, MDMA does not inhibit tryptophan hydroxylase when incubated *in vitro* (Schmidt and Taylor 1987). The mechanism of tryptophan hydroxylase inactivation may involve free radical generation. Quinone formation (from MDMA or dopamine metabolism) may bind to the sulfhydryl groups of tryptophan hydroxylase, leading to deactivation (Ratray 1991). Evidence for this proposal includes data showing tryptophan hydroxylase activity can be restored by reduction of sulfhydryl reagents under anaerobic conditions (Stone et al. 1989). The protective effect of hypothermia appears to correlate with a reduction in free radical production (Che et al. 1995b; Colado et al. 1999a).

The effects of PMA on 5-HT synthesis are not known.

1.4.1.4. MDMA and PMA effects on 5-HT metabolism

MDMA has also been shown to inhibit monoamine oxidase (MAO). It has a 10 fold greater affinity for the MAO-A subtype (low micromolar), compared to MAO-B (Leonardi and Azmitia 1994). In comparison, PMA is a significantly more potent and selective reversible inhibitor of MAO-A (sub-micromolar affinity), compared to MAO-B (high micromolar affinity), *in vitro* and *in vivo* (Green and El Hait 1980; Freezer et al. 2005). This could indicate the inhibition of MAO by PMA may contribute to the elevated 5-HT extracellular

concentrations seen after PMA administration *in vivo*, as MAO-A has greater selectivity for 5-HT than other monoamines (Garrick and Murphy 1982). Indeed, co-administration of the reversible MAO-A inhibitor moclobemide with PMA did not further potentiate the increase in striatal extracellular 5-HT content, as would be expected if MAO-A activity was already maximally inhibited by PMA (Freezer et al. 2005). However, moclobemide co-administration with MDMA did significantly potentiate the increase in striatal extracellular 5-HT content seen, indicating a significant difference in potency *in vivo* between the two drugs on MAO activity (Freezer et al. 2005).

1.4.2. Dopaminergic neurotransmission

1.4.2.1. MDMA actions on dopamine synthesis, release and reuptake processes

MDMA also produces a dose dependent release of dopamine within terminal field regions (e.g. cortex, striatum, nucleus accumbens) and from dopaminergic cell bodies (e.g. substantia nigra), as shown clearly in numerous microdialysis studies (Gough et al. 1991; Nash and Brodtkin 1991; Yamamoto et al. 1995; Gudelsky and Nash 1996; Koch and Galloway 1997; Sabol and Seiden 1998; Colado et al. 1999b; Esteban et al. 2001; Nixdorf et al. 2001; Gough et al. 2002a; Freezer et al. 2005), *in vitro* brain slice studies (Johnson et al. 1986; Schmidt 1987b; Crespi et al. 1997) synaptosomal preparations (Rothman et al. 2001) and *in vivo* voltammetric studies (Yamamoto and Spanos 1988; Iravani et al. 2000), through calcium-dependent and -independent mechanisms. Studies examining the effect of MDMA *in vivo* show a rapid increase in dopamine release in regions such as striatum (Logan et al. 1988; Yamamoto and Spanos 1988; Gough et al. 1991; Colado and Green 1994; Gough et al. 2002a). MDMA does not affect the rate-limiting enzyme in dopamine synthesis, tyrosine hydroxylase (Gibb et al. 1989).

MDMA induced [H^3] dopamine release from rat brain slices was one order of magnitude less potent than amphetamine, indicating a significant difference in potency (Fitzgerald and Reid 1993). As previously mentioned, MDMA has a 10 fold lower affinity for the DAT compared to the SERT (Steele et al. 1987; Battaglia et al. 1988b). Stereoselectivity is also evident with MDMA actions at the DAT, with again the *S*-(+) isomer being more potent (Steele et al. 1987).

The role of specific monoamine transporters in MDMA-mediated dopamine release appears to be far more complex than the mechanisms of MDMA-mediated 5-HT release, as previously described. To determine if MDMA-mediated dopamine release was carrier-mediated by the DAT, various approaches have been taken with differing results. The selective DAT inhibitor, GBR12909 blocked MDMA-mediated DA release from striatal brain slices (Koch and Galloway 1997). GBR12909 also attenuated striatal extracellular dopamine release as measured by microdialysis after local application of MDMA through the probe, again consistent with the DAT playing a pivotal role in dopamine release (Nash and Brodtkin 1991). However, GBR12909 did not block increases in dopamine extracellular concentration after peripheral MDMA administration (Mechan et al. 2002). One explanation for this result is that the concentration of GBR12909 was not high enough. However, DA release induced by the substituted amphetamine, methamphetamine, was also not blocked by mazindol, even at very high doses (Marek et al. 1990). In addition, lack of effect of GBR12909 on blockade of MDMA evoked DA release was also reported in mice (O'Shea et al. 2001).

Interestingly, MDMA appears to inactivate DAT (as shown by reduced DAT binding) for at least 24 hours after peripheral administration (Hansen et al. 2002). This also presented as a dose dependent reduction in [H^3]-dopamine release from rat striatal synaptosomes from animals 1 hour, but not 24 hours after MDMA treatment (Metzger et al. 1998). The reduction in dopamine uptake after co-incubation of striatal synaptosomes with MDMA

was prevented by treatment with protein kinase C (PKC) inhibitors (Hansen et al. 2002). This could indicate that MDMA in part, may internalise DAT from the cell membrane to reduce dopamine transport, as has been shown for amphetamine, through PKC activation and subsequent phosphorylation of the transporter (Saunders et al. 2000). As such, the complexity of MDMA interactions with the DAT to modulate dopamine release and reuptake appear to be much less clear than the relationship between SERT and MDMA-induced 5-HT release.

The role of DAT in MDMA- & PMA-mediated dopamine release is further complicated by promiscuity of other monoamine transporters (SERT and NET), that under appropriate conditions, can release and block reuptake of dopamine. For example, MDMA-mediated striatal dopamine release can also be significantly attenuated by fluoxetine (Koch and Galloway 1997), but not NET inhibitors or noradrenaline depleting agents (Shankaran and Gudelsky 1998). However, MDMA-mediated hippocampal dopamine release was significantly attenuated by NET inhibitors or a prior noradrenergic lesion (Shankaran and Gudelsky 1998). This indicates that evoked dopamine efflux may be released via interactions at the NET & SERT, in addition to the DAT in certain brain regions. Conversely, similar promiscuity is seen with extracellular 5-HT uptake via DAT. In homozygous SERT knockout mice, 5-HT can be detected immunohistochemically within dopaminergic neurons in substantia nigra and ventral tegmental area (Zhou et al. 2002b). This was further reinforced when the selective DAT inhibitor GBR-12935 blocked the aberrant 5-HT uptake into DA neurons. The findings from this emerging field have important implications for understanding the mechanism of action of amphetamines within the CNS.

1.4.2.2. PMA effects on dopamine synthesis, release and reuptake processes

As noted previously, considerably less is known about the pharmacology of PMA-mediated modulation of dopaminergic neurotransmission. The first microdialysis study has shown peripheral PMA administration resulted in an increase in striatal dopamine release only at a very high dose of 20 mg/kg (Gough et al. 2002a). Interestingly, recent studies have shown perfusion of PMA through the microdialysis probe into striatum to evoke dopamine and 5-HT release at equivalent efficacy (Romero et al. 2006).

Prior studies have shown PMA to dose-dependently evoke [H^3]-DA release from preloaded cortical and striatal brain slices, but much less potent compared to amphetamine (Tseng et al. 1974). PMA was only 14% as effective compared to amphetamine at blocking [H^3]-DA uptake into striatal slices (Tseng et al. 1974). PMA administration to rats also had negligible effects on striatal tissue content of DA (Martin-Iverson et al. 1991).

The effects of PMA on dopamine synthesis are not known.

1.4.3. Noradrenergic neurotransmission

The effects of acute MDMA administration on noradrenergic neurotransmission appear to be less dramatic than that of the other monoamines, as highlighted above. MDMA was shown *in vitro* to increase basal release of [H^3]-noradrenaline from preloaded brain slices, an effect blocked by the NET inhibitor, desipramine (Fitzgerald and Reid 1993). Interestingly, MDMA evokes noradrenaline release with a similar potency to 5-HT release, but not dopamine release (4 times less potent) from rat brain synaptosomes (Rothman et al. 2001). MDMA was also a more potent inhibitor of noradrenaline uptake (K_i :0.46 μ M) than dopamine uptake (K_i :1.5 μ M), but not 5-HT uptake (K_i :0.24 μ M) in rat synaptosomal preparations (Steele et al. 1987; Rothman et al. 2001). Differences in the potency of

inhibition of [³H]- noradrenaline uptake were not seen between the isomers of MDMA (Steele et al. 1987)

PMA is known to be an inhibitor of [³H]-noradrenaline uptake into cortex and striatum in tissue slices *in vitro* (Tseng et al. 1974). Its efficacy for release of noradrenaline was also considerably greater than its effects on dopamine release, being equipotent with amphetamine, indicating significantly greater efficacy compared to MDMA (Tseng et al. 1974; Tseng et al. 1976). This was also shown by increased brain noradrenaline concentrations (with similar potency/efficacy to amphetamine) after peripheral PMA administration in rats (Hegadoren et al. 1994; Hegadoren et al. 1995). In spite of these findings, the functional role of noradrenaline release in PMA behaviour/physiology is not clear.

1.4.4. Direct receptor interactions

In addition to the primary mode of action of MDMA, through interaction with monoamine transporters, this compound is known to have affinity for various neurotransmitter receptors. Based on the K_i value from binding studies, MDMA has a moderate affinity for 5-HT₂, α_2 adrenergic, M₁ cholinergic and H₁ histaminergic receptors (1-10 μ M). MDMA has moderate affinity (10-100 μ M) for M₁ cholinergic, 5-HT₁, α_1 and β adrenergic receptors. MDMA had negligible affinity (> 100 μ M) for opioid, benzodiazepine and D₁ and D₂ dopamine receptors (Battaglia et al. 1988b).

MDMA has also been shown to have high affinity (EC₅₀: low micromolar) for the trace amine receptor (Bunzow et al. 2001). However, this receptor is at low concentrations in rat brain, and its physiological role is still unclear.

Little is known of whether PMA actions involve direct receptor interactions in addition to interactions at monoamine transporters. PMA was shown to have low affinity

(micromolar) for 5-HT₁ and 5-HT₂ receptors, but other receptors have not been investigated (Battaglia and De Souza 1994).

1.5. Physiological/behavioural effects in animals

1.5.1. Thermoregulation

Many studies have shown MDMA to produce a dose dependent alteration in core body temperature, inducing a marked hyperthermic response at a normal (20-22°C) ambient environmental temperature (Nash et al. 1988; Schmidt et al. 1990b; Dafters 1994; Che et al. 1995b; Dafters 1995; Malberg et al. 1996; Malberg and Seiden 1998; O'Shea et al. 1998; Bexis et al. 2004). Also, MDMA produces a significant hypothermic response in a cold ambient environment (Gordon et al. 1991; Dafters 1994; Dafters and Lynch 1998; Malberg and Seiden 1998). Both metabolic rate and evaporative water loss are increased with hyperthermic responses to MDMA administration in normal or increased ambient temperatures (Gordon et al. 1991). Metabolic rate is not affected after MDMA treatment at low ambient temperatures where hypothermia was seen (Gordon et al. 1991). This was further reinforced by the work of Mechan and coworkers, showing that rat tail temperature is unchanged after MDMA doses that induce hyperthermia (Mechan et al. 2002). This indicates that a major heat loss mechanism in rodents is impaired after MDMA administration (Green et al. 2003).

The pharmacological mechanism for MDMA-mediated hyperthermia is more complex than initial conjecture that indirectly proposed MDMA-evoked acute 5-HT release could be a primary mechanism. 5-HT has been shown to mediate hyperthermia (Lin et al. 1998), as shown indirectly by administration of L-tryptophan or monoamine oxidase inhibitors (Grahame-Smith 1971). Administration of the 5-HT precursor, 5-hydroxytryptophan or fluoxetine into the anterior-preoptic hypothalamus elicits a significant hyperthermic

response, correlating with local increased 5-HT extracellular concentration (Lin et al. 1998). However, co-administration of fluoxetine with MDMA, while almost totally inhibiting 5-HT release, had no effect on the hyperthermic response (Schmidt et al. 1990b; Berger et al. 1992; Malberg et al. 1996; Mehan et al. 2002). In addition, selective 5-HT_{2A} and 5-HT_{2C} antagonists failed to block the hyperthermic response of MDMA (Mehan et al. 2002). Interestingly, the dopamine D₁ antagonist SCH23390 but not the DAT inhibitor, GBR12909, dose dependently inhibited MDMA-mediated hyperthermia, indicating the effects of MDMA on dopaminergic neurotransmission (possibly indirectly) may be critical in this response. The GABA_B agonist baclofen was also shown to block MDMA induced hyperthermia, but mechanism for this response is not clear (Bexis et al. 2004).

Again, very few studies have investigated the effects of PMA on thermoregulation. PMA was initially shown to induce hyperthermia in rats given 10 mg/kg, but not 5 mg/kg (Menon et al. 1976a). The ambient temperature was not reported, which is a critical factor in determining hypo- or hyperthermic responses after MDMA administration (Dafters 1994; Broening et al. 1995; Malberg et al. 1996; Dafters and Lynch 1998; Malberg and Seiden 1998). In the study of Nichols and coworkers, PMA at the enormous dose of 60.7 mg/kg produced a significant hyperthermic effect for the 1st 3-4 hours after administration, followed by hypothermia (Nichols et al. 1975). However, using a dose near or above the LD₅₀ of PMA (as determined in this study) is a significant confound. As such, the data reflect only the surviving animals in the study.

As such, there is a need to compare the pharmacodynamic dose-response relationship of PMA or MDMA administration on core body temperature. Additionally, the role of ambient temperature in the modulation of PMA effects on core temperature needs to be assessed, with respect to the effects seen after MDMA.

1.5.2. Serotonin syndrome and stimulant effects

Drugs that increase extracellular concentrations of 5-HT elicit a characteristic set of behaviours in animals, referred to as serotonin syndrome. These include hyperactivity, head weaving, piloerection, fore paw threading, salivation, penile erection and ejaculation. MDMA elicits a well characterised dose-dependent increase in locomotor activity (Slikker et al. 1988; Spanos and Yamamoto 1989; Callaway et al. 1990; Colado et al. 1993; McNamara et al. 1995). The other characteristic behaviours are also seen after MDMA administration (Slikker et al. 1988; Spanos and Yamamoto 1989; Colado et al. 1993; Hegadoren et al. 1995; Shankaran and Gudelsky 1999)

The pharmacological mechanism for MDMA-mediated increases in activity is complex, involving both serotonergic and dopaminergic components. Blockade of the SERT with fluoxetine prevented the dose dependent effects of MDMA on activity (Callaway et al. 1990). The role of pre- and postsynaptic 5-HT and dopamine receptors in this response is complex (Kehne et al. 1996; Bankson and Cunningham 2002). Qualitative differences in the spatial nature of the hyperactivity response exist between MDMA-mediated compared to amphetamine-mediated effects (Callaway et al. 1990). While amphetamine hyperactivity is not spatially restricted, MDMA-treated rats have increased activity around the periphery of the chamber (thigmotaxis).

These characteristic serotonin syndrome behaviours are also significantly expressed after PMA administration (Martin-Iverson et al. 1991; Hegadoren et al. 1995). However, the locomotor component of these behaviours was absent or reduced for PMA (as previously mentioned) (Hitzemann et al. 1971; Menon et al. 1976a), when compared at equimolar dose with MDMA. This probably correlates with the lack of efficacy of PMA for modulation of dopamine release. As such, further work is required to compare the dose response effects of PMA and MDMA on locomotor activity, over a dose range where maximal effects are seen. This is addressed within this thesis.

1.5.3. Cardiovascular effects

Evidence exist showing cardiovascular toxicity in some cases with both PMA and MDMA use in humans (Mas et al. 1999; de la Torre et al. 2000b; O'Cain et al. 2000; Ling et al. 2001)). Gordon and colleagues showed MDMA to mediate cardiac stimulant effects, namely tachycardia and arrhythmia in rodents (Gordon et al. 1991). MDMA was also shown to have vasoconstrictor properties in rodent aortic rings (Fitzgerald and Reid 1994). MDMA dose dependently increases mean arterial blood pressure (O'Cain et al. 2000), in addition to increasing heart rate (Bexis et al. 2004). This is consistent with the increases in mean arterial blood pressure seen in humans (Vollenweider et al. 1998).

Few studies have investigated the cardiovascular effects of PMA administration in animals. The initial study of Cheng and coworkers demonstrated PMA to be a powerful sympathomimetic in dogs, blocked by the post-ganglionic autonomic inhibitor, guanethidine (Cheng et al. 1974). This included an increase in heart rate mediated by beta-adrenergic receptors. Similar effects were also seen in cats (Nichols et al. 1975). It is not clear whether these effects are resulting from central or peripheral stimulation of sympathetic neurons.

1.6. Pharmacokinetics

MDMA has been shown to display non-linear pharmacokinetics, indicating a linear increase in dose results in an increase in blood/body concentration of drug that cannot be predicted (de la Torre et al. 2000a; de la Torre et al. 2004). Low doses in humans have been shown to have a half life of 8-9 hours, with a 30-60 minute delay in the onset of notable effects (de la Torre et al. 2004), with a duration of action of 3-4 hours (Liechti and Vollenweider 2001). There is still conjecture over what blood concentrations are considered to be toxic. Many pharmacokinetics studies in humans have used low concentrations, to avoid ethical concerns of long term neurodegenerative effects (de la

Torre et al. 2000b; Pacifici et al. 2002; de la Torre et al. 2004; de la Torre et al. 2005). A recent investigatory study taking samples from recreational users in a recreational environment in South Australia indicated that the blood concentrations were considerably higher than predicted from the pharmacokinetic studies, with all subjects reporting no adverse effects (Irvine et al. 2006). As such, further work is necessary to correlate pharmacokinetic studies with data obtained from the recreational environment. Factors such as physical exertion, environmental temperature, hydration status may have significant influences on the pharmacokinetics and metabolism of MDMA.

In contrast, PMA possesses simple linear pharmacokinetics in humans, rats, guinea pigs and dogs, with one metabolite by o-demethylation, p-hydroxyamphetamine (Hubbard et al. 1977; Kitchen et al. 1979; Kaminskis et al. 2002). 70-80% of the parent drug was excreted as conjugated metabolite within 24 hours in rats (Kitchen et al. 1979).

1.7. Metabolism

Like most amphetamines, MDMA is primarily metabolised by the liver in the periphery. Unlike other amphetamine analogues such as methamphetamine or p-methoxyamphetamine, MDMA has a very complex metabolic pathway. This complexity is thought to provide an explanation for the lack of apparent dose response relationship seen between MDMA tablet intake, and the development of side effects and acute adverse events. The primary complication lies in the fact that many of the metabolites of MDMA are active psychoactive substances in their own right (Henry et al. 1998; Gowing et al. 2002). Methylenedioxyamphetamine (MDA, also known as 'eve') is a drug of abuse in its own right, having similar psychoactive properties to MDMA and often sold as 'ecstasy'. MDA is also added to tablets containing other amphetamines (Dowling et al. 1987; Henry et al. 1992). Being similar compounds, they are also metabolised primarily by the same

isozymes of the cytochrome P450 enzymes (CYP), an extremely important class of enzymes that also breakdown most illicit and legitimate pharmaceuticals in the body.

Metabolites of MDMA are generated via 5 main biosynthetic pathways. The two most important of these, N-demethylation and O-demethylation, appear to play a primary role in rodents, non-human primates and humans (as shown in figure 6) (Lim and Foltz 1988; de la Torre and Farre 2004). Of these pathways, the three most important are N-demethylation, producing methylenedioxyamphetamine (MDA), O-demethylation, producing dihydroxymethamphetamine (HHMA) and dihydroxyamphetamine (HHA) and ring hydroxylation, producing 6-hydroxymethylenedioxyamphetamine (6-OHMDMA). O-Demethylation and N-demethylation have been shown to be major routes of metabolism in the rat liver and brain, leading to MDA, HHMA and HMMA being the primary metabolites (Tucker et al. 1994). However in humans, O-demethylation leads to the production of HHMA and HMMA as the primary metabolites of MDMA. The disparity in metabolic profile between mice, rats and even non-human primates and humans means that animal models are no longer sufficient to fully understand the pharmacokinetics and metabolism of MDMA in humans. Recent studies have used low dose administration of MDMA in humans to understand this complexity in humans directly.

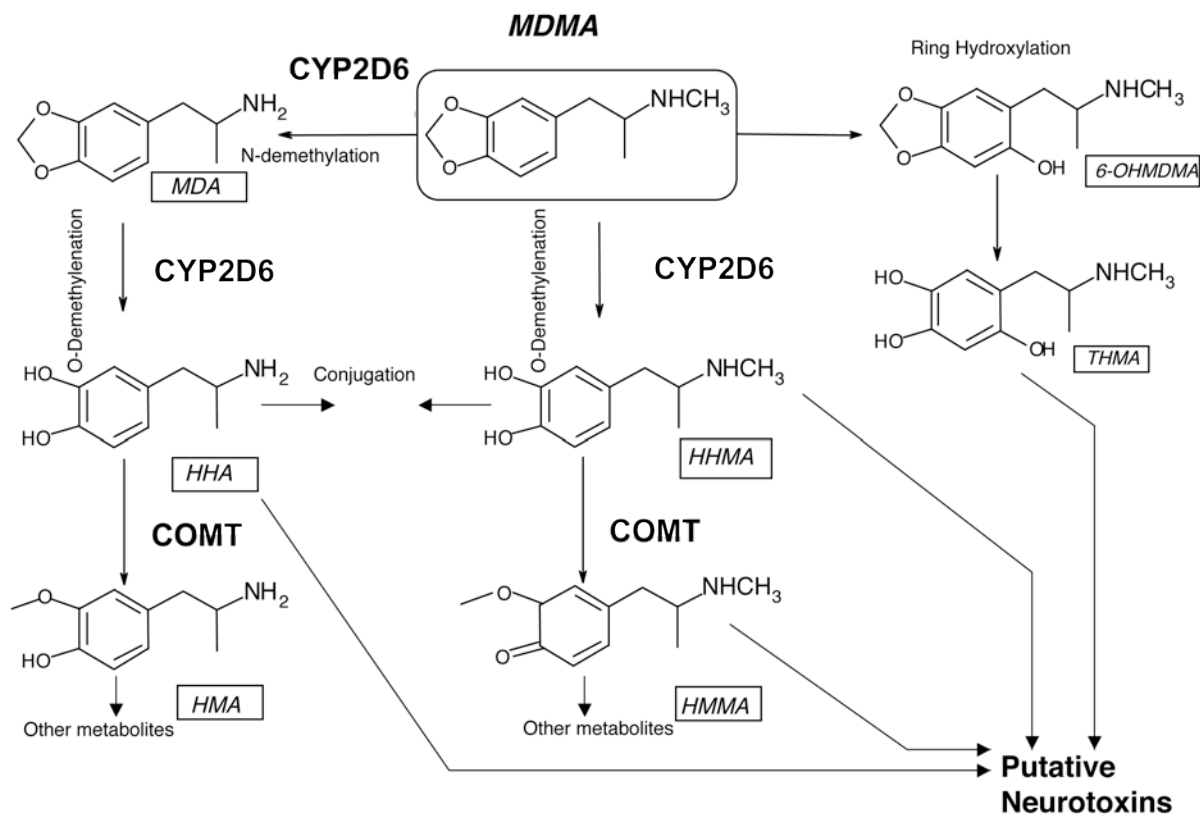


Figure 6: The primary metabolic pathways of MDMA *in vivo*. Breakdown of MDMA via O-demethylation and N-demethylation is of primary importance in the rat, non-human primates, and humans. Initial steps in the pathway are primarily controlled by the enzyme cytochrome P450 2D6, for which genetic polymorphisms exist. Later steps involve breakdown via the enzyme catechol-O-methyl transferase (COMT). HHMA, 3,4-dihydroxymethamphetamine; HHA, 3,4-Dihydroxyamphetamine; MDA, 3,4-methylenedioxyamphetamine; HMMA, 4-hydroxy-3-methoxymethamphetamine; COMT, catechol-O-methyl transferase; HMA, 3-methoxy,4-hydroxyamphetamine; 6OH-MDMA, 6 hydroxy-MDMA; THMA, 2,4,5-trihydroxymethamphetamine.

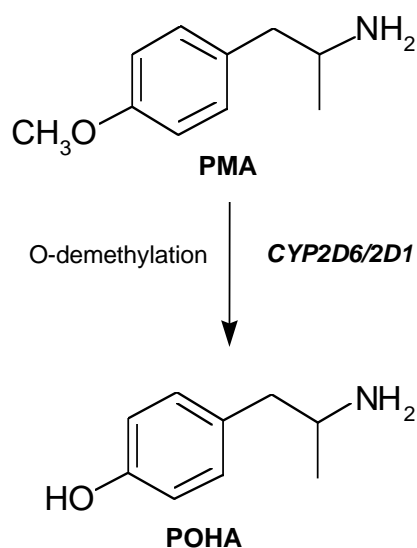


Figure 7: The primary metabolic pathways of PMA *in vivo*. Breakdown of PMA via O-demethylation by CYP isoform 2D6 is of primary importance in the rat, non-human primates, and humans. POHA, p-hydroxyamphetamine

The cytochrome P450 family of enzymes predominate in hepatic oxidation of many drugs in the body. Of the many isoforms present in the liver, the 2D6 isozyme (or its analogue in the rat, CYP2D1) has been shown mediate O-demethylation in rats (with N-demethylation) and humans. This is of interest, as polymorphisms for the CYP2D6 gene are found in humans (Lin et al. 1997). Poor metabolisers, those with a deficiency in the CYP2D6 gene, have a decreased ability to metabolise MDMA and make up ~ 7 % of the Caucasian population. Approximately 90% of Caucasians are normal metabolisers, and 3 % are extensive metabolisers. Extensive metabolisers are more efficient metabolisers of MDMA, and thus would display higher plasma concentrations of HHMA and HMMA. A rodent model for the CYP2D1 poor metaboliser phenotype exists, the female dark agouti rat (Barham et al. 1994). Despite the lack of expression in the rat homologue CYP2D1 gene in these animals, it does not have a generalised deficiency in metabolism. In every CYP2D6 substrate investigated, the female dark agouti rat has been found to have impaired metabolism (Barham et al. 1994). As expected, O-demethylation of MDMA in the female dark agouti rat is deficient (Kumagai et al. 1992). Additionally, it was shown to produce lower amounts of MDA compared to normal metabolisers (Chu et al. 1996). This model has been used to determine the effect of impairment of MDMA metabolism on various physiological parameters, *in vivo*, (see review, Green et al., 2003).

Interestingly, recent findings indicate the genetic polymorphism in this enzyme would not have a great impact on MDMA pharmacokinetics, as MDMA inhibits its own metabolism, as described below (Yang et al. 2006).

The primary metabolites in humans, HHMA and HMMA are easily broken down in the body to ortho-quinones, highly reactive compounds that are thought to lead to free radical induced brain injury (Hiramatsu et al. 1990). Thus it was thought that poor metabolisers would have higher concentrations of MDMA for longer than normal metabolisers. Interestingly, recent studies have shown that a single dose of MDMA in humans alters its

own metabolism via inhibition of the CYP2D6 isozyme for 10 days (Yang et al. 2006). This means that further doses of MDMA taken within that period of time will lead to an unpredictable and significantly higher plasma concentrations of MDMA in plasma (as well as putatively neurotoxic metabolites). As such, this provides a plausible hypothesis for why doses of MDMA that have been previously taken safely by users can suddenly result in acute adverse responses in recreational users.

Unlike MDMA, PMA has a relatively simple metabolic pathway, generating only one primary metabolite, p-hydroxyamphetamine. O-demethylation appears to be the major route of metabolism (see figure 7) of rat, guinea pig, dog and human (Hubbard et al. 1977; Kitchen et al. 1979). O-demethylation also occurred by the CYP2D6 (2D1 in rat) isozyme, indicating genetic polymorphisms alter the pharmacokinetics of the drug (Kitchen et al. 1979). However, due to its linear pharmacokinetics, this would be likely to be relatively unimportant, unless the CYP isozyme was inhibited by other means. Such studies have yet to be attempted.

1.8. Long term residual effects

The original finding that the MDMA metabolite, methylenedioxyamphetamine (MDA) produced a long term reduction in 5-HT content in brain by Ricaurte and colleagues provided initial evidence that substituted amphetamines may induce selective neurodegeneration within the brain (Ricaurte et al. 1985). This has been an important focal point of research ever since, and yet the question of whether MDMA produces a selective neurodegeneration of CNS 5-HT fibres is still contentious. Unfortunately the definition of ‘neurotoxicity’ and ‘neurodegeneration’ differ depending on the scientific background of the researchers involved. While these terms are used interchangeably in the substituted amphetamine literature, neurotoxicity is defined by toxicologists as being damage and/or

death of the neuronal cell and its axonal processes. In order to clarify this within the scope of this thesis, several terms will be defined. Long term effects are defined as those that occur after a time that the parent drug and its metabolites would no longer present *in vivo*. Selective 5-HT neurodegeneration refers to damage of serotonergic fibres, without any injury to the neuronal cell body.

In rodent models, the following factors have been shown to significantly effect the extent of neurodegenerative damage that follows substituted amphetamine administration (Itzhak and Achat-Mendes 2004; Itzhak and Ali 2006). First and foremost, the chemical nature of the substituted amphetamine itself is critical. One hypothesis proposed that substituted amphetamines, which evoked acute release dopamine (e.g. amphetamine) or 5-HT (e.g. p-methylthioamphetamine and tetralin and indan analogues of MDA) exclusively, did not have neurotoxic properties (Nichols et al. 1990; Huang et al. 1992; Marona-Lewicka and Nichols 1994). Indeed, concurrent administration of amphetamine with the non-neurotoxic indan analogue of MDMA, 5-methoxy-6-methyl-2-aminoindan (MMAI) resulted in reductions in markers for 5-HT fibres, while each dose of the drugs alone had no deleterious effects (Johnson and Nichols 1991). As such, the initial component of this thesis work was to assess relative differences in PMA and MDMA effects on dopaminergic and serotonergic neurochemistry *in vivo*. The differences in the functional profile of these drugs may provide insight into the potential mechanism(s) underlying differences in the long term effects of these drugs on biomarkers for serotonergic neurodegeneration.

Species differences are also seen in specificity and potency of substituted amphetamine-induced neurodegeneration. Rats have been used as the primary rodent model due to the fact that MDMA and MDA have been shown to reduce markers for serotonergic fibres specifically, as also seen in guinea pigs, non-human primates and somewhat controversially in humans (Ricaurte et al. 1985; Commins et al. 1987; Battaglia et al. 1988a; Ricaurte 1989; McCann et al. 2000; Green et al. 2003; Saadat et al. 2004). Non-

human primates appear to be more sensitive to the neurodegenerative properties of substituted amphetamines (Ricaurte et al. 1988a; Hatzidimitriou et al. 1999; Ricaurte et al. 2000a). Single doses of 5 mg/kg in primates result in substantial reductions in markers of serotonergic fibres in forebrain (Ricaurte et al. 1988a), while single doses of 10-20 mg/kg or multiple doses of 5 mg/kg, 1-2 hourly are required in rats (except the dark agouti strain). However, mice differ significantly, in that MDMA is 'neurotoxic' to dopaminergic forebrain fibres, but not 5-HT fibres (Stone et al. 1987a; Logan et al. 1988; O'Callaghan and Miller 1994; Colado et al. 2001). This is thought to be due to relative importance of different metabolic pathways in mice, leading to much lower plasma concentrations of dihydroxy metabolites compared with MDMA concentrations (de la Torre and Farre 2004). However within rats, strain differences are also seen in the potency of MDMA to induce acute toxic and long-term serotonergic neurodegeneration (Chu et al. 1996; Malpass et al. 1999; Green et al. 2003). A likely explanation for this is strain differences in metabolism and pharmacokinetics of MDMA in differing strains (Chu et al. 1996; Green et al. 2003). Additionally, not only the frequency but also the pattern of dosing regimen of substituted amphetamines also has a significant effect on presentation of serotonergic fibre neurodegeneration, and the severity of the loss seen (Battaglia et al. 1987; Battaglia et al. 1988a; McKenna and Peroutka 1990; Battaglia et al. 1991; Colado and Green 1994; Green et al. 2003). Initial studies characterising the neurodegenerative effects of MDMA used a dosing regimen of twice daily dosing (12 hour interval), for 4 days (see reviews: Green et al. 2003, McKenna and Peroutka, 1990). This dosing regimen, while less relevant to usage patterns of humans, has been used for the assessment of the putative neurotoxicity of most substituted amphetamines, and as such, we would be able to assess and compare the putative neurotoxicity of PMA within this dataset (Ricaurte et al. 1985; Ricaurte et al. 1987; McKenna and Peroutka 1990; Johnson and Nichols 1991; Green et al. 2003). While recent studies have assessed more relevant dosing regimens to that seen recreationally

(O'Shea et al. 1998; McGregor et al. 2003; Wang et al. 2004; Wang et al. 2005; Clemens et al. 2007), the primary aim of this study was to compare the long term effects of PMA with that of MDMA, on monoaminergic biomarkers. As mentioned earlier, the concentration of PMA and MDMA in 'ecstasy' tablets are not greatly different, so in spite of the significantly different pharmacokinetics and metabolism of these drugs, it is relevant to assess the putative neurotoxicity at equimolar doses. In addition, environmental temperature (Malberg and Seiden 1998; Esteban et al. 2001; O'Shea et al. 2006) and the magnitude of the physiological responses to the drug itself (i.e. induction of hypo/hyperthermia) (Malberg et al. 1996; Malberg and Seiden 1998) also significantly affect the magnitude of the 'neurotoxic' effect seen after substituted amphetamine administration

The long term effects of MDMA and PMA administration on important biomarkers of neurotransmitter localisation and function will be presented below, followed by evidence for neurodegenerative pathologies, and the repercussion of these effects *in vivo* in animals. Unless otherwise stated, all data pertain to rats, the most commonly used species for investigating the long term effects of MDMA. This will be compared with clinical evidence for long term functional deficits within populations of MDMA users.

1.8.1. Long term alterations in monoamine and indolamine content in the CNS

MDMA has been shown robustly in an extensive array of studies to reduce 5-HT content in most forebrain regions (see reviews Green et al., 2003; Baumann et al., 2007). In contrast, MDMA has been shown to not effect dopamine terminals in rats or primates (Stone et al. 1986; Battaglia et al. 1987; Schmidt and Kehne 1990; Lew et al. 1996; Sabol et al. 1996; Colado et al. 1997). Additionally, MDMA treatment after a prior repeated treatment regimen failed to effect dopamine release *in vivo* (Shankaran and Gudelsky 1998). As

such, further discussion will examine the effects of MDMA on long-term serotonergic neurochemistry.

MDMA was found to produce a biphasic alteration in 5-HT content in forebrain regions containing 5-HT terminals (Schmidt 1987b, a). The acute actions of MDMA induce a maximal depletion of 5-HT content in cortex 3-6 hours after a single dose, after which a recovery to pre-treatment concentrations occurs by 24 hours. This is followed by a second phase of 5-HT depletion, significantly reduced from 1 week after initial dosing. A dose dependent reduction in 5-HT & 5-HIAA content was seen in various brain regions (Stone et al. 1986; Battaglia et al. 1988a; Ricaurte et al. 1988a; Colado et al. 1993; O'Shea et al. 1998; Shankaran et al. 1999a). In addition to dose, increasing the frequency of injection of multiple doses also potentiated the effect of MDMA on reductions in 5-HT/5-HIAA content (Battaglia et al. 1988a; Ricaurte et al. 1988a; O'Shea et al. 1998).

While recovery of tryptophan hydroxylase occurs 1 week after acute MDMA administration, a potential explanation for the long term reductions in indolamine content could be reduced indole synthesis rather than neurodegeneration. As such, subsequent studies investigated the effects on other markers for serotonergic fibres.

MDMA was shown to dose dependently reduce binding to SERT in most forebrain regions containing 5-HT fibres (Battaglia et al. 1987; De Souza et al. 1990; Scanzello et al. 1993; Hewitt and Green 1994; Broening et al. 1995; Colado et al. 1995; Lew et al. 1996; Sabol et al. 1996; O'Shea et al. 1998; Obradovic et al. 1998). This resulted in functional consequences, with a significant long term reduction in high affinity [H³] 5-HT uptake in regions such as cortex and hippocampus (De Souza et al. 1990; Hewitt and Green 1994; Sabol et al. 1996). Based on initial studies, a standard dosing regimen for twice daily dosing, for 4 days, at MDMA doses greater 5 mg/kg produce significant major reductions in markers for 5-HT fibres.

From the finding of these studies, we chose dosing regimens that produced maximal and submaximal depletion of 5-HT content for our studies investigating the long term effects of MDMA and PMA on 5-HT neurotransmission. As discussed in subsequent chapters, measurement of these indices allowed MDMA to act as a positive control for our studies investigating SERT function *in vivo* using electrochemical methodologies, which has not been previously attempted for amphetamines.

Considerable evidence exists to show that only the 5-HT fibres are affected, and not the cell bodies in the raphe nuclei. Indolamine content in 5-HT cell bodies in the dorsal raphe nuclei were unaffected at doses of MDMA that significantly reduced 5-HT content in terminal field regions (Battaglia et al. 1991; Aguirre et al. 1995; Lew et al. 1996). Longitudinal studies investigating MDMA-induced serotonergic deficits over a 1 year period in rats also showed no alteration in raphe SERT binding or indolamine content (Lew et al. 1996). Likewise, prior administration of MDMA treatments that consistently reduce serotonergic markers did not alter the neuronal firing rate in dorsal raphe (Gartside et al. 1996). However, the selective serotonergic neurotoxin, 5,7-dihydroxytryptamine did reduce neuronal firing rates along with a raphe lesion, further validating the lack of MDMA effects on serotonergic cell bodies.

As mentioned, several studies have investigated the recovery of forebrain serotonergic markers in rodents over a 1-year period. After repeated MDMA administration, [H^3]-paroxetine binding to SERT and indolamine content were significantly reduced (Battaglia et al. 1988a). By 6 months, this had recovered to 75% of control values, with full recovery at 1 year post treatment. Scanzello and colleagues furthered these findings, measuring indolamine content, [H^3]-paroxetine binding and 5-HT immunohistochemistry over the same time period (Scanzello et al. 1993). A differing pattern of recovery was seen, with hypothalamic indole content recovering within 8 weeks, and striatal and hippocampal content recovered by 16 weeks. Indole content in other regions had recovered by 1 year

post administration. Recovery of SERT binding had a differing time course, with cortex and striatum recovered by 32 weeks, and hippocampal SERT binding was still reduced at 1 year. Investigation of 5-HT axonal density (using 5-HT immunohistochemistry) demonstrated recovery in most animals by 1 year. Sabol and colleagues investigated long term effects of repeated MDMA treatment on regional indole content and [H^3] 5-HT uptake into striatal and hippocampal synaptosomes (Sabol et al. 1996). Different effects on recovery of serotonergic markers were seen, with striatal 5-HT content recovering to control levels at 16 weeks, but hippocampal 5-HT did not recover by 1 year. Interestingly, 5-HT uptake into hippocampal synaptosomes was reduced at 2 weeks but had recovered by 8 weeks post treatment. Also evidence of hyper-innervation in hypothalamus and septum was seen. In a companion study, rats receiving the same dosing regimen show a differential recovery of [I^{125}]-RTI-55 binding to SERT, with only hippocampal SERT showing a full recovery (Lew et al. 1996). This anomalous finding was clarified by autoradiographic studies, showing a rostro-caudal difference in recovery of hippocampal SERT binding. These findings were at odds with those of Scanzello and colleagues, but a much higher dosing regimen (20 mg/kg, BID for 4 days cf. 10 mg/kg every 2 hours x 4) was used.

In contrast, MDMA has been shown to have no effects on long term brain dopamine content, synthesis or axonal integrity (Stone et al. 1986; Battaglia et al. 1987; Lew et al. 1996; Sabol et al. 1996; Colado et al. 1997; Colado et al. 1999b). Also, MDMA treatment after a neurotoxic dosing regimen of MDMA failed to alter extracellular release of dopamine, in spite of significant attenuation of extracellular 5-HT release (Shankaran and Gudelsky 1999). This further indicates there is not functional alteration in dopaminergic neurotransmission after MDMA treatment.

Similar reductions in markers for 5-HT fibres were seen in non-human primates (Ricaurte et al. 1988a; Ricaurte et al. 1988c; Slikker et al. 1988; Insel et al. 1989; Kleven et al. 1989; Ricaurte 1989; Ricaurte et al. 1992; Green et al. 2003). However, as previously

mentioned, primates were more sensitive to the ‘neurotoxic’ effects of MDMA. While single doses greater than 10 mg/kg, or multiple doses a day/week are required in most rat strains to significantly reduce forebrain serotonergic markers (Battaglia et al. 1988a; O’Shea et al. 1998; McGregor et al. 2003), in primates single doses as low as 2.5 mg/kg can reduce 5-HT content in cortex, striatum and hippocampus (Ricaurte et al. 1988c). Additionally, primates followed up 7 years after a single ‘neurotoxic’ dosing regimen of MDMA still displayed significant reductions in markers for forebrain serotonergic fibres (Hatzidimitriou et al. 1999). Interestingly, in regions where partial or full recovery occurred, the innervation pattern was abnormal. This was also seen by Molliver and coworkers, showing the recovery in hypothalamic and amygdaloid 5-HT content 18 months after dosing with MDMA was associated with axonal sprouting but highly abnormal innervation (Molliver et al. 1990; Fischer et al. 1995). This partial, but abnormal re-innervation, was also seen in rats (Fischer et al. 1995).

In comparison to the relatively large body of literature, little is known about the long term effects of PMA on indices of monoaminergic neurons. Only one study exists, where Steele and co-workers showed that one week after a high dose of PMA (80 mg/kg, twice daily for 4 days), forebrain 5-HT and 5-HIAA concentrations were significantly reduced (Steele et al. 1992). However, the interpretation of this finding is complicated by the dose used being well in excess of the acute LD₅₀ for PMA (Nichols et al. 1975; Steele et al. 1992). As such, we aimed to provide the first dose response study characterising the long term effects of PMA administration on monoaminergic neurons.

1.8.2. Axonal degeneration

Initial studies investigating the question of MDMA-mediated neurodegenerative effects on 5-HT fibres demonstrated a significant accumulation of argyrophilic deposits (as shown by silver staining) in rat striatal slices, 13-16 hours after administration of drug (Commins et

al. 1987). However, it was not possible to determine the neurochemical identity of the fibres that were affected. The seminal study of O'Hearn and colleagues showed a significant reduction in the intensity of 5-HT-like immunoreactivity in brain regions containing 5-HT terminal fields (O'Hearn et al. 1988). In addition, fine aborised fibres originating primarily from the dorsal raphe were more significantly affected. Raphe nuclei were unaffected, indicating the lack of cellular toxicity to 5-HT cell bodies.

Doses of MDMA that are proposed to induce 5-HT fibre degeneration also impair the transport of [H^3]-proline into 5-HT terminal fields, 3 weeks after injection into rostral raphe (Callahan et al. 2001). This reduction in axonal transport was also seen after treatment with the selective serotonergic neurotoxin, 5,7-dihydroxytryptamine, further validating this finding (Callahan et al. 2001).

Numerous studies have looked for evidence of biochemical responses to brain injury after purported 'neurotoxic' doses of MDMA, in particular gliosis. Glial acidic fibrillary protein (GFAP), an important astrocytic filament protein, has been shown to be an effective marker of astrocyte hypertrophy (O'Callaghan and Jensen 1992). Increases in GFAP expression have been shown after MDMA administration in mice, but due to the greatly differing neurotoxic profile of MDMA in mice, the findings do not directly translate to rats, primates or humans (Miller and O'Callaghan 1995; Johnson et al. 2002). Interestingly, increases in GFAP expression could be prevented by either physically or pharmacologically induced hypothermia (Miller and O'Callaghan 1995). The findings in rats are much less clear. Numerous studies have shown no effect on GFAP expression after 'neurotoxic' dosing regimens of MDMA (Pubill et al. 2003; Wang et al. 2004; Wang et al. 2005). The findings of Aguirre and colleagues are at odd this other studies, showing a significant increase in GFAP expression in hippocampus, that could be prevented by the free radical scavenger alpha-lipoic acid (Aguirre et al. 1999). This result has been difficult to explain. In addition Pubill and colleagues showed no alteration in markers for microglial

activation, namely peripheral benzodiazepine receptor (PBR) binding using [H^3]-PK11195 and OX-6 immunohistochemistry. Both PBR binding and OX-6 expression were shown to be sensitive markers in this study, with significant increases seen after methamphetamine administration (known to promote neuroinflammation). These findings will be discussed further in relation to evidence of the lack of neurotoxic effects of MDMA.

1.8.3. Evidence against serotonergic neurotoxicity after MDMA administration

Evidence exists that indicates the changes in serotonergic markers after MDMA administration do not necessarily mean the neurotoxic damage to the fibres. Initial evidence demonstrated intra-raphe injection of MDMA did not result in changes in serotonergic markers in terminal field regions (Paris and Cunningham 1992). These findings were reinforced by the study of Esteban and colleagues, where infusion of MDMA through a microdialysis probe significantly increased hippocampal 5-HT extracellular content, yet no long-term alterations in tissue content were seen (Esteban et al. 2001). However, administration of the selective serotonergic neurotoxin, 5,7-dihydroxytryptamine using the same paradigm resulted in significant reduction in hippocampal 5-HT.

Likewise, standard biomarkers of neuroinflammation that result from free-radical induced tissue damage, such as gliosis (increases in glial fibrillary acidic protein, GFAP) and microglial activation (increased peripheral benzodiazepine receptor expression and CD11b receptor), are unaffected in all but one study after MDMA dosing regimens that reduce 5-HT content (O'Callaghan and Miller 1993; Aguirre et al. 1999; Pubill et al. 2003; Wang et al. 2004; Wang et al. 2005; Baumann et al. 2007). Most interestingly, recent studies have shown that in rats after a 'neurotoxic' MDMA treatment regimen, SERT protein was not reduced, as visualised by Western blotting (Wang et al. 2004; Wang et al. 2005; Baumann et al. 2007). This was further validated by profound loss of SERT protein after treatment

with the serotonergic neurotoxin, 5,7-dihydroxytryptamine (Wang et al. 2004; Wang et al. 2005; Baumann et al. 2007). This raised the possibility that the 5-HT fibres may still exist, yet the SERT protein may have been denatured or made non-functional. Changes due to trafficking of the SERT protein from the membrane to endosomal compartments were also ruled out. A subsequent study showed reductions in SERT protein (63-68kDa), inducing conjecture over whether the protein detected in prior studies corresponded to the SERT (70kDa). Interestingly, recent studies have shown that despite high dose MDMA pre-treatment resulting in significant SERT loss (as measured by receptor autoradiography), treatment with the 5-HT precursor, 5-hydroxytryptophan resulted in a restoration in regional 5-HT depletions (Wang et al. 2007). As such, this indicates the question as to whether MDMA results in selective serotonergic lesions in the brain is still far from being answered.

1.8.4. Potential mechanisms for substituted amphetamine-mediated neurodegeneration

1.8.4.1. Role of free radical production

The primary mechanism proposed for MDMA-mediated neurodegeneration is based on studies investigating the role for free radical generation in this process (Sprague et al. 1998). Sprague and Nichols demonstrated MDMA increased lipid peroxidation, which was confirmed later by Colado and colleagues (Sprague and Nichols 1995; Colado et al. 1997). Free radical production has been demonstrated *in vivo* after acute MDMA administration (Colado et al. 1997; Aguirre et al. 1999; Colado et al. 1999a; Shankaran et al. 1999a, b; Yeh 1999; Shankaran et al. 2001). Treatment of rats with free radical scavenging compounds has also been shown to attenuate or prevent long term alterations in regional 5-HT content after MDMA treatment (Colado et al. 1997; Yeh 1999). For

example, co-administration of PBN (alpha-phenyl-N-tert-butyl nitron) partially attenuated long term serotonergic neurodegeneration, and prevented free radical production *in vivo*, as assessed by conversion of local application of salicylic acid to 2,5-dihydroxybenzoic acid within hippocampus (Colado and Green 1995; Colado et al. 1997; Yeh 1999). Ascorbic acid co-administration with MDMA was also shown to prevent subsequent loss of 5-HT content, also blocking free radical production (Shankaran et al. 2001). Administration of α -lipoic acid also suppressed MDMA mediated increase in free radical production, preventing long term reductions in 5-HT content in hippocampus and striatum but without any effect on the acute MDMA effects on hyperthermia (Aguirre et al. 1999). PBN administration also failed to alter MDMA-induced hyperthermia (Yeh 1999). Other antioxidants such as ascorbic acid, L-cystine and vitamin E have also been shown to reduce MDMA induced free radical production, and attenuate long term serotonergic deficits. (Gudelsky 1996; Shankaran et al. 2001). Evidence also exists that the free radical production occurs at the axonal terminals. Prior administration of fenfluramine to induce selective 5-HT terminal degeneration prevented MDMA induced free radical production *in vivo* (Colado et al. 1997). This was further supported by the finding that MDMA free radical production could also be attenuated by fluoxetine (Shankaran et al. 1999b).

Evidence also exists that peroxynitrite (ONOO⁻) radicals are produced *in vivo* at psychoactive doses of MDMA and PMA (Colado et al. 2001; Imam et al. 2001a; Imam et al. 2001b; Darvesh et al. 2005). Peroxynitrite is formed by the interaction of the gaseous neurotransmitter, nitric oxide (NO) and superoxide radicals, and is a known neurotoxic species, shown to play a significant role in methamphetamine-induced dopaminergic neurotoxicity (Beckman et al. 1990; Itzhak and Achat-Mendes 2004; Itzhak and Ali 2006). Psychostimulants such as MDMA are known to activate glutamatergic neurotransmission, in turn stimulating release of NO by neuronal nitric oxide synthase (nNOS), often by activation of NMDA glutamate receptors (Itzhak and Ali 2006). It is thought that NO plays

an important role as a second messenger within dopaminergic-glutamatergic interactions in mesolimbic structures. For example, the nNOS inhibitor 7-nitroindazole blocked methamphetamine induced behavioural sensitisation and dopaminergic neurotoxicity (Itzhak and Ali 1996; Imam et al. 2000). Additionally, nNOS knockout mice are resistant to methamphetamine-induced neurotoxicity (Itzhak et al. 1998). Peroxynitrite production (measured by production of the precursor nitrotyrosine) is also involved in MDMA-induced long term reductions in striatal 5-HT content, also being attenuated by the NOS inhibitor S-methyl-l-thiocitrulline (Darvesh et al. 2005). Interestingly, the only study investigating *in vivo* PMA-induced peroxynitrite production showed PMA induced significantly more peroxynitrite production than MDMA, at equimolar doses, in mice (Imam et al. 2001a). As such, this provided evidence for the potential for PMA to possess long term ‘neurotoxic’ properties, like MDMA.

1.8.4.2. Role of dopamine in serotonergic neurodegeneration

One potential mechanism for acute MDMA-mediated increases in free radical production revolves around increased extracellular dopamine (in addition to 5-HT) being transported via SERT into 5-HT-depleted presynaptic terminals, where it is metabolised to the neurotoxin, 6-hydroxydopamine by MAO-B (see Sprague et al, 1998). Initial studies have shown agents that deplete dopamine (alpha methyl-para-tyrosine and reserpine) attenuated MDMA-induced 5-HT depletion (Stone et al. 1988). Interestingly, a prior 6-hydroxydopamine lesion of dopamine terminals blocked MDMA-mediated reductions in tryptophan hydroxylase activity in hippocampus and cortex, but not in striatum (Stone et al. 1988). The dopamine D₂ antagonist, haloperidol, DAT inhibitors and MAO inhibitors were also neuroprotective against MDMA induced deficits (Stone et al. 1988; Schmidt et al. 1990a; Hewitt and Green 1994; Sprague and Nichols 1995; Shankaran et al. 1999a). L-deprenyl blocked the generation of lipid peroxidation products induced by MDMA

administration (Sprague and Nichols 1995). However, a significant confound exist in the interpretation of these findings, in that many of these compounds induce hypothermia, which in itself is neuroprotective (Green et al. 2003). In fact, free radical formation after MDMA administration is inhibited when the normal hyperthermic response is blocked (Colado et al. 1999a; Colado et al. 1999b)

However, significant evidence indicates this theory may not be the only explanation. L-DOPA was shown to further potentiate MDMA-mediated increases in extracellular striatal dopamine, but no increase in free radical production was seen in hippocampus (Colado et al. 1999b). This was despite a potentiation in the acute hyperthermic effect of MDMA. Also, co-administration of MDMA at a sub-neurotoxic dose with L-DOPA did not reduce serotonergic markers post-administration. The study of Yuan and colleagues addressed these anomalous findings with those of the prior literature. If the body temperature of the control rats was raised to the same extent as those having received MDMA treatment, the neuroprotective effects of alpha methyl-para-tyrosine and reserpine was negated (Yuan et al. 2002). Thus it appears likely the neuroprotective effect of these drugs is related to their hypothermic properties rather than depletion of dopamine. Indeed, lowering the core temperature of rats with MDMA-induced hyperthermia in fact reduced production of free radicals (Colado et al. 1999a).

Another confounding factor in this theory is that MDMA depletes 5-HT content and reduces binding to SERT in regions where there is negligible dopamine content (Commins et al. 1987; Schmued 2003).

Additionally, blockade of the SERT (with SERT inhibitors) has been protective against the long term alterations in brain 5-HT content (Malberg et al. 1996; Sanchez et al. 2001). Interestingly, SERT inhibitors such as fluvoxamine and fluoxetine do not alter the acute effects of MDMA on hyperthermia, as has been discussed above. Potentially, inhibition of SERT may limit dopamine access to the 5-HT terminals, and hence prevent free radical

generation. This view was supported by the work of Shankaran and coworkers, where fluoxetine administration prior to, or up to 4 hours after MDMA treatment prevented extracellular free radical production and long term 5-HT depletion in rats (Shankaran et al. 1999b).

Some evidence suggests DAT inhibitors may also be neuroprotective. For example, the DAT inhibitor, GBR12909 was also neuroprotective after MDMA administration. Co-administration of mazindol with MDMA also attenuated free radical generation and long term reductions in 5-HT content, with no alteration in the acute hyperthermic response, as seen with fluoxetine (Shankaran et al. 1999a).

1.8.4.3. Role of substituted amphetamine metabolites in serotonergic neurodegeneration

Many of the postulated mechanisms mentioned so far for MDMA-mediated serotonergic neurodegeneration cannot explain why local application of MDMA within brain does not induce damage (Paris and Cunningham 1992; Esteban et al. 2001). In addition there is evidence of neuronal degeneration in other non-serotonergic brain regions such as somatosensory and insular cortex and ventral thalamic regions (Commins et al. 1987; Schmued 2003). The fact that systemic administration of MDMA is required for presentation of serotonergic neurodegeneration indicates that metabolites of MDMA may cross through the blood brain barrier and could mediate neurodegeneration (de la Torre and Farre 2004; Monks et al. 2004).

MDMA metabolites (HHMA & HMA) that undergo further breakdown by monoamine oxidases can lead to formation of ortho-quinones, highly reactive compounds that lead to generation of reactive oxygen and nitrogen species (Monks and Lau 1997). Recent work has shown thioether metabolites of MDMA, formed by *in vivo* by glutathione and N-acetylcysteine conjugation, can be transported via the blood brain barrier into brain

(Miller et al. 1997). Peripheral administration of these compounds also increased the severity of 5-HT depletion, neurodegeneration and increase glial markers such as GFAP (Miller et al. 1997; Monks et al. 2004). They have been shown to be detected *in vivo*, via microdialysis after MDMA administration, and be potent ligands for the SERT (Jones et al. 2004; Jones et al. 2005; Erives et al. 2008). Of particular interest, these thioether compounds increase dopamine uptake via SERT while inhibiting 5-HT uptake via SERT, in cell culture (Jones et al. 2004). This has been a proposed mechanism of MDMA-induced neurodegeneration for some time, yet up until this point the evidence was not strong. These metabolites were shown to more efficacious neurotoxins in primary cortical cultures, compared to other MDMA metabolites (Capela et al. 2007).

Such mechanisms would not be likely for PMA effects, due to its simple metabolism, and its primary metabolite not having known neurotoxic properties (Hubbard et al. 1977; Kitchen et al. 1979; Law and Moody 1994; Kaminskas et al. 2002).

1.8.5. Physiological/behavioural effects after prior substituted amphetamine administration in animal models

It is not clear whether prior treatment of rats with MDMA leads to an attenuation of degree of MDMA-mediated hyperthermia. An attenuation was seen in the acute hyperthermic response to a subsequent MDMA dose after a neurotoxic dose of MDMA or fenfluramine (Colado et al. 1997; Shankaran and Gudelsky 1999). However, Dafters showed a sensitisation effect, with increases in the hyperthermic and locomotor activity responses to an acute dose after a prior chronic treatment of MDMA (Dafters 1995). In a subsequent study, treatment of the rats with higher doses of MDMA initially did result in an attenuation of hyperthermic effects to a subsequent MDMA challenge (Dafters and Lynch 1998). This indicates the sensitisation effect may be related to the frequency interval and dose of the prior MDMA treatments, as seen with MDMA-induced sensitisation effects to

activity and serotonin syndrome behaviours (Spanos and Yamamoto 1989). Mehan and colleagues extended these observations, showing MDMA pre-treated rats had a reduced capacity to thermoregulate in the absence of drug in addition to when challenged with a single dose of MDMA (Mehan et al. 2001). This reduced capacity to lose heat was only seen if subsequent MDMA doses were given at high ambient temperatures. Interestingly, prior MDMA administration impaired the ability of an untreated rat to lower its body temperature in a cool room after being maintained at a high ambient temperature. As such, subsequent MDMA treatment at high ambient temperatures after a ‘neurotoxic’ treatment of MDMA resulted in an enhanced hyperthermic response, implicating the resultant serotonergic loss as a mechanism for the impaired heat loss.

1.8.6. Repeated MDMA use in humans: Clinical studies

The pertinent question as to whether the apparent MDMA-induced neurodegeneration of serotonergic fibres, demonstrated in animal models, translates to the clinical realm has been one of great interest. It has also been limited by the lack of precise tools to measure potential neurochemical deficits until recent years. Three primary approaches have been used: investigation of potential neuropsychological and cognitive changes among regular and infrequent users; assessment of changes in direct pharmacodynamic responses to serotonergic drug challenges; assessment of neurochemical changes, using nuclear medicine with specific ligands for receptors/transporters critical in serotonergic neurotransmission. There are several significant caveats with the interpretation of this clinical data, most of which apply to clinical illicit drug use research as a whole. Firstly, most regular ‘ecstasy’ users are poly-drug users (Schifano et al. 1998). Secondly, variation in the constituents of an ‘ecstasy’ tablet over time and location can complicate findings, as other common ‘contaminants’, such as methamphetamine are also known to be neurotoxic. As such, this had necessitated clinical research where users are given low doses of

MDMA, thought to be below the threshold for putative neurodegenerative effects (Vollenweider et al. 1998; Liechti et al. 2000; Liechti and Vollenweider 2000; Vollenweider et al. 2001; Vollenweider et al. 2002). These doses were determined initially using the interspecies dosing scale, for extrapolating the ‘safe’ doses in rats and monkeys to those to be used in humans. As such, clinical research investigating ‘ecstasy’ naïve, drug naïve users, with regular and infrequent users appears to be the safest approach for determining the extent of neurochemical and cognitive changes for which ‘ecstasy’ may be responsible.

1.8.6.1. Changes in serotonergic function *in vivo* amongst regular ‘ecstasy’ users

Homeostatic control mechanisms controlling endocrine function that are modulated by 5-HT have been shown to be affected by prior MDMA use. Administration of l-tryptophan, d-fenfluramine or m-chlorophenylpiperazine (m-CPP) resulted in an altered response in plasma prolactin, cortisol and growth hormone concentrations in a cohort of MDMA users (Price et al. 1989; McCann et al. 1994; Gerra et al. 1998; Gerra et al. 2000; McCann et al. 2000; Verkes et al. 2001). These effects are consistent with those seen in rodent models (Nash et al. 1988). In addition, when MDMA was administered to healthy volunteers, a dose of 75 mg or greater increased plasma cortisol and prolactin concentrations. At these doses, MDMA also had dose dependent sympathomimetic effects (Mas et al. 1999; de la Torre et al. 2000b).

1.8.6.2. Changes in serotonergic biomarkers *in vivo* amongst regular ‘ecstasy’ users

Measurement in CSF changes in 5-HIAA, followed by *in vivo* measurement of changes in 5-HT transporter binding using PET/SPECT imaging techniques have been the first approaches for quantifying the long term effects of ‘ecstasy’ use. Significant reductions in

CSF 5-HIAA were seen between regular users (but not currently using) and non-users (Ricaurte et al. 1990; Bolla et al. 1998; McCann et al. 1999). As these changes could be due to various other processes, early studies modelled changes in CSF 5-HIAA concentration, and correlated these to direct regional brain 5-HT/5-HIAA content in primates (Ricaurte et al. 1988b). Indeed two weeks after a ‘neurotoxic’ dosage of MDMA, there was a 60% reduction in CSF 5-HIAA, correlating with a 73-94% reduction in brain 5-HT/5-HIAA content.

PET studies in humans also demonstrated a significant reduction in cortical and subcortical SERT binding using the ligand [C^{11}]-McN-5652 between current MDMA users and non-users (McCann et al. 1998). The reduction in SERT binding was also found to correlate with the extent of previous ‘ecstasy’ use (Ricaurte et al. 2000b). Cortical SERT binding was also reduced, measured using the ligand [I^{123}]-carbomethoxy-3 β -(4-iodophenyl)-tropane ([I^{123}]- β CIT) with SPECT imaging, between current users and non-users (Semple et al. 1999). These reductions in SERT binding *in vivo* using PET/SPECT studies have been validated in animal models, correlating well with reductions in regional 5-HT content after ‘neurotoxic’ MDMA treatment regimens in baboons, indicating the usefulness of this technique in the clinical setting (Scheffel et al. 1998). The most common reasons for discrepancies between the PET and SPECT studies include methodological differences in imaging, differences in the definition of what makes a high and moderate regular user, as well as the definition of a “former user”, i.e. duration of abstinence (McCann et al. 1998; Semple et al. 1999; Ricaurte et al. 2000b; Reneman et al. 2001; Buchert et al. 2004; McCann et al. 2005; Reneman et al. 2006).

The time frame for recovery of CSF 5-HIAA content and *in vivo* SERT binding after abstinence from MDMA use is not clear. However, all of the current studies indicate the changes in SERT binding are transient, with recovery after long term abstinence of use (McCann et al. 1998; Semple et al. 1999; Ricaurte et al. 2000b; Reneman et al. 2001;

Buchert et al. 2004; McCann et al. 2005). In all but one of these studies, SERT binding correlated with duration of abstinence. (McCann et al. 1998; Semple et al. 1999; Ricaurte et al. 2000b; Reneman et al. 2001; Buchert et al. 2004; McCann et al. 2005).

1.9. Research aims:

As outlined in the previous section, a considerable literature exists investigating the clinical implications of acute substituted amphetamine use, but more so discriminating what residual physiological/psychological deficits can occur. Within this dissertation, two fundamental questions were addressed. Firstly, *how do substituted amphetamines modulate monoaminergic and serotonergic neurotransmission to produce their acute adverse physiological (i.e. hyperthermia and cardiovascular dysfunction) and mood altering effects (i.e. positive and negative emotional responses, cognitive dysfunction)*. Most research to this point has concentrated on the pharmacology of MDMA, the most common drug sold as ‘ecstasy’. In contrast, little is currently known about the pharmacology of PMA *in vivo*. The neurochemical basis for the increased incidence of acute adverse events associated with PMA vs. MDMA use is still unclear. The aim of this component of the thesis is to *compare and understand the differences in acute neurochemical mechanisms between PMA and MDMA*.

The second aspect of substituted amphetamine pharmacology of major clinical relevance concerns *the long term residual neurochemical changes that occur with regular substituted amphetamine use*. The question of whether substituted amphetamines cause selective serotonergic neurodegeneration in the CNS needs to be addressed for each ‘new’ compound, and this was not investigated for PMA prior to this work. The relevance of these studies relates to the ability to predict whether PMA is likely to induce residual psychological and/or psychiatric illness, as seen with methamphetamine, or controversially, with MDMA. Such knowledge allows the updating of harm minimisation

education approaches to potentially reduce the use of a drug, and thus reduce incidence of long term illness. These studies were approached to answer the following questions:

- *Does repeated PMA use result in long term neuroadaptive or neurodegenerative changes in monoamine and/or serotonergic neurotransmission?*
- *Does repeated PMA or MDMA use result in alterations in one of the key processes in serotonergic neurotransmission, function of SERT in vivo?*

The latter question of whether functional reductions in serotonin clearance are seen *in vivo*, correlating with reductions in the *in vitro* serotonergic biomarkers, has never been investigated for any substituted amphetamine previously.

1.9.1. Project aims

- Investigate the pharmacology of the acute effects of the substituted amphetamines PMA and MDMA, on monoaminergic neurotransmitter release and reuptake *in vivo*, within brain regions with dense dopaminergic (striatum) and serotonergic (hippocampus) innervation.
- To investigate the potential for repeated PMA or MDMA administration leading to reductions in serotonergic function, as measured by both *in vivo* and *in vitro* methodologies

1.9.2. Publication 1: “Differential behavioural and neurochemical effects of para-methoxyamphetamine and 3,4-methylenedioxymethamphetamine in the rat”.

The aim of this study was two fold. The first was to characterise and compare potency and efficacy of PMA and MDMA on their two primary pharmacodynamic effects relevant to this study, locomotor activity and body temperature. Secondly, the acute effects of PMA

on serotonergic and dopaminergic neurotransmission (release and reuptake) *in vivo*, were compared with those of MDMA, within striatum

The first limitation of the previous literature concerns the relative paucity of data concerning the neurochemical mechanism of action of PMA. Additionally, previous studies investigating evoked release from *in vivo* and *in vitro* preparations did not have the greater spatial and temporal resolution afforded by voltammetric methodologies used in this study. Likewise, investigation of drug effects on 5-HT and dopamine uptake in real time *in vivo*, were only possible using voltammetry, and had not been investigated previously for either drug. Using local application of PMA and MDMA within striatum allowed characterisation of the effects of the parent drug, without the presence of active metabolites. This increased spatial resolution that showed heterogeneity in responses through the dorsoventral extent of striatum. This had not been previously demonstrated for either PMA or MDMA.

A further limitation of the previous literature was the lack of comparison of potency and efficacy of the pharmacodynamic responses of PMA and MDMA. Previous reports of the greater acute hyperthermic effects of PMA compared to other substituted amphetamines come from the clinical literature.

1.9.3. Publication 2: “Differences in the *in vivo* dynamics of neurotransmitter release and serotonin uptake after acute para-methoxyamphetamine and 3,4-methylenedioxymethamphetamine revealed by chronoamperometry”.

The aim of this publication was to further examine the differences in potency and selectivity between PMA and MDMA to evoke release of monoamines, and inhibit monoamine uptake, in striatum and hippocampus. This work extended from the previous study, investigating the role of SERT in PMA/MDMA evoked neurotransmitter release. Additionally, investigation of the dose response relationship of PMA and MDMA on 5-HT

clearance, *in vivo*, had not previously been investigated. Other *in vivo* techniques (e.g. microdialysis) lack the temporal resolution to measure these high-speed phenomena. This paper was published in ‘Neurochemistry International’.

- 1.9.4. Publication 3: “Repeated administration of the substituted amphetamine p-methoxyamphetamine produces reductions in cortical 5-HT transporter binding but not 5-HT content, unlike 3,4-methylenedioxy-methamphetamine”.

The next two publications indicate a change in focus, moving to investigation of the ability of PMA or MDMA to induce long term damage to serotonergic fibres, as assessed by changes in cortical SERT binding (as measured by [H^3]-paroxetine binding) and 5-HT content, *in vitro*. One considerable limitation of previous work was the lack of confirmation of what the long term effects of PMA on indices of serotonergic fibre viability were. Only one previous study existed, using doses of PMA considerably above its LD₅₀ concentration.

Additionally, previous studies had not investigated whether a 6-hour period of elevated ambient temperature during PMA/MDMA administration could potentiate the long term effects of PMA or MDMA on SERT binding. Previous studies had shown greater periods of increased ambient temperature during drug dosing to potentiate the ‘neurotoxic’ effects of MDMA. This time point was thought to simulate the time spent in night clubs by MDMA users, where they would be likely to be exposed to high ambient temperatures. Published in the ‘European Journal of Pharmacology’.

- 1.9.5. Publication 4: “In vivo analysis of serotonin clearance in rat hippocampus reveals that repeated administration of p-methoxyamphetamine (PMA) but not 3,4-methylenedioxymethamphetamine (MDMA) leads to long lasting deficits in serotonin transporter function”.

This study follows on and extends considerably from the findings in the previous publication. The primary aim of this study was to determine if the reduction in serotonergic markers seen 2 weeks after either PMA or MDMA repeated treatment, resulted in a functional change in synaptic 5-HT clearance mechanisms *in vivo*. These responses were compared to the changes in *in vitro* markers of 5-HT fibre neurodegeneration (SERT binding, tissue 5-HT content, *in vitro* 5-HT clearance). No previous work has investigated if functional changes in synaptic 5-HT clearance mechanisms *in vivo* occur after prior treatment of animals with MDMA or PMA. This also allowed correlation of *in vivo* 5-HT clearance with *in vitro* 5-HT clearance from tissue in the same experimental subject. Published in the ‘Journal of Neurochemistry’.

2. Publication 1

Daws L. C., Irvine R. J., **Callaghan P. D.**, Toop N. P., White J. M. and Bochner F. (2000) Differential behavioural and neurochemical effects of para-methoxyamphetamine and 3,4-methylenedioxyamphetamine in the rat. *Prog Neuropsychopharmacol Biol Psychiatry* 24, 955-977.

The aim of this study was to characterise the pharmacodynamic differences of PMA effects *in vivo*, compared to MDMA, and compare differences in neurochemical mechanisms *in vivo* that may explain the disparity in these pharmacodynamic effects.

The two pharmacodynamic effects investigated, locomotor activity and body temperature, relate to the sympathomimetic and thermoregulatory effects that result clinically in most acute adverse events seen with ‘ecstasy’ use. While previous studies have investigated the pharmacodynamic effects of these drugs, separately, this study extended beyond previous work by comparing both drugs (at different doses and ambient temperatures) using *in vivo* telemetric implants. Thus, this high-resolution technique was able to eliminate temperature/activity changes due to handling stress required for measurement of rectal temperatures. These data showed significantly greater potency and efficacy for PMA induced changes in thermoregulation, with respect to MDMA. Additionally, PMA was shown to have minimal effects on activity, correlating with clinical reports from users who have been known to be taking PMA, rather than MDMA.

The second component of this work investigated the two primary neurochemical systems that modulate thermoregulation and locomotor activity, namely 5-HT and dopamine respectively. In this study, the potency and efficacy of PMA/MDMA effects on neurotransmitter release and reuptake were investigated *in vivo*, within striatum.

This extended beyond previous studies investigating evoked release from *in vivo* and *in vitro* preparations, in that the greater spatial and temporal resolution afforded by

voltammetric methodologies allowed *in vivo* measurement of responses only previously shown using *in vitro* approaches. The drug effects on 5-HT and dopamine uptake and evoked neurotransmitter release, in real time (on a millisecond time scale) *in vivo*, had not been investigated previously for either drug. Additionally, local application of PMA and MDMA within striatum allowed characterisation of the effects of the parent drug, without the presence of active metabolites. This increased spatial resolution showed heterogeneity in responses through the dorsoventral extent of striatum. This had not been previously demonstrated for either PMA or MDMA. The data showed minimal effects of PMA on dopamine uptake, and reduced efficacy compared to MDMA, for drug-induced dopaminergic efflux. PMA appeared to also evoke release of 5-HT, but further confirmatory pharmacology was required to substantiate this finding (see publication 2).



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**DIFFERENTIAL BEHAVIOURAL AND NEUROCHEMICAL EFFECTS OF
PARA-METHOXYAMPHETAMINE AND
3,4-METHYLENEDIOXYMETHAMPHETAMINE IN THE RAT**

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(Final form, July 2000)

Abstract

Daws, Lynette C., Rodney J. Irvine, Paul D. Callaghan, Natasha P. Toop, Jason M. White and Felix Bochner: Differential Behavioural and Neurochemical Effects of para-Methoxyamphetamine and 3,4-Methylenedioxyamphetamine in the Rat. Prog. Neuro-Psychopharmacol. & Biol. Psychiat. 2000, **24**, pp. 955-977. ©2000 Elsevier Science Inc.

1. This study was prompted by recent deaths that have occurred after recreational administration of the substituted amphetamine para-methoxyamphetamine (PMA). Because relatively little is known regarding its mechanism(s) of action, its effects on physiological, behavioural and neurochemical parameters were compared with the well known effects of 3,4-methylenedioxyamphetamine (MDMA).
2. Equivalent doses of PMA (5-20 mg/kg) produced greater hypothermia than MDMA at an ambient temperature of 20 °C. At 30 °C, PMA continued to evoke hypothermia except the highest dose where hyperthermia ensued. MDMA altered body temperature only at the highest dose where hyperthermia also resulted.
3. At both 20 and 30 °C, MDMA stimulated locomotor activity whereas PMA had modest effects and then, only at high doses.
4. *In vivo* chronoamperometry was used to measure the effect of MDMA and PMA on release, and inhibition of uptake, of serotonin (5-HT) and dopamine (DA) in the dorsal striatum of anaesthetised rats. As expected, MDMA evoked release of DA and inhibited uptake of both DA and 5-HT. By contrast, PMA was a relatively weak releasing agent and did not inhibit DA uptake. However, PMA potently inhibited uptake of 5-HT.
5. Taken together these data suggest that the acute adverse effects of PMA are more likely to be associated with alterations in serotonergic rather than dopaminergic neurotransmission.

Keywords: core body temperature, dopamine, *in vivo* chronoamperometry, locomotor activity, 3,4-methylenedioxyamphetamine, para-methoxyamphetamine, serotonin.

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Daws, L. C., Irvine, R. J., Callaghan, P. D., Toop, N. P., White, J. M. & Bochner, F. (2000) Differential behavioural and neurochemical effects of para-methoxyamphetamine and 3,4-methylenedioxymethamphetamine in the rat. *Progress in Neuropsychopharmacology & Biological Psychiatry*, v. 24 (6), pp. 955-977

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3. Publication 2

Callaghan P. D., Irvine R. J. and Daws L. C. (2005) Differences in the *in vivo* dynamics of neurotransmitter release and serotonin uptake after acute para-methoxyamphetamine and 3,4-methylenedioxymethamphetamine revealed by chronoamperometry. *Neurochem Int* 47, 350-361.

The aim of this publication was to further examine the differences in potency and selectivity between PMA and MDMA to evoke release of monoamines, and inhibit monoamine uptake. This work extended significantly beyond publication 1 in several aspects. Maximal effects on PMA and MDMA on neurotransmitter release were compared, revealing unique differences in both the qualitative and quantitative nature of striatal evoked-release for each drug. Local application of PMA in striatum resulted in a single discrete release event, characterised electrochemically as having both a serotonergic and dopaminergic component. Confirmatory pharmacological experiments showed that a component of PMA-evoked release was indeed blocked by prior application of a SERT inhibitor. Inhibition of SERT did not alter MDMA-evoked release, indicating evoked release to be primarily dopaminergic in nature. This indicated a fundamental difference in the nature of PMA effects within striatum, compared to MDMA. Also, local application of MDMA resulted in multiple release events, over the minute's time scale, after a single local application in striatum. This had not ever been seen previously. This is probably due to the need for preservation of local neural circuitry for the 'feed forward' or 'feed back' loop, which would be destroyed *in vitro*. Additionally, techniques such as microdialysis and differential pulse voltammetry have a temporal resolution between samples that is too great to see these effects. The underlying mechanism is not known, but worthy of further investigation. These studies also demonstrated significant SERT and DAT promiscuity in striatum, indicating that blockage of DAT can in fact significantly reduce the clearance of

5-HT *in vivo*. As such, the actions of MDMA to inhibit DAT and SERT should synergistically increase extrasynaptic and synaptic concentrations of 5-HT (but this was not assessed in these experiments), again showing a mechanistic difference compared to PMA. Thus, *in vivo* neurochemical data from this study further explains differences in the sympathomimetic profile of MDMA, with that of PMA.

The following experiments were conducted in the CA3 region of hippocampus, where 5-HT innervation is of similar density to striatum, but minimal dopaminergic innervation is present. No effect of either drug was seen on *in vivo* 5-HT release. This may be due to technical limitations of the chronoamperometric methodology (limit of detection is higher than baseline 5-HT concentration in hippocampus). As such, this area was chosen to characterise the potency and efficacy of PMA effects on 5-HT uptake *in vivo*, and contrasted with MDMA. PMA was shown to inhibit 5-HT uptake with greater potency and efficacy than MDMA, yet the differences were relatively subtle, compared to differences in the pharmacodynamic effects of these drugs on core body temperature. Thus it appears that differences in the acute actions of PMA on 5-HT neurotransmission alone cannot explain pharmacodynamic differences with MDMA. Further studies need to investigate other aspects of PMA and MDMA pharmacology to answer this question.



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Differences in the in vivo dynamics of neurotransmitter release and serotonin uptake after acute *para*-methoxyamphetamine and 3,4-methylenedioxymethamphetamine revealed by chronoamperometry

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Abstract

Illicit use of *p*-methoxyamphetamine (PMA) is rapidly increasing. However, little is known about the acute effects of PMA on neurotransmission in vivo. High-speed chronoamperometry was used to monitor neurotransmitter release and clearance in anesthetized rats after local application of PMA or 3,4-methylenedioxymethamphetamine (MDMA). In striatum, PMA caused less neurotransmitter release than MDMA. PMA-evoked release could be partially blocked by pre-treatment with a serotonin (5-HT) reuptake inhibitor, suggesting that evoked 5-HT release contributed to the electrochemical signal and was mediated by the 5-HT transporter (SERT). MDMA-evoked release was not blocked by a SERT inhibitor, suggesting that primarily DA was released. To study the effect of these amphetamines on clearance of 5-HT mediated specifically by the SERT, clearance of exogenously applied 5-HT was measured in the CA3 region of the hippocampus. In contrast to the striatum where 5-HT is cleared by both the SERT and the dopamine transporter (DAT), 5-HT is cleared primarily by the SERT in the CA3 region. This is also a region where neither PMA nor MDMA evoked release of neurotransmitter. The maximal inhibition of 5-HT clearance was greater after PMA than MDMA.

These data demonstrate in vivo (1) brain region variability in the ability of PMA and MDMA to evoke release of neurotransmitter; (2) that clearance of 5-HT in the striatum is mediated by both the SERT and the DAT; (3) distinct differences in the amount and nature of neurotransmitter released in the striatum after local application of PMA and MDMA and (4) that PMA is a more efficacious inhibitor of 5-HT clearance in the hippocampus than MDMA. These fundamental differences may account for the more severe adverse reactions seen clinically after PMA, compared to MDMA.

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Keywords: *Para*-methoxyamphetamine; 3,4-Methylenedioxymethamphetamine; Serotonin transporter; Dopamine transporter; Chronoamperometry; Release; Uptake

Callaghan, P. D., Irvine, R. J. & Daws, L. C. (2005) Differences in the *in vivo* dynamics of neurotransmitter release and serotonin uptake after acute para-methoxyamphetamine and 3,4-methylenedioxymethamphetamine revealed by chronoamperometry.

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4. Publication 3

Callaghan P. D., Farrand K., Salem A., Hughes P., Daws L. C. and Irvine R. J. (2006) Repeated administration of the substituted amphetamine p-methoxyamphetamine produces reductions in cortical 5-HT transporter binding but not 5-HT content, unlike 3,4-methylenedioxymethamphetamine. *Eur J Pharmacol* 546, 74-81.

The remaining publications shift in focus to investigate whether residual reductions in serotonergic *in vitro* biomarkers, and indeed serotonergic transporter function *in vivo*, are seen after prior treatment of animals with PMA. These effects were contrasted with those of MDMA. These studies address the question of whether PMA has residual selective neurotoxic properties, as seen with methamphetamine, and controversially, MDMA.

In this publication, the two most widely used serotonergic *in vitro* biomarkers, SERT binding (as measured by [³H]-paroxetine binding) and 5-HT content were chosen. The residual effects on 5-HT biomarkers and neurotransmission after PMA administration were not known. While one previous study existed, the doses of PMA used were considerably greater than its LD₅₀ concentration. These doses had minimal pharmacological relevance, based on pharmacokinetic studies of blood and brain concentrations using behaviourally active doses in rats.

Both drugs (at both doses) significantly reduced cortical SERT binding with respect to controls, with the highest dose regimen of MDMA having greater reductions than seen with equivalent doses of PMA. Elevation of room temperature to 28°C for 6 hours after treatment had no additive effects on the reductions in [³H]-paroxetine binding seen.

Interestingly, use of a more frequent dosing regimen of PMA did not reduce cortical 5-HT content, despite significant reductions after MDMA administration. As such, the question of whether PMA can induce serotonergic neurodegeneration required further study. This was addressed in publication 4.



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Repeated administration of the substituted amphetamine *p*-methoxyamphetamine produces reductions in cortical 5-HT transporter binding but not 5-HT content, unlike 3,4-methylenedioxyamethamphetamine

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Abstract

Worldwide growth in *p*-methoxyamphetamine (PMA) usage amongst 'ecstasy' users indicates a proportionally greater incidence of acute toxicity compared to 3,4-methylenedioxymethamphetamine (MDMA). While longer-term use of MDMA appears to produce degeneration of 5-hydroxytryptamine (5-HT, serotonin) neurons, PMA effects are poorly understood. The aim of this study was to determine the effect of repeated PMA administration on two indices of 5-HT axonal degeneration, cortical brain 5-HT transporter (SERT) density and 5-HT/5-hydroxyindolacetic acid (5-HIAA) content. Treatment of male rats once daily for 4 days (10 or 20 mg/kg) with PMA or MDMA resulted in significant reductions (20 mg/kg: 53% and 23% of vehicle treatment respectively) in [³H]-paroxetine binding (SERT density) one week after final drug administration. When rats were housed at a higher ambient temperature (28 °C vs. 22 °C) for 6 h after dosing, no additive effect was seen for either drug. A more intensive dosing regimen (10 or 20 mg/kg twice daily for 4 days) was used to examine PMA/MDMA effects on cortical 5-HT content. Two weeks after MDMA treatment, significant reductions in cortical 5-HT content (20 mg/kg: 39% of vehicle treatment) were seen. However, PMA did not alter cortical 5-HT content, yet reduced cortical 5-HIAA content (20 mg/kg: 72% of vehicle treatment). These data suggest PMA has severe long-term implications clinically for alteration of 5-HT neurotransmission that may differ from MDMA, but may not necessarily be interpreted as a degeneration of 5-HT fibres.

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Keywords: *Para*-methoxyamphetamine; 3,4-methylenedioxyamethamphetamine; Serotonin transporter; Neurodegeneration; 5-HT

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5. Publication 4

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This work extends considerably beyond the findings of publication 3. The aim of this study was to investigate whether prior PMA/MDMA administration resulted in a residual reduction in serotonergic biomarkers (assessed using *in vitro* methodologies), and a functional reduction in serotonergic clearance *in vivo*. This study was comprised of two aspects. Firstly, does PMA administration have the capacity to induce neurodegeneration of serotonergic fibres in the CNS? This was assessed using the comparison of residual changes in both *in vivo* and *in vitro* biomarkers, in the same study, paired to the same animals, after prior drug administration. MDMA was a positive control in this study in some respects, as the doses chosen were known to either not effect, or significantly reduce a wide range of serotonergic biomarkers, as assessed using *in vitro* methodologies. The second question of whether prior MDMA treatment would reduce SERT function *in vivo*, had not been previously investigated, nor for any other substituted amphetamines. A considerable number of studies have shown physiological and behavioural functional deficits after ‘neurodegenerative’ dosing regimens of MDMA, but the mechanism underlying these effects was not clear.

Two doses were chosen, a low dose that had been previously been shown for MDMA to not effect SERT binding two weeks post drug administration, and a moderate dosage regimen, known to induce acute pharmacodynamic effects, and alter all measured serotonergic biomarkers. At the low dose, neither PMA nor MDMA altered SERT

binding, nor SERT function *in vivo*, as expected. After moderate dosing, PMA administration resulted in reductions in hippocampal SERT binding and reduced [³H]-5HT uptake into hippocampal synaptosomes, indicating reductions in SERT using *in vitro* biomarkers previously used to indicate neurodegeneration of 5-HT fibres. This correlated with reductions in SERT function *in vivo* using chronoamperometric techniques, as would be predicted. However, hippocampal 5-HT content was not reduced, indicating that selective neurodegeneration of 5-HT fibres may not in fact be occurring.

After MDMA administration, all *in vitro* serotonergic biomarkers (hippocampal SERT binding, reduced [³H]-5HT uptake into hippocampal synaptosomes and 5-HT content) were significantly reduced, to the extent reported previously in the literature. However, SERT function *in vivo* was completely unaffected. This indicates that despite the reductions in biomarkers for localisation of serotonergic fibres, the primary site of action for MDMA, the serotonin transporter, remains functional. This could mean that there is enough ‘transporter reserve’ to maintain normal function under the experimental conditions used, and/or that other potential compensating mechanisms are able to maintain normal SERT function. Interestingly, these data indicate that reductions in *in vitro* biomarkers of 5-HT axonal degeneration do not necessarily predict the potential compensatory mechanisms that maintain SERT function *in vivo*. Compensatory mechanisms appear to exist *in vivo* to maintain clearance of extracellular 5-HT that may be disrupted or eliminated during tissue preparation for *in vitro* assays.

In vivo analysis of serotonin clearance in rat hippocampus reveals that repeated administration of p-methoxyamphetamine (PMA), but not 3,4-methylenedioxymethamphetamine (MDMA), leads to long-lasting deficits in serotonin transporter function

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Abstract

p-Methoxyamphetamine (PMA) has been implicated in fatalities as a result of 'ecstasy' (MDMA) overdose worldwide. Like MDMA, acute effects are associated with marked changes in serotonergic neurotransmission, but the long-term effects of PMA are poorly understood. The aim of this study was to determine the effect of repeated PMA administration on *in vitro* measures of neurodegeneration: serotonin (5-HT) uptake, 5-HT transporter (SERT) density and 5-HT content in the hippocampus, and compare with effects on *in vivo* 5-HT clearance. Male rats received PMA, MDMA (4 or 15 mg/kg s.c., twice daily) or vehicle for 4 days and 2 weeks later indices of SERT function were measured. [³H]5-HT uptake into synaptosomes and [³H]cyanoimipramine binding to the SERT

were significantly reduced by both PMA and MDMA treatments. 5-HT content was reduced in MDMA-, but not PMA-treatment. In contrast, clearance of locally applied 5-HT measured *in vivo* by chronoamperometry was only reduced in rats treated with 15 mg/kg PMA. The finding that 5-HT clearance *in vivo* was unaltered by MDMA treatment suggests that *in vitro* measures of 5-HT axonal degeneration do not necessarily predict potential compensatory mechanisms that maintain SERT function under basal conditions.

Keywords: chronoamperometry, 3, 4-methylenedioxymethamphetamine, neurodegeneration, para-methoxyamphetamine, serotonin, serotonin transporter.

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6. Discussion and Conclusions

The complexities of understanding how the actions of illicit drugs classed under the banner of 'ecstasy', lead to unpredictable acute toxicity and potential long term neuropsychological/psychiatry issues, has remained an elusive goal over the last 30 years of intense research. The goal of this thesis has been to examine both the acute and residual neuropharmacological effects of an unusual substituted amphetamine, p-methoxyamphetamine, a compound that has been associated with significant acute toxicity. Within these studies, the question of how PMA differs from the drug usually sold as 'ecstasy', 3,4-methylenedioxymethamphetamine (MDMA) has been paramount. PMA use has spread from a specific locus within Australia, to usage world-wide since 1994, repeatedly associated with significant morbidity, and thus is most worthy of further understanding to potentially aid in development of novel acute treatments in cases of overdose.

This goal has been approached in two distinct ways. The initial component of this thesis investigated whether differences between potency and efficacy of *in vivo* pharmacodynamic effects could be explained by differences in key underlying monoaminergic neurochemical processes. The second component investigated the potential for residual neurochemical injury after repeated administration of PMA. The question of whether PMA use results in selective neurodegeneration of monoaminergic and/or serotonergic systems, like methamphetamine, or controversially, MDMA, had not been previously investigated. These studies extrapolated beyond previously used *in vitro* biomarkers of serotonergic neurodegeneration, to use a novel methodological approach to investigate serotonin transporter function *in vivo* after prior PMA or MDMA administration. The direct measurement of a functional deficit in SERT action had not been previously shown in substituted amphetamine-induced serotonergic neurodegeneration.

A unique approach was taken within most studies in this thesis, using an *in vivo* electrochemical methodology, chronoamperometry, to measure monoamine concentrations within specific brain regions in anaesthetised animals. This allowed examination of the acute, local effects of drug on monoamine release and uptake processes. The temporal resolution (millisecond time frame) afforded comparison of the pharmacology of the parent compound, without the confound of the presence of active metabolites. As such, this approach was considered an optimal methodology for realising the aims of this study.

The initial study (publication 1) characterised the dose response relationship of PMA or MDMA administration on the two most relevant acute pharmacodynamic responses, core body temperature and locomotor activity. In terms of thermoregulatory modulation, PMA had greater efficacy compared to MDMA, producing greater magnitude alterations in core body temperature when treated at room or high ambient temperatures. This is consistent with the increased incidence of acute hyperthermia reported by health professionals in cases where PMA is detected in blood drug screens, compared to MDMA (Felgate et al. 1998; Ling et al. 2001; Caldicott et al. 2003). Also consistent with clinical reports, PMA had lesser efficacy compared with MDMA for increasing locomotor activity, an important marker for comparing stimulant effects amongst substituted amphetamines in animal models.

The second component of this study compared the effects of PMA and MDMA on monoamine release and re-uptake processes in real time using *in vivo* electrochemistry. PMA was a relatively weak dopamine releasing agent, and did not inhibit dopamine uptake, consistent with its minimal pharmacodynamic effects on locomotor activity. This was in contrast to MDMA significantly increasing dopamine release, and blockage of dopamine uptake. Both drugs significantly inhibited 5-HT uptake. These data provide an initial step in understanding the neurochemical basis for the differing effects of PMA and MDMA on locomotor activity.

The second publication extended upon these findings, investigating differences in the potency and efficacy of PMA and MDMA to alter 5-HT neurochemistry through dose-response studies. Regional anatomical heterogeneity of effects were also assessed in dorsal striatum and the CA3 region of hippocampus. A component of PMA-evoked neurotransmitter release (likely to be primarily dopaminergic) was shown to be mediated by serotonergic fibres in striatum (attenuated by prior application of the SERT inhibitor, zimelidine), which did not occur for MDMA. Neither PMA nor MDMA were able to evoke release of neurotransmitter in the CA3 region of hippocampus, a region with similar density of serotonergic innervation to the striatum (Benmansour et al. 1992), but minimal dopaminergic innervation. PMA was shown to be a more efficacious inhibitor of 5-HT uptake in hippocampus than MDMA. The studies demonstrated novel differences in the both the qualitative and quantitative nature of PMA and MDMA evoked neurotransmitter release in striatum that had not previously been reported.

The remaining component of this thesis shifted focus to characterising the longer term residual neurochemical alterations that occur after repeated treatment with PMA and MDMA. These studies were designed to determine the potential for PMA to have 'neurodegenerative' properties. As such, MDMA, which has been extensively studied in this regard, was also a useful positive control, in terms of alternations of *in vitro* biomarkers of neurodegeneration. A more intense treatment protocol (twice daily administration, for 4 days) was chosen, based on the methodology initially used to characterise the 'neurodegenerative' potential of a range of substituted amphetamines. Thus a comparison of PMA with other substituted amphetamines could be made (Ricaurte et al. 1985; Stone et al. 1987c; Battaglia et al. 1988a; Insel et al. 1989; Battaglia et al. 1991; Johnson and Nichols 1991; Huang et al. 1992; Bowyer et al. 2003).

In publication 3, the effect of repeated treatment of PMA on two biomarkers commonly used to indicate residual serotonergic 'neurodegeneration' (as seen with several other

substituted amphetamines) were assessed: cortical 5-HT/5-HIAA content and [H^3]-paroxetine binding, to the SERT. While repeated MDMA treatments reduced both markers significantly, PMA reduced cortical SERT binding and 5-HIAA content, yet cortical 5-HT content was not altered 1-2 weeks after drug treatment. Neither drug altered cortical dopamine content, as expected. As such, this indicated PMA may not have 'neurodegenerative' properties, and may still produce more complex alterations in serotonergic function.

Publication 4 extended considerably beyond these findings, to clarify if PMA did indeed have neurodegenerative properties, and whether prior PMA or MDMA treatment results in any functional change in clearance of 5-HT *in vivo*. The investigation of residual changes in 5-HT clearance *in vivo* after a prior treatment regimen of MDMA (or PMA) was entirely novel. The implications of these data are two-fold. Firstly, this approach allows the assessment of neurochemical function with polysynaptic pathways intact, and thus any alternate compensating mechanisms should remain functional. This addresses a common scientific criticism concerning the use of *in vitro* approaches to model *in vivo* processes in neuroscience, and physiology in general. Secondly, this gives great insight into the question of whether the alterations in markers of 5-HT fibre localisation after 'neurodegenerative' treatments of substituted amphetamines do in fact have any functional consequences. Such an experimental approach has previously shown reductions in 5-HT clearance *in vivo* in the CA3 region of hippocampus after graded 5-HT lesions, gene dose in SERT knockout mice, or chronic administration with an SSRI (Benmansour et al. 2002; Montanez et al. 2003a; Montanez et al. 2003b; Daws et al. 2005). Thus the validity of this approach has been previously established.

Rats were given a 'neurodegenerative' or a 'non-neurodegenerative' repeated treatment regimen of PMA or MDMA, as quantified by alterations in hippocampal SERT autoradiography and 5-HT content. Again, as reported in publication 3, no change in

hippocampal 5-HT content after a prior PMA treatment regimen was observed. After a 2 week washout, rats were anaesthetised and 3 concentrations ranges ('low', 'medium' and 'high') of 5-HT were exogenously applied within the CA3 region of hippocampus, where an *in vivo* electrochemical electrode recorded the rate of clearance of exogenously applied 5-HT. Prior moderate dose PMA treatment significantly reduced *in vivo* clearance of 5-HT, consistent with the reduced SERT binding shown, and reduced *in vitro* 5-HT uptake in synaptosomes were prepared from the contralateral hippocampus of animals post mortem. However, prior MDMA treatment had paradoxical effects on *in vivo* 5-HT clearance. As has been extensively shown previously, the moderate dose MDMA treatment resulted in significant reductions in hippocampal 5-HT content, SERT binding, and *in vitro* synaptosomal 5-HT uptake. *Despite this, there was no alteration in the in vivo clearance of 5-HT.* This finding suggests that *in vitro* measures of 5-HT axonal degeneration may not necessarily predict potential compensating mechanisms *in vivo* that maintain SERT function under basal conditions. A particular strength of this study was that all *in vitro* data was paired with *in vivo* assessment of 5-HT clearance, in the same animals.

Differences in the acute neurochemical responses after acute PMA and MDMA administration.

As has been established through an extensive literature, substituted amphetamines mediate their individual pharmacodynamic profiles through differences in potency and efficacy to modulate the three key processes in synaptic neurotransmission: reverse transport of monoamines from the presynaptic terminal, blockade of monoamine uptake and inhibition of monoamine metabolism. Differences in ability to modulate serotonergic, dopaminergic and noradrenergic neurotransmission are thought to be defining characteristics for the functional 'phenotype' of individual substituted amphetamines. A primary hypothesis of this component of the thesis was to determine if differences in acute pharmacodynamic

profiles of PMA and MDMA could be explained by differences in acute potency/efficacy of these drugs on their primary neurochemical targets: 5-HT and dopamine systems. This thesis extended upon previous findings to reveal novel quantitative and qualitative differences in PMA and MDMA mediated neurotransmitter efflux, *in vivo*.

While the acute actions of MDMA on these systems have been extensively characterised over the last 20 years, little is known of the neurochemistry of PMA. Characterisation of PMA effects on monoamine release and reuptake using *in vitro* brain slices and synaptosomal preparations, indicated PMA to be a potent 5-HT releasing agent and reuptake inhibitor (Hitzemann et al. 1971; Tseng et al. 1976; Nichols et al. 1982; Hegadoren et al. 1994). Despite differences in the potency of PMA effects on SERT in some studies, all demonstrate much less potent actions on dopamine release and reuptake processes compared to other amphetamines (Tseng et al. 1974; Hegadoren et al. 1994).

For some time, the only *in vivo* assessment of PMA-mediated modulation of serotonergic function was the indirect measure of brain 5HT release, the myoclonic twitch activity (MTA) of the suprahyoideal muscle (Menon et al. 1976b; Tseng 1978a, 1979). Gough and co-workers conducted the first study to compare the actions of PMA and MDMA using microdialysis (after the commencement of these doctoral studies) in freely moving rats on *in vivo* 5-HT release, showing greater efficacy for MDMA (Gough et al. 2002a). This finding may be explained by the significant contribution of pharmacologically active metabolites of MDMA to this release event, and also recent novel findings showing MDMA-mediated inhibition of its own metabolism (Lim and Foltz 1988; Green et al. 2003; Yang et al. 2006). PMA does have one primary metabolite, p-hydroxyamphetamine, yet while psychoactive it does not appear to contribute greatly to PMA's pharmacodynamic effects, due to rapid clearance and elimination (Kitchen et al. 1979; Kaminskis et al. 2002). These studies were confirmed and extended by recent studies from Freezer and coworkers and Stanley and coworkers to show that PMA-mediated

increases in extracellular fluid (ECF) 5-HT involved significant inhibition of monoamine oxidase A (Freezer et al. 2005; Stanley et al. 2007). Due to increases in 5-HT in the extracellular fluid being measured over the 15-30 minute sampling period using microdialysis, conclusions can not be drawn about which neurochemical processes are being modulated by PMA and MDMA. As such, to investigate monoamine transporter kinetics of monoamine uptake, only a fast sampling voltammetric technique had the temporal resolution required.

In summary, it was not possible to interpret from the prior literature whether differences in potency and efficacy exist between MDMA and PMA at monoamine transporter sites that could explain differences seen in pharmacodynamic responses.

Additionally, the use of chronoamperometric methodologies in this study revealed unique qualitative and quantitative properties of both PMA and MDMA evoked striatal neurotransmitter efflux (publication 1 and 2). Firstly, an increasing dorsal to ventral gradient was seen in the proportion of 5-HT content compared to dopamine for both PMA and MDMA evoked efflux, as the electrode/micropipette complex was stepped down through striatum. This reflects the increasing density of serotonergic innervation seen in ventral aspects of striatum (Soghomonian et al. 1987). Medio-lateral gradients have also been shown in the alteration of dopamine clearance *in vivo* after systemic cocaine-administration, consistent with microanatomical differences in dopaminergic innervation (Glynn and Yamamoto 1989; Cline et al. 1995). Also micropunch neurochemical analyses of dorsal and ventral regions of striatum indicate that a ventrally increasing serotonergic gradient exists (Beal and Martin 1985).

Antagonist studies (publication 2) indicated that blockade of the SERT did not alter MDMA-evoked striatal release, consistent with the resulting neurochemical identity of the recorded currents being predominantly dopaminergic. PMA-evoked release was significantly attenuated by SERT blockade, indicating that PMA had greater efficacy for 5-

HT release compared to MDMA. This finding for MDMA initially seems inconsistent with the extensive literature outlining MDMA-induced 5-HT release *in vivo* and *in vitro* (Nichols et al. 1982; Johnson et al. 1986; Gough et al. 1991; Johnson et al. 1991; Gudelsky and Nash 1996; Crespi et al. 1997; Esteban et al. 2001; Gough et al. 2002a; Freezer et al. 2005; Stanley et al. 2007). The only studies using local application of MDMA *in vivo* (by reverse dialysis through the microdialysis probe) also demonstrated dopamine release in striatum (Nash and Brodtkin 1991; Esteban et al. 2001). MDMA induced 5-HT release may require peripheral administration, due to the increased number of active metabolites, which may mediate this effect. Alternatively, the lack of 5-HT release may be a consequence of the limit of detection of our system. It is worth noting though that MDMA-induced increases in striatal 5-HT could be due to primarily inhibition of SERT rather than 5-HT efflux in combination with SERT inhibition. The relative contributions of these processes were not delineated in the microdialysis studies previously mentioned. Interestingly, MDMA evoked neurotransmitter release was undetectable *in vitro* from striatal, nigral or raphe brain slices, using local application of drug coupled with high speed voltammetric detection (Iravani et al. 2000). This further reinforces the requirement for *in vivo* circuitry to be intact in order to observe MDMA-induced neurochemical responses (Iravani et al. 2000). Using the same experimental approach, Iravani and coworkers quantified neurotransmitter efflux after local application of amphetamine, indicating the lack of MDMA effects on evoked neurotransmitter release are unlikely to be due to methodological issues (Iravani and Kruk 1995).

The critical role of *in vivo* connectivity in mediation of MDMA-evoked neurotransmitter release was also seen in the differing qualitative characteristics of the efflux signals, compared to PMA. A single local application of MDMA within striatum produced a composite of continuous multiple discrete release events rather than a single event, as was seen with PMA. This may be explained by MDMA itself, or post-synaptic effects due to

MDMA-mediated neurotransmitter release acting at receptors to initiate further neurotransmitter release. It would appear likely that some form of positive feedback loop could mediate such effects, yet the mechanism for such phenomena is not clear. The reason that this has not been previously seen most likely relates to methodological differences. It is worth noting the sampling time required for measuring *in vivo* monoamine release using techniques such as microdialysis would mask such responses. Two other studies have used voltametric approaches for investigating MDMA effects on monoamine release *in vivo*, both after a single peripherally administered treatment. Firstly, the onset of drug action was greatly reduced compared to local application of drug *in vivo*, the approach used in studies reported here. In the study of Gazzara and coworkers, the 10 minute interval between voltogram measurement (using semidifferential voltammetry, over 40 sec period) would not allow the detection of multiple release events (Gazzara et al. 1989). This time period is required for ECF equilibration after the local monoamine depletion from the 40 sec voltage scan. The study of Yamamoto and Spanos showed, using a similar voltametric approach to Gazzara and colleagues, the first evidence for time, dose and region dependent increases in dopamine release after peripheral administration of MDMA (Yamamoto and Spanos 1988). Again this technique only allowed the collection of cumulative data at 5 minute intervals, so multiple release events would likely go undetected.

In summary, these data indicate that significant qualitative and quantitative differences exist between PMA and MDMA neurotransmitter efflux *in vivo*. PMA did have greater efficacy compared to MDMA for modulation of striatal 5-HT release. However the neurochemical processes underlying PMA and MDMA induced striatal neurotransmitter release were different. Additionally, the complexity of MDMA-evoked striatal efflux, being composed of multiple release events, was also an unexpected finding.

The second aspect of these studies (publication 2) concentrated on a comparison of drug potency and efficacy to alter 5-HT clearance, another key process whereby substituted amphetamines modulate monoamine neurochemistry. Local administration of both PMA and MDMA potently inhibited clearance of exogenously applied 5-HT in striatum, but only MDMA altered striatal uptake of exogenously applied dopamine. Within the CA3 region of hippocampus, neither drug evoked endogenous release (Daws et al. 1998), allowing potency and efficacy of the drug effects on *in vivo* 5-HT clearance to be assessed. Additionally, prior data had shown concentrations of exogenously applied 5-HT used in this study were cleared only by active transport by the SERT, with a minor diffusional component (Daws et al. 1998; Daws et al. 2005). Indeed PMA was shown to have an EC₅₀ for inhibition of 5-HT clearance at a lower dose than MDMA, in addition to a significantly greater maximal response. This indicated PMA had greater potency and efficacy compared to MDMA. The maximal response was similar in magnitude to that of the selective SSRI, fluvoxamine, using a similar voltammetric experimental paradigm (Daws et al. 2000a). This indicates both drugs are clearly efficacious inhibitors of 5-HT uptake, and as such these differences are likely to have pharmacological relevance.

During the course of these studies, the role of monoamine transporter promiscuity in the acute actions of both PMA and MDMA became apparent. Evidence exists to show functional clearance *in vivo* of 5-HT by NET in CA3 region of hippocampus (Daws et al. 1998; Daws et al. 2005). Additionally 5-HT has been shown to be cleared by DAT in various brain regions, under basal conditions in SERT knockout mice (Zhou et al. 2002a). Of most relevance to this thesis, 5-HT can be stored as a 'false neurotransmitter' in dopaminergic terminals, and released via reversal of DAT with MDMA (Stamford et al. 1990; Jackson and Wightman 1995). Transporter promiscuity is thought to be a mechanism for the generation of free radicals (from dopamine ortho-quinones, after dopamine uptake via SERT) that leads to damage of 5-HT fibres with MDMA

administration (Sprague et al. 1998; Green et al. 2003). Considerable heterogeneity in the time course of 5-HT clearance between brain nuclei was seen after local application of exogenous 5-HT. Similar amounts of 5-HT were cleared in less than half the time in striatum, compared with the CA3 region of hippocampus. The rate of 5-HT clearance *in vivo* has been clearly shown to be dependent on SERT density, with 5-HT clearance being reduced by graded lesion of 5-HT fibres, or gene dose in 5-HT knockout mice (Montanez et al. 2002; Montanez et al. 2003a; Montañez et al. 2003; Daws et al. 2005). As both regions have comparable concentrations of 5-HT transporter (Benmansour et al. 1992), the most probable explanation for this finding is that another active transport process(s) is clearing 5-HT in striatum. This was clearly demonstrated (publication 2), as it was shown that striatal 5-HT clearance was significantly inhibited by local application of either a DAT inhibitor (GBR12909) or a SERT inhibitor (zimeclidine). This indicates that 5-HT is indeed taken up into dopamine terminals via DAT, *in vivo*. The relation of this finding to the neurochemical mode of action of PMA and MDMA within striatum is complex. As MDMA was shown to interact with both DAT and SERT, this indicates that MDMA can increase striatal 5-HT extrasynaptic concentration by blockade of both active transport processes. On the other hand, PMA primarily modulates 5-HT clearance through interactions with the SERT. This again is a key difference in the mode of action of these drugs, and a novel finding *in vivo*.

One potential complication for interpretation of these data related to how relevant the concentration of MDMA locally applied was compared with brain concentrations attained with pharmacologically active doses, administered peripherally. Measurement of MDMA concentrations in hippocampus by microdialysis indicate the doses we used here are equivalent to pharmacologically relevant doses of 10-15 mg/kg of MDMA (Esteban et al. 2001). Likewise, a recent study showed reverse dialysis of 100 μ M PMA and MDMA into striatum also resulted in equivalent increases in both extracellular 5-HT and dopamine

(Romero et al. 2006). This is within the range of the barrel concentrations of both drugs used in the studies reported here, indicating indirectly that concentrations of the drug used are within a physiologically relevant range.

The primary hypothesis of this component of the thesis predicted that the differing pharmacodynamic profiles of PMA and MDMA would be reflected in significant differences in the potency and efficacy to increase 5-HT/DA release and inhibit reuptake, *in vivo*. While significant differences were noted, the greater efficacy and potency of PMA for increasing 5-HT release and inhibition of 5-HT uptake did not appear great enough to explain the disproportionate pharmacodynamic effects on body temperature that are seen clinically. Other neurochemical and/or pharmacokinetic processes must also contribute to the actions of PMA to explain this difference. Recent studies have shown that the potent actions of PMA to inhibit monamine oxidase A (MAO-A) contribute to its pharmacodynamic profile (Freezer et al. 2005; Stanley et al. 2007). Administration of MDMA after the MAO-A inhibitor, moclobemide, significantly potentiated increases in 5-HT concentration within striatal microdialysate samples. Interestingly, administration of PMA after moclobemide did not further potentiate the increase in striatal 5-HT, indicating that inhibition of MAO-A is a key component of the mode of action of PMA.

Are PMA/MDMA neurotoxic to CNS serotonergic fibres?

The key question of whether repeated MDMA use leads to long term neurodegeneration of forebrain 5-HT fibres has been controversial for more than 20 years. In isolation, reductions in each of the serotonergic biomarkers that have been linked with selective neurotoxicity could be equally well explained by functional downregulation in serotonergic mechanisms. However, when viewed holistically, the most likely explanation for the data is that repeated MDMA use produces anatomical and functional deficits in biomarkers of serotonergic neurochemistry, consistent with selective serotonergic neurodegeneration.

This thesis extended beyond previous findings, investigating whether repeated MDMA or PMA administration could produce a *residual functional change in 5-HT clearance in vivo* (publication 4). In order to understand the resultant physiological changes, a clear understanding of the mechanisms of the causal processes is paramount. While linking causality of functional changes in hippocampal serotonergic function after MDMA/PMA use with residual psychological/psychiatry deficits is well beyond the scope of this thesis, it does underline the need for future experiments targeted to this unexplored area. *In vitro* measures ($[^3\text{H}]5\text{-HT}$ uptake into synaptosomes; SERT binding; tissue 5-HT content) have long been established to be reduced after the high dose MDMA treatment regimen chosen for this study. These *in vitro* indices were also investigated to validate the conditions that are usually considered indicative of serotonergic neurodegeneration. These studies were paired with the measurement of the kinetics of 5-HT clearance *in vivo*, again using voltammetric methodologies. It was expected that reduced SERT density, which follows MDMA treatment, would result in reduced 5-HT clearance *in vivo*. *In vivo* 5-HT clearance is correlated with SERT density. This is shown through regional heterogeneity in 5-HT clearance that correlates with SERT density in the respective brain regions (Montanez et al. 2002; Daws et al. 2005). This was also elegantly demonstrated in gene dose responses in 5-HT knockout mice, using *in vivo* electrochemical detection of exogenously applied 5-HT (Montanez et al. 2003b). Reductions in SERT density by chronic administration of an SSRI also reduced 5-HT clearance *in vivo* (Benmansour et al. 1999; Benmansour et al. 2002). As such, it was most intriguing to see that MDMA treatment did not alter 5-HT clearance *in vivo*. This provides the first evidence for functional compensation by alternative mechanisms for 5-HT clearance, despite reductions in hippocampal synaptosomal 5-HT uptake, reduced SERT binding and 5-HT tissue content. The magnitude of these reductions of *in vitro* indices also correlated with previously published findings (Battaglia et al. 1988a; Green et al. 2003).

Under basal conditions, the concentrations of 5-HT exogenously applied within CA3 region of hippocampus in this study are cleared primarily by active transport through the SERT (Daws et al. 1998; Daws et al. 2005). However, higher concentrations of locally applied 5-HT have been shown to be cleared by an additional process, a low affinity, high capacity uptake system via the noradrenaline transporter (Daws et al. 1998; Daws et al. 2005). To determine if this could be a potential compensating mechanism, *in vitro* measurement of [H^3]-NE uptake into hippocampal synaptosomes was measured in MDMA and PMA treated animals, but no differences were seen compared to control groups. This suggests that NET may not upregulate to maintain normal *in vivo* 5-HT clearance in MDMA treated animals, but due to its high capacity uptake, this may not be necessary. In addition, the reduction in residual SERT binding after PMA or MDMA administration was similar, and as such any resultant upregulation in NET expression would be similar for both drugs (if this was a key compensating mechanism for maintenance of 5-HT clearance). Other potential mechanisms exist that may explain the functional compensation in 5-HT uptake *in vivo* after MDMA administration. Recent studies indicate a novel target, the organic cation transporter (OCT), can mediate clearance of extracellular 5-HT under certain conditions. An increase in OCT expression was reported in SERT knockout mice (Watts et al. 2006; Baganz et al. 2007). OCT proteins have been shown to transport 5-HT *in vitro* (Grundemann et al. 1998; Wu et al. 1998). Additionally, OCT mRNA is upregulated in SERT knockout mice, in brain and within the enteric nervous system, in regions where 5-HT is present (Chen et al. 2001; Schmitt et al. 2003).

Pharmacological data has shown the OCT antagonist, decynium 22, was able to block 5-HT clearance in hippocampus, but the most marked inhibition of clearance occurred in heterozygote and homozygous SERT knockout mice, consistent with increased OCT expression in these genotypes. Investigation of alternative mechanisms for maintenance of

extracellular 5-HT concentrations is of critical importance for understanding the way antidepressants function, in addition understand the neurochemistry of drug abuse.

On the other hand, PMA treatment resulted in reductions in clearance of 5-HT *in vivo*, together with significant reductions in *in vitro* synaptosomal uptake of 5-HT and SERT binding. However, there was an incongruous lack of reduction in hippocampal 5-HT content, indicating that PMA produces a functional change in serotonergic neurotransmission, but not necessarily selective serotonergic neurodegeneration. Again, few studies exist to compare against. The only prior study investigating the residual effects after repeated doses of 80 mg/kg of PMA showed reductions in hippocampal, hypothalamic and cortical 5-HT content, but these changes were lesser in magnitude than those observed after much lower doses of MDMA (Steele et al. 1992). A major confound of this study was the doses used were far in excess of the LD₅₀ of PMA (Nichols et al. 1975). Initial MDMA studies using high doses showed increases in non specific regional neurotoxicity, rather than selective serotonergic deficits (Commins et al. 1987). The utilisation of such high doses has limited clinical relevance. The lack of a residual effect of repeated PMA administration on forebrain 5-HT content was replicated in two studies (publication 3 and 4), indicating it was a consistent effect. However, the recent study by Straiko and colleagues saw reductions in striatal 5-HT content 1 week after administration of 4 doses of PMA (10 mg/kg), every two hours, as opposed to 4 doses every 12 hours, the regimen used in this thesis. Considering the much shorter half life, and simple linear pharmacokinetics of PMA compared to MDMA, it is logical that a shorter dosing interval is required to produce longer term alterations in serotonergic function (Kitchen et al. 1979; Kaminskis et al. 2002). This has been shown to be the case for methamphetamine also, where a much shorter interdosing interval is required for presentation of neurotoxicity (Wallace et al. 1999; Cho et al. 2001; Clemens et al. 2004). Indeed, with a reduction in the

interdosing interval, much lower individual doses of MDMA can produce decreases in markers of serotonergic neurotransmission (O'Shea et al. 1998; McGregor et al. 2003; Clemens et al. 2004). This is again thought to be related to pharmacokinetic interactions accentuating plasma MDMA levels in a non-linear fashion (Yang et al. 2006). This dosing regimen has far greater ethological validity, more closely modelling the usage patterns seen clinically. However, care must be taken with extrapolating human dosing pattern to those in animals, as the pharmacokinetics of MDMA are not the same across species (de la Torre and Farre 2004).

Recent studies by Wang and colleagues uncovered the novel finding that MDMA treatment regimens that alter the standard gamut of biomarkers for 5-HT neurodegeneration, may in fact not alter expression of SERT (Wang et al. 2004; Wang et al. 2005). Instead, SERT protein expression, as detected by Western blot analysis, was not different between groups of rats treated with MDMA or vehicle. These findings lead to the conclusion that deficits in 5-HT uptake may be attributable to loss of SERT function rather than loss of the protein itself. This conclusion has reinvigorated the debate on whether MDMA is neurotoxic, or just results in a transient downregulation in serotonergic neurotransmission. All but one study (Aguirre et al. 1999) has failed to find increases in routinely used markers of axonal degeneration, such as astroglial activation using GFAP immunohistochemistry after MDMA treatment (O'Callaghan and Miller 1993; Pubill et al. 2003; Wang et al. 2004; Wang et al. 2005). Likewise, no effects of MDMA have been seen on microglial activation, as measured by increases in heat shock protein or peripheral benzodiazepine receptor binding (Pubill et al. 2003; Wang et al. 2004; Wang et al. 2005). Microglial activation has been shown to correlate with peripheral benzodiazepine binding in the mitochondria of the microglia themselves after wide range of neuroinflammatory insults. These include experimental autoimmune encephalitis, excitotoxic brain lesions, strokes and nerve crush injuries (Benavides et al. 1990; Karchewski et al. 2004; Papadopoulos

2004; Mattner et al. 2005; Boutin et al. 2007). In particular, methamphetamine treatment regimens that reduce serotonergic biomarkers induce significant astroglial and microglial activation (Pubill et al. 2003). Changes in SERT trafficking did not appear to be a likely explanation for altered SERT protein expression, as changes were not seen in the localisation of glycosylated and non-glycosylated SERT protein to fractionated plasma membrane and endosomal preparations respectively, between MDMA and vehicle treatments (Wang et al. 2005). Additionally, treatment with the selective serotonergic neurotoxin, 5,7-dihydroxytryptamine resulted in a loss of SERT protein, further validating these findings (Wang et al. 2004). The recent study of Straiko and coworkers has extended these studies to show that treatment regimens of PMA or MDMA do not alter the expression of cleaved microtubule-associated protein tau, another marker of neurotoxicity (Straiko et al. 2007). The study of Xie and colleagues recently revisited the findings of Wang and colleagues, suggesting that the SERT was a slightly smaller molecular weight protein than previously reported, and was reduced by MDMA, p-chloroamphetamine, 5,7-dihydroxytryptamine and fenfluramine administration (Xie et al. 2006). However, much higher concentrations of protein (~10 fold) were necessary to visualise SERT in the latter study, indicating the sensitivity of the antibody may not be optimal. It was recently proposed that standard heat denaturing of protein with SERT Western blotting may lead to dispersion of the signal, leading to the differing results reported (McLane et al. 2007). Most interestingly, very recent studies from Wang and coworkers have shown that depletion of 5-HT content after 'neurodegenerative' MDMA dosing regimens can be restored by subsequent treatment with the 5-HT precursor, 5-hydroxytryptophan (Wang et al. 2007). These exciting findings indicate that remaining 5-HT fibres are functional, as suggested by our studies showing no deficit in 5-HT uptake *in vivo* after repeated MDMA. Further studies are required to clarify this issue, which is key to understanding this

controversy. These findings may have major implications for treatment of MDMA-induced neuropsychological perturbations.

Limitations and Future directions

In the comparison of the neurochemical mechanisms of action of PMA, certain limitations were noted. The use of *in vivo* electrochemistry allowed the investigation of MDMA- and PMA-induced neurotransmitter release on a time scale that has never been investigated (milliseconds, as opposed to 5-15 minutes with complementary techniques such as microdialysis). While this has shown significant differences in the qualitative nature of neurotransmitter release induced by MDMA *in vivo*, certain trade offs are apparent with the use of this methodology. As chronoamperometry relies on oxidation of all electroactive species at the potential chosen (+0.55V was used for detection of monoamines in this study), the increased temporal resolution comes at the cost of chemical specificity. The relative index of the amount of current induced during oxidation and reduction of the chemical species at each data point is the only measure available to determine chemical identity. As such, chronoamperometry requires the use of confirmatory pharmacology and repetition of studies in animals with selective lesions of either the dopaminergic or serotonergic systems to confirm chemical identity. This was a factor in the choice of the striatum and hippocampus as model brain nuclei to investigation of PMA/MDMA effects on dopaminergic and serotonergic neurotransmission, respectively. These regions, while ideal of the terms of the neural circuitry, have been extensively investigated in terms of their neurochemistry, also using chronoamperometry. As such, this provided an ideal paradigm.

On the basis of the studies in this thesis, an extension of this work would investigate the effects of PMA and MDMA on serotonin release within the primary thermoregulatory control regions of the brains, the median preoptic hypothalamic regions. It is hypothesised

that MDMA-induced 5-HT release within this region mediates, directly or indirectly, thermoregulatory dysfunction that underlies the primary cause of acute toxicity in 'ecstasy' users. As such, characterisation of the differing acute potency and efficacy of PMA and MDMA within this region would give a direct indication of the relevance of this hypothesis to understand the mechanism of acute hyperthermia. However, this question is composed of several levels of complexity. In terms of the *in vivo* promiscuity of monoamine transporters, shown in this thesis, as well as the work of others, any alteration in 5-HT synaptic concentrations would involve serotonergic, dopaminergic (DAT) and noradrenergic transporters (NET), all of which are present within median preoptic hypothalamus. Due to the lack of prior voltammetric studies in this region, a considerable number of validation experiments would be required. Initially, the role of DAT and NET in 5-HT clearance *in vivo* within median preoptic regions would need to be determined. This would indicate the degree of transporter promiscuity that occurs in normal neurotransmission in the region, a key finding in understanding the pharmacology of this region. Subsequently, the comparison of PMA/MDMA potency and efficacy to modulate 5-HT release in animals with selective lesions of dopamine and noradrenergic systems would be required. Such studies would be a considerable undertaking and are beyond the scope of this dissertation.

Another limitation is the use of anaesthetised animals as the primary experimental preparation within this study. Considerable advances have occurred in the use of *in vivo* electrochemistry in freely moving animals during the course of this dissertation, both for the investigation of dopaminergic and serotonergic neurochemistry (Sabeti et al. 2002).

One strength of this study was the local application of substituted amphetamines *in vivo*, allowing the characterisation of the effects of the parent drug, without the complications of the presence of other active metabolites. This was of particular relevance for PMA, due to its simple metabolic pathway. Studies within our laboratory extended from my initial work

to show the primary active metabolite of PMA, p-hydroxyamphetamine, had similar efficacy to PMA in modulation of dopaminergic and serotonergic neurotransmission, but was not present in high enough concentrations in pharmacokinetic studies to contribute of the neurochemical effects of PMA *in vivo* (Kaminskas et al. 2002). The situation is significantly different for MDMA, possessing many active metabolites. Ideally, *in vivo* characterisation of the neurochemical effects of these metabolites is required as well, particularly given that the properties of MDMA-induced release appear to be mediated in part by polysynaptic pathways, unlike PMA. These properties cannot be quantified using standard methodologies (microdialysis, evoked release from brain slices, synaptosomal release), due to the lack temporal resolution. Indeed, the unique properties of MDMA to induce monoamine release via a composite of multiple small release effects (rather than a single discrete event, as seen with PMA), only seen in an *in vivo* preparation, is of considerable interest. This also warrants further investigation.

Investigation of the residual effects of PMA administration on serotonergic function *in vivo*, and *in vitro* markers, indicated PMA did indeed possess effects consistent with potential neurodegenerative effects, except for one inconsistency. In two separate studies, all *in vitro* markers from 5-HT fibres, except 5-HT content in forebrain regions, were reduced. This issue has subsequently been addressed in the study of Straiko and coworkers (Straiko et al. 2007). Using a much shorter dosing interval, PMA did reduce forebrain 5-HT content after a 2 week washout. This is consistent with a pharmacokinetic explanation, with a shorter dosing interval leading to higher blood/brain levels of PMA. As such, it does indeed appear that PMA has potential neurodegenerative properties. Such findings are important for harm minimisation education programs, but unfortunately this is rarely helpful due to PMA rarely being sold as a drug in its own right. Due to the unknown amount of PMA usage amongst 'ecstasy' users, no clinical evidence yet exists to concerning potential psychological/psychiatric issues.

The investigation of the residual effects of MDMA treatment on *in vivo* 5-HT clearance raises many questions that warrant further investigation. Considering the measurement of *in vitro* biomarkers for 5-HT fibres was consistent with the extensive literature on the long term effect of MDMA on serotonin neurotransmission, this finding was most unexpected. To further validate this finding, it needs to be determined if *in vivo* 5-HT clearance is unaltered at time points beyond the two week post-drug time point chosen for this study. Ideally, these findings should also be replicated using a dosing regimen with greater ethological validity. Such regimens use lower doses (2.5-5 mg/kg ip.) at much shorter intervals (1-2 hrs, for 2-4 doses), yet still produce significant reductions in the *in vitro* serotonergic markers used in this study (5-HT content, SERT concentration, 5-HT synaptosomal uptake) (McGregor et al. 2003). Of great interest is a novel transporter, the organic cation transporter 3 (OCT3) that upregulates in heterozygote SERT knockout mice to maintain 5-HT clearance *in vivo*. Evidence indicates that injection of antisense OCT3 accentuated methamphetamine-induced locomotor activity and extracellular dopamine concentrations in nucleus accumbens, indicating OCT3 is also capable of transporting dopamine (Nakayama et al. 2007). It would be of great interest to determine if OCT3 inhibitors, such as D22 and corticosterone, could selectively inhibit the apparently normal 5-HT clearance *vivo* seen in rats after prior doses of MDMA that produce an apparent 'neurodegeneration'.

Conclusion

The clinical implications of the data generated within this dissertation are twofold. With continuing growth in 'ecstasy' use worldwide, reducing acute toxicity after drug use, and minimising potential psychological/psychiatric pathology after long term use are going to be needed to reduce the increasing burden on the health care professions. Firstly, the characterisation of the acute neurochemistry of PMA and MDMA has shown significant fundamental mechanistic differences, yet has not revealed the 'smoking gun', the fundamental difference in mode of action that results in the significantly higher risk of acute toxicity seen clinically for PMA. This indicates that either subtle differences in pharmacological mechanisms are in fact paramount, or more likely, the additive effects of subtle differences have major clinical effects. Current studies following this question are investigating the role of monoamine oxidase in PMA and MDMA pharmacology. Further knowledge of the acute pharmacology of these drugs is driving experimental targeted pharmacotherapeutic treatments to reverse 'ecstasy'-induced acute hyperthermia, such as the use of the atypical antipsychotic, clozapine (Blessing 2003).

Secondly, while great progress has been made in understanding the acute neurochemical mechanisms of action that underlie the key pharmacodynamic effects of substituted amphetamines over the last 25 years, the question of whether 'ecstasy' induces long term neurodegeneration is still as elusive as ever. Significant progress has also been made in the use of novel biomarkers for long term anatomical changes in the brains of regular 'ecstasy' users, and in particular, tracking recovery in abstinent users. This has the potential to lead to novel targets for nuclear medicine to image and monitor disease states. The work in this dissertation has extended beyond previous animal studies, to investigate whether fundamental changes in *key functional processes* of substituted amphetamines are altered by prior MDMA/PMA exposure. While such studies are only possible in animal models, human studies have extended to investigate whether serotonergic function is altered with

prior 'ecstasy' use, and whether abstinence leads to recover in function over time. Such data are suggested to be as a result of the alteration in these fundamental neurochemical mechanisms. The data presented in this dissertation indicate, under the controlled parameters of the study, residual MDMA-induced reductions in serotonergic biomarkers did not result in reductions in serotonin transporter *function*. These findings need to be validated under a wider range of experimental conditions. Determination of the mechanism of this functional compensation may lead to more targeted treatments to modulate potential psychological/psychiatric deficits that occur in regular 'ecstasy' users.

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