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Caffeine exacerbates the toxicity of 3,4 methylenedioxymethamphetamine (MDMA, “Ecstasy”): A role for dopamine.

Thesis submitted for the degree of Doctor of Philosophy at the University of Dublin, Trinity College

by

Natacha Vanattou-Saïfoudine

Thesis submitted April 2010

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DECLARATION

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Natacha Vanattou-Saifoudine
To my Dearest parents, Mr and Mme Saïfoudine,  
and my sisters Nadia and Nalina.

À mes très chers parents et mes très chères sœurs.
ABSTRACT

Co-administration of caffeine profoundly enhances the acute toxicity of the substituted amphetamine 3, 4-methylenedioxymethamphetamine (MDMA “Ecstasy”) in rats, evidenced by high body temperature, tachycardia and increased mortality. This project set out to determine the mechanisms underlying the ability of caffeine to exacerbate the toxicity of MDMA.

A mechanism comprising 5-HT and catecholamines was found to mediate MDMA-induced hyperthermia and combination of adenosine $A_{2A}$ receptor antagonism and phosphodiesterase inhibition was observed to account for caffeine’s ability to exacerbate MDMA-induced hyperthermia. In particular as central catecholamines and dopamine $D_1$ receptor blockade was found to attenuate the ability of caffeine to exacerbate MDMA-induced hyperthermia, the role of dopamine $D_1$ and $D_2$ receptors in mediating caffeine’s effects on MDMA-induced changes in body temperature, heart rate and locomotor activity were further assessed. MDMA induced hypothermia, locomotor hyperactivity and reduced heart rate in individually housed rats and this was assessed by the use of radiotelemetric technique. While caffeine alone did not influence these parameters, co-administration with MDMA provoked a switch from MDMA-induced hypothermia and bradycardia to hyperthermia and tachycardia without influencing MDMA-induced hyperlocomotion. Pre-treatment with dopamine $D_1$ and $D_2$ receptor antagonists revealed a differential role for dopamine $D_1$ and $D_2$ receptors in shaping the behavioural and physiological responses to MDMA and suggested that the ability of caffeine to provoke MDMA-induced toxicity is associated with a switch from dopamine $D_2$ to $D_1$ receptor related responses.

As MDMA provokes the release of dopamine in the brain and caffeine has also been reported to influence central dopamine release, the ability of caffeine to influence MDMA-induced dopamine release in superfused brain tissue slices was also determined. Caffeine enhanced MDMA-induced $[^3H]$dopamine release from superfused striatal tissue slices, provoking a greater response than that obtained following either caffeine or MDMA applications alone. The synergistic effects observed in the striatum were in contrast to those obtained in hypothalamic slices where caffeine attenuated MDMA-induced $[^3H]$dopamine release. DPCPX, a selective adenosine $A_1$ receptor antagonist, enhanced and attenuated MDMA-induced dopamine release from both striatal and
hypothalamic slices respectively. These results suggest that caffeine differentially regulates MDMA-induced dopamine release in striatal and hypothalamic tissue slices, via inhibition of adenosine A1 receptors.

Finally, due to the earlier demonstrated role for dopamine D1 receptor in mediating the ability of caffeine to exacerbate the toxicity of MDMA and as dopamine D1 receptors are coupled to cyclic AMP production, it was proposed that the interaction observed between caffeine and MDMA converges on this intracellular signalling pathway. Consequently, intracellular markers of dopamine D1 receptor activation were assessed in both the striatum and hypothalamus, regions associated with the acute behavioural and physiological response observed following co-administration of caffeine with MDMA. In tandem with behavioural and temperature measures, phosphorylation of the dopamine D1 related intracellular markers cAMP response element binding protein (CREB), cAMP regulated phosphoprotein of 32 kDa (DARPP-32) and expression of the immediate early gene c-fos were determined in the striatum and hypothalamus. Co-administration with caffeine potentiated MDMA-induced 5-HT syndrome, locomotor and stereotyped behaviours and caffeine and MDMA-induced hyperthermia is in conjunction with increased p-CREB, p-DARPP-32 and c-fos expression in the hypothalamus and increased p-CREB and c-fos expression in the striatum, when compared to either treatment alone. Pre-treatment with SCH-2339 blocked MDMA-induced hyperthermia and its exacerbation by caffeine and attenuated the changes in p-CREB, p-DARPP-32 and c-fos expression in the hypothalamus and striatum. These results support a dopamine D1 receptor related mechanism convergent on intracellular cAMP signalling pathways mediating the acute toxicity associated with the combined treatment of caffeine and MDMA.

In conclusion the experiments have elucidated a dopamine D1 related mechanism underlying a potentially serious drug interaction between caffeine and MDMA and suggest that similar risks may be associated with the concurrent consumption of caffeine and other drugs which influence dopaminergic transmission in the central nervous system.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor Dr. Andrew Harkin for all his help and guidance over the past few years. Thanks for your patience, especially when correcting my manuscripts, and also for bearing with my numerous questions. Your availability and prompt answers help me a lot in the completion of my PhD.

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A big and sincere thanks to Peter who morally and affectionately helped me a lot in the accomplishment of this PhD thesis. Thanks to you, for all your advice and for being there when I needed you the most.

Finally, I’d like to thank my family and friends (especially Manu), in Trinity and back home (in France), for supporting and listening to me for these last 3 memorable years through all the highs and lows.

Buíochas le gach duine!!!

Merci à tous!!!!
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declaration</td>
<td>i</td>
</tr>
<tr>
<td>Dedication</td>
<td>ii</td>
</tr>
<tr>
<td>Abstract</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>v</td>
</tr>
<tr>
<td>Table of contents</td>
<td>vi</td>
</tr>
<tr>
<td>List of figures</td>
<td>xiii</td>
</tr>
<tr>
<td>List of tables</td>
<td>xix</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xx</td>
</tr>
<tr>
<td><strong>Chapter 1</strong> <strong>Introduction</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1 3, 4 Methyleneoxymethamphetamine (MDMA, “Ecstasy”)</td>
<td>2</td>
</tr>
<tr>
<td>1.1.1 Acute pharmacological effects of MDMA</td>
<td>7</td>
</tr>
<tr>
<td>1.1.2 Pharmacokinetics of MDMA</td>
<td>8</td>
</tr>
<tr>
<td>1.1.3 Acute physiological and toxicological effects of MDMA</td>
<td>10</td>
</tr>
<tr>
<td>1.1.4 Role of 5-HT in MDMA-induced hyperthermia</td>
<td>14</td>
</tr>
<tr>
<td>1.1.5 Role of dopamine in MDMA-induced hyperthermia</td>
<td>15</td>
</tr>
<tr>
<td>1.1.6 Long term neurotoxicity associated with MDMA</td>
<td>16</td>
</tr>
<tr>
<td>1.1.7 MDMA administration to rats: a useful model to predict toxicity in humans</td>
<td>18</td>
</tr>
<tr>
<td>1.2 Consumption of caffeine</td>
<td>20</td>
</tr>
<tr>
<td>1.2.1 Pharmacology of caffeine</td>
<td>20</td>
</tr>
<tr>
<td>1.2.2 Adenosine-Dopamine interactions mediate the psychotropic effects of caffeine</td>
<td>22</td>
</tr>
<tr>
<td>1.2.3 Interaction between caffeine and MDMA</td>
<td>26</td>
</tr>
<tr>
<td>1.3 Aims and objectives</td>
<td>29</td>
</tr>
</tbody>
</table>
Chapter 2  Materials and Methods

2.1 Materials

  2.1.1 Animals
  2.1.2 Experimental treatments
  2.1.3 HPLC: Reagents and plastics
  2.1.4 ELISA: Kits and plastics
  2.1.5 Western (immuno)blotting: Reagents and antibodies
  2.1.6 Molecular Reagents
  2.1.7 Tissue slice superfusion
  2.1.8 General Laboratory Chemicals
  2.1.9 General Laboratory Plastics

2.2 Methods

  2.2.1 Animal husbandry
  2.2.2 Physiological assessments
    2.2.2.1 Surgical implantation of radiotelemetric transmitters
    2.2.2.2 Heart rate monitoring
    2.2.2.3 Body temperature monitoring
    2.2.2.4 Locomotor activity monitoring
  2.2.3 Behavioural assessments
  2.2.4 Drug administration
  2.2.5 Serotonin (5-HT) and catecholamine depletions
  2.2.6 Brain dissection
  2.2.7 BCA protein assay
  2.2.8 Sandwich ELISA for analysis of cAMP concentrations in the rat hippocampus and striatum
  2.2.9 Neurotransmitter analysis by HPLC
    2.2.9.1 Preparation of HPLC mobile phase and standards
    2.2.9.2 Tissue preparation for HPLC
    2.2.9.3 HPLC analysis of rat brain biogenic amines
  2.2.10 Western (immuno)blotting for CREB and DARPP-32 proteins
    2.2.10.1 Tissue preparation for Western (immuno)blotting
2.2.10.2 SDS-PAGE
2.2.10.3 Western (immuno)blotting

2.2.11 Preparation of samples for real-time polymerase chain reaction (PCR)
  2.2.11.1 RNA extraction procedure
  2.2.11.2 Assessment of RNA quality
  2.2.11.3 RNA quantification
  2.2.11.4 Reverse Transcription of RNA

2.2.12 Real-time PCR
  2.2.12.1 Plate set-up for single target (singleplex) QPCR
  2.2.12.2 Real-time PCR analysis

2.2.13 Tissue slice superfusion
  2.2.13.1 Drug preparation
  2.2.13.2 Superfusion setup
  2.2.13.3 $[^3]H$-DA release from striatal and hypothalamic tissue slices

2.3 Statistical analysis of the results

Chapter 3 Mechanisms mediating the ability of caffeine to influence MDMA (Ecstasy)-induced hyperthermia in rats

3.1 Introduction
3.2 Experimental design
3.3 Results

Study 1: Can central 5-HT or catecholamine depletion influence the ability of caffeine to exacerbate MDMA-induced hyperthermia?
  (a) Effect of caffeine on MDMA-induced hyperthermia.
  (b) PCPA-induced 5-HT depletion in the frontal cortex and in the hypothalamus.
  (c) 5-HT depletion does not influence MDMA-induced hyperthermia or its exacerbation by caffeine.
  (d) α-methyl-p tyrosine and reserpine induced catecholamine depletion.
(e) Catecholamine depletion blocks MDMA-induced hyperthermia and its exacerbation by caffeine. 68

Study 2: Can caffeine influence the metabolism of MDMA? 70

Study 3: Can caffeine influence the thermoregulatory response to D-fenfluramine and D-amphetamine alone or in combination?

(a) Co-administration of caffeine does not alter D-fenfluramine-induced hypothermia. 72

(b) Co-administration of caffeine does not alter D-amphetamine-induced hyperthermia. 74

(c) Co-administration of caffeine with D-amphetamine and D-fenfluramine is a lethal combination. 75

(d) Co-administration of caffeine exacerbates D-amphetamine + D-fenfluramine-induced hyperthermia. 76

Study 4: Can caffeine influence the thermoregulatory response to DOI and apomorphine alone or in combination?

(a) Co-administration of caffeine attenuates DOI-induced hypothermia. 78

(b) Co-administration of caffeine fails to alter apomorphine-induced hypothermia. 80

(c) Caffeine provokes hyperthermia following treatment with a combination of apomorphine and DOI. 81

Study 5: Effect of antagonist receptors pre-treatment on the ability of caffeine to exacerbate MDMA-induced hyperthermia.

(a) Prior administration of SCH 23390 blocks MDMA-induced hyperthermia and its exacerbation following the co-administration of caffeine. 84

(b) Prior administration of ketanserin and prazosin, but not ritanserin, blocks MDMA-induced hyperthermia and its exacerbation following the co-administration of caffeine. 86

Study 6: Can the adenosine receptor antagonists CGS 15943, DPCPX or SCH 58261 exacerbate MDMA-induced hyperthermia? 91
Study 7: Can the phosphodiesterase (PDE) inhibitors pentoxyfylline, rolipram or zaprinast exacerbate MDMA-induced hyperthermia? 96
Study 8: Can co-treatment with a combination of CGS 15943, DPCPX or SCH 58261 and rolipram influence MDMA-induced hyperthermia? 101

3.4 Discussion 105

Chapter 4 Caffeine promotes a switch from dopamine D\textsubscript{2} to D\textsubscript{1} receptor -mediated heart rate, body temperature and behavioural responses to MDMA ("Ecstasy") administration to rats. 112

4.1 Introduction 113
4.2 Experimental design 117
4.3 Results 120
Study 1: Role of dopaminergic receptors (D\textsubscript{1} and D\textsubscript{2}) and serotonergic 5HT\textsubscript{2} receptors on MDMA and caffeine -induced body temperature changes. 121
Study 2: Effect of MDMA and caffeine on heart rate in rats, following pre-treatment with SCH 23390, sulpiride and ketanserin. Does caffeine influence DOI/apomorphine -induced heart rate changes? 129
Study 3: Role of dopaminergic receptors (D\textsubscript{1} and D\textsubscript{2}) and serotonergic receptors (5HT\textsubscript{2}) on MDMA and caffeine -induced locomotor changes. 136

4.4 Discussion 143

Chapter 5 Brain region specific modulatory actions of caffeine on MDMA "Ecstasy" -induced dopamine release: A role for adenosine A\textsubscript{1} receptor blockade. 151

5.1 Introduction 152
5.2 Experimental design

5.3 Results

Study 1: MDMA provokes [$^3$H] dopamine release and caffeine potentiates MDMA-induced [$^3$H] dopamine release from striatal tissue slices.

(a) Caffeine provokes [$^3$H] dopamine release from striatal tissue slices. 155
(b) MDMA provokes [$^3$H] dopamine release from striatal tissue slices. 157
(c) Caffeine (100 μM) potentiates MDMA (30 μM)-induced [$^3$H] dopamine release.

Study 2: MDMA provokes [$^3$H] dopamine release and caffeine attenuates MDMA-induced [$^3$H] dopamine release from hypothalamic tissue slices.

(a) Caffeine provokes [$^3$H] dopamine release from hypothalamic tissue slices. 161
(b) MDMA provokes [$^3$H] dopamine release from hypothalamic tissue slices. 163
(c) Caffeine (100 μM) attenuates MDMA-induced [$^3$H] dopamine release from hypothalamic tissue slices. 165

Study 3: DPCPX simulates the effect of caffeine on MDMA-induced release of [$^3$H] dopamine from striatal and hypothalamic tissue slices. 167

5.4 Discussion

Chapter 6 Dopamine mediated intracellular changes associated with the ability of caffeine to influence MDMA (“Ecstasy”)-induced toxicity in rats. 175

6.1 Introduction 176
6.2 Experimental design 180
6.3 Results 182

Study 1: Caffeine enhances MDMA-induced hyperthermia and behaviour.

(a) Body temperature
(b) Locomotion
Study 2: Caffeine potentiates MDMA-induced phosphorylation of CREB but not DARPP-32 in the striatum.

Study 3 Combined treatments with caffeine and MDMA increases phosphorylation of CREB and DARPP-32 in the hypothalamus.

Study 4: Caffeine potentiates MDMA-induced c-fos expression in striatum but not in the hypothalamus.

Study 5: Pre-treatment with SCH 23390 blocks proteins phosphorylation induced by caffeine and MDMA in the striatum and hypothalamus.

Study 6: Pre-treatment with SCH 23390 blocks the intracellular effects induced by caffeine and MDMA, and associated with c-fos, in the striatum and hypothalamus.

6.4 Discussion

Chapter 7 General discussion

7.1 Introduction
7.2 Mechanisms of the interaction
7.3 Limit of the study
7.4 Future Studies
7.5 Summary of the results and conclusion

Appendix
References
Publications and Presentations
# LIST OF FIGURES

## Chapter 1

| Figure 1.1 | The chemical structures of amphetamine and some of its derivatives including MDMA. | 3 |
| Figure 1.2 | Different routes for MDMA synthesis. | 4 |
| Figure 1.3 | Different types of ecstasy tablets. | 5 |
| Figure 1.4 | MDMA metabolism and major metabolites. | 10 |
| Figure 1.5 | Caffeine and its metabolites. | 21 |
| Figure 1.6 | cAMP pathway and relationship with DARPP-32 and CREB proteins. | 24 |
| Figure 1.7 | Adenosine receptors regulating striatal dopamine release. | 25 |

## Chapter 2

| Figure 2.1 | Radiotelemetry experimental design. | 39 |
| Figure 2.2 | Transfer proteins from gel to PVDF membrane ("Sandwich"). | 47 |
| Figure 2.3 | Tissue slice superfusion experimental design. | 55 |

## Chapter 3

| Figure 3.1 | Caffeine potentiates MDMA-induced hyperthermia. | 63 |
| Figure 3.2 | 5-HT depletion does not influence MDMA-induced hyperthermia or its exacerbation by caffeine. | 66 |
| Figure 3.3 | Catecholamine depletion blocked MDMA-induced hyperthermia. | 69 |
Influence of caffeine on the metabolism of MDMA. 71

Caffeine fails to influence D-fenfluramine-induced hypothermia. 73

Caffeine fails to influence D-amphetamine-induced hyperthermia. 74

Caffeine provokes hyperthermia following treatment with a combination of D-fenfluramine and D-amphetamine. 77

Caffeine does influence DOI-induced hypothermia. 79

Caffeine influences apomorphine-induced hypothermia. 81

Caffeine provokes hyperthermia following treatment with a combination of DOI and apomorphine. 83

Influence of pre-treatment of dopamine D₁ antagonist SCH 23390 on MDMA-induced hyperthermia and its exacerbation by caffeine. 85

Prior administration of the 5HT₂A receptor antagonist ketanserin blocks the interaction between caffeine and MDMA. 87

Prior administration of the 5HT₂A receptor antagonist ritanserin did not block the interaction between caffeine and MDMA. 88

Prior administration of the α₁ adrenoceptor antagonist prazosin blocks the interaction between caffeine and MDMA. 90

Co-administration of CGS-15943 fails to alter MDMA-induced hyperthermia. 92

Co-administration of DPCPX fails to alter MDMA-induced hyperthermia. 94

Co-administration of SCH 58261 fails to alter MDMA-induced hyperthermia. 95
Co-administration of pentoxyfylline fails to influence MDMA-induced hyperthermia. 97

Co-administration of rolipram fails to influence MDMA-induced hyperthermia. 99

Co-administration of zaprinast fails to influence MDMA-induced hyperthermia. 100

Rolipram in combination with CGS 15943 exacerbates MDMA-induced hyperthermia. 102

Rolipram in combination with DPCPX does not exacerbate MDMA-induced hyperthermia. 103

Rolipram in combination with SCH 58261 exacerbates MDMA-induced hyperthermia. 104

Hyperthermic response to MDMA after co-administration of caffeine. 122

Prior treatment with SCH 23390 attenuates the ability of caffeine to promote hyperthermia following co-administration with MDMA. 123

Prior treatment with sulpiride promotes hyperthermia in response to MDMA administration alone or in combination with caffeine. 124

Effect of apomorphine, alone or in combination with caffeine, on body temperature. 125

Effect of DOI, alone or in combination with caffeine, on body temperature. 126

Effect of apomorphine and DOI, alone or in combination with caffeine, on body temperature. 127
Figure 4.1.7  Prior administration of ketanserin blocks the switch from MDMA-induced hypo- to hyperthermia following co-administration with caffeine. 128

Figure 4.2.1  Tachycardic response to MDMA following co-administration of caffeine. 129

Figure 4.2.2  Prior treatment with SCH 23390 attenuates the ability of caffeine to promote tachycardia following co-administration with MDMA. 130

Figure 4.2.3  Prior treatment with sulpiride promotes tachycardia in response to MDMA administration alone or in combination with caffeine. 131

Figure 4.2.4  Effect of apomorphine, alone or in combination with caffeine on heart rate. 132

Figure 4.2.5  Effect of DOI, alone or in combination with caffeine on heart rate. 133

Figure 4.2.6  Effect of apomorphine and DOI, alone or in combination with caffeine on heart rate. 134

Figure 4.2.7  Prior administration of ketanserin blocks MDMA-induced reduction in heart rate following co-administration with caffeine. 135

Figure 4.3.1  Locomotor response to MDMA following co-administration of caffeine. 136

Figure 4.3.2  Effect of MDMA and caffeine on locomotor activity in rats following pre-treatment with SCH 23390. 137

Figure 4.3.3  Effect of MDMA and caffeine on locomotor activity in rats following pre-treatment with sulpiride. 138

Figure 4.3.4  Effect of apomorphine, alone or in combination with caffeine, on locomotor activity. 139

xvi
Figure 4.3.5  Effect of DOI, alone or in combination with caffeine locomotor activity. 140

Figure 4.3.6  Effect of apomorphine and DOI, alone or in combination with caffeine, on locomotor activity. 141

Figure 4.3.7  Effect of MDMA and caffeine on locomotor activity in rats following pre-treatment with ketanserin. 142

Chapter 5

Figure 5.1.1  Caffeine (100 μM) induces [³H] dopamine release from striatal slices. 156

Figure 5.1.2  MDMA (300 μM) induces [³H] dopamine release from striatal slices. 158

Figure 5.1.3  Caffeine (100 μM) potentiates MDMA (30 μM)-induced [³H] dopamine release from striatal slices. 160

Figure 5.2.1  Caffeine (100 μM) induces [³H] dopamine release from hypothalamic. 162

Figure 5.2.2  MDMA (100 and 300 μM) induces [³H] dopamine release from hypothalamic slices. 164

Figure 5.2.3  Caffeine (100 μM) attenuates MDMA (100 μM)-induced [³H] dopamine release from hypothalamic slices. 166

Figure 5.3.1  DPCPX (1 μM) potentiates MDMA (30 μM)-induced [³H] dopamine release from striatal tissue slices. There was no difference in [³H] dopamine outflow. 168

Figure 5.3.2  DPCPX (1 μM) attenuates MDMA (100 μM)-induced [³H] dopamine release: from hypothalamic tissue slices. 170
Chapter 6

Figure 6.1 SCH 23390 blocks the ability of caffeine to exacerbate MDMA-induced hyperthermia.

Figure 6.2.1 Caffeine potentiates MDMA-induced hyperlocomotion.

Figure 6.2.2 Caffeine potentiates MDMA-induced stereotypy.

Figure 6.2.3 Caffeine potentiates MDMA-induced 5-HT syndrome.

Figure 6.3 Caffeine potentiates the MDMA related increase in striatal p-CREB but not p-DARPP-32.

Figure 6.4 Co-administration of caffeine and MDMA provokes an increase in hypothalamic p-CREB and p-DARPP-32.

Figure 6.5 MDMA-induced c-fos expression is potentiated by the co-administration of caffeine in the striatum but not the hypothalamus.

Figure 6.6 SCH 23390 pretreatment reduces body temperature in MDMA-treated group and blocks caffeine potentiation.

Figure 6.7 SCH 23390 blocks MDMA induced striatal p-CREB alone or in combination with caffeine.

Figure 6.8 SCH 23390 blocks the increase in hypothalamic p-CREB and p-DARPP-32 associated with the co-administration of caffeine with MDMA.

Figure 6.9 SCH 23390 blocks the increase in striatal and hypothalamic c-fos expression associated with the co-administration of caffeine with MDMA.

Chapter 7

Figure 7.1 Schematic representation of the MDMA and caffeine interaction
# LIST OF TABLES

## Chapter 1

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Summary Statistic of Lab Testing results of Ecstasy Tablets composition.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

## Chapter 2

<table>
<thead>
<tr>
<th>Table 2.1</th>
<th>Behavioural scoring table</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Table 2.2</td>
<td>List of drugs administered</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Table 2.3</td>
<td>Retention times and peak heights</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Table 2.4</td>
<td>Antibodies used for Western (immuno)blotting</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Table 2.5</td>
<td>Example of the ΔΔCT method of analysis</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

## Chapter 3

<table>
<thead>
<tr>
<th>Table 3.1</th>
<th>Summary of drug challenges undertaken to determine the mechanisms mediating the ability of caffeine to influence MDMA-induced hyperthermia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Table 3.2</td>
<td>HPLC verification of the 5-HT-depleting regime using PCPA</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Table 3.3</td>
<td>HPLC verification of the catecholamine depleting regime using AMPT and reserpine</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Table 3.4</td>
<td>Caffeine induces lethality when administered with D-amphetamine and D-fenfluramine.</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
ABBREVIATIONS

°C  degrees centigrade
\(\alpha\)-MPT/AMPT  alpha-methyl-para-tyrosine
4-MTA  4-methylthioamphetamine
5-HIAA  5-hydroxyindoleacetic acid
5-HT  Serotonin/5-hydroxytryptamine
5-HT_2  type-2 serotonin receptor
5-HTP  5-hydroxytryptophan
8-OH-DPAT  8-hydroxy-2-(Di-n-propylamino) tertralin
A_1  adenosine A_1 receptor
A_{2A}  adenosine A_{2A} receptor
Ab  antibody
ANOVA  analysis of variance
APS  ammonium persulfate
ATP  adenosine 5'-triphosphate
BCA  bicinchoninic acid
BDNF  brain derived neurotrophic factor
BSA  bovine serum albumin
BZP  benzodiazepine
Ca^{2+}  calcium
CaCl_2  calcium chloride
cAMP  cyclic adenosine monophosphate
cDNA  complementary DNA
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<td>CREB</td>
<td>cAMP response element binding protein</td>
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<td>Egr</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbant assay</td>
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<td>ERK</td>
<td>extracellular regulated kinase (MAPK)</td>
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xxi
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<td>high performance liquid chromatography</td>
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<td>HRP</td>
<td>horse radish peroxidise</td>
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<td>homovanillic acid</td>
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<td>immediate early genes</td>
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<td>immunoglobulin G</td>
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<td>intraperitoneal</td>
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<td>kDa</td>
<td>kilo Dalton</td>
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<td>μm</td>
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<td>mA</td>
<td>milliamp</td>
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xxii
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<tr>
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<td>monoamine oxidase</td>
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<td>MAOI</td>
<td>monoamine oxidase inhibitors</td>
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<td>MAPK</td>
<td>mitogen activated protein kinase (ERK)</td>
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<td>MDA</td>
<td>methylenedioxyamphetamine</td>
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<td>MDE</td>
<td>3, 4-methylendioxyethylamphetamine</td>
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<td>MDMA</td>
<td>3, 4-methylendioxymethamphetamine</td>
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<td>MDOH</td>
<td>N-hydroxy-3,4-methylenedioxyamphetamine</td>
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<td>MDP2P</td>
<td>3, 4-methylenedioxyphenyl-2 propanol</td>
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<td>MAPK/ERK kinase</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>NA</td>
<td>noradrenaline</td>
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<td>Na$_2$HPO$_4$</td>
<td>di-sodium hydrogen orthophosphate</td>
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<td>NaOH</td>
<td>sodium hydroxide</td>
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<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
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NSB non-specific binding
OD Optical density
L-DOPA 1-3-4-Dihydroxyphenylamine
μl microlitre
μM micromolar
NA noradrenaline
NaH₂PO₄ sodium dihydrogen phosphate
PAGE polyacrylamide gel electrophoresis
PBS phosphate-buffered saline
PBST phosphate-buffered saline tween
PCA para-chlorophenylalanine
PCP phencyclidine
PCPA parachlorophenylalanine
PCR polymerase chain reaction pcr
p-CREB phosphorylated cAMP response element binding protein
p-DARPP-32 phosphorylated dopamine- and cAMP- regulated phosphoprotein 32 kD
PDE phosphodiesterase
PKA cAMP-dependent protein kinase A
PMA paramethoxyamphetamine
PMSF phenylmethyl sulfonyl fluoride
PTX pentoxifylline
QPCR quantitative PCR
RNA  
ribonucleic acid

rpm  
revolution per minute

RT  
room temperature

s.c.  
subcutaneous

SB  
specific binding

SCH 23390  
7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol

SCH-58251  
5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo [1,5-c] pyrimidine

SDS  
sodium dodecyl sulphate

SDS-page  
sodium dodecylsulphate-polyacrylamine gel electrophoresis

SEM  
standard error of the mean

SERT  
5-HT reuptake transport

SSRI  
selective serotonin reuptake inhibitor

TB  
total binding

TBS  
tris-buffered saline

TBS-T  
tris-buffered saline-Tween

TFMPP  
3-trifluromethylphenylpiperazine

Tris-HCl  
trizma-hydrochloride

TTX  
tetrodotoxin

VMAT  
vesicular monoamine transporter

v/v  
volume per volume

v/w  
volume per weight

xxv
CHAPTER 1

INTRODUCTION
1.1 3, 4 Methyleneoxymethamphetamine (MDMA, “Ecstasy”)

MDMA (3, 4-methylenedioxymethamphetamine), is a ring-substituted amphetamine, structurally similar to methamphetamine and mescaline (Figure 1.1). MDMA, first made by Merck Pharmaceuticals, was patented in 1914 as a precursor for therapeutically active compounds and was never used as an appetite suppressant as it has often been said (Cohen, 1998). Its toxicology was first analysed by the US army as part as chemical warfare program (Hardman et al., 1973) while MDMA started to be used in psychotherapy only from the 1980’s as it was said to increase self-esteem and facilitated therapeutic communication. For this purpose, it was administered orally at a dose of 75 to 175 mg inducing some notable sympathomimetic effects such as increase of heart rate and blood pressure and transient anxiety (Greer et al., 1985; Grinspoon et al., 1986). Shortly after, this synthetic psychedelic drug of the phenethylamine family was classified as a Schedule 1 drug in the United States of America and as a Class A drug in the United Kingdom, due to its toxicity, abuse potential and lack of clinical application and especially due to the fact that its direct related compound 3,4-methylenedioxyamphetamine (MDA) has been shown to induce long term 5-HT loss in human (Ricaurte et al., 1985). Therefore, class A drug MDMA was classified as a potential threatening drug for recreational users in whom MDMA induces a feeling of closeness to others, empathy and facilitates interpersonal relationships. Illegal in most countries, its possession, manufacture or sale may result in criminal prosecution. Worldwide, almost all MDMA is supplied by illegal clandestine laboratories. The synthesis of MDMA is more complex than that of other amphetamine analogues such as methamphetamine. But, the most difficult part of the synthesis is obtaining the necessary chemical precursors.
Safrole extracted from the sassafras plants is the primary precursor for manufacture of MDMA. There are numerous synthetic methods available in the literature to convert Safrole into MDMA (Figure 1.2). One common route is via the MDP2P (3, 4-methylenedioxyphenyl-2-propanone, also known as piperonyl acetone) intermediate. This intermediate can be produced in at least two different ways. One method is to isomerise Safrole to isosafrole and then oxidize isosafrole to MDP2P. Another, reportedly better method, is to make use of the Wacker process to oxidize safrole directly to MDP2P. Once the MDP2P intermediate has been produced it is then consumed via a reductive amination to form MDMA as the product (Renton et al., 1993).
Figure 1.2 Different routes for MDMA synthesis (from Renton et al., 1993).

PMK = 3, 4-Methylenedioxyphenyl-2-propanone; MDMA = 3, 4-Methylenedioxymethamphetamine; MDA = 3, 4-Methylenedioxyamphetamine; MDPBP = 1-(3, 4-methylenedioxyphenyl)-2-bromopropane.

“Ecstasy”, the street name for MDMA, commonly appears in tablet form (Figure 1.3), usually imprinted with a monogram. It can also be found in pressed pills, or in capsules containing powder or crystal forms. The typical recreational dose of 100-150 mg creates a “high”, which takes effect within 30-60 minutes and lasts 4-6 hours. Because of its illegality in most countries, the typical recreational user is unable to verify the purity of the substance sold as MDMA. In fact, an important percentage of ecstasy pills are prepared with various substances (Table 1). Other active substances commonly found in ecstasy tablets include amphetamine, methamphetamine, caffeine, and inactive filler materials. In some cases, tablets sold as ecstasy do not contain MDMA, chemicals in the
Ecstasy family, or even any kind of stimulant drug. Instead they may contain drugs such as the analgesic acetaminophen or anesthetic, ketamine.

Figure 1.3 Different types of ecstasy tablets (from Erowid chemicals, http://www.erowid.org/chemicals/show_image.php?i=mdma/ecstasy_pill_collage1.jpg).
### Chapter 1: Introduction: MDMA

Table 1: Summary Statistic of Lab Testing results of Ecstasy Tablets composition (data from EcstasyData.org, expressed as percentage)

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| Totals                     | 100.0| 0    | 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0| 100.0 |

- MDMA Only: MDMA
- MDMA + Something: Results containing MDMA and at least one other non-inert substance / No MDMA: Results containing no MDMA
- No Chemicals Detected: None Detected
- Ecstasy-like Chemicals: MDA, MDE
- Psychedelics: 5-MeO-DiPT, DOB, PMA
- Dissociatives: DXM, Ketamine, PCP
- Stimulants: Amphetamines, BZP, Caffeine, Cocaine, Methamphetamine, Pseudo/Ephedrine, TFMPP/Depressants/Tranquilizers: Amitriptyline, Butabarbital, Carisoprodol, Codeine, Diazepam, Diphenhydramine
- Mostly Inactives: Acetaminophen, Aspirin, Chlorpheniramine, Guaifenesin, Methyl Salicylate, Methandrostenolone, Phenylpropanolamine
Chapter 1: Introduction: MDMA

1.1.1 Acute pharmacological effects of MDMA

The psychotropic properties of MDMA are attributed to its action as an indirect monoaminergic agonist in the brain, binding to all three of the monoamine presynaptic transporters. It acts by activating the release of and inhibiting the reuptake of serotonin (5-HT) primarily, but also dopamine, noradrenaline, acetylcholine and histamine (Berger et al., 1992; Fischer et al., 2000; Green et al., 1995; Rothman et al., 2001) and therefore, elevating synaptic concentrations of these monoamine neurotransmitters.

MDMA primarily inhibits 5-HT re-uptake and stimulates an acute and rapid release of 5-HT. This has been demonstrated by studying neurotransmitter release by the use of in vivo microdialysis (Mechan et al., 2002b; Nixdorf et al., 2001; Shankaran et al., 1999) superfusion techniques using tissue slices (Berger et al., 1992; Crespi et al., 1997; Koch et al., 1997) and synaptosomal preparations (Berger et al., 1992; O'Loinsigh et al., 2001). The levels of 5-HT are further increased by MDMA through the inhibition of enzymes responsible for the metabolism of monoamines and also from the expulsion of 5-HT from intracellular vesicles (Fitzgerald et al., 1993b; Tatsuta et al., 2005). It has been shown that MDMA inhibits tryptophan hydroxylase, the rate-limiting enzyme in the synthesis of 5-HT in a calcium-dependent manner. MDMA also affects the metabolism of 5-HT and dopamine by its inhibition of the catabolic enzyme monoamine oxidase (MAO).

In contrast to 5-HT, more moderate alterations are observed in brain dopamine levels and its metabolites di-hydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) following MDMA administration in rats (Schmidt et al., 1986). Yamamoto and Spanos in 1988 were the first to report that MDMA induced dopamine release using in vivo voltammetry and high performance liquid chromatography (HPLC). Nash, in 1991, reported that MDMA induced a dose dependent increase in the extracellular concentration of striatal dopamine an effect that has been reproduced by other groups. In fact, Colado et al., in 1994 demonstrated that MDMA injected peripherally (20 mg/kg) produced an increase in striatal dopamine and a decrease of the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) while Gough and co-workers (1991) showed, by microdialysis in freely moving rats, that MDMA (10 mg/kg, i.p.) exhibited an amphetamine-like increase in dopamine concentrations in caudate nuclei. MDMA-
induced 5-HT release in the striatum and hippocampus has been shown to be attenuated by pre-treatment with the 5-HT reuptake transport (SERT) inhibitor fluoxetine, implicating SERT in MDMA-induced 5-HT release. On the other hand, the dopamine uptake site is not thought to be involved in MDMA-induced dopamine release. Other possibilities such as MDMA itself entering the dopaminergic terminal by diffusion and affecting the release of dopamine have been proposed. Additionally, 5-HT appears to be involved in MDMA-induced dopamine release as pre-treatment with fluoxetine has been shown to attenuate MDMA-induced striatal dopamine release (Koch et al., 1997). An important point in MDMA-induced dopamine release is its regulation by 5-HT. 5-HT receptors influence dopamine release which influences dopamine-mediated responses to MDMA (Doly et al., 2008; Gudelsky et al., 2008). Stimulation of postsynaptic 5-HT receptors after SERT-dependent release has been shown to be crucial in MDMA-induced dopamine release as Gudelsky et al., in 1996 demonstrated that blocking the access of SERT by the selective serotonin reuptake inhibitor (SSRI) fluoxetine, inhibited MDMA-induced dopamine efflux in the striatum, as well as, the infusion in the striatum of tetrodotoxin (TTX). TTX is a neurotoxin which blocks voltage dependent sodium channels and therefore interferes with axonal conduction. MDMA induced a nonexocytotic transmitter release and therefore, occurs in absence of cell firing. If TTX is able to reduce MDMA-induced dopamine efflux, one conceivable hypothesis is that TTX is interfering with dopamine pathway synthesis. In fact, tyrosine hydroxylase is activated by a Ca^{2+}/calmodulin-dependent phosphorylation sensitive to cell firing and calcium influx.

1.1.2 Pharmacokinetics of MDMA

MDMA reaches maximal concentrations in the blood between 1.5 and 3 hours after ingestion. Its half-life increases with higher doses, and is estimated to be between 6 to 10 hours at doses of 40 to 125 mg. MDMA is a chiral compound and is almost exclusively available as a racemate. Given as the racemate, MDMA has a half-life of around 8 hours. MDMA shows non-linear pharmacokinetics in humans (de la Torre et al., 2000), and it seems that MDMA can inhibit its own metabolism by interacting with cytochrome P-450 2D6 (CYP2D6) (Delaforge et al., 1999). Due to the inhibition of its own metabolism, a
first dose can modify the pharmacokinetic parameters and metabolic profile of the following dose.

Metabolites of MDMA (Figure 1.4) are 3, 4-methylenedioxymphetamine (MDA), 4-hydroxy-3-methoxy-methamphetamine (HMMA), 4-hydroxy-3-methoxyamphetamine (HMA), 3, 4-dihydroxyamphetamine (DHA) (also called alpha-methyladopamine), 3, 4-methylenedioxynphenylacetone (MD2P2), and N-hydroxy-3, 4-methylenedioxymphetamine (MDOH) but MDMA is known to be metabolised via different pathways:

- O-demethylation followed by catechol-O-methyltransferase (COMT)-catalyzed methylation and/or glucuronide/sulfate conjugation
- N-dealkylation, deamination, and oxidation to the corresponding benzoic acid derivatives conjugated with glycine.
- N-demethylation (through which important actives metabolites are produced, i.e. MDA) (Escobedo et al., 2005).

The fact that the polymorphic enzyme CYP2D6 partially regulates the O-demethylation pathway gave rise to the expectation that subjects who display a poor metaboliser phenotype may be at higher risk of acute toxicity episodes. While such a risk factor has not been yet established definitively, the current leading theory is that metabolism of MDMA produces reactive oxygen species, such as hydroxyl radicals (Colado et al., 1997; Shankaran et al., 2001) and subsequent lipid peroxidation (Colado et al., 1997; Sprague et al., 1995) and these are important events in the loss of 5-HT function after MDMA treatment in rats. This metabolism is primarily mediated by the cytochrome P450, enzymes CYP2D6 (in humans, but CYP2D1 in mice), and CYP3A4. 65% of MDMA is excreted unchanged in the urine. Additionally 7% is converted into the active metabolite MDA over 24 hours following consumption.
1.1.3 Acute physiological and toxicological effects of MDMA

Effects of MDMA on humans have been well characterised. Vollenweider and co-workers (2002) showed that a dose of 1.7 mg/kg orally (per os, p.o.) of MDMA on healthy subjects produced an affective state with increased mood, well-being and increase of emotional sensitiveness. Increase of closeness, openness and responsiveness to emotions has been reported in many studies performed in human volunteers (Downing, 1986; Greer et al., 1986; Vollenweider et al., 2002). Visual illusion and thought disturbance such as difficulty to concentrate warranted MDMA and its derivatives to be considered as a new class of psychoactive substance (Nichols, 1986; Vollenweider et al., 2002). The physiological effects of MDMA include an increase in blood pressure, cardiac activity and body temperature and side effects such as jaw clenching, lack of appetite, thirst and loss of weight (Liechti et al., 2000a; Liechti et al.,
Chapter 1: Introduction: MDMA

2000b; Mas et al., 1999; Vollenweider et al., 1998). In the list of acute negative effects induced by MDMA, aggression, impulsion and irrational behaviour have been also associated with use of this drug. Somatic toxic events occurred with the use of MDMA such as thrombotic or haemorrhagic strokes (Rothwell et al., 1993; Shearman et al., 1992), leukoencephalopathy (Bertram et al., 1999), myocardial infarction (Qasim et al., 2001), arrhythmias (Dowling et al., 1987; Henry et al., 1992) and pneumothorax (Mazur et al., 2001). Idiosyncraticity of these symptoms is evident which may be accounted for, at least in part, by the presence of impurities in tablets from the manufacturing process.

In England and Wales, in the years between 2001 and 2005, there was an average of 27 deaths per year attributed to MDMA alone. In the same period, the average deaths due to heroin (including morphine), methadone, and cocaine abuse alone were 575, 99, and 45 respectively. Comparison of the number of Ecstasy pills estimated to be consumed in England and Wales per year and the number of deaths resulting from Ecstasy use, suggests that the risk of death from taking Ecstasy is approximately 1 death per 100,000 users per year (report of the Royal Society of Arts Commission on Illegal Drugs, Communities and Public Policy, March 2007). This is approximately the same risk of death as is associated with adverse drug reactions to oestrogen-containing forms of hormonal contraception (Jick et al., 1983).

The short-term health risks of taking MDMA include hyperthermia, hyponatremia, multi-organ failure, rhabdomyolysis (muscle breakdown), disseminated intravascular coagulation and 5-HT syndrome (muscle rigidity, hyper-reflexia and hyperthermia), which can ultimately lead to death (Cami et al., 2003; Kalant, 2001). Hyperthermia and dehydration are notable in the rave context of dancing for long periods of time, as the drug’s stimulatory effects can mask the body’s normal sense of exhaustion and thirst. The number one cause of death following MDMA use is hyponatremia which is a decrease of blood sodium levels as a result of drinking too much water (Ben-Abraham et al., 2003; Budisavljevic et al., 2003). While it is important to maintain fluid intake, especially when out dancing in a hot environment, too much fluid can also be dangerous. Although many cases involved individuals drinking large amounts of water, there are cases where there is no evidence of excessive water consumption. Death, in such cases, might have been caused by MDMA-induced release of the antidiuretic hormone vasopressin by the pituitary gland (Wolff et al., 2006). The action of vasopressin on the
rinal tubules leads to the retention of water, resulting in a difficulty to urinate. This phenomena is commonly referred to as E-wee phenomena.

An especially vulnerable population to the acute toxicity of MDMA are small children who can die following ingestion of just one single adult dose. People who have obesity problems, or who have diabetes, high blood pressure or heart conditions have a greater risk of overdose related death from any stimulant. However, most fatal Ecstasy overdoses occur with children who find their parent’s pills (Duffy et al., 2006).

The standard treatment for Ecstasy overdose given in hospitals includes a range of drugs such as cyproheptadine (antihistaminic and antiserotonergic agent) or chlorpromazine (anticholinergic, antihistaminic and antidopaminergic effects) (Rusyniak et al., 2005) but these are often of limited efficacy. MDMA overdose mainly results in hyperthermia and hyponatremia, which leads to convulsions which can be treated with benzodiazepines such as diazepam or lorazepam (Duffy et al., 2007). Hyperthermia is the second most important cause of death by MDMA use, resulting from core body temperature rising too high until the major organs shut down at about 42°C (Dar et al., 1996; Mallick et al., 1997). Human recreational users of MDMA can suffer an acute hyperthermic response which, if severe, can result in death (Schifano, 2004). In such cases MDMA provokes a severe reaction akin to malignant hyperthermia, a neurogenetical disease with symptoms observed during and after an anesthetic period. Such a reaction has been observed in drug users who have consumed Ecstasy or Ecstasy analogues such as paramethoxyamphetamine (PMA) (Kraner et al., 2001), 4-methylthioamphetamine (4-MTA) (De Letter et al., 2001), 5-hydroxytryptophan (5HTP), or if additional stimulants are involved, such as methamphetamine or cocaine. Sustained hyperpyrexia leads to rhabdomyolysis, which in turn can cause renal failure and death. Rhabdomyolysis can generally be successfully treated with dantrolene, a skeletal muscle relaxant which acts by binding ryanodine receptors and decreasing intracellular calcium (Krause et al., 2004) if diagnosed early enough, but often the characteristic symptoms may not be apparent until the condition is already severe.

Dance parties are an obvious hyperthermia risk environment, the venue often being hot and crowded, and the attending public dancing whilst on stimulant drugs. The temperature inside the dance rooms should be maintained in the range between 24 and 27°C because Ecstasy affects the body’s ability to regulate temperature and it is easy to
become either too hot or too cold in temperatures outside this range. MDMA appears to decrease heat loss in the body by causing constriction of blood vessels near the skin (Petersen et al., 2001). In addition, it can increase heat production by muscles and the brain (Bexis et al., 2006). These effects may be amplified when people become dehydrated and are therefore unable to cool by sweating. An important point is that MDMA can mask the body's normal thirst and exhaustion responses, particularly if a user is dancing or is otherwise physically active for long periods of time without hydration. Because of these effects, MDMA can temporarily reduce the body's ability to regulate its core temperature so that high-temperature surroundings combined with physical effort may lead to hyperpyrexia if precautions are not taken to remain cool. Although dantrolene is the primary pharmacological line of defense in MDMA-induced hyperthermia, it fails to adequately control the hyperthermic response seen after MDMA ingestion (Dar et al., 1996).

Overdose on MDMA alone is rare but some drug combinations can render it more dangerous: the anti-ulcer anti-histamine cimetidine which blocks a liver enzyme CYP2D6, and the monoamine oxidase inhibitors (MAOIs) which block monoamine oxidase (e.g. moclobemide, tranylcypromine) are notable examples. People who take MDMA whilst also taking these drugs have greatly increased drug effects, but also increased side effects, which can lead to lethal overdose at normal doses. Some people can overdose more easily from MDMA because they have Gilbert syndrome, a genetic condition where the liver's glucuronidation system is impaired, inhibiting the metabolism and excretion of MDMA.

Allergic reactions are extremely uncommon, but a few rare cases have been reported of people dying by anaphylactic shock after taking MDMA and were allergic to MDMA. Liver damage, which may have an immunological cause, has been seen in a small number of users (Nunez et al., 2002). Animal studies suggest a risk of liver damage brought about by high body temperature (Carvalho et al., 2002).

In animals, MDMA provokes a behavioural syndrome referred to as the 5-HT syndrome induced by the release of 5-HT and includes hyperactivity associated with head-weaving, fore-paw treading and piloerection (Colado et al., 1993; De Souza et al., 1997; Marston et al., 1999; McNamara et al., 1995; Shankaran et al., 1999). The main cardiovascular effects induced by MDMA are an increase in heart rate (tachycardia) and
abnormal cardiac electrical activity (arrhythmia) (Gordon et al., 1991b). MDMA is also known to induce core body temperature changes dependent on environmental temperature (Broening et al., 1995; Dafters, 1994; Dafters, 1995; Green et al., 2005; Malberg et al., 1998; Saadat et al., 2005), dose of MDMA administered and strain of rats used (Malberg et al., 1998; Marston et al., 1999). Normally, MDMA induces hyperthermia in rats (+ 1°-2°C) at normal ambient temperature (20 to 22°C) with a maximal response around one hour following drug administration (Colado et al., 1993; Dafters, 1994; Green et al., 2003; Green et al., 2004; Malberg et al., 1996; McNamara et al., 2006; O'Shea et al., 1998).

Gordon and co-workers (1991) proposed that MDMA induces a dysfunction in the thermoregulatory mechanisms of the central nervous system that are influenced by ambient temperature. Several laboratories have reported an increase of body temperature when MDMA is given to rats in an environment maintained at 24°C or greater (Gordon et al., 1991b; Schmidt et al., 1990b). However, if the ambient temperature is lowered to 10°C, a hypothermic response occurs (Gordon et al., 1991b). The set point for either a hyperthermic or hypothermic response seems to be above or below 20-22°C (Gordon et al., 1991b; Malberg et al., 1998).

To determine the mechanisms of thermogenesis induced by MDMA, studies have been performed with hypophysectomised and thyroparathyroidectomised rats treated with MDMA where an interaction between the hypothalamic-pituitary-thyroid axis and the sympathetic nervous system has been reported. In fact, such animals did not become hyperthermic and displayed a significant hypothermia following MDMA administration (Sprague et al., 2003).

1.1.4 Role of 5-HT in MDMA-induced hyperthermia

5-HT has been associated with the regulation of body temperature (Myers, 1981; Salmi and Ahlenius, 1988). Many studies have proposed that an increase of extracellular 5-HT results in hyperthermia (Colado et al., 1993; Grahame-Smith, 1971a; Grahame-Smith, 1971b; Yamawaki et al., 1983). However, observations such as the lack of an effect of fluoxetine on the hyperthermic response induced by MDMA in rats, despite totally
inhibiting the increase in extracellular 5-HT, imply that other neurotransmitters are involved (Meehan et al., 2002b). A more in-depth study of MDMA-induced hyperthermia in rats, reported that administration of methysergide and ritanserin, selective 5-HT$_{2A}$ and 5-HT$_{2C}$ antagonists respectively, fail to block its development (Meehan et al., 2002b). In other studies of the mechanisms mediating the hyperthermic effects of MDMA in animals, a combination of prazosin (alpha-adrenergic receptor blocker) and pindolol (5HT$_{1A}$ antagonist) quickly and completely blocks MDMA-induced hyperthermia (Sprague et al., 2003). Pizotyline (also called Pizotifen), a non selective 5-HT receptor antagonist use to treat migraine, has also been shown to reduce the discriminative stimulus of MDMA (Young et al., 2005).

1.1.5 Role of dopamine in MDMA-induced hyperthermia

As MDMA is a potent releaser of dopamine and noradrenaline and as dopamine plays a role in mediating the hyperthermia associated with methamphetamine (Bronstein and Hong, 1995), dopamine may also be involved in MDMA-induced hyperthermia. Dopamine is involved in thermoregulation, especially in the pre-optic area and anterior hypothalamus and involved in the regulation of body temperature (Hasegawa et al., 2005). Dopamine D$_1$ and D$_2$ receptor subtypes are implicated in MDMA-induced changes in body temperature. MDMA induced hypothermia in rats housed at 15°C can be blocked by pre-treatment with the D$_2$ receptor antagonist remoxipride, but not by the D$_1$ receptor antagonist SCH 23390 (Green et al., 2005). Meehan and co-workers (2002) provided evidence in support of a role for dopamine D$_1$ receptors in MDMA-induced hyperthermia while pre-treatment with the dopamine D$_1$ receptor antagonist SCH 23390 inhibits MDMA-induced hyperthermia in rats. It has been proposed that D$_2$ stimulation predominates in animals housed individually or at low ambient temperatures which is why hypothermia is observed in these animals in response to MDMA.

To support these observations proposing a role for dopamine in MDMA-induced hyperthermia, hyperthermia associated with administration of the 5-HT-releasing drug parachloroamphetamine (PCA) to rats was blocked by SCH 23390 while it was unaffected by fluoxetine or parachlorophenylalanine (PCPA), which is used to deplete 5-HT concentrations in the brain (Sugimoto et al., 2001).
Chapter 1: Introduction: MDMA

1.1.6 Long term neurotoxicity associated with MDMA

MDMA produces reductions in 5-HT axon terminal markers which last for months or years after cessation of drug exposure (Battaglia et al., 1988; Lew et al., 1996; Sabol et al., 1996; Hatzidimitriou et al., 1999; Ricaurte et al., 2000). The particular effects of MDMA are to produce a dose-related reduction of 5-HT concentrations and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), a reduction of the density of 5-HT uptakes sites or transporters which are correlated to 5-HT axon terminal destruction (Huether et al., 1997; Sprague et al., 1998), and a sustained reduction in the activity of tryptophan hydroxylase, the rate limiting enzyme for 5-HT synthesis (Che et al., 1995; Johnson et al., 1992; Schmidt et al., 1988). The long term effect of MDMA is also highly selective for 5-HT neurons. A neurotoxic dose of MDMA (15 mg/kg) to rats decreased cortical and hippocampal 5-HT content by approximately 30% seven days later. Administration of high or repeated doses of MDMA produces a long-term neurotoxic loss of 5-HT in the forebrain (Green et al., 2003).

Preclinical studies have revealed that methamphetamine (METH), MDMA and 3,4-methylenedioxyamphetamine (MDA) produce long-term damage to dopaminergic and 5-HT nerve terminals (Axt et al., 1991; Colado et al., 1995; Fukui et al., 1989; Gibb et al., 1990; Lorez, 1981; Mamounas et al., 1991; O’Hearn et al., 1988; Ricaurte et al., 1982) in multiple brain areas. Specifically, early reports indicated that MDMA-induced depletions have typically been specific to 5-HT terminal markers, while METH administration has been shown to produce long-term toxicity to both dopaminergic and 5-HT systems (Green et al., 1992; Schmidt et al., 1990b). The doses of MDMA required to produce long-term effects on brain 5-HT neurons were lower than those required to produce comparable effects with other amphetamine derivatives such as methamphetamine in rat brains (Ricaurte et al., 1985; Commins et al., 1987; Schmidt et al., 1987).

Increasing evidence suggests that MDMA-induced neurotoxicity involves the production of reactive oxygen and reactive nitrogen species (Stephans et al., 1994) and a subsequent induction of oxidative stress. The excessive extracellular dopamine concentrations resulting from the ability of substituted amphetamines to induce dopamine release may be oxidised enzymatically and non-enzymatically to form highly reactive dopamine
phosphate and reactive oxygen species, leading to an increase in oxidative stress (Michel et al., 1990).

Analysis by immunocytochemistry of 5-HT containing nerve fibres for up to 1 year post-treatment showed that the earliest recovery of 5-HT content was observed in the hypothalamus 8 weeks after drug administration, while hippocampal and striatal levels recovered by 16 weeks. All the other brain regions examined by the authors showed complete recovery of 5-HT within 1 year of drug treatment (Scanzello et al., 1993).

Recently, MDMA effects have been shown to implicate neuroinflammation as MDMA injection in rats was associated with an increase of interleukin 1β production and microglial activation (O'Shea et al., 2005; Orio et al., 2004). Activation of microglial cells by MDMA seems to be independent on hyperthermia and there is so far no direct implication of microglial activation on MDMA-induced long term neurotoxicity (Orio et al., 2004). However, O'Shea et al. (2004) demonstrated that interleukin 1β production was partially due to MDMA-induced hyperthermia and associated with its long term 5-HT neurotoxicity. In fact, i.c.v injection of IL-1β increase long lasting reduction of 5-HT transporters and 5-HT concentration induced by MDMA (O'Shea et al., 2005). Cannabinoid CB2 receptor over-expressed in microglia during non-immune and immune pathological conditions are known to be involved in controlling the production of neurotoxic factors such as proinflammatory cytokines. Recently, the expression on these receptors in the microglia has been shown to be increased after MDMA injection. A CB2 agonist, JWH-015, decreased MDMA -induced microglial activation and IL-1β release. It also slightly decreased MDMA-induced 5-HT neurotoxicity which demonstrated that CB2 receptor activation reduces the neuroinflammatory response induced by MDMA and provides partial neuroprotection against MDMA (Torres et al., 2010).

Evidence from rodent and non-human primate studies suggests that MDMA-induced long-term 5-HT neurotoxicity is linked to hyperthermia (Broening et al., 1995; Farfel and Seiden, 1995; Green et al., 2004; Malberg et al., 1996). In addition, hypophysectomised and thyroparathyroidectomised animals are resistant to the 5-HT neurotoxic effects of MDMA, as protection was observed in the striatum and hippocampus up to 7 days following drug administration (Sprague et al., 2003), showing
that the hypothalamus and thyroid are important in mediating MDMA-induced long-term toxicity.

1.1.7 MDMA administration to rats: a useful model to predict toxicity in humans

Previous reports on the acute physiological and toxicological effects of MDMA in laboratory animals especially in rats have demonstrated a correlation with observations in humans. MDMA provokes an increase in blood pressure, cardiac activity (Gordon et al., 1991b) and vasoconstriction (Fitzgerald et al., 1994b) in rats. Similar effects of MDMA have been reported in humans with an increase in arterial pressure (Vollenweider et al., 1998); (O'Cain et al., 2000). Similarly numerous studies have reported an MDMA-induced increase in body temperature in both rats and humans (Broening et al., 1995; Che et al., 1995; Colado et al., 1993; Dafters, 1994; Malberg et al., 1996; O'Shea et al., 1998; Schmidt et al., 1990b). Increase of core body temperature induced after MDMA administration or ingestion have to be carefully interpreted since both species differ in their thermoregulation systems and such hyperthermia can be altered by ambient temperature. Rats possess a specific way of thermoregulating their body temperature via their tail which is considered to be a heat-loss organ (Grant, 1963; Romanovsky et al., 2002) and contributes to dissipating 17 % of the rat’s body heat while human skin can dissipate 90 % of body heat illustrating that humans are better predisposed for heat dissipation and thermoregulation than rats. Another issue is that MDMA-induced hyperthermia has been show to be influenced by ambient temperature. Placed in high ambient temperature (between 20-30°C) rats treated with MDMA show an enhanced hyperthermia and neurotoxicity (Dafters, 1994; Malberg et al., 1998; Mechan et al., 2001). Human studies on the effect of ambient temperature on MDMA responses are limited on the basis that such experiments are difficult to carry out on ethical grounds. Freedman and co-workers (2005) however reported that MDMA induced hyperthermia in recreational users despite control over the room temperature, and proposed that MDMA provoked an impairment in the heat loss regulating systems in humans. However, this study was limited to a single exposure of MDMA and at a very low dose compared to the actual range of doses consumed by recreational users. One study performed to analyse the influence of ambient room temperature in night clubs
reported that environmental conditions were conducive to the control of body
temperature and heat loss (Cole et al., 2005).

Despite the differences between animals and humans and potential limitations in
extrapolating from animal to human, there is no real alternative to using animals to
determine the acute toxicity risk of MDMA or related drugs in humans. As experimental
human research directed to study the safety profile of MDMA is often unethical and the
limited number of clinical studies performed to date refer to heterogeneous populations
in terms of estimated numbers of prior exposures and the type, amount and pattern of co-
ingestions of other recreational substances, it is impossible to identify simple
relationships associated with combinatorial drug use in retrospective human studies of
Ecstasy users. The behavioural, physiological, neurochemical and long-term effects of
MDMA however are well established in laboratory rodents. Similar physiological effects
occur in animals following MDMA administration as have been reported in human
abusers supporting a view that an animal model would serve as a useful analogy to the
human situation. In the current body of work experiments were undertaken in both group
and individually housed rats in attempts to model MDMA-induced physiological effects
and optimise the conditions by which mechanisms underlying such effects might be
determined. In particular, responses obtained in group-housed animals were aimed at
simulate the environmental conditions in which recreational users encounter and
consume the drug such as crowded night club environments.
Chapter 1: Introduction: Caffeine

1.2 Consumption of caffeine

Caffeine is the most widely consumed psychoactive substance in the world. It is found in coffee (Frary et al., 2005), tea, chocolate, soft drinks, and also in capsule or tablet forms for the treatment of asthma, nasal congestion, and headache or to improve athletic endurance and facilitate weight loss. Typically, caffeine consumption is estimated at 76 mg/per person per day in habitual tea/coffee drinkers (Cauli & Morelli, 2005). Caffeine content in a single serving of coffee ranges from 40 to 100 mg of caffeine. Tea contains a lower caffeine content from 10-50 mg whereas commercially available high energy drinks such as Red Bull contain 80 mg of caffeine per serving. Several natural sources of caffeine also possess other xanthine alkaloids including theophylline and theobromine which are pharmacologically active and contribute to the overall physiological effects of caffeine.

1.2.1 Pharmacology of caffeine

The psychostimulant properties of caffeine are due to its ability to interact with neurotransmission in different regions of the brain, promoting behavioral functions, such as vigilance or attention (Nehlig, 1999). Caffeine produces behavioral effects that are similar, but weaker, to those of typical psychomotor drugs, whose effects are known to be dopaminergically mediated such as amphetamines and cocaine. Due to its widespread use and low abuse potential, it is considered as an atypical drug of abuse. Its popularity comes from its stimulant properties, which depend on its ability to interact with the dopamine system. In human studies, the reinforcing effect of caffeine is limited to the intake of low or moderate doses and has been shown in caffeine users attempting to avoid withdrawal symptoms (Griffiths et al., 2000). An interruption of habitual heavy caffeine consumption provokes symptoms including headache, lethargy and anxiety, irritability and depression, increased fatigue and sleepiness (Griffiths et al., 1986; Griffiths et al., 1988; Strain et al., 1995; Stringer et al., 1987) which are manifestations of physical dependence (Garrett et al., 1998). Similar withdrawal symptoms, although less severe, are also observed following withdrawal from habitual abuse of amphetamines and cocaine (Watson et al., 1992).
Chapter 1: Introduction: Caffeine

Chemically, caffeine is a xanthine molecule: 1, 3, 7-trimethylxanthine. It is metabolised in the liver by the cytochrome P450 isoenzyme 1A2 (or CYP1A2). The first products of the metabolism are all dimethylxanthines: paraxanthine (84%; 1, 7-dimethylxanthine), theobromine (12%; 3, 7-dimethylxanthine), and theophylline (4%; 1, 3-dimethylxanthine) and they are all pharmacologically active (Figure 1.5). The half-life for caffeine metabolism is typically 5 to 6 hours in adult humans.

Figure 1.5 Caffeine and its metabolites

Under normal physiological conditions, caffeine’s mechanism of action is primarily through its antagonism of adenosine receptors (Ferre, 2008; Fisone et al., 2004; Fredholm et al., 1999). Adenosine is a purine that functions as a general inhibitor of neuronal activity; four heptahelical G-protein-coupled receptors, expressed in the brain, have been cloned and characterised as A1, A2A, A2B, A3 (Fisone et al., 2004). The affinity of caffeine for the adenosine A2B and A3 receptors is low and their basal activation is not significant. On the other hand, caffeine binds with high affinity adenosine to A1 and A2A receptors and these are activated by nanomolar concentrations (Fisone et al., 2004). Concentrations of caffeine required to antagonise adenosine receptors fall within the concentration range associated with the dietary intake of caffeine (10-100 µM or 152-1520 mg which equates to 1-10 cups of brewed coffee).

The methylxanthines family is known to possess a similar structure to cyclic nucleotides and therefore caffeine possesses the ability to interact with cyclic nucleotide phosphodiesterase (Butcher et al., 1962). Caffeine is a competitive inhibitor of cyclic nucleotide phosphodiesterase isoenzymes at concentrations of 100 µM (Cardinali, 1980)
which can therefore be reached following several ingestion of caffeine. Furthermore, caffeine has been shown to play an important role as respiratory stimulant prominently mediated by type IV phosphodiesterase (Howell, 1993).

In addition, caffeine can mobilise calcium from intracellular stores via activation of ryanodine-sensitive channels (McPherson et al., 1991; Sitsapesan et al., 1995) and inhibits benzodiazepine, binding to the \( \gamma \)-aminobutyric acid (GABA)\(_A\) receptor (Ferre, 2008). However, the ability of caffeine to mobilise intracellular \( \text{Ca}^{2+} \) and to influence GABA receptors is considered physiologically irrelevant as the circulating concentrations of caffeine necessary for such activity would result in lethal intoxication (millimolar range) (Ferré, 2008). Lethal intoxication with caffeine can be reached with a blood concentration of 500 \( \mu \)M (Dews, 1982) but after consumption of three cups of coffee (equal to 300 mg of caffeine approximately), the peak concentration of caffeine in the blood reaches approximately 30 \( \mu \)M (Bonati et al., 1982). Consequently, caffeine’s ability to regulate calcium mobilization cannot account for its physiological effects.

### 1.2.2 Adenosine-Dopamine interactions mediate the psychotropic effects of caffeine

In animal studies, the behavioral profile of caffeine seems to be similar to those of amphetamines and cocaine. Caffeine increases locomotive activity (Nehlig, 1999), produces stimulant-like discriminative effects (Gauvin et al., 1994), and supports self-administration in animals (Horger et al., 1991; Schenk et al., 1994; Worley et al., 1994). Several reports have implicated dopamine in caffeine’s central effects (Daly et al., 1993; Ferré et al., 1992) but the cellular mechanisms are unclear (Nehlig et al., 1992). The stimulant effects of caffeine are related to increased activity in dopaminergic neurotransmission via dopamine-adenosine interaction (Cauli et al., 2005; Ferre et al., 1992; Fisone et al., 2004; Garrett et al., 1997). The stimulant effects of a series of adenosine antagonists including caffeine in rodents are correlated with their potencies as adenosine receptor antagonists (Popoli et al., 1991). Caffeine can also modify the psychomotor effects of other drugs of abuse, such as nicotine, cocaine and amphetamines (Shoaib et al., 1999; Gasior et al., 2000; Green and Schenk, 2002; Cauli et al., 2003). Such effects of caffeine are attributed to an indirect action on dopamine transmission secondary to antagonism of adenosine receptors.
Dopamine interacts with dopamine D₁ and dopamine D₂ receptors. Dopamine D₁ receptors are positively coupled to adenylyl cyclase and activation leads to increased cyclic adenosine monophosphate (cAMP) production (Herve et al., 2001; Zhuang et al., 2000). Therefore, the activity of cAMP-dependent protein kinase (i.e. Protein Kinase A (PKA)) is increased and leads to the phosphorylation of several intracellular substrates (Cooper et al., 2003). Conversely, dopamine D₂ receptors are coupled via Gᵢ and Gₒ proteins, effecting a reduction in adenylyl cyclase activity and cAMP expression (Stoof et al., 1981).

PKA is known to mediate phosphorylation of cAMP response element binding protein (CREB) on Ser 133 (Sands & Palmer, 2007), which, in turn, enables the activation of target gene transcription by p-CREB. Another major target of PKA is dopamine- and cAMP-regulated phosphoprotein molecular weight 32 kDa (DARPP-32), an important modulator of the cAMP pathway found in high levels in the striatum (Svenningsson et al., 2005; Borgkvist & Fisone, 2006). Phosphorylation of threonine 34 (Thr³⁴) by PKA converts DARPP-32 into a potent inhibitor of protein phosphatase 1 (PP-1) thereby amplifying PKA-mediated reactions (Svenningsson et al., 2005). Activation of D₁ receptors decreases the phosphorylation of DARPP-32 at threonine 75 (Thr⁷⁵) via an apparent PKA-dependent activation of protein phosphatase 2A (PP-2A) which is then, facilitating activation of the PKA/Thr³⁴-DARPP-32/PP-1 cascade (Svenningsson et al., 2005) (Figure 1.6).

DARPP-32 can also be phosphorylated at Thr⁷⁵ by the proline-directed kinase Cdk5. Phosphorylation of Thr⁷⁵ has a major inhibitory effect on the phosphorylation of Thr³⁴ by PKA as well as an inhibitory effect on the phosphorylation of other PKA substrates (Svenningsson et al., 2005). Cdk5 itself is activated by two neuron-specific proteins: p35 and p39, as well as by a proteolytic product of p35 called p25 (Guo, 2006) (Figure 1.6).
Figure 1.6 cAMP pathway and relationship with DARPP-32 and CREB proteins.

(AC: adenylyl cyclase; CREB: cAMP response element binding protein; DARPP-32: dopamine- and cAMP- regulated phosphoprotein 32 kDa (Thr\(^{34}\) or Thr\(^{72}\); phosphorylation on threonine 34 or 72); PP-2A: protein phosphatase 2A; PPase 1: protein phosphatase 1; PP-2B/CalnA1: protein phosphatise 2B/Calcineurin A1; CK1: casein kinase 1; CDK5: cyclin-dependent kinase 5).
Chapter 1: Introduction: Caffeine

The adenosine A<sub>1</sub> receptor is coupled to pertussis toxin-sensitive G<sub>i</sub> and G<sub>o</sub> proteins, widely expressed in the brain, whose activation leads to inhibition of adenylyl cyclase and some voltage-gated Ca<sup>2+</sup>-channels, such as the N- and Q-channels (Fredholm et al., 1999; Fisone et al., 2004). The majority of adenosine A<sub>1</sub> receptors are located singularly on presynaptic nerve terminals, mediating inhibition of transmitter release. Adenosine A<sub>1</sub>-A<sub>2A</sub> receptors are known to form presynaptic heteromers on striatal glutamatergic terminals, the activation of which has been shown to affect dopaminergic transmission (Figure 1.7 -A) (Fisone et al., 2004; Ferré et al., 2008). An exception to this is their postsynaptic co-localisation with dopamine D<sub>1</sub> receptors on striatonigral neurons in the ‘direct pathway’ of the striatum (Ferré, 2008) (Figure 1.7 -B).

Caffeine is able to modify striatal dopaminergic release presynaptically in 2 ways: (a) via A<sub>1</sub> receptor- or via heterodimeric A<sub>1</sub>-A<sub>2A</sub> receptor localized on dopaminergic ending (pink) and glutamatergic endings (blue) respectively. (b) Interactions with the A<sub>1</sub>-D<sub>1</sub> receptor heteromer. Adenosine binds to the A<sub>1</sub>R and inhibits D<sub>1</sub>R-associated mechanisms. Caffeine is an antagonist at A<sub>1</sub>R and could reverse such an inhibition resulting in an increase in cAMP and PKA activity, and an increase in the phosphorylation of several PKA dependent substrates (from Ferré, 2008).
Adenosine $A_{2A}$ receptors are mostly localized in the striatum (Fredholm et al., 2001; Schiffmann et al., 2007) and are predominantly present on GABAergic enkephalinergic neurons co-localised with dopamine $D_2$ receptors (Agnati et al., 2003; Ferre et al., 2007b; Ferre et al., 1997; Schiffmann et al., 2007; Schiffmann et al., 1991). These receptors interact with each other and therefore modulate the function of the GABAergic and enkephalinergic neurons. Antagonism effects between dopamine $D_2$ and adenosine $A_{2A}$ receptors have been demonstrated in both human and rat striatum, in membrane preparations, where selective $A_{2A}$ receptor agonists decrease the binding of selective dopamine $D_2$ receptor ligands (Dasgupta et al., 1996; Díaz-Cabiale et al., 2001; Dixon et al., 1997; Ferre et al., 1991c; Salim et al., 2000). In vivo microdialysis experiments conducted by Ferre and co-workers (1993) showed that perfusion of an adenosine $A_{2A}$ receptor agonist completely blocks the effect of a dopamine $D_2$ receptor agonist, with $A_{2A}$ receptor agonism acting to reduce the activity of dopamine $D_2$ receptors. In a similar way, a selective $A_{2A}$ receptor agonist or antagonist inhibited or potentiated, respectively, the motor activation induced by dopamine $D_2$ receptor agonist (Ferre et al., 1991a; Ferre et al., 1991b; Rimondini et al., 1998; Stromberg et al., 2000). Furthermore, at a cellular level, adenosine $A_{2A}$ receptors are known to stimulate adenylyl cyclase and therefore activate the cAMP-PKA signalling pathway with phosphorylation of DARPP-32, CREB or activation of immediate early genes such as $c-fos$ in GABAergic and enkephalinergic neurons (Agnati et al., 2003; Ferre et al., 2007a; Ferre et al., 2007b; Schiffmann et al., 2007). Dopamine $D_2$ receptors inhibit adenosine $A_{2A}$ receptor stimulation at the level of adenylyl cyclase and dopamine $D_2$ receptor agonists block the effects induced by adenosine $A_{2A}$ receptor stimulation on CREB phosphorylation and $c-fos$ expression. Thus, this confirms a reciprocal antagonistic $A_{2A}$-$D_2$ receptor interaction (Kull et al., 1999).

### 1.2.3 Interaction between caffeine and MDMA

Severe adverse reactions to MDMA are idiosyncratic, as there is a poor correlation between the doses ingested, and blood concentration with severity of the symptoms. A potential factor contributing to the idiosyncratic nature of adverse reactions to MDMA is the presence of other psychoactive substances in “Ecstasy” tablets (O’Connell et al., 2000). In addition, epidemiological studies demonstrate that the majority of “Ecstasy” users are poly-drug abusers for whom co-use of drugs such as LSD, D-amphetamine,
McNamara and co-workers have reported that concurrent administration of caffeine with MDMA or MDA to rats results in a profound response characterised by hyperthermia, tachycardia and increased mortality, which are not observed following administration of equivalent doses of these amphetamines alone (McNamara et al., 2006; McNamara et al., 2007). This is a serious finding and if it translates to the human drug use setting, has important health consequences for recreational “Ecstasy” users. It is appropriate to independently refer to hyperthermia and lethality: in fact, the increase in body temperature may not be the sole contributing factor to mortality but hyperthermia is nevertheless a common feature in cases presenting with MDMA-induced toxicity and is a causative factor in long-term 5-HT loss (Green et al., 2004). As a result, we place a particular emphasis on the body temperature response to MDMA in our experiments.

As previously discussed, it is widely reported that the pharmacological actions of MDMA result in biogenic amine (particularly 5-HT and dopamine) release in several regions of the brain (Green et al., 2003). Caffeine has also been reported to induce an increase in the release of 5-HT and dopamine in the cortex, hippocampus and striatum of freely-behaving rats (Acquas et al., 2002; Okada et al., 1997; Okada et al., 1996). It is therefore conceivable that administration of caffeine could result in an augmentation of MDMA-induced 5-HT or dopamine release. In this study, using neurotransmitter depletion strategies, we examined the role of 5-HT and catecholamines in the hyperthermic response to caffeine and MDMA. It is also noteworthy that co-administration of caffeine with amphetamine or cocaine leads to a dramatic increase in seizures and mortality in rats in comparison to administration of amphetamine or cocaine alone (Derlet et al., 1992). As amphetamine and cocaine increase extracellular dopamine, such interactions support a role for dopamine as an integral factor, mediating severe adverse reactions associated with the concurrent use of caffeine.

McNamara and co-workers (2006) have reported that MDMA in individually housed animals induced hypothermia and bradycardia while co-administration with caffeine provoked a profound hyperthermia and tachycardia. Dopamine and dopamine receptors are known to play an important role in cardio- and thermoregulation. The role of
dopamine D₁ and D₂ receptors in mediating such effects of MDMA in rats were investigated in this study.

As previously discussed, dopamine D₁ and D₂ receptors have opposing actions at the biochemical level on the activity of adenyl cyclase and protein kinase A (PKA). The dopamine and cAMP regulated phosphoprotein of 32 kDa (DARPP-32) is abundant in neurons which receive dopaminergic input. Activation of PKA and the subsequent phosphorylation of DARPP-32 on Thr³⁴ occur in response to dopamine acting on D₁ receptors. In contrast, activation of dopamine D₂ receptors results in inhibition of PKA activation, the activation of protein phosphatase 2B and the subsequent dephosphorylation of DARPP-32. Phosphorylated DARPP-32 is a potent inhibitor of protein phosphatase-1 (Greengard et al., 1998). Thus, the phosphorylation status of DARPP-32 provides an intra-neuronal marker of D₁/D₂ receptor activation, and may be useful to study the influence of caffeine on MDMA-induced intracellular changes associated with D₁/D₂ receptor activation.

Caffeine is known to be an adenosine receptor antagonist (Fredholm et al., 1999; Nehlig, 1999), and also a weak inhibitor of cAMP phosphodiesterase (PDE) as mentioned earlier. Therefore, it may interact indirectly with dopaminergic neurotransmission. However, experiments carried out in the laboratory to date show that co-treatment of MDMA with adenosine antagonists failed to provoke a caffeine-like interaction with MDMA, indicating that the blockade of adenosine receptors alone is unlikely to mediate caffeine's interaction with MDMA. Interestingly, we have found that concurrent administration of low doses of the cAMP PDE inhibitor rolipram with MDA provokes a marked caffeine-like hyperthermic response. The role of adenosine receptor, inhibition in addition to PDE inhibition, in mediating the ability of caffeine to influence MDMA-induced toxicity is further explored in the experiments carried out in this thesis.
1.3 Aims and objectives

The overall objective of the work described in this thesis was to assess the role of dopamine in a potentially lethal interaction between caffeine and MDMA in a model of acute MDMA toxicity in rats. Moreover, this study examined if the interaction between caffeine with MDMA generalises to other drugs which enhanced dopamine transmission in the brain.

The specific objectives of the thesis are to determine:

(1) the mechanisms involved in MDMA-induced hyperthermia and its exacerbation by caffeine and to assess the role of dopamine $D_1$ receptors in the ability of caffeine to promote hyperthermia with MDMA.

(2) the role of dopamine $D_1$ and $D_2$ receptors in the ability of caffeine to promote hyperthermia and tachycardia following MDMA administration.

(3) the influence of MDMA and caffeine alone or in combination on $[^3H]$ dopamine release from striatal and hypothalamic tissue slices and the mechanisms involved in this release.

(4) Intracellular mechanisms associated with the behavioural and hyperthermia induced by MDMA alone or in combination with caffeine.
2.1 MATERIALS

2.1.1 Animals
Sprague-Dawley rats Harlan, UK

2.1.2 Experimental treatments
Alpha-methyl-para-tyrosine Sigma Aldrich Ireland
Apomorphine Sigma Aldrich Ireland
Caffeine Sigma Aldrich Ireland
CGS 15943 (9-chloro-2-(2-furanyl)-[1,2,4]triazolo [1,5-C]quinazolin-5-amine) Sigma Aldrich Ireland
D-amphetamine Sigma Aldrich Ireland
D-fenfluramine Sigma Aldrich Ireland
DOI (1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane) Sigma Aldrich Ireland
DPCPX (1,3-dipropargyl-8-cyclopenthyxanthine) Sigma Aldrich Ireland
MDMA NIDA, USA
GBR 12909 Sigma Aldrich Ireland
Ketanserin Sigma Aldrich Ireland
Para-chlorophenylalanine (PCPA) Sigma Aldrich Ireland
Pentoxifylline (PTX) Sigma Aldrich Ireland
Prazosin Sigma Aldrich Ireland
Raclopride Sigma Aldrich Ireland
Reserpine Sigma Aldrich Ireland
Ritanserin Sigma Aldrich Ireland
Rolipram Sigma Aldrich Ireland
SCH 23390 Sigma Aldrich Ireland
SCH-58251 (5-amino-7-(2-phenylethyl)-2-(2-furyl)
-pyrazolo-[4,3-c]-1,2,4-triazolo [1,5-c] pyrimidine) Sigma Aldrich Ireland
SKF 38393 Sigma Aldrich Ireland
Zaprinast Sigma Aldrich Ireland
## Chapter 2: Materials

### 2.1.3 HPLC: Reagents and plastics

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3-4-Dihydroxyphenylamine (L-DOPA)</td>
<td>Sigma Aldrich Ireland</td>
</tr>
<tr>
<td>3,4-Dihydroxyphenyl-acetic acid (DOPAC)</td>
<td>Sigma Aldrich Ireland</td>
</tr>
<tr>
<td>5-Hydroxyindole-3-acetic acid (5-HIAA)</td>
<td>Sigma Aldrich Ireland</td>
</tr>
<tr>
<td>Citric acid (C₆H₈O₇)</td>
<td>BDH Chemicals, Poole, U.K.</td>
</tr>
<tr>
<td>Dopamine hydrochloride (DA)</td>
<td>Sigma Chemical Co., UK</td>
</tr>
<tr>
<td>Ethylenediaminetetra-acetic acid (EDTA)</td>
<td>BDH Chemicals, Poole, U.K.</td>
</tr>
<tr>
<td>Homovanillic acid (HVA)</td>
<td>Sigma Aldrich Ireland</td>
</tr>
<tr>
<td>Methanol, 100%</td>
<td>Lab-Scan, Ireland</td>
</tr>
<tr>
<td>N-methyl-5-HT</td>
<td>Sigma Chemical Co., UK</td>
</tr>
<tr>
<td>Noradrenaline (NA)</td>
<td>Sigma Chemical Co., UK</td>
</tr>
<tr>
<td>Octane-1-sulfonic acid</td>
<td>Sigma Chemical Co., U.K.</td>
</tr>
<tr>
<td>Perchloric acid</td>
<td>BDH Chemicals, Poole U.K.</td>
</tr>
<tr>
<td>Serotonin (5-HT)</td>
<td>Sigma Aldrich Ireland</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate (NaH₂PO₄)</td>
<td>BDH Chemicals, Poole UK</td>
</tr>
</tbody>
</table>

### 2.1.4 ELISA: Kits and plastics

<table>
<thead>
<tr>
<th>Kit Name</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat cAMP Immunoassay</td>
<td>Promega Ireland</td>
</tr>
</tbody>
</table>

### 2.1.5 Western (immuno)blotting: Reagents and antibodies

<table>
<thead>
<tr>
<th>Antibody Name</th>
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</thead>
<tbody>
<tr>
<td>Anti-mouse IgG</td>
<td>Sigma Aldrich Ireland</td>
</tr>
<tr>
<td>Anti-Rabbit IgG</td>
<td>Amersham, UK</td>
</tr>
<tr>
<td>Anti-rat DARPP-32</td>
<td>Cell Signalling Ireland</td>
</tr>
<tr>
<td>Anti-rabbit IgG</td>
<td>Amersham, UK</td>
</tr>
<tr>
<td>Anti-rat phospho DARPP-32</td>
<td>Cell Signalling Ireland</td>
</tr>
<tr>
<td>Monoclonal anti-β-actin antibody</td>
<td>Sigma Aldrich Ireland</td>
</tr>
<tr>
<td>Phospho CREB antibody rat</td>
<td>Cell signalling Ireland</td>
</tr>
<tr>
<td>Total rat CREB antibody</td>
<td>Cell Signalling Ireland</td>
</tr>
<tr>
<td>Anode buffer I: 0.3 M Tris, pH 10.4, 10% methanol</td>
<td>Sigma Aldrich Ireland</td>
</tr>
<tr>
<td>Anode buffer II: 25 mM Tris, pH 10.4, 10% methanol</td>
<td>Sigma Aldrich Ireland</td>
</tr>
<tr>
<td>Cathode buffer: 25 mM Tris, 40 mM 6-amino-n-caproic acid</td>
<td>Sigma Aldrich Ireland</td>
</tr>
<tr>
<td>CL-Xposure X-ray film</td>
<td>Pierce</td>
</tr>
<tr>
<td>Broad range molecular weight marker (Precision Plus)</td>
<td>BioRad, Ireland</td>
</tr>
<tr>
<td>Filter paper</td>
<td>Whatman Ltd.</td>
</tr>
</tbody>
</table>
### Chapter 2: Materials

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immobilon PVDF transfer membrane</td>
<td>Millipore Ireland</td>
</tr>
<tr>
<td>Photographic film (Hyperfilm)</td>
<td>Amersham, UK</td>
</tr>
<tr>
<td>Restore&lt;sup&gt;TM&lt;/sup&gt; Western Blot stripping solution</td>
<td>Pierce</td>
</tr>
<tr>
<td>Supersignal® West Dura extended substrate solution</td>
<td>Pierce</td>
</tr>
<tr>
<td>Whatman 3MM filter paper</td>
<td>Millipore Ireland</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>Sigma Aldrich Ireland</td>
</tr>
</tbody>
</table>

#### 2.1.6 Molecular Reagents

- Absolute ethanol
- Agarose
- Biosphere filter tips (1000, 100 and 20μl)
- Diethyl pyrocarbonate (DEPC)
- Ethidium bromide
- High Capacity cDNA Archive Kit
- Loading dye (6X)
- Molecular grade H<sub>2</sub>O
- Nucleospin RNA II Extraction Kit
- Optical adhesive covers
- PCR tubes
- RNase Away
- RNase Out ribonuclease
- RNase-free (2 ml) microfuge tubes
- RNAlater™
- RNase Zap wipes
- TaqMan gene expression assays
- TaqMan universal PCR master mix
- 10X TBE buffer
- 96-well optical reaction plates
- Sigma Aldrich Ireland
- Condra
- Sarstedt, Ireland
- Sigma Aldrich Ireland
- Sigma Aldrich Ireland
- Applied biosystems Ireland
- Promega Ireland
- Sigma Aldrich Ireland
- Macherey-Nagel, Germany
- Applied biosystems Ireland
- Sarstedt, Ireland
- Invitrogen, Ireland
- Invitrogen, Ireland
- Ambion, UK
- Ambion, UK
- Ambion, UK
- Applied biosystems Ireland
- Applied biosystems Ireland
- Invitrogen Ireland
- Applied biosystems Ireland

#### 2.1.7 Tissue slice superfusion

Krebs buffer: 122 mM NaCl, 3.1 mM KCl, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>(H<sub>2</sub>O), 25 mM NaHCO<sub>3</sub>, 10 mM Glucose, 1.3 mM CaCl<sub>2</sub>, 10 μM pargyline, [³H] dopamine

- Sigma Aldrich Ireland
- GE Healthcare, Ireland
2.1.8 General Laboratory Chemicals

- Acrylamide: Sigma Aldrich Ireland
- Ammonium persulfate (APS): Sigma Aldrich Ireland
- Bicinchoninic acid (BCA) protein kit: Pierce
- Bovine serum albumin 96% (BSA): Sigma Aldrich Ireland
- Bromophenol blue: Sigma Aldrich Ireland
- DL-Dithiothreitol (DTT): Sigma Aldrich Ireland
- Diethyl Pyrocarbonate (DEPC): Sigma Aldrich Ireland
- Di-Sodium hydrogen orthophosphate (Na$_2$HPO$_4$): BDH Chemicals, Poole UK
- N- Ethylenediaminetetra acetic acid (EDTA): Sigma Aldrich Ireland
- (1-naphthyl)-ethylene diamine dihydrochloride: Sigma Aldrich Ireland
- N’N’ Bis acrylamide: Sigma Aldrich Ireland
- Glycerol: Sigma Aldrich Ireland
- Glycine: Sigma Aldrich Ireland
- Hydrochloric acid (HCl): BDH Chemicals, Poole UK
- Magnesium chloride (MgCl$_2$): Sigma Aldrich Ireland
- Methanol: BDH Chemicals, Poole UK
- NP-40: Amersham, UK
- Phosphatase inhibitor cocktail I and II: Sigma Aldrich Ireland
- Potassium chloride (KCl): Sigma Aldrich Ireland
- 2-Propanol: Sigma Aldrich Ireland
- Protease inhibitor: Sigma Aldrich Ireland
- Sodium bicarbonate (NaHCO$_3$): Sigma Aldrich Ireland
- Sodium carbonate (Na$_2$CO$_3$): BDH Chemicals, Poole UK
- Sodium chloride (NaCl): BDH Chemicals, Poole UK
- Sodium dodecyl sulfate (SDS) 99%: Sigma Aldrich Ireland
- Sodium hydroxide (NaOH): Sigma Aldrich Ireland
- Sulphuric acid (98%) (H$_2$SO$_4$): BDH Chemicals, Poole UK
- N’N’N’-Tetramethylethylenediamine (TEMED): Sigma Aldrich Ireland
- Tris-HCl: Sigma Aldrich Ireland
- Trizma base: Sigma Aldrich Ireland
- Tween-20: Sigma Aldrich Ireland
### 2.1.9 General Laboratory Plastics

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood collection tubes (12 ml)</td>
<td>Sarstedt, Ireland</td>
</tr>
<tr>
<td>Eppendorfs</td>
<td>Sarstedt, Ireland</td>
</tr>
<tr>
<td>Parafilm Laboratory rolls</td>
<td>Sarstedt, Ireland</td>
</tr>
<tr>
<td>Microtest 96-well flat bottomed plates</td>
<td>Sarstedt, Ireland</td>
</tr>
<tr>
<td>Pasteur pipettes</td>
<td>Sarstedt, Ireland</td>
</tr>
<tr>
<td>Pipette tips</td>
<td>Sarstedt, Ireland</td>
</tr>
<tr>
<td>50 ml Yellow capped containers</td>
<td>Sarstedt, Ireland</td>
</tr>
<tr>
<td>24-well plates (superfusion)</td>
<td>Sarstedt, Ireland</td>
</tr>
<tr>
<td>96-well plates (protein)</td>
<td>Sarstedt, Ireland</td>
</tr>
</tbody>
</table>
2.2 METHODS

2.2.1 Animal husbandry

Male Sprague Dawley rats (200-300 g upon arrival) were obtained from Harlan laboratories UK. Animals were housed four to a cage under standard laboratory conditions, with a 12 hour light: 12 hour dark cycle (lights on 08.00 hours, lights off 20.00) and in a temperature-controlled room maintained at 20-24°C. Animals had access to food and water *ad libitum* (standard laboratory diet), except during periods of behavioural testing. Animals were handled at regular time periods throughout the course of *in vivo* treatment periods. All procedures were approved by the Ethics Committee in Trinity College Dublin and were in accordance with the European Council Directive 1986 (86/806/EEC).

2.2.2 Physiological assessments

Body weight measurements were routinely recorded and were taken as an indicator of the general well-being of the animals. The animals were allowed an acclimatization period of at least 2 weeks prior to commencement of any treatment. In experiments where core body temperatures were recorded, the animals were lightly restrained by hand and rectal temperature measurements were taken using a lubricated digital rectal thermometer (Omron) inserted 3 cm into the rectum. Cages and bedding were changed the day prior to drug challenges to minimize any possible effects on temperature measurements. In experiments where core body temperatures were obtained manually, a temperature measurement was taken 60 minutes and immediately prior to drug administration. Temperatures were subsequently taken every 30 minutes for up to 120 minutes and after this, every 60 minutes for up to 300 minutes following drug administration. Further details of individual experiments may be found in the results sections of this thesis.

Radiotelemetry was used to record body temperature, heart rate and locomotor activity in freely behaving animals using a MiniMitter radiotelemetric system.
2.2.2.1 Surgical implantation of radiotelemetric transmitters

Intraperitoneal implantation of HR E-Mitters (PDT-4000 HR) was performed in rats anaesthetised with a mix of 2% xylazine (10 mg/kg; i.p.) and ketamine (100 mg/kg, i.p.) using an injection volume of respectively 0.75 ml/kg and 1.25 ml/kg. The ventral surface of the abdomen and the thorax to the area of the axilla, slightly cranial on the right side was shaved. The shaved area was swabbed with Betadine® and the animal was secured to a heated sterile surgical surface with adhesive tape. The abdomen was opened by making a 2-cm incision along the linea abla (the white line of fascia where the abdominal muscles join on the midline). The bowel was now visible immediately under the surface. The body of the E-Mitter was slipped into the abdominal cavity along the sagittal and dorsal plan to the digestive organs so that both leads were situated in the direction of the head of the animal. The abdominal cavity was then massaged gently to allow the internal organs to settle. For heart lead placement, the heart leads were threaded through two small holes in the abdominal wall (external oblique) made to the right and left of the incision. For negative lead attachment, using a trochar and sleeve, the lead was passed under the skin to an incision made near the clavicle and left resting on the pectoralis superficialis. Similarly, for positive lead placement the lead was passed under the skin to an incision made to the left of the xiphoid process, cranial to the last rib and left resting on the posterior pectoralis superficialis. Steel ferrules were placed over the tips of the leads and crimped into tabs. These tabs at the tip of the stainless steel spiral typically detect the heart signal. Heart lead placement follows the lead II configuration as described by others for determination of heart rate in small laboratory animals (Brockway et al., 1993; Stohr, 1988). The leads were then secured against the chest muscles by attaching a steel suture just behind the tabs. At this time the heart signal was checked using the system's heart signal monitor. To finish the surgical procedure the abdominal opening and the smaller two lead access points were closed with polyester sutures. After surgery, animals were returned to their cage. Analgesic (Disprol: 0.5 mg/ml) was placed in their drinking water for the immediate 24 hours following surgery. Disprol also contains sodium saccharine and lime flavor to give a sweet and pleasant taste to the water. The animals were allowed 10–14 days to recover following surgery before any drug challenge.
2.2.2.2. Heart rate monitoring
Heart rate and others parameters are transmitted by the intraperitoneal placed implant to the receiver and acquired by the software program VitalView™. The R-wave of the cardiac electric cycle (QRS complex) detected by proper leads positioning, creates a voltage which is triggering a pulse detected by the ER-4000 receiver. The heart rate is then reported by the VitalView™ system as beats per minutes (BPM) value.

2.2.2.3. Body temperature monitoring
Prior to data collection, calibration values (specific to each transmitter and provided by the manufacturer) have to be entered into the system configuration. The signal emitted by the implant is a train of pulses whose period are dependent on temperature. The receiver converts this pulse frequency into a serial bit stream readable by the computer. E-Mitters are accurate to 0.1°C and are normally calibrated over the range of 32-42°C.

2.2.2.4. Locomotor activity monitoring
For the detection of activity, the transponder has to move. Any change in signal strength from a transponder is interpreted by the system as an indication that the transponder has moved and is scored as an activity count. With the transponder implanted in the peritoneal cavity, behaviour involving finer movement such as occurs with feeding or grooming, for example, will not be registered as activity. Instead, behaviours primarily including exploratory behaviour and ambulation are cumulated in activity counts.

The Mini Mitter telemetry system used comprises an acquisition hardware including implantable series E-mitter-4000, series 4000 receivers (placed underneath the home cage) and VitalView™, the integrated software and hardware system for data acquisition (see Figure 2.1).
2.2.3 Behavioural assessments

Behaviours were recorded in some experiments by observation every 15 minutes following drug administration over a 5 hour observation period. Animals were observed continuously for 1 minute every 15 minutes and assigned a score as detailed in the table below (see Table 2.1). Behaviours associated with the 5-HT syndrome were scored as either (0) absent or (1) present or, in the case of head weaving, according to the following scale (1) behaviour present for less than 30 seconds (2) behaviour present for more than 30 seconds (3) behaviour present continuously. Behavioural experiments were single-blind trials at least.

2.2.4 Drug administration

All drugs were prepared in 0.89% saline or in 0.89% saline with 1% Tween 80 or 1% dimethyl sulfoxide (DMSO) in cases where there was difficulty with drug solubility and were prepared on the day of administration. The route of administration used was either intraperitoneal or subcutaneous (see Table 2.2).
### Methods

<table>
<thead>
<tr>
<th>Score</th>
<th>5-HT Syndrome Scale</th>
<th>Locomotion Scale</th>
<th>Stereotypic Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absence (0) or presence (1) of the behavior</td>
<td>Piloerection</td>
<td>Inactive, Asleep</td>
<td>Inactive, Asleep</td>
</tr>
<tr>
<td>1-3</td>
<td>Sweating</td>
<td>Awake Stationary</td>
<td>Awake Stationary</td>
</tr>
<tr>
<td></td>
<td>Low body posture</td>
<td>Normal ((^1))</td>
<td>Locomotion with sniffing, rearing or grooming((^2))</td>
</tr>
<tr>
<td></td>
<td>Head weaving</td>
<td>Rapid and continuous locomotion</td>
<td>Continuous Sniffing or rearing((^3)) over a wide area</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rapid locomotion with leaping, jumping or rapid darting around the perimeter of the test cage</td>
<td>Continuous Sniffing((^4)) / Gnawing in one location</td>
</tr>
</tbody>
</table>

**Table 2.1: Behavioral scoring table (5-HT syndrome, locomotion and stereotypy).**

Rating scales were introduced for approximation of the 5-HT syndrome (Spanos et al., 1989) and amphetamine -induced behaviours (Mumford et al., 1979). \(^1\)Normal locomotion: some locomotion with some sniffing, rearing and grooming; \(^2\)Grooming: often a discontinuous process broken at intervals by locomotion and exploratory activity. It is initially a head and snout grooming with the front paws followed by burrowing of the snout into the body; \(^3\)Rearing: when the rat raises both fore paws from the floor of the test area; \(^4\)Sniffing: rhythmic movement of the snout and head along the cage, wall or floor, accompanied by rapid movement of vibrissae; \(^5\)Chewing: jaw movement not directed at any stimulus; \(^6\)Gnawing: where the bedding on the floor was gripped between the teeth; \(^7\)Licking: tongue protrusion against the cage floor or wall; \(^8\)Continuous and compulsive stereotypies: stereotypies that occurred regularly and were not interrupted for more than 5 seconds are considered as continuous.
### Methods

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Route of administration</th>
<th>Solubility</th>
<th>Volume of injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apomorphine</td>
<td>i.p.</td>
<td>0.89% saline</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>CGS 15943</td>
<td>i.p.</td>
<td>0.89% saline with 1% Tween 80</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>D-amphetamine</td>
<td>i.p.</td>
<td>0.89% saline</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>D-fenfluramine</td>
<td>i.p.</td>
<td>0.89% saline</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>DOI</td>
<td>i.p. or s.c.</td>
<td>0.89% saline with 1% Tween 80</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>DPCPX</td>
<td>i.p.</td>
<td>0.89% saline with 1% Tween 80</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>Ketanserin</td>
<td>i.p. or s.c.</td>
<td>0.89% saline</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>MDMA</td>
<td>i.p. or s.c.</td>
<td>0.89% saline</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>PCPA</td>
<td>i.p.</td>
<td>0.89% saline</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>Prazosin</td>
<td>i.p.</td>
<td>0.89% saline</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>PTX</td>
<td>i.p.</td>
<td>0.89% saline</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>Reserpine</td>
<td>i.p.</td>
<td>0.89% saline</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>Ritanserin</td>
<td>i.p.</td>
<td>0.89% saline</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>Rolipram</td>
<td>i.p.</td>
<td>0.89% saline with 1% Tween 80</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>SCH 23390</td>
<td>i.p. or s.c.</td>
<td>0.89% saline</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>SCH 58261</td>
<td>i.p.</td>
<td>0.89% saline with 1% DMSO</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>Sulpiride</td>
<td>i.p.</td>
<td>0.89% saline</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>Zaprinast</td>
<td>i.p.</td>
<td>0.89% saline</td>
<td>1ml/kg</td>
</tr>
<tr>
<td>α-MPT</td>
<td>i.p.</td>
<td>0.89% saline</td>
<td>1ml/kg</td>
</tr>
</tbody>
</table>

**Table 2.2: List of drugs administered.**

Drugs were subcutaneously (s.c.) administered only in the individually housed study (see Chapter 4) while the remaining drugs were administered intraperitoneally (i.p.).

#### 2.2.5 Serotonin (5-HT) and catecholamine depletions.

Central 5-HT depletion was induced in rats by intraperitoneal administration of the tryptophan hydroxylase inhibitor para-chlorophenylalanine (PCPA) (150 mg/kg) once
daily for 3 days. Central catecholamine depletion was induced by intraperitoneal administration of reserpine (5 mg/kg), an inhibitor of the vesicular monoamine transporter with the tyrosine hydroxylase inhibitor alpha-methyl-para-tyrosine (α-MPT) (150 mg/kg). Regional brain 5-HT and catecholamine (dopamine and noradrenaline) concentrations were determined by HPLC (high performance liquid chromatography) coupled to electrochemical detection, described below. Both produce transient effects and recovery of 5-HT or catecholamine levels occurs from 7 days withdrawal from the treatment regimes.

2.2.6 Brain dissection

*Harvesting brain tissue for HPLC and protein assays*

Following decapitation the brain was removed and dissected quickly on ice. Samples from a number of brain regions were dissected, as described below and snap-frozen on dry ice. Brain regions were dissected following Harkin *et al.* (2001) descriptions. The brain was turned ventral side uppermost and the hypothalamus dissected. For the striatal section, the brain was turned ventral side uppermost and cut horizontally, just anterior to the position of the optic chiasma, at a point on the brain adjacent to the thalamus. The striatal and frontal cortex were then separated from surrounding cortex. Samples were stored at -80°C until analysis was completed.

2.2.7 BCA protein assay

Protein content for ELISA and western blotting were determined using the BCA protein assay kit (Pierce). A 2000 μg/ml stock solution of bovine serum albumin (BSA) was prepared using lysis buffer that the tissue sample was prepared in. Dilutions were made from the stock solution to produce a standard curve of concentration range: 2000, 1500, 1000, 750, 500, 250, 125, 25, 0 μg/ml BSA protein. 25 μl of each protein standard and sample (diluted as appropriate to fall within the standard curve range) were added in triplicate to the wells of a 96-well plate. 200 μl of BCA working reagent (supplied in kit, prepared from 50:1 parts of the BCA working reagents A:B) was added to each sample and standard to allow detection of protein. The plate was covered and incubated for 30 minutes at 37°C. The absorbance was read at 560 nm on a microtitre plate reader. A
standard curve was then plotted from the protein standards and absorbance readings. The unknown sample protein concentrations were then determined from the standard curve and results were expressed as μg/ml of protein.

2.2.8 Sandwich ELISA for analysis of cAMP concentrations in the rat hypothalamus and striatum

Samples were prepared by homogenising hypothalamic and striatal tissues in 1 ml of lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% (v/v) NP-40, 1 mM PMSF(phenylmethyl sulfonil fluoride), 10 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM sodium vanadate) with a polytron tissue disrupter (PT 2100, Kinematica). The homogenates were then centrifuged at 14,000 g for 15 minutes at 4°C and a BCA protein assay was carried out on the supernatants.

cAMP concentrations were determined in the hypothalamus and in the striatum using a cAMP competitive enzyme immunoassay system (Amersham Bioscience). Standards were prepared by serial dilutions to produce a standard curve of concentration range: 3200, 1600, 800, 400, 200, 100, 50, 25, 12.5 and 0 fmol/well cAMP. 100 μl of each standard and supernatant sample were added in duplicates to the 96-well pre-coated plate (with donkey anti-rabbit IgG). 100 μl of antiserum (rabbit anti cAMP) was added in each wells except the blank and NSB (non specific binding) wells, which was then covered and incubated for 2 hours at 3-5°C. Then, 50 μl of cAMP-peroxidase conjugate was added into all wells except the blank and the plate was incubated at 3-5°C for 60 minutes. Following this, the plate was washed four times with wash buffer, blotted on tissue paper ensuring any residual volume was removed. 150 μl of enzyme substrate (cAMP horseradish peroxidase) was dispensed into all wells and the plate was covered and placed in a microplate shaker for 60 minutes at room temperature. A blue color development was stopped after 10 minutes incubation at RT by the addition of 100 μl of 1 M sulfuric acid. The absorbance measured at 450 nm with a microtitre plate reader and a standard curve was constructed by plotting the cAMP standard concentrations against the absorbance. This was then used to determine the cAMP concentrations of the unknown samples, which were expressed as fmol/μg of protein.
2.2.9 Neurotransmitter analysis by HPLC

2.2.9.1 Preparation of HPLC mobile phase and standards

HPLC mobile phase (100 mM citric acid, 100 mM NaH$_2$PO$_4$, 1.4 mM octane-1-sulfonic acid, 0.1 mM EDTA and 10% (v/v) methanol) was prepared using double-distilled NANOpure H$_2$O. The pH was adjusted to 2.8 by the addition of 4M NaOH and the mobile phase was vacuum filtered to reduce any residual solute. Neurochemical standards of the biogenic amines were also prepared as standard calibration points and to assess retention times on the HPLC column. Standard amines noradrenaline (NA), levodopa (L-DOPA), 3, 4-dihydroxyphenylacetic acid (DOPAC), dopamine (DA), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT) and N-methyl-5-HT were dissolved individually in 10 ml HPLC mobile phase to give solutions of 1 mg/ml of each amine and these were then used to prepare 10ml of standard mixture containing 2 ng/20 μl of each compound in HPLC mobile phase. A standard “mix” solution was also prepared containing all the standard amines (2 ng/20 μl) in mobile phase buffer.

2.2.9.2 Tissue preparation for HPLC

Tissue samples were weighted and then homogenized by sonication (MSE sonicator) for 5-10 seconds at 10 microns in 0.5 ml of ice-cold homogenization buffer (HPLC mobile phase with 2 ng/20 μl N-methyl-5-HT used as an internal standard (IS)). The homogenate was then centrifuged at 15,000 g for 15 minutes at 4°C and stored at -80°C until HPLC analysis was carried out.

2.2.9.3 HPLC analysis of rat brain biogenic amines

Tissue samples for HPLC were thawed and re-centrifuged at 15,000 g for 15 minutes at 4°C. 150 μl of the resulting supernatant fluid was transferred into an insert within a vial and was then capped. Air bubbles, if present, were removed by tapping. Vials containing 150 μl of standard “mix” were placed every 5 samples. These acted to recalibrate the system and minimize any drift that occurred in amine retention times during sampling. The samples and standards were analyzed using an automated HPLC system (Shimadzu ADVP module) and an autosampler was used to inject 20 μl into the HPLC reverse
phase column (LI Chrosorb RP-18, 25cm x 4mm internal diameter, particle size 5μm).
Neurotransmitter concentrations were quantified by electrochemical detection (Shimadzu) and chromatograms were generated using a Merck Hitachi D-2000 integrator. Inclusion of the internal standard in the samples allowed for correction of processing losses. These data together, with the data obtained for the external standards, and brain tissue weights were used to calculate the neurotransmitter concentration in the samples. Results are expressed as ng of dopamine, noradrenaline and 5-HT per g fresh weight of tissue (Table 2.3).

![Chromatograph of standard mixture of amines containing 2ng/20μl of each compound in HPLC Buffer containing 2ng/20μl Internal Standard (N-methyl-5-HT)](image)

<table>
<thead>
<tr>
<th>Amine</th>
<th>Retention Time (mins)</th>
<th>Peak Height</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>4.068</td>
<td>16714</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>5.539</td>
<td>10385</td>
</tr>
<tr>
<td>DOPAC</td>
<td>6.753</td>
<td>12030</td>
</tr>
<tr>
<td>Dopamine</td>
<td>8.063</td>
<td>15800</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>11.329</td>
<td>7904</td>
</tr>
<tr>
<td>HVA</td>
<td>16.017</td>
<td>4563</td>
</tr>
<tr>
<td>5-HT</td>
<td>19.879</td>
<td>9074</td>
</tr>
<tr>
<td>N-methyl 5-HT (IS)</td>
<td>24.901</td>
<td>7411</td>
</tr>
</tbody>
</table>

Table 2.3: Retention times and peak heights from the chromatogram obtained from a standard mix of biogenic amines and their metabolites.
2.2.10 Western (immuno)blotting for CREB and DARPP-32 proteins

2.2.10.1 Tissue preparation for Western (immuno)blotting

Samples were prepared by homogenizing the hypothalamus and striatal tissues in 1 ml of lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% (v/v) NP-40, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM sodium vanadate) with a polytron tissue disrupter (PT2100, Kinematica). Samples were then incubated on a rock ‘n roller for 15 minutes on ice to ensure complete lysis. The lysate was then centrifuged at 14,000 g for 15 minutes at 4°C and the supernatant was transferred to a new eppendorf. Supernatant proteins were then determined with a BCA protein assay and samples were equalized. Supernatants were diluted in a 1:1 ratio with Laemmli sample buffer (500 mM Tris-HCl (pH 6.8), 10% (w/v) SDS, 50 mM DTT, 20% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.05% (w/v) bromophenol blue) and were vortexed thoroughly. Samples were boiled for 10 minutes at 90-100°C before being aliquoted and stored at -80°C.

2.2.10.2 SDS-PAGE

Sodium dodecylsulphate-polyacrylamine gel electrophoresis (SDS-PAGE) was performed using a “BioRad” SDS-PAGE rig and electrophoresis was completed with a 10% (depending on the size of the protein of interest) separating gel and a 4% stacking gel. 10 μl aliquots of the samples (in sample buffer) were loaded into each well and 10 μl of a broad range molecular weight marker was added into the outside wells of the gel, to allow determination of the molecular weight of separated proteins. Proteins were separated out by applying a constant 50mV to the gel for 30-45 minutes in a gel electrophoresis unit with electrode running buffer (125 mM Tris base, 960 mM glycine, 0.5% (w/v) SDS).

2.2.10.3 Western (immuno)blotting

Following electrophoresis, proteins were transferred to 0.45 μm PVDF (Polyvinylidene fluoride: Immobilon P) membrane using a semi-dry blotter. The PVDF membrane and six sheets of filter paper were cut to the size of the gel. Two pieces of filter paper were
soaked in anode buffer I for at least 30 seconds. One piece of filter paper was soaked in anode buffer II for at least 30 seconds. Three pieces of filter paper were soaked in cathode buffer for at least 30 seconds.

The membrane was activated in methanol for 15 seconds. The membrane should uniformly change from opaque to semi-transparent. It was then placed in Milli-Q water and soaked for 2 minutes. Then, the membrane was soaked in anode buffer II and letted equilibrate for at least 5 minutes. A sandwich was constructed by placing two pieces of filter paper soaked in anode buffer 2 in the center of the anode electrode plate. The filter paper soaked in anode buffer II is placed on top of the first two sheets and under the PVDF membrane. The gel was positioned on top of the PVDF and the three other piece of filter paper soaked in cathode buffer was placed on top of the gel. The lid, which contains the cathode, is then placed on top of the sandwich. A current of 225 mA was applied for 90 minutes to transfer the proteins to the PVDF membrane (Figure 2.2).

![Diagram of protein transfer](image)

**Figure 2.2 Transfer proteins from gel to PVDF membrane ("Sandwich")**

After transferring, the gel was disposed of and the membrane was washed briefly in Buffer Saline Tween (PBS-T; 20mM Tris HCl (pH 7.5), 150mM NaCl, 0.05% (v/v) Tween). The membrane was then blocked for non-specific binding with 10ml of 5% (w/v) BSA in TBS-T for 90 minutes at RT. The membrane was then washed with PBS-T four times for 10-15 minutes at RT on a rock 'n roller, and probed overnight with an antibody raised against the appropriate protein at 4°C (see Table 2.4 for summary of antibodies used and appropriate dilutions). Following incubation the primary antibody was washed off as described previously, and membrane was incubated with secondary
Methods

antibody (horse-radish peroxidase (HRP)-conjugated) diluted to the requisite concentration in 2% (w/v) BSA in TBS-T for 1 hour at RT. The membrane was again washed in TBS-T and 1ml of a Supersignal chemiluminescent detection chemical was added for 1 minute. The membrane was then placed in a light proof box and exposed to photographic film (Hyperfilm) in the dark room and developed using a Fuji X-ray film processor (Model RGII, FUJI Film Medical Systems, Stanford, USA). The membrane was then stripped by incubation with 10mls of stripping solution for 10 minutes at RT and blocked as before. Following blocking, the membrane was washed as before and re-probed with another primary antibody and the procedure was completed as previously described.

Densitometry was performed on photographic film using MCID Analysis (7.0) software to quantify band intensity.

<table>
<thead>
<tr>
<th>Protein Target</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Protein Band Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-p-CREB (Ser133)</td>
<td>Rabbit polyclonal IgG (#9191, Cell signaling) 1:2000 dilution</td>
<td>Donkey anti-rabbit IgG (NA934V, Amersham) 1:20000 dilution</td>
<td>43kDa</td>
</tr>
<tr>
<td>Anti-CREB mAb</td>
<td>Rabbit polyclonal IgG (#9197, Cell Signaling) 1:2000 dilution</td>
<td>Donkey anti-rabbit IgG (NA934V, Amersham) 1:20000 dilution</td>
<td>43kDa</td>
</tr>
<tr>
<td>Anti-phospho-DARPP-32 (Thr34)</td>
<td>Rabbit polyclonal IgG (#2304, Cell Signaling) 1:2000 dilution</td>
<td>Donkey anti-rabbit IgG (NA934V, Amersham) 1:20000 dilution</td>
<td>32kDa</td>
</tr>
<tr>
<td>Anti-DARPP-32 (Thr34)</td>
<td>Rabbit polyclonal IgG (#2302, Cell Signaling) 1:2000 dilution</td>
<td>Goat anti-rabbit IgG (NA934V, Amersham) 1:20000 dilution</td>
<td>32kDa</td>
</tr>
<tr>
<td>Anti-β-Actin</td>
<td>Mouse monoclonal IgG (A5441, Sigma) 1:1000 dilution</td>
<td>Goat anti-mouse IgG (A8924, Sigma) 1:10000 dilution</td>
<td>42kDa</td>
</tr>
</tbody>
</table>

Table 2.4: Antibodies used for Western (immuno)blotting
2.2.11 Preparation of samples for real-time polymerase chain reaction (PCR)

To prepare samples for PCR, they were placed in RNase-free tubes containing 300 µL of RNAlaterTM solution and stored for two weeks at 4°C. Samples were then transferred to fresh RNase-free tubes and frozen at -80°C until RNA extraction took place.

2.2.11.1 RNA extraction procedure

RNA was extracted from sample tissue using a total RNA isolation kit (Macherey-Nagel). Briefly, samples were thawed and put in 350 µl of RA1 buffer (supplied in kit) and 3.5 µl of β-mercaptoethanol and homogenized using a polytron tissue disrupter (PT2100, Kinematica). Sample homogenate was then added to NucleoSpin® Filter units and filtered by centrifugation at 13,000 rpm for 1 minute. 350 µl of 70% ethanol was added to each sample lysate and mixed by pipetting up and down approximately 5 times. Each sample mix was placed in NucleoSpin® RNA II columns and centrifuged at 13,000rpm for 30 seconds to bind the RNA to the silica column. Following centrifugation the column was placed in a new collecting tube and 350 µl of membrane desalting buffer (supplied) was added. The column was then centrifuged at 13,000 rpm for 1 minute. DNA was digested using rDNase and DNase Reaction Buffer (supplied). rDNase was diluted 1:10 in DNase Reaction Buffer and 95µl of this solution was pipetted directly onto the centre of the silica column. Samples were incubated with DNase mix for 15 minutes at RT. 200 µl of buffer RA2 (supplied) was added to the column and centrifuged at 13,000 rpm for 30 seconds following which the column was placed in a new collecting tube. 600 µl of RA3 buffer (supplied, 50ml of ethanol added to 25 ml of RA3 buffer concentrate) was added to each column and centrifuged at 13,000 rpm for 30 seconds. The flow-through was discarded and the collecting tube reused for the second RA3 wash. 250 µl of RA3 buffer was added to each column and centrifuged at 13,000 rpm for 2 minutes. The column was placed in a fresh RNase-free microtube and RNA was eluted by addition of 60 µl of H2O and centrifugation at 13,000 rpm for 1 minute. Eluted RNA was then frozen and stored at -85°C for qualification, quantification and reverse transcription.
2.2.11.2 Assessment of RNA quality

RNA was separated on a 1% agarose gel to check integrity of extracted RNA samples. 1.3 g of agarose was added to 130 ml of TBE (1M Tris, 900mM Boric acid, 10mM EDTA) and fully dissolved by heating in microwave. The solution was allowed to cool such that container could be hand-held before 1.3 µl of ethidium bromide was carefully added and swirled to mix. The agarose solution was poured into a sealed agarose gel tray containing a comb and allowed to solidify for approximately 30 minutes. The tray was then transferred to a gel tank (OWL model B2) and covered with 1X TBE running buffer. 3 µl of RNA sample was mixed with 2 µl DEPC H₂O and 1 µl of loading dye, and 4 µl of this mixture loaded onto gel. RNA was separated using 90 volts for 30 minutes or up to a period of 90 minutes (or until yellow dye at front reached the end of the gel). The gel was then taken to a UV transilluminator and RNA was visualised. Only extracted RNA that demonstrated visible 28S and 18S ribosomal RNA bands were used as this indicated that the RNA had not been degraded during the extraction process.

2.2.11.3 RNA quantification

Optical density (OD) of RNA was measured using a spectrophotometer (UV/vis Beckman Coulter Du730) to determine RNA concentration and purity. Concentration of RNA can be measured due to its ability to absorb light at 260nm. As an OD reading of 1.0 at 260nm is equivalent to an RNA concentration of 40 µg/ml, sample RNA concentrations can be quantified using the following equation: RNA = OD\textsubscript{260} X dilution factor X 40 µg/ml. The purity of RNA may also be established by measuring absorbance at 280nm. A ratio of OD\textsubscript{260}/OD\textsubscript{280} of approx. 1.8-2.1 is indicative of pure RNA. All RNA samples used had ratios of >1.5. RNA concentrations were then equalized so that equal concentrations of RNA could be used as template for cDNA transcription. Samples were aliquoted in equal volumes until reverse- transcribed.

2.2.11.4 Reverse Transcription of RNA

A high capacity cDNA archive kit (Applied Biosystems) was used to reverse transcribe samples extracted using the Total RNA isolation kit (Macherey-Nagel). Briefly, 20-50 µl of 0.5-2.5 µg of RNA was mixed in a PCR mini-tube with an equal volume of 2X master mix (supplied) that was made up as follows: 1:5 dilution of 10X Reverse Transcription Buffer, 1:12.5 dilution of 25X dNTPs, 1:5 dilution of Random Primers,
**1:10 dilution of MultiScribe Reverse Transcriptase and 1:2.381 dilution of H2O.** Samples were then placed in thermal cycler and incubated at 25°C for 10 minutes followed by 2 hour of incubation at 37°C. Resultant cDNA was frozen at –20°C until ready for RT-PCR analysis.

### 2.2.12 Real-time PCR

Gene expression of c-fos (assay number: Rn02396759_m1) was assessed using off the shelf Taqman gene expression assays (Applied Biosystems) which contain forward and reverse primers, and a 6-carboxy fluorescein (FAM)-labelled minor groove binders (MGB) Taqman. β-actin gene expression was used to normalize gene expression between samples, and was quantified using a β-actin endogenous control gene expression assay containing specific primers, and a calibration dye for rat β-actin (order number 4352341E).

#### 2.2.12.1 Plate set-up for single target (singleplex) QPCR

Briefly, cDNA was diluted 1:5 and 10 µl of diluted cDNA was pipetted onto a PCR plate, to which 1µl of target or endogenous primer/probe and 10µl of Taqman master mix was added (21µl reaction volume). Samples were run in duplicate, and electronic pipettes (EDP3 20-200 µl, 2-20 µl and 10-100 µl) were used to ensure pipetting accuracy. Samples were placed in the real-time PCR thermocycler (Applied Biosystems 7300) using the following programme; step 1: 95°C for 10 minutes, step 2: 95°C for 15 seconds followed by 1 minute at 60°C. Step two was repeated 40 times, and fluorescence read during the annealing and extension phase (60°C) for the duration of the programme. During step two of the PCR reaction, the double stranded cDNA is denatured at 95°C for 15 seconds. As the temperature begins to fall to 60°C (annealing and extension) the target probe is first to anneal to the single-stranded cDNA as it has a higher melting temperature than the target primers (Applied Biosystems). This probe contains a FAM/VIC dye and a proprietary non-fluorescent quencher (NFQ) dye, this quencher prevents the dye from emitting a fluorescent signal by fluorescence resonance energy transfer (FRET) technology (Applied Biosystems). At 60°C the primers anneal and the strand is extended by 5' nuclease activity of the Taq polymerase. This displaces the FAM/VIC-labelled probe causing the FRET between the dye and quencher to be broken, and the generation of a fluorescent signal. Due to the specificity of the probe and primers
for the cDNA sequence, one fluorescent signal is generated for each new cDNA copy and measured during the annealing stage of the PCR cycle (60°C).

2.2.12.2 Real-time PCR analysis

The ΔΔCT method (Applied Biosystems RQ software, Applied Biosystems, UK) was used to assess gene expression for all real-time PCR analysis. This method is used to assess relative gene expression by comparing gene expression of treated/experimental samples to a normal or untreated sample (control), rather than quantifying the exact copy number of the target gene. In this manner the fold difference (increase or decrease) can be assessed between treated and control samples. The fold-difference is assessed using the cycle number (CT) difference between samples. Briefly, a threshold for fluorescence is set, against which CT is measured. To accurately assess differences between gene expressions, the threshold is set when the PCR reaction is in the exponential phase, when the PCR reaction is optimal or 100% efficient. Thus, samples with low CT readings demonstrate high fluorescence, indicating greater amplification and hence, greater gene expression. When a PCR is 100% efficient a one-cycle difference between samples means a 2-fold difference in copy number (2^1), similarly a 5-fold difference is a 32-fold difference (2^5).

To measure this fold-difference relative to control, the CT of the endogenous control (β-actin) is subtracted from the CT of the target gene for each sample, thus accounting for any difference in cDNA quantity that may exist. This normalized CT value is called the (ΔCT). The CT difference (ΔCT) of the control is subtracted from itself to give 0, and subtracted from all other samples, which is the ΔΔCT value. The ΔΔCT (cycle difference corrected for β-actin) is then converted into a fold-difference. As a one-cycle difference corresponds to a two-fold increase or decrease relative to control, 2 to the power of the -ΔΔCT (difference in control and sample CT corrected for β-actin) gives the fold-difference in gene expression between the control and treated samples. The control sample always has a ΔΔCT value of 0, thus 0^-2 gives a 2^-ΔΔCT of 1, against which all other samples are referenced, as outlined in Table 2.5 below.
**Methods**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Target CT</th>
<th>β-actin CT</th>
<th>ΔCT</th>
<th>ΔΔCT</th>
<th>$2^{-ΔΔCT}$ Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>16</td>
<td>20 - 16 = 4</td>
<td>4 - 4 = 0</td>
<td>$2^{-0} = 1$</td>
</tr>
<tr>
<td>Sample 1</td>
<td>21</td>
<td>16</td>
<td>21 - 16 = 5</td>
<td>5 - 4 = 1</td>
<td>$2^{-1} = 0.5$</td>
</tr>
<tr>
<td>Sample 2</td>
<td>22</td>
<td>16</td>
<td>22 - 16 = 6</td>
<td>6 - 4 = 2</td>
<td>$2^{-2} = 0.25$</td>
</tr>
<tr>
<td>Sample 3</td>
<td>19</td>
<td>16</td>
<td>19 - 16 = 3</td>
<td>3 - 4 = -1</td>
<td>$2^{-(1)} = 2$</td>
</tr>
<tr>
<td>Sample 4</td>
<td>18</td>
<td>16</td>
<td>18 - 16 = 2</td>
<td>2 - 4 = -2</td>
<td>$2^{-(2)} = 4$</td>
</tr>
<tr>
<td>Sample 5</td>
<td>17</td>
<td>16</td>
<td>17 - 16 = 1</td>
<td>1 - 4 = -3</td>
<td>$2^{-(3)} = 8$</td>
</tr>
</tbody>
</table>

*Table 2.5 Example of the ΔΔCT method of analysis*
2.2.13 Tissue slice superfusion.

2.2.13.1 Drug preparation

All the drugs (MDMA, caffeine, DPCPX) were dissolved in KREB’s buffer (containing 122 mM NaCl, 3.1 mM KCl, 0.4 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$(H$_2$O), 25 mM NaHCO$_3$, 10 mM Glucose, 1.3 mM CaCl$_2$, adjusted to pH 7.3 and added 10 μM pargyline).

2.2.13.2 Superfusion setup

After decapitation, brain of naive rat was quickly removed and transferred into ice-cold KREB’s buffer containing constituents listed above. Pargyline is added in KREB’s buffer to ensure that $[^{3}]$H outflow represented primarily $[^{3}]$H-DA rather than its metabolites. Either striata or hypothalamus were dissected out from the brain and cut in 750 μM-thick slices and incubated for 30 min at room temperature (21°C) in a shaker with up to 3 slices per well containing 2ml of superfusion buffer. The resulting slices were incubated with the 0.1 μM of $[^{3}]$H dopamine during 30min of incubation at room temperature. After incubation, brain slices were transferred into Brandel superfusion chambers (up to 3 slices / chamber) bound by filters and superfused continuously with drug free superfusate buffer (oxygenated KREB’s buffer) at 37°C at a flow rate of 0.75ml/min. The reaction chambers were heated using a circulating water bath through the superfusion chamber surrounding the reaction chambers (type FH15; Grant). After 1hour of superfusion, the collection of fractions was started (see Figure 2.3).

2.2.13.3 $[^{3}]$H-DA release from striatal and hypothalamic tissue slices

To determine basal $[^{3}]$H-DA outflow, superfusion was adjusted to 0.5ml/min for 8 min and 2 min-fractions were collected. During the following 32 minutes, the superfusion rate was reduced to 0.25 ml/min and slices were exposed to KREB’s buffer containing MDMA in concentrations of 0 μM, 30 μM, 100 μM or 300 μM and/or caffeine in the concentrations of 0 μM, 10 μM, 30 μM or 100 μM or DPCPX 1 μM. 4 min-fractions were collected. After the drug challenge, slices were superfused with drug free superfusate buffer for an additional 12 min at 0.5 ml/min and 2 min-fractions were collected to see if the $[^{3}]$H-DA outflow returned to basal amounts. Radioactivity in the superfusion samples and residual radioactivity in the slices/filters was measured by liquid scintillation spectroscopy (Packard Scintillation Counter Tri Carb 2100) using 5
ml counting medium at the end of the experiment. The tissue and filters were dissolved by sonication using 300 µl tissue solubiliser (Soluene 350, Packard). Fractional release is calculated by dividing the amount of radioactivity released by the residual radioactivity to correct for differences in the amount of tissue in each chamber and normalised by dividing each value by the average radioactivity in the perfusate before exposing the slices to any drug.

Figure 2.3 Tissue slice superfusion setup
2.3 Statistical analysis of results

All data was analyzed using the statistical software package GB-STAT. Statistical comparisons were performed using a one, two, or three-way analysis of variance (ANOVA) (with or without repeated measures) or a Student’s t-test, as indicated in the experimental results. If significant changes were observed, the data was further analyzed using a Student Newman-Keuls or Fishers LSD post hoc comparison test. Data were deemed significant when $P<0.05$ or $P<0.01$ where appropriate and results are expressed as means, mean change from baseline or mean percentage control with standard error of the mean (SEM).
CHAPTER 3: RESULTS

Mechanisms mediating the ability of caffeine to influence MDMA (Ecstasy)-induced hyperthermia in rats
3.1 INTRODUCTION

Recently we and others have reported that co-administration of caffeine exacerbates the acute toxicity of MDMA characterised by hyperthermia, tachycardia and lethality at higher doses (Camarasa et al., 2006; McNamara et al., 2006; McNamara et al., 2007). This is a potentially serious drug interaction especially if it is translated to humans, the mechanism of which warrants further investigation. Hyperthermia is a major feature in MDMA-induced toxicity where high body temperatures have been reported in human users (Green et al., 2003; Henry et al., 1992) which can lead to several clinical complications such as liver damage, renal failure and cardiotoxicity. Therefore, mechanisms mediating the interaction between caffeine and MDMA will be investigated by the use of body temperature record as an output.

There are several ways in which caffeine could interact with MDMA. As metabolism of both caffeine and substituted amphetamines is catalysed by hepatic cytochrome enzymes such as CYP1A2 (Carrillo et al., 2000; Maurer et al., 2000), it is possible that co-administration of caffeine could inhibit the metabolism of MDMA. Caffeine may also affect other processes including absorption, distribution or elimination processes. Importantly, MDMA has a non-linear pharmacokinetic profile, which has been attributed to a saturable or inhibitable metabolic pathway (de la Torre et al., 2004; Farre et al., 2004), making it especially vulnerable to pharmacokinetic drug-drug interactions. For this reason, the effect of co-administered caffeine on the bioavailability of MDMA was determined.

It is widely reported that the pharmacological actions of MDMA result in serotonin (5-HT) and dopamine release in several regions of the brain (El-Mallakh et al., 2007; Green et al., 2003; Gudelsky et al., 2008). Caffeine has also been reported to induce an increase in the release of 5-HT and dopamine in the cortex, hippocampus and striatum of freely-behaving rats (Acquas et al., 2002; Okada et al., 1999; Okada et al., 1997; Okada et al., 1996). It is therefore conceivable that administration of caffeine could result in an augmentation of MDMA-induced 5-HT or dopamine release. It is also noteworthy that co-administration of caffeine with amphetamine or cocaine leads to a dramatic increase in seizures and mortality in rats in comparison to administration of amphetamine or
cocaine alone (Derlet et al., 1992). As amphetamine and cocaine increase extracellular dopamine levels, such interactions support a role for dopamine as an integral factor mediating severe adverse reactions associated with the concurrent use of caffeine.

Caffeine is an adenosine receptor antagonist (Fredholm et al., 1999; Nehlig, 1999) and therefore could mediate its effect on MDMA toxicity via a dopamine dependent pathway (by either dopamine receptor activation or dopamine release). In rats, caffeine induces an increase in motor activity, blood pressure and heart rate. These effects of caffeine are mediated primarily through adenosine A1 and A2 receptors. Caffeine is also a weak inhibitor of phosphodiesterase (PDE) (Fredholm et al., 1999), and therefore may augment intracellular cAMP/cGMP concentrations following MDMA administration, subsequent dopamine release and the activation of receptors which couple to adenylate/guanylate cyclase.

This study set out to determine mechanisms that influence the ability of caffeine to exacerbate the hyperthermia associated with MDMA. The effects of MDMA alone or in combination with caffeine were examined in rats manipulated to influence central 5-HT and/or dopaminergic transmission in different ways. The roles of adenosine receptor antagonism and PDE inhibition were also assessed. The results show that both 5-HT and catecholaminergic mechanisms are relevant to the mechanism of this interaction. In addition, the ability of caffeine to exacerbate MDMA-induced hyperthermia relates to its action on adenosine A2A receptors and PDE inhibition.
Chapter 3: Experimental design

3.2 EXPERIMENTAL DESIGN

Experiments were carried out as follows:

Study 1: Can central 5-HT or catecholamine depletion influence the ability of caffeine to exacerbate MDMA-induced hyperthermia?

Study 2: Can caffeine influence the metabolism of MDMA?

Study 3: Can caffeine influence the thermoregulatory response to D-fenfluramine and D-amphetamine alone or in combination?

Study 4: Can caffeine influence the thermoregulatory response to DOI and apomorphine alone or in combination?

Study 5: Effect of receptor antagonist pre-treatment on the ability of caffeine to exacerbate MDMA-induced hyperthermia

Study 6: Can the adenosine receptor antagonists CGS 15943, DPCPX or SCH 58261 exacerbate MDMA-induced hyperthermia?

Study 7: Can the phosphodiesterase (PDE) inhibitors pentoxyfylline, rolipram or zaprinast exacerbate MDMA-induced hyperthermia?

Study 8: Can co-treatment with a combination of CGS 15943, DPCPX or SCH 58261 and rolipram influence MDMA-induced hyperthermia?

Records of body temperature were performed following the procedure detailed in the Methods section and data were analysed with a three-way ANOVA.

A summary of drug challenges, undertaken to determine the mechanisms mediating the ability of caffeine to influence MDMA-induced hyperthermia, is provided in Table 3.1.
Chapter 3: Experimental design

### Target and rationale

| Role of central 5-HT or catecholamines in mediating the ability of caffeine to exacerbate the hyperthermic response to MDMA |
| Role of central 5-HT or catecholamines in simulating the ability of caffeine to exacerbate the hyperthermic response to MDMA |
| Role of 5-HT or dopamine receptor activation in simulating the ability of caffeine to exacerbate the hyperthermic response to MDMA |
| Role of Dopamine D<sub>1</sub> receptor blockade on the ability of caffeine to exacerbate MDMA-induced hyperthermia |
| Role of 5-HT<sub>2</sub> and α<sub>1</sub> adrenoceptor blockade on the ability of caffeine to exacerbate MDMA-induced hyperthermia |
| Can adenosine receptor blockade exacerbate MDMA-induced hyperthermia? |
| Can PDE inhibition exacerbate MDMA-induced hyperthermia? |
| Can combined adenosine receptor blockade and PDE inhibition influence MDMA-induced hyperthermia? |

### Drugs

| PCPA; Reserpine in combination with α-MPT |
| 5-HT releaser: D-fenfluramine, Catecholamine releaser: D-amphetamine or D-fenfluramine & D-amphetamine in combination |
| 5-HT<sub>2</sub> receptor agonist, DOI: Dopamine receptor agonist, Apomorphine alone or in combination |
| Dopamine D<sub>1</sub> receptor antagonist, SCH 23390 |
| 5-HT<sub>2A</sub> and α<sub>1</sub> adrenoceptor antagonist, Ketanserin or 5-HT<sub>2</sub> receptor antagonist, Ritanserin or α<sub>1</sub> adrenoceptor antagonist, Prazosin |
| A<sub>1</sub> adenosine receptor antagonist CGS 15943 or A<sub>1</sub> adenosine receptor antagonist, DPCPX or A<sub>2A</sub> adenosine receptor antagonist or SCH 58261 |
| Non selective PDE inhibitor, Pentoxyffline PDE-4 inhibitor, Rolipram or PDE-5 inhibitor, Zaprinast |
| Rolipram in combination with CGS 15943, DPCPX or SCH 58261 |

### Table 3.1: Summary of drug challenges undertaken to determine the mechanisms mediating the ability of caffeine to influence MDMA-induced hyperthermia
3.3 RESULTS

Study 1: Can central 5-HT or catecholamine depletion influence the ability of caffeine to exacerbate MDMA-induced hyperthermia?

Control group-housed rats received a single administration of caffeine (10 mg/kg, i.p.) and MDMA (15 mg/kg, i.p.) alone or in combination. 24 hours following the last treatment with PCPA (50 mg/kg, i.p.) or α-MPT (50 mg/kg, i.p.), rats received a single administration of caffeine (10 mg/kg, i.p.) and MDMA (15 mg/kg, i.p.) alone or in combination. Core body temperatures were recorded 1 hour before drug administration and immediately prior to administration and 30 minutes, 1, 1.5, 2, 3 and 5 hours following drug administration. Cortical and hypothalamic tissue was obtained immediately following the last temperature measurement for the determination of 5-HT, dopamine and noradrenaline concentrations.

(a) Effect of caffeine on MDMA-induced hyperthermia

ANOVA of body temperature showed effects of MDMA \( [F(1,20) = 73.09, P<0.001] \), caffeine \( [F(1,20) = 7.47, P = 0.018] \), time \( [F(8,160) = 63.54, P<0.001] \), MDMA x time \( [F(8,160) = 19.81, P<0.001] \) and caffeine x time \( [F(8, 160) = 2.99, P<0.01] \). Post hoc comparisons revealed that MDMA increased body temperature 0.5, 1, 1.5 and 2 hours following drug administration when compared to vehicle-treated controls. Caffeine alone did not significantly alter body temperature when compared to vehicle-treated controls. Caffeine increased MDMA-induced hyperthermia 0.5, 1, 1.5, 2 and 3 hours following drug administration compared to the MDMA alone treated group (Figure 3.1).
Chapter 3: Study 1: Can central 5-HT or catecholamine depletion influence the ability of caffeine to exacerbate MDMA-induced hyperthermia?

Figure 3.1 Caffeine potentiates MDMA-induced hyperthermia. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean with standard error of the mean. *P<0.01 vs. Vehicle Control; +P<0.01 vs. Vehicle + MDMA. N= 12 animals per group.
PCPA induced a > 90% depletion of cortical and hypothalamic 5-HT. For the frontal cortex, two-way ANOVA of 5-HIAA concentrations showed an effect of PCPA \([F(1,56) = 2143, P<0.001]\), drug treatment \([F(3,56) = 104.95, P<0.001]\) and a PCPA x drug treatment interaction \([F(3,56) = 105.47, P<0.001]\). Two-way ANOVA of 5-HT concentrations revealed an effect of PCPA \([F(1,56) = 718, P<0.001]\), drug treatment \([F(3,56) = 90.9, P<0.001]\) and a PCPA x drug treatment interaction \([F(3,56) = 71.2, P<0.001]\). For the hypothalamus, two-way ANOVA of 5-HIAA concentrations showed an effect of PCPA \([F(1,56) = 645, P<0.001]\), drug treatment \([F(3,56) = 27.48, P<0.001]\) and a PCPA x drug treatment interaction \([F(3,56) = 27.24, P<0.001]\). Two-ANOVA of 5-HT concentrations showed an effect of PCPA \([F(1,56) = 452, P<0.001]\), drug treatment \([F(3,56) = 15.0, P<0.001]\) and a PCPA x drug treatment interaction \([F(3,56) = 13.0, P<0.001]\). Neither PCPA nor MDMA influenced noradrenaline or dopamine concentrations in the hypothalamus or cortex (Table 3.2).
Table 3.2: HPLC verification of the 5-HT-depleting regime using PCPA.

<table>
<thead>
<tr>
<th>Group</th>
<th>5-HIAA</th>
<th>5-HT</th>
<th>5-HIAA</th>
<th>5-HT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FRONTAL CORTEX</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>317 ± 7</td>
<td>474 ± 20</td>
<td>784 ± 71</td>
<td>1040 ± 103</td>
</tr>
<tr>
<td>Vehicle + Vehicle</td>
<td>395 ± 7</td>
<td>491 ± 25</td>
<td>986 ± 42</td>
<td>1108 ± 45</td>
</tr>
<tr>
<td>Caffeine + Vehicle</td>
<td>150 ± 9'</td>
<td>153 ± 131'</td>
<td>454 ± 28'</td>
<td>625 ± 32'</td>
</tr>
<tr>
<td>Vehicle + MDMA</td>
<td>183 ± 17</td>
<td>135 ± 25</td>
<td>502 ± 34</td>
<td>619 ± 72</td>
</tr>
<tr>
<td>Caffeine + MDMA + PCPA</td>
<td>6 ± 4''</td>
<td>36 ± 5''</td>
<td>75 ± 14''</td>
<td>120 ± 21''</td>
</tr>
<tr>
<td><strong>HYPOTHALAMUS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Not Detected</td>
<td>Not Detected</td>
<td>77 ± 8</td>
<td>100 ± 11</td>
</tr>
<tr>
<td>Vehicle + Vehicle</td>
<td>Not Detected</td>
<td>13 ± 6</td>
<td>73 ± 4</td>
<td>100 ± 10</td>
</tr>
<tr>
<td>Caffeine + MDMA</td>
<td>5 ± 3</td>
<td>9 ± 3</td>
<td>73 ± 4</td>
<td>100 ± 10</td>
</tr>
</tbody>
</table>

Animals received a pre-treatment with saline or PCPA (150 mg/kg once daily for 3 days, i.p.). On the fourth day, MDMA (15 mg/kg, i.p.) was administered to animals alone or in combination with caffeine (10 mg/kg, i.p.) or vehicle. All data expressed as mean ± SEM (ng/g brain tissue). *P<0.05 compared to vehicle-treated group; ** P<0.05 compared to vehicle-treated, non-depleted counterparts (Student Newman Keuls post hoc comparison). Differences produced by MDMA were not noted in the table.
Chapter 3: Study 1: Can central 5-HT or catecholamine depletion influence the ability of caffeine to exacerbate MDMA-induced hyperthermia?

(c) 5-HT depletion does not influence MDMA-induced hyperthermia or its exacerbation by caffeine.

ANOVA of body temperature showed effects of MDMA \([F(1,28) = 35.73, P<0.001]\), caffeine \([F(1,28) = 3.34, P = 0.018]\), time \([F(6,168) = 42.84, P<0.001]\), MDMA \(\times\) time \([F(6,168) = 36.18, P<0.001]\) caffeine \(\times\) time \([F(6,168) = 3.62, P<0.01]\) and MDMA \(\times\) caffeine \(\times\) time \([F(6,168) = 2.22, P<0.05]\). Post hoc comparisons revealed that MDMA increased body temperature 0.5, 1 and 2 hours following administration when compared to vehicle-treated controls. Caffeine alone did not significantly alter body temperature when compared to vehicle-treated controls. Caffeine increased MDMA-induced hyperthermia 1, 2 and 3 hours following drug administration (Figure 3.2).

Figure 3.2 5-HT depletion does not influence MDMA-induced hyperthermia or its exacerbation by caffeine. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean with standard error of the mean. *P<0.01 vs. Vehicle Control; +P<0.01 vs. Vehicle + MDMA. N= 6-8 animals per group.
(d) \( \alpha \)-methyl-\( p \) tyrosine and reserpine induced catecholamine depletion.

\( \alpha \)-MPT and reserpine induced a depletion of central noradrenaline and dopamine concentrations. For the frontal cortex, two-way ANOVA of cortical noradrenaline concentrations showed effects of reserpine + \( \alpha \)-MPT \( [F(1,56) = 792, P<0.001] \) and drug challenge \( [F(3,56) = 4.98, P = 0.004] \). ANOVA of cortical dopamine concentrations failed to reach significance. For the hypothalamus, two-way ANOVA of noradrenaline concentrations showed effects of reserpine + \( \alpha \)-MPT \( [F(1,56) = 74.8, P<0.001] \). ANOVA of hypothalamic dopamine concentrations showed effects of reserpine + \( \alpha \)-MPT \( [F(1,56) = 75.1, P<0.001] \). Reserpine + \( \alpha \)-MPT induced a 91% reduction in cortical noradrenaline concentrations when compared to vehicle-treated controls. Reserpine + \( \alpha \)-MPT induced a 72% reduction in hypothalamic noradrenaline concentrations when compared to vehicle-treated controls. Reserpine + \( \alpha \)-MPT induced a 70% reduction in hypothalamic dopamine concentrations when compared to their corresponding vehicle-treated controls (Table 3.3).
Chapter 3: Study 1: Can central 5-HT or catecholamine depletion influence the ability of caffeine to exacerbate MDMA-induced hyperthermia?

Table 3.3: HPLC verification of the catecholamine depleting regime using α-MPT and reserpine

<table>
<thead>
<tr>
<th>Group</th>
<th>FRONTAL CORTEX</th>
<th>HYPOTHALAMUS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NA</td>
<td>DA</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle + Vehicle</td>
<td>466 ± 12</td>
<td>240 ± 97</td>
</tr>
<tr>
<td>Caffeine + Vehicle</td>
<td>478 ± 9</td>
<td>427 ± 145</td>
</tr>
<tr>
<td>Vehicle + MDMA</td>
<td>430 ± 16</td>
<td>357 ± 108</td>
</tr>
<tr>
<td>Caffeine + MDMA</td>
<td>420 ± 32</td>
<td>351 ± 108</td>
</tr>
<tr>
<td>+ α-methyl-p-tyrosine + reserpine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle + Vehicle</td>
<td>88 ± 32**</td>
<td>168 ± 105</td>
</tr>
<tr>
<td>Caffeine + Vehicle</td>
<td>177 ± 22</td>
<td>221 ± 70</td>
</tr>
<tr>
<td>Vehicle + MDMA</td>
<td>Not detected</td>
<td>105 ± 51</td>
</tr>
<tr>
<td>Caffeine + MDMA</td>
<td>15 ± 15</td>
<td>337 ± 73</td>
</tr>
</tbody>
</table>

Catecholamine depletion was induced by administration of reserpine (5 mg/kg). 24 hours later, animals were treated with a tyrosine hydroxylase inhibitor alpha-methyl-para-tyrosine (α-MPT) administration (150 mg/kg once daily for 2 days). On the fourth day, rats received a single administration of vehicle (0.89% saline), MDMA (15 mg/kg, i.p.), caffeine (10 mg/kg, i.p.) alone or in combination with MDMA. All data expressed as mean ± SEM (ng/g brain tissue). **P<0.05 compared to vehicle-treated, non-depleted counterparts (Student Neuman-Keuls post hoc comparisons).
(e) Catecholamine depletion blocks MDMA-induced hyperthermia and its exacerbation by caffeine

ANOVA of body temperature showed effects of caffeine [F(1,28) = 5.52, P = 0.026], time [F(6,168) = 5.42, P<0.001], MDMA x time [F(6,168) = 5.74, P<0.001] and caffeine x time [F(6,168) = 3.24, P = 0.005] on body temperature. Post hoc comparisons revealed that MDMA decreased body temperature 2 hours following administration when compared to vehicle-treated controls. Caffeine alone did not significantly alter body temperature when compared to vehicle-treated controls. MDMA did not produce hypothermia in caffeine + MDMA-treated animals when compared to MDMA treatment alone (Figure 3.3).

Figure 3.3: Catecholamine depletion blocked MDMA-induced hyperthermia. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean with standard error of the mean. *P<0.01 vs. Vehicle Control. N= 8 animals per group.
Chapter 3: Study 2

Study 2: Can caffeine influence the metabolism of MDMA?

Rats received caffeine (10 mg/kg, i.p) and MDMA (15 mg/kg, i.p.) alone or in combination. Animals were euthanised by decapitation 30 minutes, 1, 2, 4, 8 and 24 hours following drug administration. Brain tissue was prepared for determination of MDMA and MDA concentrations (in ng/mg of brain tissue) as described earlier.

ANOVA of MDMA and MDA concentrations showed an effect of time only, \([F(5, 36) = 127.5, P < 0.0001]\) and \([F(5, 36) = 157, P < 0.0001]\), respectively. Brain concentrations of MDMA were maximal 30 minutes after administration, reaching over 12 ng/mg (65 nmol/g). Peak levels fell by over 50% 2 hours following administration, and only minute concentrations were quantifiable 24 hours later. Brain concentrations of MDA were maximal (16 nmol/g) 1 hour following MDMA administration and had fallen by nearly a third from peak levels at 4 hours. Concentrations of MDA were not detectable 24 hours following administration. Co-administration of caffeine did not significantly affect brain concentrations of MDMA or MDA following drug treatment, compared to MDMA administration alone (Figures 3.4A and B).
Chapter 3: Study 2: Can caffeine influence the metabolism of MDMA?

Figure 3.4 Influence of caffeine on the metabolism of MDMA. Brain tissue concentrations of (A) MDMA and (B) MDA following administration of MDMA (15 mg/kg; i.p.) alone or in combination with caffeine (10 mg/kg; i.p.). All data expressed as mean ± SEM (ng/mg of brain tissue) of 4 rats per group.
Study 3: Can caffeine influence the thermoregulatory response to D-fenfluramine and D-amphetamine alone or in combination?

We further investigated if the ability of caffeine to exacerbate MDMA-induced hyperthermia could generalise to D-fenfluramine, a synthetic amphetamine which selectively induces the release of central 5-HT. Rats received a single administration of caffeine (10 mg/kg, i.p.) and D-fenfluramine (5 mg/kg, i.p.) alone or in combination. The dose of D-fenfluramine was selected from the descending limb of the dose-related core body temperature response in rats (Cryan et al., 2000). To determine if the synergistic effects of caffeine with MDMA could generalise to D-amphetamine, which has predominant actions on central catecholamine systems, rats received a single administration of caffeine (10 mg/kg, i.p.) and D-amphetamine (5 or 15 mg/kg, i.p.) alone or in combination. As caffeine failed to influence the hyperthermic response to D-amphetamine alone, we examined the effect of co-administration of caffeine with D-fenfluramine (5 mg/kg, i.p.) in combination with D-amphetamine (1 and 2.5 mg/kg, i.p.). Animals were observed continuously following drug administration. Core body temperatures were obtained as previously described and the incidence of lethality was recorded.

(a) Co-administration of caffeine does not alter D-fenfluramine-induced hypothermia

ANOVA of body temperature showed effects of D-fenfluramine \( F(1,28) = 90.97, P<0.001 \), time \( F(8,224) = 3.4, P = 0.001 \) and a D-fenfluramine x time interaction \( F(8,224) = 33.07, P<0.001 \). Post hoc comparisons revealed that D-fenfluramine reduced body temperature 1, 1.5, 2, 3 and 4 hours following administration when compared to vehicle-treated controls. Caffeine alone did not significantly alter body temperature when compared to vehicle-treated controls. Caffeine did not influence D-fenfluramine-induced hypothermia (Figure 3.5.1).
Figure 3.5.1 Caffeine fails to influence D-fenfluramine-induced hypothermia. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 7-8 rats with standard error of the mean *P<0.01 vs. Vehicle Control.
Chapter 3: Study 3: Can caffeine influence the thermoregulatory response to D-fenfluramine and D-amphetamine alone or in combination?

(b) Co-administration of caffeine does not alter D-amphetamine-induced hyperthermia

Three-way ANOVA of body temperature showed effects of D-amphetamine \([F(2,41) = 19.07, P < 0.0001]\), time \([F(8,328) = 56.03, P < 0.0001]\) and D-amphetamine x time \([F(16,328) = 13.06, P < 0.0001]\). D-amphetamine (15 mg/kg) induced hyperthermia 0.5, 1, 1.5 and 2 hours following drug administration when compared to vehicle-treated controls. Caffeine alone, or co-injected with D-amphetamine (5 or 15 mg/kg), did not significantly alter body temperature when compared to D-amphetamine treatment alone (Figure 3.5.2).

![Figure 3.5.2 Caffeine fails to influence D-amphetamine-induced hyperthermia. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 7-8 rats with standard error of the mean *P<0.01 vs. Vehicle Control.](image)

74
Chapter 3: Study 3: Can caffeine influence the thermoregulatory response to D-fenfluramine and D-amphetamine alone or in combination?

(c) Co-administration of caffeine with D-amphetamine and D-fenfluramine is a lethal combination

The number of fatalities/the total number of subjects (and time of death following drug administration) was 0/8, 0/8 and 1/8, 0/8 and 0/8 in animals receiving D-fenfluramine (5 mg/kg) alone, D-amphetamine (5 and 15 mg/kg) alone, D-fenfluramine (5 mg/kg) in combination with D-amphetamine (5 and 15 mg/kg) respectively. Co-administration of caffeine (10 mg/kg) with D-fenfluramine (5 mg/kg) / D-amphetamine (15 mg/kg) increased the number of fatalities from 0/8 to 5/8. Seizures were not apparent in animals receiving either D-fenfluramine or D-amphetamine alone or in combination. Co-administration of caffeine with D-fenfluramine + D-amphetamine increased the incidence of seizures from 0 alone to 8/8 with caffeine (Table 3.4).

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence of lethality</th>
<th>Time of death</th>
<th>Seizure occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle + D-amphetamine (15mg/kg) + D-fenfluramine (5mg/kg)</td>
<td>0/8</td>
<td>N/A</td>
<td>None</td>
</tr>
<tr>
<td>Caffeine (10mg/kg) + D-amphetamine (15mg/kg) + D-fenfluramine (5mg/kg)</td>
<td>5/8</td>
<td>30 min</td>
<td>8/8 (30 min)</td>
</tr>
<tr>
<td>Caffeine (10mg/kg) + D-amphetamine (5mg/kg) + D-fenfluramine (5mg/kg)</td>
<td>6/8</td>
<td>30 min</td>
<td>3/8 (30 min)</td>
</tr>
<tr>
<td>Caffeine (10mg/kg) + D-amphetamine (2.5mg/kg) + D-fenfluramine (5mg/kg)</td>
<td>4/8</td>
<td>1h-2h 30 min</td>
<td>3/8 (2h)</td>
</tr>
</tbody>
</table>

Table 3.4 Caffeine induces lethality when administered with D-amphetamine and D-fenfluramine. Lethality incidence is expressed as the number of fatalities/total subjects, while time of death is given as hours (h) and minutes (min) following drug administration.
Chapter 3: Study 3: Can caffeine influence the thermoregulatory response to D-fenfluramine and D-amphetamine alone or in combination?

(d) Co-administration of caffeine exacerbates D-amphetamine + D-fenfluramine-induced hyperthermia

Three-way ANOVA of body temperature showed effects of D-amphetamine + D-fenfluramine [$F(1,28) = 14.53, P < 0.0001$], caffeine [$F(1,28) = 33.68, P < 0.0001$], D-amphetamine + D-fenfluramine x caffeine [$F(1,28) = 9.24$, time [$F(8,224) = 30.66, P<0.001$], caffeine x time [$F(8,224) = 2.08, P = 0.039$], D-amphetamine + D-fenfluramine x time [$F(8,224) = 11.08, P<0.001$], and a D-amphetamine + D-fenfluramine x caffeine x time interaction [$F(8,224) = 15.91, P<0.001$]. Post hoc comparisons revealed that the combination of D-amphetamine (1 mg/kg) + D-fenfluramine (5 mg/kg) increased body temperature 30 minutes, 1, 1.5, 2, and 3 hours after challenge when compared to vehicle-treated controls. Caffeine alone did not increase body temperature but exacerbated D-amphetamine + D-fenfluramine-induced hyperthermia 30 minutes, 1, 1.5 and 2 hours after drug administration when compared to D-amphetamine + D-fenfluramine treatment alone (Figure 3.5.3).
Figure 3.5.3 Caffeine provokes hyperthermia following treatment with a combination of D-fenfluramine and D-amphetamine. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 7-8 rats with standard error of the mean *P<0.01 vs. Vehicle Control; +P<0.01 vs. Vehicle + D-fenfluramine/D-amphetamine.
Study 4: Can caffeine influence the thermoregulatory response to DOI and apomorphine alone or in combination?

(a) Co-administration of caffeine attenuates DOI-induced hypothermia

We investigated whether the ability of caffeine to exacerbate MDMA-induced hyperthermia might be simulated by co-administration with the non-selective 5-HT$_2$ receptor agonist DOI. Rats received a single administration of caffeine (10 mg/kg, i.p.) and DOI (2 mg/kg, i.p.) alone or in combination. The dose of DOI was selected from previous reports of 5-HT$_2$ receptor-mediated effects on core body temperature in rats (Mazzola-Pomietto et al., 1995).

Three-way ANOVA of body temperature showed effects of DOI [F(1,19) = 20.78, P<0.001], caffeine [F(1,19) = 41.75, P<0.001], time [F(8,152) = 12.73, P<0.001], DOI x time [F(8,152) = 2.21, P = 0.029] and a DOI x caffeine x time interaction [F(8, 152) = 4.81, P<0.001]. Post hoc comparisons revealed that DOI decreased body temperature 1, 1.5 and 2 hours following administration when compared to vehicle-treated controls. Caffeine attenuates DOI-induced hypothermia 1, 1.5 and 2 hours following drug administration compared to DOI treatment alone (Figure 3.6.1).
Figure 3.6.1 Caffeine does influence DOI-induced hypothermia. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 4-8 rats with standard error of the mean. *P<0.01 vs. Vehicle control, +P<0.01 vs. Caffeine + Vehicle or DOI-treated groups.
Chapter 3: Study 4: Can caffeine influence the thermoregulatory response to DOI and apomorphine alone or in combination?

(b) Co-administration of caffeine did alter apomorphine-induced hypothermia.

As caffeine did influence the hypothermic response to DOI, we determined if caffeine might also influence the thermoregulatory response to the non-selective dopamine receptor agonist, apomorphine. Rats received a single administration of caffeine (10 mg/kg, i.p.) and apomorphine (1 mg/kg, i.p.) alone or in combination. The dose of apomorphine was selected from the descending limb of the dose-related core body temperature response in rats (Harkin et al., 2000).

Three-way ANOVA of body temperature showed effects of apomorphine \( [F(1,28) = 14.96, P<0.001] \), caffeine \( [F(1,28) = 9.40, P = 0.005] \), time \( [F(8,224) = 6.50, P<0.001] \), apomorphine x time \( [F(8,224) = 3.34, P = 0.001] \) and an apomorphine x caffeine x time interaction \( [F(8, 224) = 4.41, P<0.001] \). Post hoc comparisons revealed that apomorphine decreased body temperature 1 hour following administration when compared to vehicle-treated controls. Caffeine provoked hypothermia in animals 30 minutes following apomorphine administration but did not influence apomorphine-induced hypothermia 1 hour following drug administration compared to apomorphine treatment alone (Figure 3.6.2).
Chapter 3: Study 4: Can caffeine influence the thermoregulatory response to DOI and apomorphine alone or in combination?

![Graph showing temperature changes over time with different groups.](image)

**Figure 3.6.2 Caffeine influences apomorphine-induced hypothermia.** There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 4-8 rats with standard error of the mean. *P<0.01 vs. Vehicle control, +P<0.01 vs. Caffeine + vehicle or Apomorphine-treated groups.
Chapter 3: Study 4: Can caffeine influence the thermoregulatory response to DOI and apomorphine alone or in combination?

(c) Caffeine provokes hyperthermia following treatment with a combination of apomorphine and DOI.

As caffeine did influence the hypothermic response to apomorphine alone, we examined the effect of co-administration of caffeine with DOI (2 mg/kg, i.p.) in combination with apomorphine (1 mg/kg, i.p.). Core body temperatures were obtained as previously described.

Three-way ANOVA of body temperature showed effects of caffeine \([F(1,19) = 21.43, P<0.001]\), time \([F(8,152) = 21.07, P<0.001]\), DOI + apomorphine x time \([F(8,152) = 4.68, P<0.001]\), caffeine x time \([F(8,152) = 3.26, P = 0.002]\) and DOI + apomorphine x caffeine x time interaction \([F(8,152) = 4.22, P<0.001]\). Post hoc comparisons revealed that treatment with DOI + apomorphine did not alter core body temperature when compared to vehicle-treated controls. When DOI + apomorphine were co-administered with caffeine (10 mg/kg), there was an increase in body temperature 30 minutes, 1 and 1.5 hour following administration when compared to caffeine or DOI + apomorphine-treated controls (Figure 3.6.3).
Chapter 3: Study 4: Can caffeine influence the thermoregulatory response to DOI and apomorphine alone or in combination?

Figure 3.6.3 Caffeine provokes hyperthermia following treatment with a combination of DOI and apomorphine. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 4-8 rats with standard error of the mean. *P*<0.01 vs. caffeine + vehicle or DOI + Apomorphine-treated groups.


Chapter 3: Study 5

Study 5: Effect of pre-treatment with receptor antagonists on the ability of caffeine to exacerbate MDMA-induced hyperthermia.

(a) Prior administration of SCH 23390 blocks MDMA-induced hyperthermia and its exacerbation following the co-administration of caffeine

Rats were pre-treated with a single administration of SCH 23390 (1 mg/kg, i.p.), a selective dopamine D<sub>1</sub>/D<sub>5</sub> receptor antagonist, and 30 minutes later animals received a single administration of caffeine (10 mg/kg, i.p.) and MDMA (15 mg/kg, i.p.) alone or in combination. SCH 23390 has previously been shown to attenuate MDMA-induced hyperthermia in rats (Mechan et al., 2002b). Core body temperatures were recorded as previously described.

Without SCH 23390, ANOVA of body temperature showed effects of MDMA \( [F(1,36) = 54.1, \ P<0.001] \), caffeine \( [F(1,36) = 36.72, \ P<0.001] \), time \( [F(7,252) = 71.90, \ P<0.001] \), MDMA x caffeine \( [F(1,36) = 4.73, \ P = 0.036] \), MDMA x time \( [F(7,252) = 4.93, \ P<0.001] \) and caffeine x time \( [F(7, 252) = 2.33, \ P<0.001] \). Post hoc comparisons revealed that MDMA increased body temperature 0.5, 1 and 2 hours following administration when compared to vehicle-treated controls. Caffeine alone did not significantly alter body temperature when compared to vehicle-treated controls. Caffeine increased MDMA induced hyperthermia 0.5, 1, 2, 3 and 4 hours following drug administration compared to the MDMA alone treated group (Figure 3.7A).

With SCH 23390, three-way ANOVA of body temperature showed effects of MDMA \( [F(1,44) = 31.64, \ P<0.0001] \), time \( [F(7,308) = 45.97, \ P<0.0001] \), MDMA x time \( [F(7,308) = 13.48, \ P<0.0001] \) and a MDMA x caffeine x time interaction \( [F(7,308) = 4.20, \ P<0.001] \). Pre-treatment with SCH 23390 blocked any increase in core body temperature following MDMA administration alone or in combination with caffeine. Post hoc comparisons revealed that MDMA induced hypothermia 1 hour following drug administration. Caffeine alone did not significantly alter body temperature when administered alone or in combination with MDMA-treated groups (Figure 3.7B).
Chapter 3: Study 5: Effect of antagonist receptors pre-treatment on the ability of caffeine to exacerbate MDMA-induced hyperthermia

Figure 3.7: Influence of pre-treatment of dopamine D₁ antagonist SCH 23390 on MDMA-induced hyperthermia and its exacerbation by caffeine. In each experiment there was no difference in body temperature between the groups at T0 prior to challenge. Figure depicts (A) response without antagonist and (B) SCH 23390 blocks the interaction between caffeine and MDMA. Values represent mean of 7-12 rats with standard error of the mean. * P<0.01 vs. Vehicle Control. + P<0.01 vs. Vehicle + MDMA.
Chapter 3: Study 5: Effect of antagonist receptors pre-treatment on the ability of caffeine to exacerbate MDMA-induced hyperthermia

(b) Prior administration of ketanserin and prazosin, but not ritanserin, blocks MDMA-induced hyperthermia and its exacerbation following the co-administration of caffeine

Rats were pre-treated with the 5-HT₂ receptor antagonists ketanserin (5 mg/kg, i.p.) or ritanserin (1 mg/kg, i.p.) and 30 minutes later, animals received a single administration of caffeine (10 mg/kg, i.p.) and MDMA (15 mg/kg, i.p.) alone or in combination. The time interval and dose of ketanserin were selected from previous studies. We have previously reported that ketanserin (5 mg/kg) attenuates D-fenfluramine-induced hypothermia in rats (Cryan et al., 2000). Ritanserin was used at a dose of 1 mg/kg that is estimated to be 20 times the ED₅₀ for inhibiting 5-HT₂-mediated behaviours (Goodwin et al., 1985) and has previously been shown to reduce the benzodiazepine withdrawal syndrome in rats (Begg et al., 2005).

**Ketanserin:** ANOVA of body temperature showed effects of caffeine \( [F(1,28) = 6.58, P = 0.016] \), MDMA x time \( [F(8,224) = 4.55, P<0.001] \) and caffeine x time \( [F(8,224) = 7.66, P<0.001] \). Pre-treatment with ketanserin blocked MDMA-induced hyperthermia. A reduction in core body temperature was observed in the MDMA-treated group 1 hour following drug administration when compared to vehicle-treated controls. Moreover co-administration of caffeine with MDMA did not produce a change in body temperature when compared to MDMA treatment alone (Figure 3.8.1).
Chapter 3: Study 5: Effect of antagonist receptors pre-treatment on the ability of caffeine to exacerbate MDMA-induced hyperthermia

Figure 3.8.1 Prior administration of the 5HT₂₄ receptor antagonist ketanserin blocks the interaction between caffeine and MDMA. There was no difference in body temperature between the groups at T₀ prior to challenge. Values represent mean of 7-12 rats with standard error of the mean. * P<0.01 vs. Vehicle Control.
Chapter 3: Study 5: Effect of antagonist receptors pre-treatment on the ability of caffeine to exacerbate MDMA-induced hyperthermia

**Ritanserin:** ANOVA of body temperature showed effects of caffeine \([F(1,31) = 5.15, P = 0.03]\), MDMA \([F(1,31) = 73.74, P<0.001]\), time \([F(8,248) = 129.29, P<0.001]\) MDMA x time \([F(8,248) = 21.62, P<0.001]\), caffeine x time \([F(8,248) = 9.18, P<0.001]\) and MDMA x caffeine x time interaction \([F(8,248) = 3.39, P = 0.001]\). MDMA provoked an increase in core body temperature 0.5, 1, 1.5 and 2 hours post administration when compared to vehicle-treated controls. Co-treatment with caffeine potentiated this response 3 hours following MDMA administration when compared to the MDMA alone treated group (Figure 3.8.2).

![Graph showing temperature changes](image)

**Figure 3.8.2** Prior administration of the 5HT\(_{2A}\) receptor antagonist ritanserin did not block the interaction between caffeine and MDMA. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 7-12 rats with standard error of the mean. * P<0.01 vs. Vehicle Control. + P<0.01 vs. Vehicle + MDMA.
As ketanserin is known to block $\alpha_1$ adrenoceptors (McCall et al., 1984) rats were also pre-treated with the $\alpha_1$ adrenoceptor antagonist prazosin (0.2 mg/kg, i.p.) and 30 minutes later, animals received caffeine (10 mg/kg, i.p.) and MDMA (15 mg/kg, i.p.) alone or in combination. Furthermore, MDMA is known to induce effects on cardiovascular function, inducing tachycardia, and arrhythmia but also facilitates vasoconstriction (Fitzgerald et al., 1994a; Gordon et al., 1991a). Binge administration of MDMA in rats has been reported to induce cardiotoxicity (Badon et al., 2002a). It is also well established that this drug of abuse can displace noradrenaline from adrenergic nerve terminals (Fitzgerald et al., 1993a) and appears to have direct adrenergic mediating action both in the periphery (Lavelle et al., 1999) and centrally (McDaid et al., 2001).

Furthermore, Blessing et al., 2003, demonstrates that clozapine, atypical psychotic agent, established $\alpha_1$ adrenergic receptor antagonist reverses MDMA-induced increase in tail artery vasoconstriction (Blessing et al., 2003) and a previous report has shown that prazosin attenuates MDMA-induced hyperthermia in rats (Sprague et al., 2003). The rat tail is a crucial organ in the regulation of heat loss and a recent study has shown that the tail temperature was unchanged following a hyperthermic dose of MDMA (Mechan et al., 2002a). Taken together, these data suggest that MDMA might interfere with normal heat loss mechanisms via adrenergic mediated mechanisms.

**Prazosin:** ANOVA of body temperature showed effects of caffeine [$F(1,27) = 10.99, P = 0.003$], time [$F(8,216) = 3.69, P<0.001$] caffeine x time [$F(8,216) = 3.19, P = 0.002$] and MDMA x caffeine x time interaction [$F(8,216) = 2.99, P = 0.003$]. Caffeine provoked an increase in core body temperature 3 hours post administration when compared to vehicle-treated controls. MDMA provoked a decrease in core body temperature 1.5 and 2 hours post administration when compared to vehicle-treated controls. There were no other differences found between the treatment groups (Figure 3.8.3).
Chapter 3: Study 5: Effect of antagonist receptors pre-treatment on the ability of caffeine to exacerbate MDMA-induced hyperthermia

Figure 3.8.3 Prior administration of the $\alpha_1$ adrenoceptor antagonist prazosin blocks the interaction between caffeine and MDMA. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 7-12 rats with standard error of the mean. * $P<0.01$ vs. Vehicle Control. + $P<0.01$ vs. Vehicle + MDMA.
Study 6: Can the adenosine receptor antagonists CGS 15943, DPCPX or SCH 58261 exacerbate MDMA-induced hyperthermia?

Co-treatment with the adenosine receptor antagonists CGS-15943, DPCPX or SCH 58261 fails to influence MDMA-induced hyperthermia

We further investigated if the ability of caffeine to exacerbate MDMA-induced hyperthermia might be simulated by co-administration with the non-xanthine adenosine receptor antagonist CGS 15943. Rats received a single administration of CGS 15943 (10 mg/kg, i.p.) and MDMA (15 mg/kg, i.p.) alone or in combination. Core body temperatures were obtained as previously described.

**CGS-15943:** Three-way ANOVA of body temperature showed effects of MDMA \([F(1,36) = 22.25, P<0.001]\), time \([F(8,288) = 15.94, P<0.001]\) and a CGS x MDMA x time interaction \([F(8,288) = 5.20, P<0.001]\). MDMA induced hyperthermia 0.5, 1, 1.5 and 2 hours following drug administration when compared to vehicle-treated controls. CGS alone reduced body temperature 30 minutes post challenge when compared to vehicle-treated controls. Co-administration of CGS with MDMA did not significantly alter body temperature when compared to MDMA treatment alone (Figure 3.9.1).
Chapter 3: Study 6: Can the adenosine receptor antagonists CGS 15943, DPCPX or SCH 58261 exacerbate MDMA-induced hyperthermia?

Figure 3.9.1 Co-administration of CGS-15943 fails to alter MDMA-induced hyperthermia. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 5-10 rats with standard error of the mean. *P<0.01 vs. Vehicle control group.
Chapter 3: Study 6: Can the adenosine receptor antagonists CGS 15943, DPCPX or SCH 58261 exacerbate MDMA-induced hyperthermia?

As CGS 15943 failed to influence MDMA-induced hyperthermia, we next determined if co-treatment with the selective adenosine A_1 or A_2A receptor antagonists might influence the thermoregulatory response to MDMA. Rats received a single administration of the selective adenosine A_1 receptor antagonist DPCPX (1 mg/kg, i.p.) or the selective A_2A receptor antagonist SCH 58261 (2 mg/kg, i.p.) and MDMA (15 mg/kg, i.p.) alone or in combination. Dose of SCH 58261 was selected following several significant effect on parkinsonian animal model (Simola et al., 2004) and persistent persisting action after chronic ingestion of caffeine (Popoli et al., 2000).

The doses of CGS 15943 and DPCPX employed were chosen based on several previous experiments in which their central effects were investigated and demonstrated in rodents. Both antagonists possess a greater potency than caffeine as adenosine receptor antagonists (Jacobson et al., 1996; Marston et al., 1998; Ongini et al., 1999). CGS 15943 possesses some selectivity for the adenosine A_2A receptor over the adenosine A_1 receptor (Jarvis et al., 1989a; Williams et al., 1987). Like caffeine, it is a behavioural stimulant in animals: it dose dependently increases the locomotor activity of rats (Holtzman, 1991) and the rate of which squirrel monkeys press a lever to avoid an aversive stimulus (Howell et al., 1993). Unlike caffeine, CGS 15943 does not inhibit the activity of PDE (Williams et al., 1987), increasing the possibility that effects that the two drugs have in common are mediated by the blockade of adenosine receptors. SCH 58261 is known as an adenosine A_2A antagonist with moderate activity for A_1 receptors. This antagonist is responsible of neuroprotection from ischemia injury on rats by its inhibition of phosphoo-p38 MAPK (Melani et al., 2006) and prevented from quinolinic acid-induced increase in striatal BDNF and prevented increased of COX-2 expression, which expression is linked to excitotoxic processes (Minghetti et al., 2007). For the purpose of this study, we investigated a role for adenosine A_1 and A_2A receptors only, as caffeine mainly binds adenosine A_1 and A_2A receptors with high affinity compared to adenosine A_2B or A_3 receptors (Fisone et al., 2004).

**DPCPX:** Three-way ANOVA of body temperature showed effects of MDMA \( [F(1,32) = 56.69, P<0.001] \), time \( [F(8,256) = 40.18, P<0.001] \) and a DPCPX x MDMA x time interaction \( [F(8,256) = 2.76, P = 0.006] \). MDMA induced hyperthermia 0.5, 1, 1.5, 2 and 3 hours following drug administration when compared to vehicle-treated controls. DPCPX did not influence body temperature when compared to vehicle-treated controls.
Chapter 3: Study 6: Can the adenosine receptor antagonists CGS 15943, DPCPX or SCH 58261 exacerbate MDMA-induced hyperthermia?

Co-administration of DPCPX with MDMA did not significantly alter body temperature when compared to MDMA treatment alone (Figure 3.9.2).

*Figure 3.9.2 Co-administration of DPCPX fails to alter MDMA-induced hyperthermia. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 5-10 rats with standard error of the mean. *P<0.01 vs. Vehicle control group.*
Chapter 3: Study 6: Can the adenosine receptor antagonists CGS 15943, DPCPX or SCH 58261 exacerbate MDMA-induced hyperthermia?

**SCH-58261**: Three-way ANOVA of body temperature showed effects of MDMA [F(1,19) = 52.86, P<0.001], time [F(8,152) = 67.88, P<0.001], MDMA x time [F(8,152) = 19.35, P<0.001], SCH x time [F(8,152) = 3.33, P = 0.002] and SCH x MDMA x time interaction [F(8,152) = 2.14, P = 0.035]. MDMA induced hyperthermia 0.5, 1, 1.5 and 2 hours following drug administration when compared to vehicle-treated controls. Co-administration of SCH with MDMA did not significantly alter body temperature when compared to MDMA treatment alone (Figure 3.9.3).

![Graph](image)

**Figure 3.9.3** Co-administration of SCH 58261 fails to alter MDMA-induced hyperthermia. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 5-10 rats with standard error of the mean. *P<0.01 vs. Vehicle control group.
Study 7: Can the phosphodiesterase (PDE) inhibitors pentoxyfylline, rolipram or zaprinast exacerbate MDMA-induced hyperthermia?

As adenosine receptor antagonists failed to influence MDMA-induced hyperthermia we next tested to see whether the ability of caffeine to exacerbate MDMA-induced hyperthermia might be simulated by co-administration with the non-selective xanthine-based inhibitor of PDE, pentoxyfylline. Pentoxyfylline has a lower potency than caffeine as a PDE inhibitor (Kruuse et al., 2000; Meskini et al., 1994). Rats received a single administration of pentoxyfylline (50 mg/kg, i.p.) and MDMA (15 mg/kg, i.p.) alone or in combination. Core body temperatures were obtained as previously described.

**Pentoxyfylline:** ANOVA of body temperature showed effects of MDMA [$F(1,32) = 17.53, P<0.001$] and time [$F(8,256) = 32.43, P<0.001$]. MDMA induced hyperthermia 0.5 and 1 hour following drug administration when compared to vehicle-treated controls. Pentoxyfylline alone did not influence body temperature when compared to vehicle-treated controls. Co-administration of pentoxyfylline with MDMA did not significantly alter body temperature when compared to MDMA treatment alone (Figure 3.10.1).
Chapter 3: Study 7: Can the phosphodiesterase (PDE) inhibitors pentoxyfylline, rolipram or zaprinast exacerbate MDMA-induced hyperthermia?

Figure 3.10.1 Co-administration of pentoxyfylline fails to influence MDMA-induced hyperthermia. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 8-10 rats with standard error of the mean. *P<0.01 vs. Vehicle control.
Chapter 3: Study 7: Can the phosphodiesterase (PDE) inhibitors pentoxyfylline, rolipram or zaprinast exacerbate MDMA-induced hyperthermia?

As pentoxyfylline failed to influence MDMA-induced hyperthermia, we next determined whether or not co-treatment with rolipram, a potent and selective inhibitor of the PDE-4 isoform, abundant in the brain, might influence the thermoregulatory response to MDMA. Rolipram is structurally unrelated to the methylxanthines and is metabolised by a different pathway to caffeine (Bian et al., 2004; Muller et al., 1996). Symptomatology induced by systemic administration of rolipram (e.g. hypothermia, hypoactivity, forepaw shaking, grooming) have been associated with an enhanced availability of brain cAMP (Wachtel, 1982). The characteristic behavioural syndrome associated with this selective inhibitor of phosphodiesterase IV (spontaneous motility, increases maintenance activity, vocalization on touch) has been shown to be mimicked by dibutyryladenosine cAMP, an analogue of cAMP. This indicates that this syndrome may be the result of increases amount of cAMP. The behavioural effect of rolipram are also associated with enhanced central adrenergic signal transduction (Wachtel, 1982).

**Rolipram:** Three-way ANOVA of body temperature showed effects of MDMA \([F(1,32) = 52.44, P<0.001]\), time \([F(8,256) = 18.21, P<0.001]\) and a rolipram x MDMA x time interaction \([F(8,256) = 15.55, P<0.001]\). MDMA induced hyperthermia 0.5, 1, 1.5 and 2 hours following drug administration when compared to vehicle-treated controls. Rolipram alone reduced body temperature 30 minutes and 1 hour following challenge when compared to vehicle-treated controls. Co-administration of rolipram (0.5 mg/kg) with MDMA did not significantly alter body temperature when compared to MDMA treatment alone (Figure 3.10.2).
Chapter 3: Study 7: Can the phosphodiesterase (PDE) inhibitors pentoxyfylline, rolipram or zaprinast exacerbate MDMA-induced hyperthermia?

Figure 3.10.2 Co-administration of rolipram (0.5 mg/kg) fails to influence MDMA-induced hyperthermia. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 8-10 rats with standard error of the mean. *P<0.01 vs. Vehicle control.

Finally, co-administration of the archetypical PDE-5 inhibitor zaprinast allowed investigation of the functional role of PDE-5 (Lugnier, 2006) in the interaction. Rats received a single administration of rolipram (0.025 mg/kg, i.p.) or zaprinast (1 mg/kg, i.p.) and MDMA (15 mg/kg, i.p.) alone or in combination.

Zaprinast: Three-way ANOVA of body temperature showed effects of MDMA [F(1,32) = 21.16, P<0.001] zaprinast [F(1,32) = 10.94, P = 0.002] and time [F(8,256) = 34.16, P<0.001]. MDMA induced hyperthermia 0.5, 1, 1.5 and 2 hours following drug administration when compared to vehicle-treated controls. Zaprinast alone increased body temperature 90 minutes and 2 hours following drug administration when compared to vehicle-treated controls. Co-administration of zaprinast with MDMA did not significantly alter body temperature when compared to MDMA treatment alone (Figure 3.10.3).
Chapter 3: Study 7: Can the phosphodiesterase (PDE) inhibitors pentoxyfylline, rolipram or zaprinast exacerbate MDMA-induced hyperthermia?

Figure 3.10.3 Co-administration of zaprinast fails to influence MDMA-induced hyperthermia. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 8-10 rats with standard error of the mean. *P<0.01 vs. Vehicle control.
Study 8: Can co-treatment with a combination of CGS 15943, DPCPX or SCH 58261 and rolipram influence MDMA-induced hyperthermia?

As pentoxyphyline, rolipram and zaprinast alone failed to influence the hyperthermic response to MDMA, we finally examined the effect of co-administration of rolipram (0.025 mg/kg, i.p.) with each of the adenosine receptor antagonists CGS 15943 (10 mg/kg, i.p.), DPCPX (10 mg/kg, i.p.) or SCH 58261 (2 mg/kg, i.p.) in combination with MDMA (15 mg/kg, i.p.). Lower dose of rolipram (0.025 instead of 0.5mg/kg) were used in this study in order to recreate a caffeine-like effect where no rolipram-induced hypothermia will be observed. Core body temperatures were obtained as previously described.

**CGS-15943 with Rolipram:** ANOVA of body temperature showed effects of CGS/Rolipram \[F(1,28) = 173.4, P<0.001\], MDMA \[F(1,28) = 13.36, P = 0.001\], a CGS/Rolipram x MDMA interaction \[F(1,28) = 14.23, P<0.001\], time \[F(8,224) = 60.85, P<0.001\] and CGS/Rolipram x time \[F(8,224) = 40.89, P<0.001\], MDMA x time \[F(8,2244) = 2.42, P = 0.016\] and CGS/Rolipram x MDMA x time interactions \[F(8,24) = 10.43, P<0.001\]. MDMA induced hyperthermia 0.5, 1, 1.5, 2 and 3 hours following drug administration when compared to vehicle-treated controls. CGS/Rolipram alone did not influence body temperature when compared to vehicle-treated controls. Co-administration of CGS/Rolipram with MDMA potentiated MDMA-induced hyperthermia 0.5, 1, 1.5, 2 and 3 hours post administration when compared to MDMA treatment alone (**Figure 3.11.1**).
**Figure 3.11.1 Rolipram in combination with CGS 15943 exacerbates MDMA-induced hyperthermia.** There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 5-8 rats with standard error of the mean. *P<0.01 vs. Vehicle control group. + P<0.01 vs. MDMA + Vehicle-treated group.
Chapter 3: Study 8: Can co-treatment with a combination of CGS 15943, DPCPX or SCH 58261 and rolipram influence MDMA-induced hyperthermia?

DPCPX with Rolipram: ANOVA of body temperature showed effects of MDMA \[F(1,26) = 75.59, P<0.001\], time \[F(8,208) = 51.03, P<0.001\], MDMA x time \[F(8,208) = 65.31, P<0.001\], and rolipram + DPCPX x time \[F(8,208) = 9.20, P<0.001\] and rolipram + DPCPX x MDMA x time interactions \[F(8,208) = 10.07, P<0.001\]. MDMA induced hyperthermia 0.5, 1, 1.5, 2 and 3 hours following drug administration when compared to vehicle-treated controls. Rolipram + DPCPX alone reduced body temperature 0.5 and 1 hour following challenge when compared to vehicle-treated controls. Co-administration of rolipram + DPCPX with MDMA did not significantly alter body temperature when compared to MDMA treatment alone (Figure 3.11.2).

![Graph showing body temperature changes](image)

Figure 3.11.2 Rolipram in combination with DPCPX does not exacerbate MDMA-induced hyperthermia. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 5-8 rats with standard error of the mean. *P<0.01 vs. Vehicle control group.
Chapter 3: Study 8: Can co-treatment with a combination of CGS 15943, DPCPX or SCH 58261 and rolipram influence MDMA-induced hyperthermia?

**SCH-58261 with Rolipram:** ANOVA of body temperature showed effects of MDMA \( [F(1,18) = 68.09, P<0.001] \), SCH/rolipram \( [F(1,18) = 12.12, P = 0.003] \), a SCH/rolipram x MDMA interaction \( [F(1,18) = 4.46, P<0.05] \), time \( [F(8,144) = 68.53, P<0.001] \), and SCH/rolipram x time \( [F(8,144) = 22.04, P<0.001] \), MDMA x time \( [F(8,144) = 2.49, P = 0.014] \) and SCH/rolipram x MDMA x time interactions \( [F(8,144) = 2.49, P = 0.014] \). MDMA induced hyperthermia 0.5, 1, 1.5 and 2 hours following drug administration when compared to vehicle-treated controls. SCH/rolipram alone did not influence body temperature when compared to vehicle-treated controls. Co-administration of SCH/Rolipram with MDMA potentiated MDMA-induced hyperthermia 1, 1.5 2 and 3 hours post administration when compared to MDMA treatment alone (Figure 3.11.3).

![Graph](https://via.placeholder.com/150)

**Figure 3.11.3 Rolipram in combination with SCH 58261 exacerbates MDMA-induced hyperthermia.** There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 5-8 rats with standard error of the mean.

\( *P<0.01 \) vs. Vehicle control group. \( + P<0.01 \) vs. MDMA + Vehicle-treated group.
3.4 DISCUSSION

To resume, a mechanism comprising 5-HT and catecholamines is proposed to mediate MDMA-induced hyperthermia. A combination of adenosine $A_{2A}$ receptor antagonism and PDE inhibition can account for caffeine's ability to exacerbate MDMA-induced hyperthermia.

Central catecholamine depletion but not 5-HT depletion blocked MDMA-induced hyperthermia and its exacerbation by caffeine. In animal pre-treated with reserpin and $\alpha$-MPT, MDMA was inducing a transient but notable hypothermia which can be associated by the sole activation of 5-HT receptors, as MDMA is known to predominantly induce 5-HT release (Green et al., 1995) and activation of these receptors have associated with a decrease of body temperature in rodent model (Aguirre et al., 1998; Rusyniak et al., 2008; Rusyniak et al., 2007).

Caffeine provoked a hyperthermic response when the catecholamine releaser D-amphetamine was combined with the 5-HT releaser D-fenfluramine or the non selective dopamine receptor agonist apomorphine was combined with the 5-HT$_2$ receptor agonist DOI but not following either agents alone. Pre-treatment with the dopamine D$_1$ receptor antagonist SCH 23390, the 5-HT$_2$ receptor antagonist ketanserin or $\alpha_1$-adreno- receptor antagonist prazosin but not with the selective 5-HT$_2$ receptor antagonist ritanserin blocked MDMA-induced hyperthermia and its exacerbation by caffeine. Co-administration of a combination of MDMA with the PDE-4 inhibitor rolipram and the adenosine A$_{1/2}$ receptor antagonist CGS 15943 or the A$_{2A}$ receptor antagonist SCH 58261 but not the A$_1$ receptor antagonist DPCPX provoked a caffeine-like response and exacerbated MDMA-induced hyperthermia.

**Caffeine exacerbates MDMA-induced hyperthermia but does not influence brain concentrations of MDMA or its metabolite MDA.**

In line with our previous observations, caffeine exacerbates MDMA-induced hyperthermia in rats (McNamara et al., 2006). Since caffeine is a well-known inhibitor of CYP 1A2 (Carrillo et al., 2000; Kot et al., 2008) and N-demethylation of MDMA to methylenedioxyamphetamine (MDA) may be catalysed in rats and humans by CYP 1A2
(Maurer et al., 2000), it was conceivable that altered metabolism of MDMA by caffeine might lead to a higher than usual plasma concentration and account for the observed effect on core body temperature. However, co-administration of caffeine with MDMA did not influence the concentration of MDMA or its metabolite MDA in the brain. As caffeine did not alter MDMA and MDA concentrations in the brain, we propose that the interaction between the drugs is pharmacodynamic in nature.

**A role for catecholamines and 5-HT in the interaction.**

The present report provides evidence for a dual role of 5-HT and dopamine in MDMA-induced hyperthermia and its exacerbation by caffeine. Initially, depletion of endogenous catecholamines attenuated MDMA-induced hyperthermia. Furthermore, a specific role for dopamine is suggested as pre-treatment with the dopamine D₁ receptor antagonist, SCH 23390, blocked MDMA-induced hyperthermia and inhibited the ability of caffeine to exacerbate MDMA-induced hyperthermia. By contrast, depletion of central 5-HT failed to block MDMA-induced hyperthermia and exacerbation by caffeine. To further understand 5-HT and catecholaminergic mechanisms mediating the hyperthermic response to MDMA and its exacerbation by caffeine, we examined the effect of caffeine co-administered with two other amphetamines on core body temperature response. D-fenfluramine induces hypothermia in rats due to its selective interactions with the 5-HT system (Cryan et al., 1999) whereas D-amphetamine increases core body temperature in rats (Jaehne et al., 2005) via catecholamine-dependent mechanisms (Glaser et al., 2005). We first determined whether co-administration of caffeine with D-fenfluramine would provoke hyperthermia. Caffeine failed to alter the hypothermic response to D-fenfluramine, suggesting that 5-HT release does not play a role in mediating the ability of caffeine to promote hyperthermia. However, upon further exploration of the mechanisms, we observed that caffeine failed to increase D-amphetamine-induced hyperthermia and provoked an MDMA-like response only when D-amphetamine was co-administered with D-fenfluramine. Such a response indicates that, despite the lack of interaction following 5-HT depletion, 5-HT release is nevertheless an important contributing factor to the interaction between caffeine and MDMA.
Chapter 3: Discussion

As the 5-HT system plays a major role in mammalian thermoregulation and is the one of the primary targets of the substituted amphetamines, it has often been assumed that this neurotransmitter is responsible for the hyperthermia seen following MDMA administration (Shankaran et al., 1999). This theory is supported by studies showing that 5-HT$_2$ receptor antagonists can prevent MDMA-induced hyperthermia (Nash et al., 1988; Schmidt et al., 1990a). More recently however, Meehan et al. (2002) suggests that 5-HT may not be such a key player in MDMA-induced hyperthermia as pre-treatment with 5-HT$_2$ receptor antagonists such as methysergide, MDL 100,907, SB 242084 and ritanserin or the 5-HT re-uptake inhibitors zimeldine and fluoxetine failed to influence MDMA-induced hyperthermia in rats. The present study also suggests that 5-HT does not play a primary role in either MDMA-induced hyperthermia or its exacerbation by caffeine as central depletion of 5-HT did not influence the response to drug challenge. Conversely, catecholamine depletion blocked the hyperthermia and provoked a switch to hypothermia in response to MDMA. A similar finding has been previously reported by Dafters et al., in 2003, where acute co-administration of a-MPT or the dopaminergic antagonist haloperidol, reversed MDMA-induced hyperthermia to produce a hypothermic response. Interestingly, despite the profound effect of catecholamine depletion on MDMA-induced hyperthermia here, it did not fully prevent the interaction between caffeine and MDMA. As reserpine and a-MPT treatment only resulted in ~70% depletion in noradrenaline and dopamine, it is possible that the remaining catecholamine content was sufficient to mediate an interaction between caffeine and MDMA.

Dopamine is involved in thermoregulation, especially in the pre-optic area and anterior hypothalamus which are the primary loci for maintenance of body temperature (Hasegawa et al., 2005). In this regard, both dopamine D$_1$ and D$_2$ receptor subtypes are implicated in MDMA-induced changes in body temperature. MDMA-induced hypothermia in rats housed at 15°C can be blocked by pre-treatment with the dopamine D$_2$ receptor antagonist remoxipride, but not the dopamine D$_1$ receptor antagonist SCH 23390 (Green et al., 2005). This is the converse of the hyperthermic response, which is blocked by SCH 23390 but unaltered by remoxipride (Mechan et al., 2002). It has been proposed that dopamine D$_2$ receptor stimulation predominates in animals housed individually or at low ambient temperatures, which is why hypothermia is observed in such animals in response to MDMA. We have previously described how co-administration of caffeine switches the hypothermic response to MDMA in individually
housed animals to a profound hyperthermia (McNamara et al., 2006). Under such conditions caffeine may override dopamine D$_2$ receptor-mediated hypothermia, and promote a switch to D$_1$ receptor-mediated hyperthermia.

Such a mechanism is consistent with a number of responses obtained following drug challenge in the current study. MDMA provoked a hypothermic response following catecholamine depletion or dopamine D$_1$ receptor blockade. Dopamine itself has a greater affinity for dopamine D$_2$-like receptors, in particular D$_3$ and D$_4$ (Missale et al., 1998) and it is possible that in the depletion study, although there was insufficient dopamine release to provoke an overt dopamine D$_1$ receptor-mediated hyperthermic response to MDMA, a D$_2$-like hypothermia occurred instead. Co-administration with caffeine may overcome the dopamine D$_2$ receptor-mediated response by amplifying the dopamine signal. However, a switch to hyperthermia is not obtained in animals pre-treated with SCH 23390 as dopamine D$_1$ receptors are blocked under these conditions. Similar responses were obtained where the combination of D-fenfluramine and D-amphetamine provoked a hypothermic response following dopamine D$_1$ receptor blockade (Supplementary data 1) and co-administration of caffeine failed to provoke a switch to hyperthermia. As MDMA provokes the release of dopamine in the brain (Green et al., 2003), and caffeine has also been reported to influence central dopamine release (Cauli et al., 2005; Ferré, 2008), dopamine release may represent a mechanism whereby caffeine effects a change from a D$_2$ to a D$_1$ receptor-mediated response. Co-administration of caffeine with MDMA may provoke dopamine release sufficient to induce a switch from a D$_2$ receptor-mediated hypothermic response to a D$_1$ receptor-mediated hyperthermic response due to the enhanced availability of dopamine in the synapse. Further work, however, is required to clarify such a mechanism.

Despite clear effects obtained following catecholamine depletion and dopamine D$_1$ receptor blockade, and the lack of effect following central 5-HT depletion, a role for 5-HT cannot be ruled out. This is evident where the co-administration of caffeine with a combination of D-fenfluramine and D-amphetamine provokes a toxic response, akin to that described following the co-administration of caffeine with MDMA (McNamara et al., 2006). A role for 5-HT is further supported by evidence that pre-treatment with the
preferential 5-HT2 receptor antagonist ketanserin attenuates the hyperthermic response to MDMA and its exacerbation by caffeine, and that co-administration of the 5-HT- and dopamine-selective agonists, DOI and apomorphine respectively, with caffeine, provokes hyperthermia but not when either agonist is administered with caffeine alone. MDMA has direct agonist actions at 5-HT receptors which may account for its ability to provoke toxicity in the absence of endogenous 5-HT. Such actions include the ability of presynaptic 5-HT receptors to influence dopamine release and thereby augment dopamine-mediated responses to MDMA (Doly et al., 2008; Gudelsky et al., 2008). 5-HT2 receptors play an important role in the regulation of central dopaminergic function (Di Matteo et al., 2008). It is therefore not unreasonable to suggest that ketanserin may act to reduce MDMA-induced dopamine release resulting in the attenuation of MDMA-induced hyperthermia. Conversely, the effect of DOI may be accounted for an enhancement of central dopamine release.

As ketanserin is also known to interact with α1 adrenoreceptors, and α1 blockade rather than 5-HT2 blockade by ketanserin has been implicated in the physiological actions of ketanserin (Centurion et al., 2006; Orallo et al., 2000) including MDMA-induced hyperthermia (Mechan et al., 2002), we examined the effects of pre-treatment with the selective 5-HT2 receptor antagonist ritanserin and the α1 adrenoreceptor antagonist prazosin. Prior administration of ketanserin and prazosin, but not ritanserin, blocks MDMA-induced hyperthermia and its exacerbation by caffeine, suggesting that α1 adrenergic blockade plays a significant role in mediating the actions of ketanserin. In support, there is substantial evidence that noradrenaline mediates MDMA-induced hyperthermia via both peripheral and central mechanisms (Bianco et al., 1988; Sprague et al., 2004).

A role for adenosine receptors and PDE inhibition

Under normal physiological conditions, the mechanism of action of caffeine is primarily via antagonism of adenosine receptors (Ferre et al., 2008; Fisone et al., 2004; Fredholm et al., 1999). Modulation of dopamine transmission through adenosine receptors has
been implicated in the psychostimulant effects of caffeine (Cauli et al., 2005; Fuxe et al., 1998) and represents a putative mechanism whereby caffeine exacerbates MDMA-induced toxicity. Antagonistic A1-D1 and A2A-D2 heteromeric receptor complexes reduce dopamine receptor recognition, coupling and signalling in the basal ganglia. Moreover, caffeine is proposed to influence dopamine release via an adenosine A1 receptor-mediated mechanism (Cauli et al., 2005; Quarta et al., 2004; Solinas et al., 2002). In studies conducted to date, co-treatment with adenosine antagonists failed to provoke a caffeine-like interaction with MDMA, indicating that blockade of adenosine receptors alone does not mediate the interaction between caffeine and MDMA. While it has been reported that the inhibitory effect of caffeine on PDE is of little relevance at the concentrations of caffeine administered in vivo (Fredholm et al., 1999), the weak PDE-inhibiting properties of caffeine might well be relevant against a background of increased intracellular cAMP/cGMP availability following MDMA-induced biogenic amine release in the brain. Dulloo and co-workers have extensively investigated the effects of caffeine on thermogenesis induced by ephedrine. Like MDMA, ephedrine stimulates catecholamine release, its primary effect being on noradrenaline, and caffeine exacerbates ephedrine-induced hyperthermia. Following a study of the mechanisms mediating the ability of caffeine to influence the thermogenic effects of ephedrine, PDE inhibition and not adenosine receptor antagonism resulted in a potentiation of the effects of ephedrine (Dulloo et al., 1994; Dulloo et al., 1992). In the current investigation however, similar to the adenosine receptor antagonists tested, co-treatment with PDE inhibitors failed to provoke a caffeine-like interaction with MDMA, indicating that inhibition of PDE alone is unlikely to mediate the interaction between caffeine and MDMA. The lack of interaction between MDMA and the PDE-5 inhibitor zaprinast is in line with a previous study reporting that treatment with the PDE-5 inhibitor sildenafil failed to influence MDMA-induced hyperthermia in rats (Puerta et al., 2009). In a final step, to more fully simulate the pharmacology of caffeine, we combined treatment of the adenosine receptor antagonists with the PDE inhibitor rolipram and report that co-treatment with a low dose of the PDE-4 inhibitor rolipram and the non-selective adenosine receptor antagonist CGS 15943, or the selective adenosine A2A receptor antagonist SCH 58261, exacerbate MDMA-induced hyperthermia. Thus, inhibition of PDE coupled to adenosine A2A receptor blockade provokes a caffeine-like interaction with MDMA, suggesting that these targets mediate the ability of caffeine to exacerbate MDMA-induced hyperthermia.
Although the main mechanisms of action of caffeine are adenosine receptor antagonism and PDE inhibition, caffeine has also been found to increase calcium release from the sarcoplasmic reticulum through an interaction with the ryanodine receptor. Intracellular calcium release can itself induce hyperthermia and occurs in drug-induced malignant hyperthermia (Fiege et al., 2002; Penner et al., 1989). Such a mechanism may be relevant in light of human studies which have reported that MDMA intoxication and hyperthermia is associated with an elevation in myoplasmic calcium concentrations (Denborough et al., 1997). In the present study circulating concentrations of caffeine at their peak following drug administration were estimated to fall between 30-40 μM. Circulating caffeine concentrations following caffeine ingestion in humans rarely exceeds 100 μM. Whilst caffeine can mobilize intracellular calcium, such a mechanism is unlikely to be applicable either to human consumption or to the current study as a minimal concentration of 250 μM is necessary to generate detectable effects on calcium shifts (Nehlig, 1992). Moreover, caffeine at the dose given did not induce significant hyperthermia although the possibility remains that co-administration of caffeine with MDMA could influence the ability of caffeine to provoke calcium release. However, as structurally related xanthines which influence the ryanodine receptor (Xu et al., 1998), including DPCPX and pentoxyphylline, failed to influence MDMA-induced hyperthermia, it is likely that the principle mechanism by which caffeine influences MDMA-induced hyperthermia is via the proposed mechanism involving the inhibition of adenosine A$_{2A}$ receptors coupled to the inhibition of PDE.

In conclusion, the results of this study show that caffeine enhances the hyperthermic response to drugs which target both serotonergic and dopaminergic transmission but not where either system is targeted alone. Such a mechanism may account for the ability of caffeine to more readily exacerbate the acute toxicity of MDMA when compared to other amphetamines. The ability of caffeine to exacerbate MDMA-related hyperthermia may be associated to inhibitory actions on adenosine A$_{2A}$ receptors and PDE-4. Determination of the mechanisms mediating the toxicity associated with co-ingestion of caffeine with MDMA is an important step towards the treatment of severe hyperthermic reactions to Ecstasy that can occur in some users. In accordance with our results and due to the fact that agents such as prazosin and ketanserin are available for human therapy, such agents or similar may be useful candidates for testing in the treatment of hyperthermia associated with MDMA ingestion alone or in combination with caffeine.
CHAPTER 4

RESULTS

Caffeine promotes a switch from dopamine D$_2$ to dopamine D$_1$ receptor-mediated heart rate, body temperature and behavioural responses to MDMA ("Ecstasy") administration to rats.
MDMA provokes notable behavioural effects such as enhanced locomotor activity (Atkins et al., 2009; Spanos et al., 1989) and physiological effects including changes in core body temperature (Dumont et al., 2006; Gowing et al., 2002), blood pressure and heart rate (Badon et al., 2002). As MDMA induces the release of central monoamine neurotransmitters (Green et al., 2004), many studies have reported a role for 5-HT, dopamine and noradrenaline in these responses (Callaway et al., 1992b; Green et al., 2004; Rusyniak et al., 2007). MDMA induced 5-HT release has been extensively studied in both human and animal models. As 5-HT2 receptors play an important role in the regulation of central dopaminergic function (Di Matteo et al., 2008) and MDMA-induced dopamine release has been shown to be 5-HT dependent (Koch et al., 1997; Green et al., 2003; Gudelsky et al., 2008) such actions implicate dopamine as a potential mediator of MDMA-induced toxicity (Doly et al., 2008; Gudelsky et al., 2008).

As previously described, MDMA may be consumed alone or in combination with other drugs including caffeine, which can influence its psychostimulant and physiological effects (Gouzoulis-Mayfrank et al., 2006; Scholey et al., 2004; Winstock et al., 2001). Caffeine has multiple effects on behaviour, cardiovascular and body temperature regulation (Armstrong et al., 2007; Cornells et al., 2007; Nawrot et al., 2003). Recently, we and others have reported that co-administration of caffeine exacerbates the acute toxicity of MDMA in rats, which is characterised by hyperthermia, tachycardia and lethality at higher doses (Camarasa et al., 2006; McNamara et al., 2006; McNamara et al., 2007). This is a serious drug interaction, the mechanism of which warrants further investigation.

A role for the catecholamines, and in particular dopamine, has been proposed to mediate the ability of caffeine to influence MDMA-induced hyperthermia in rats. Vanattou-Saïfoudine and co-workers (2010) reported that central catecholamine depletion but not 5-HT depletion blocked MDMA-induced hyperthermia in rats and its exacerbation by caffeine. Moreover, pre-treatment with the dopamine D1 receptor antagonist SCH 23390...
Chapter 4: Introduction

blocked MDMA-induced hyperthermia and its exacerbation by caffeine. It is well known that dopamine is involved in core body temperature regulation by the pre-optic area and anterior hypothalamus (Hasegawa et al., 2005). In this regard, MDMA-induced changes in core body temperature involve both dopamine D₁ and D₂ receptors. MDMA-induced hypothermia in rats housed individually or at low ambient temperatures (15°C) can be blocked by pre-treatment with the dopamine D₂ receptor antagonist remoxipride, but not by the dopamine D₁ receptor antagonist SCH 23390 (Green et al., 2005) Conversely, Meehan and co-workers (2002) have shown that MDMA-induced hyperthermia in group-housed animals can be blocked by SCH 23390 but not by remoxipride. Thus, a leading role for dopamine D₂ and D₁ receptors in MDMA-induced hypo- and hyperthermia, respectively, has been proposed.

The role of dopamine in mediating the cardiovascular effects of MDMA, either alone or in combination with caffeine is less well characterised, but in both animal and human studies, MDMA has been found to induce cardiovascular toxicity (Badon et al., 2002b; Burgess et al., 2000; Downing et al., 1985; Milroy et al., 1996). It has been established that dopamine plays an important role in cardiovascular homeostasis mediated through α and β adrenergic receptors, but also dopamine D₁ and D₂ receptors which are found in the heart and in the vascular system (Cavero et al., 1982) and play a role in the regulation of heart rate. Dopamine D₂ receptor agonists including bromocriptine and pergoline provoke a decrease in heart rate whereas dopamine D₁ receptor agonists including fenoldopam or SKF 3893 induce an increase in heart rate in addition to a decrease in peripheral resistance and reduced arterial pressure leading to a further increase in heart rate (Velasco et al., 1998).

Dopamine receptor agonists evoke a complex locomotor and stereotypic response in laboratory animals (Jackson et al., 1994; Kiyatkin, 2002; Wise et al., 1987). For example, the non-selective dopamine receptor agonist apomorphine reduces locomotor activity at low doses but at higher doses produces hypermotility and stereotypy. The reduction in locomotor activity is believed to reflect the activation of presynaptic dopamine D₂ autoreceptors and a consequent attenuation of presynaptic dopamine release whereas apomorphine-induced hypermotility is mediated by postsynaptic
dopamine $D_{1/2}$ receptors. Dopamine antagonists such as haloperidol and sulpiride can block these locomotor effects and inhibit stereotyped behaviours when apomorphine activates postsynaptic dopamine receptors (Harkin et al., 2000). MDMA administration produces a dose-dependent hyperlocomotor response together with 5-HT syndrome. The role of 5-HT in the hyperlocomotor response to MDMA has been investigated extensively and 5-HT$_{1B}$, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor subtypes have been shown to influence the locomotor effects of MDMA (Green et al., 2003). In addition blockade/inhibition of dopamine $D_1$ and $D_2$ receptors significantly attenuates MDMA-induced locomotor activity (Ball et al., 2003; Benturquia et al., 2008; Risbrough et al., 2006).

We have previously described how co-administration of caffeine switches the hypothermic response to MDMA to a profound hyperthermia in individually-housed animals (McNamara et al., 2006). In addition, caffeine provokes a profound tachycardia when co-administered with MDMA (McNamara et al., 2007). As a role for dopamine receptors has been established in mediating MDMA-induced changes in body temperature, and caffeine is known to influence dopamine neurotransmission in the brain, we investigated if caffeine may override dopamine $D_2$ receptor-mediated hypothermia and promote a switch to dopamine $D_1$ receptor-mediated hyperthermia.

While central 5-HT depletion is insufficient to block MDMA-induced hyperthermia, or the ability of caffeine to exacerbate MDMA-induced hyperthermia a role for 5-HT cannot be ruled out. This is evident where the co-administration of caffeine with a combination of the 5-HT releasing agent D-fenfluramine and the catecholamine releasing agent D-amphetamine provokes a hyperthermic response similar to that described following the co-administration of caffeine with MDMA. Moreover, pre-treatment with the preferential 5-HT$_2$ receptor antagonist ketanserin attenuates the hyperthermic response to MDMA and its exacerbation by caffeine, and co-administration of the 5-HT and dopamine selective agonists DOI and apomorphine, respectively, with caffeine, provokes hyperthermia but not when each agonist is administered with caffeine alone (Vanattou-Saifoudine et al., 2010).
In tandem, through the use of passive automated monitoring facilitated by implantable bioradiotelemetric devices, the role of dopamine D<sub>1</sub>, D<sub>2</sub> and 5-HT<sub>2</sub> receptors in mediating changes in body temperature, heart rate and locomotor activity responses to caffeine and MDMA alone, and in combination, were determined. Our results support a mechanism consistent with a caffeine-induced switch from dopamine D<sub>2</sub>- to D<sub>1</sub>-mediated body temperature and heart rate responses following MDMA administration. A role for 5-HT<sub>2</sub> receptors is also described in MDMA-induced changes in heart rate and caffeine and MDMA-induced hyperthermia.
Heart rate, body temperature and locomotor activity were recorded by radiotelemetry (see **Methods** Chapter section Physiological assessments) as previously described (Harkin *et al.*, 2002; McNamara *et al.*, 2006; 2007). Following the surgical implantation of these devices animals were individually-housed and permitted a 2 weeks-recovery period prior to experimentation. Due to the abdominal position of the devices, all injections have been performed sub-cutaneously in this chapter. In our laboratory, we have previously observed hypothermia in MDMA-treated animals in individually housed condition and for those animals, temperatures were measured rectally which led us to the conclusion that the change in method of measurement was not the cause under which hypothermia occurs. Moreover, studies have shown that a dose of 10 mg/kg of MDMA induced hyperthermia in grouped housed rats (Blessing *et al.*, 2003; Colado *et al.*, 1995) emphasising that modifying housing rather than modifying the dose is the cause of the change from hyper- to hypothermia. The dose of MDMA was selected based on previous work performed previously in our laboratory in individually housed animals (McNamara *et al.*, 2007).

Experiments were carried out as follows:

**Study 1: Caffeine promotes hyperthermia and tachycardia following co-administration with MDMA.**

Rats received a single administration of caffeine (10 mg/kg, s.c.) and MDMA (10 mg/kg, s.c.) alone or in combination. Core body temperature, heart rate and locomotor activity were recorded, every 5 minutes, 1 hour prior to and for 4 hours following drug administration. Doses of caffeine and MDMA were chosen from previous reports of the interaction between caffeine and MDMA in rats (McNamara *et al.*, 2006; 2007).

**Study 2: Effect of SCH 23390 on the ability of caffeine to promote hyperthermia and tachycardia following co-administration with MDMA.**

Rats were pre-treated with a single administration of SCH 23390 (1 mg/kg, s.c.) and 30 minutes later, animals received a single administration of caffeine (10 mg/kg, s.c.) and MDMA (15 mg/kg, s.c.) alone or in combination. Core body temperature, heart rate and
locomotor activity were recorded, every 5 minutes, 1 hour prior to and for 4 hours following drug administration. The selective dopamine D_{1/5} antagonist, SCH 23390, was used in this study at a dose of 1 mg/kg which blocks MDMA-induced hyperthermia and its exacerbation by caffeine in group-housed rats (Vanattou-Saifoudine et al., 2010).

**Study 3:** *Effect of sulpiride on the ability of caffeine to promote hyperthermia and tachycardia following co-administration with MDMA*

To further investigate the role of dopamine D_{2} receptors, rats were pre-treated with a single administration of the dopamine D_{2} antagonist sulpiride (100 mg/kg, s.c.), and 30 minutes later animals received a single administration of caffeine (10 mg/kg, s.c.) and MDMA (10 mg/kg, s.c.) alone or in combination. Core body temperature, heart rate and locomotor activity were recorded, every 5 minutes, 1 hour prior to and for 4 hours following drug administration. Sulpiride is a highly selective antagonist commonly used to block central dopamine D_{2} receptor-mediated responses in rats at parenteral doses up to 100 mg/kg (Andersen, 1986; Kaneno et al., 2001; O'Tuathaigh et al., 2004). Shioda and co-workers (2008) have previously reported, by contrast to SCH 23390 (0.5 mg/kg), that pre-treatment with sulpiride (50 mg/kg) does not prevent MDMA-induced hyperthermia. In the present study a dose of 70 mg/kg of sulpiride was initially tested, where sulpiride blocked MDMA-induced hypothermia, and provoked a switch to hyperthermia. However the ability of caffeine to potentiate MDMA-induced hyperthermia, following pre-treatment with sulpiride, was not apparent (Supplementary data 2). A higher dose of supiride (100 mg/kg) was subsequently tested and the results from this challenge are presented here.

**Study 4:** *Effect of caffeine on the response to DOI and apomorphine alone or in combination.*

MDMA is known to induce the release of serotonin and dopamine. Thus, we proposed that the ability of caffeine to exacerbate MDMA-induced toxicity might be simulated by co-administration with the non-selective 5-HT_{2} receptor agonist DOI. Rats received a single administration of caffeine (10 mg/kg, s.c.) and DOI (2 mg/kg, s.c.) alone or in combination. As caffeine failed to influence the hypothermic and the bradycardic
responses to DOI, we determined if caffeine might influence the responses to the non-selective dopamine receptor agonist, apomorphine. Rats received a single administration of caffeine (10 mg/kg, s.c.) and apomorphine (1 mg/kg, s.c.) alone or in combination. As caffeine failed to influence the response to apomorphine, to more closely simulate the conditions following MDMA administration, we further investigated the effect of co-administration of caffeine with a combined challenge of DOI and apomorphine. Core body temperatures, heart rate and locomotor activity were obtained as previously described.
Study 5: Effect of pre-treatment with ketanserin on the ability of caffeine to promote hyperthermia and tachycardia following co-administration with MDMA.

Rats were pre-treated with the 5-HT$_2$ receptor antagonist ketanserin (5 mg/kg, s.c.) and 30 minutes later, animals received a single administration of caffeine (10 mg/kg, s.c.) and MDMA (10 mg/kg, s.c.) alone or in combination. As ketanserin is known to block $\alpha_1$ adrenoceptors (McCall and Schuette, 1984), non-implanted but individually housed animals were pre-treated with the $\alpha_1$ adrenoceptor antagonist prazosin (0.2 mg/kg, s.c.) and 30 minutes later animals received caffeine (10 mg/kg, s.c.) and MDMA (10 mg/kg, s.c.) alone or in combination. Pre-treatment with prazosin failed to influence the ability of caffeine to provoke a switch from MDMA-induced hypothermia to MDMA-induced hyperthermia (Supplementary data 3).
4.3 RESULTS

The results of this study can be summarised as follows:

(1) MDMA (10 mg/kg) induced hypothermia, reduced heart rate and increased locomotor activity. Caffeine (10 mg/kg) alone did not influence these parameters whereas co-administration of caffeine with MDMA provoked a switch from MDMA-induced hypothermia and bradycardia to hyperthermia and tachycardia without influencing MDMA-induced hyperlocomotion.

(2) Pre-treatment with SCH 23390 (1 mg/kg) blocked MDMA-induced hyperactivity, enhanced MDMA-induced hypothermia and blocked the ability of caffeine to provoke a switch from MDMA-induced hypothermia to hyperthermia. Furthermore, a persistent increase in heart rate was observed in SCH 23390-treated animals following MDMA administration, which was not apparent when caffeine was co-administered with MDMA.

(3) Pre-treatment with sulpiride (100 mg/kg) blocked MDMA-induced hypothermia and induced a late onset hyperthermia. Furthermore, bradycardia observed in MDMA-treated animals switched to a persistent tachycardia when pre-treated with sulpiride. Finally sulpiride increased locomotor activity in the control, caffeine and MDMA-treated groups. Caffeine produced a delayed enhancement of MDMA-induced hyperthermia but did not influence MDMA-induced changes in heart rate and locomotor activity in sulpiride pre-treated animals.

(4) Caffeine did not influence either DOI (2 mg/kg) or apomorphine (10 mg/kg) responses on body temperature and heart rate, while DOI- and apomorphine-induced MDMA-like effects (such as reduced heart rate and hypothermia) were reduced by co-administration of caffeine.

(5) Ketanserin (5 mg/kg) blocked the ability of caffeine to promote a switch from MDMA-induced hypo- to hyperthermia yet provoked a sustained increase in body temperature in caffeine-treated animals. Ketanserin also inhibited the ability of MDMA
to reduce heart rate and provoked an increase in locomotor activity in caffeine, MDMA and MDMA/caffeine-treated groups when compared to the appropriate-treated group.

**Study 1: Role of dopaminergic receptors (D₁ and D₂) and serotonergic receptors (5HT₂) on MDMA and caffeine-induced body temperature changes.**

Body temperature was monitored for 1 hour pre- and for 4 hours post-challenge with MDMA (10 mg/kg), DOI (2 mg/kg) and apomorphine (1 mg/kg), alone or in combination with caffeine (10 mg/kg). Pre-treatments with SCH 23390 (1 mg/kg), sulpiride (100 mg/kg) and ketanserin (5 mg/kg) were administered 30 min prior to caffeine/MDMA challenge.

**Caffeine/MDMA:** ANOVA of change in body temperature showed an effect of caffeine \([F(1,16) = 10.36, P = 0.005]\), MDMA x caffeine \([F(1,16) = 5.43, P = 0.033]\), time \([F(49,784) = 3.49, P<0.001]\), MDMA x time \([F(49,784) = 10.12, P<0.001]\), caffeine x time \([F(49,784) = 2.79, P<0.001]\) and a MDMA x caffeine x time interaction \([F(49,784) = 1.62, P = 0.005]\). Post hoc comparisons revealed that treatment with MDMA reduced body temperature 30 minutes following drug challenge for 25 minutes compared to vehicle-treated controls \((P<0.01)\). Co-administration with caffeine attenuated MDMA-induced hypothermia for up to 35 minutes following drug administration that switched to hyperthermia for the remainder of the trial compared to caffeine or MDMA treatments alone \((P<0.01)\). Caffeine treatment alone failed to influence body temperature when compared to vehicle-treated controls (Figure 4.1.1).
Chapter 4: Study 1: Role of dopaminergic receptors ($D_1$ and $D_2$) and serotonergic receptors ($5HT_2$) on MDMA and caffeine-induced body temperature changes.

Figure 4.1.1 Hyperthermic response to MDMA following co-administration with caffeine. Values represent mean with standard error of the mean change from baseline of 5-6 animals. Baseline averages were as follows: Vehicle + Vehicle, $37.2 \pm 0.4$ °C; Caffeine + Vehicle, $36.7 \pm 0.2$ °C; Vehicle + MDMA, $36.8 \pm 0.2$ °C; Caffeine + MDMA, $36.8 \pm 0.2$ °C. Prior to drug challenge there was no baseline difference found in body temperature between the treatment groups.
Chapter 4: Study 1: Role of dopaminergic receptors ($D_1$ and $D_2$) and serotonergic receptors ($5HT_2$) on MDMA and caffeine-induced body temperature changes.

**SCH 23390 pre-treatment:** ANOVA of change in body temperature showed an effect of MDMA [$F(1, 20) = 35, P<0.001$], time [$F(49,980) = 26.63, P<0.001$], MDMA x time [$F(49,980) = 23.76, P<0.001$] and caffeine x time [$F(49,980) = 4.68, P<0.001$] only. Post hoc comparisons revealed that MDMA reduced body temperature in SCH 23390 pre-treated animals, 15 minutes following MDMA administration and for 3 hours compared to vehicle-treated controls ($P<0.01$). Co-administration of caffeine attenuated MDMA-induced hypothermia 1 hour and 40 minutes following drug administration and for the remainder of the trial when compared to MDMA treatment alone ($P<0.01$). Caffeine alone did not influence core body temperature (Figure 4.1.2).

**Figure 4.1.2** Prior treatment with SCH 23390 attenuates the ability of caffeine to promote hyperthermia following co-administration with MDMA. Values represent mean with standard error of the mean change from baseline of 5-6 animals. Baseline of 1 hour points averages prior any drug challenge were as follows: Vehicle + Vehicle, 36.6 ± 0.25 °C; Caffeine + Vehicle, 37.1 ± 0.12 °C; Vehicle + MDMA, 36.9 ± 0.19 °C; Caffeine + MDMA, 37 ± 0.17 °C. Prior to drug challenge there was no baseline difference found in body temperature between the treatment groups.
Chapter 4: Study 1: Role of dopaminergic receptors (D₁ and D₂) and serotonergic receptors (5HT₂) on MDMA and caffeine-induced body temperature changes.

**Sulpiride pre-treatment**: ANOVA of change in body temperature showed an effect of MDMA \([F(1, 20) = 23.63, P<0.001]\), caffeine \([F(1, 20) = 8.53, P = 0.008]\), time \([F(49,980) = 5.57, P<0.001]\), MDMA x time \([F(49,980) = 13.52, P<0.001]\) and caffeine x time \([F(49,980) = 1.75, P = 0.001]\) only. Post hoc comparisons revealed that treatment with caffeine increased body temperature in sulpiride pre-treated animals 3 hours post-drug administration when compared to vehicle-treated controls \((P<0.01)\). Treatment with MDMA increased body temperature in sulpiride pre-treated animals 2 hours and 40 minutes post-drug challenge and for the remainder of the trial when compared to vehicle-treated controls \((P<0.01)\). By contrast to the temperature response presented in Figure 4.1.1, pre-treatment with sulpiride attenuated MDMA-induced hypothermia. Co-administration of caffeine with MDMA in sulpiride pre-treated animals promoted MDMA-induced hyperthermia 2 hours and 5 minutes post-drug challenge and for 20 minutes compared to MDMA treatment alone \((P<0.01)\) (Figure 4.1.3).

![Figure 4.1.3](image-url)  
*Figure 4.1.3 Prior treatment with sulpiride promotes hyperthermia in response to MDMA administration alone or in combination with caffeine. Values represent mean with standard error of the mean change from baseline of 5-6 animals. Baselines of 1 hour points average prior any drug challenge were as follows: Vehicle + Vehicle, 36.9 ± 0.2 °C; Caffeine + Vehicle, 36.9 ± 0.1 °C; Vehicle + MDMA, 36.6 ± 0.1 °C; Caffeine + MDMA, 36.7 ± 0.14 °C. Prior to drug challenge there was no baseline difference found in body temperature between the treatment groups.*
Chapter 4: Study 1: Role of dopaminergic receptors (D₁ and D₂) and serotonergic receptors (5HT₂) on MDMA and caffeine-induced body temperature changes.

Apomorphine: ANOVA of change in body temperature showed an effect of apomorphine \[ F(1, 16) = 8.31, P = 0.01 \], time \[ F(49,784) = 1.98, P<0.001 \] and apomorphine x time \[ F(49,784) = 5.17, P<0.001 \] only. Post hoc comparisons revealed that treatment with caffeine increased body temperature 1 hour following drug administration and for 50 minutes compared to control-treated rats (P<0.01). Apomorphine decreased body temperature 5 minutes following drug administration and for 40 minutes when compared to vehicle-treated group (P<0.01). Apomorphine-induced hypothermia was not affected by caffeine treatment when compared to apomorphine treatment alone (Figure 4.1.4).

![Figure 4.1.4 Effect of apomorphine, alone or in combination with caffeine, on body temperature.](image)

Values represent mean with standard error of the mean change from baseline of 5 animals. Baseline averages for body temperature were as follows: Vehicle + Vehicle, 36.4 ± 0.1 °C; Caffeine + Vehicle, 36.3 ± 0.1 °C; Vehicle + Apomorphine, 36.6 ± 0.2 °C; Caffeine + Apomorphine, 36.6 ± 0.1 °C. Prior to drug challenge there was no baseline difference found in body temperature between the treatment groups.
Chapter 4: Study 1: Role of dopaminergic receptors ($D_1$ and $D_2$) and serotonergic receptors ($5HT_2$) on MDMA and caffeine-induced body temperature changes.

**DOI:** ANOVA of change in body temperature showed an effect of caffeine [$F(1, 8) = 5.6, P = 0.04$], time [$F(49,392) = 15.8, P<0.001$] and DOI x time [$F(49,392) = 2.39, P<0.001$] only. Post hoc comparisons revealed that neither caffeine nor DOI influenced body temperature (Figure 4.1.5).

![Figure 4.1.5 Effect of DOI, alone or in combination with caffeine, on body temperature. Values represent mean with standard error of the mean change from baseline of 3 animals. Baseline averages for body temperature were as follows: Vehicle + Vehicle, 36.7 ± 0.1 °C; Caffeine + Vehicle, 36.8 ± 0.1 °C; Vehicle + DOI, 36.7 ± 0.3 °C; Caffeine + DOI, 36.8 ± 0.2 °C. Prior to drug challenge there was no baseline difference found in body temperature between the treatment groups.](image-url)
Chapter 4: Study 1: Role of dopaminergic receptors (D₁ and D₂) and serotonergic receptors (5HT₂) on MDMA and caffeine-induced body temperature changes.

**Apomorphine/DOI:** ANOVA of change in body temperature showed an effect of apomorphine/DOI [F(1, 18)= 30.33, P<0.001], caffeine [F(1,18)= 14.47, P<0.001], time [F(49,882) = 37.8, P<0.001], apomorphine/DOI x time [F(49,882) = 8.95, P<0.001], caffeine x time [F(49,882) =1.99, P<0.001] and apomorphine/DOI x caffeine x time interaction [F(49,882)= 2.69, P<0.001]. Post hoc comparisons revealed that caffeine increased body temperature 45 minutes following drug administration and for 65 minutes compared to vehicle-treated group (P<0.001). Co-administration of apomorphine with DOI reduced body temperature 15 minutes post-drug administration and for the remainder of the trial, when compared to vehicle-treated group (P<0.001). Co-administration of caffeine in apomorphine/DOI-treated rats attenuated apomorphine/DOI-induced hypothermia 45 minutes following drug administration and for the remainder of the trial compared to apomorphine/DOI-treated rats (P<0.001) (Figure 4.1.6).

![Figure 4.1.6](image)

**Figure 4.1.6** Effect of apomorphine and DOI, alone or in combination with caffeine, on body temperature. Values represent mean with standard error of the mean change from baseline of 5-6 animals. Baseline averages for body temperature were as follows: Vehicle + Vehicle, 36.4 ± 0.1 °C; Caffeine + Vehicle, 36.6 ± 0.1 °C; Vehicle + Apomorphine/DOI, 36.7 ± 0.1 °C; Caffeine + Apomorphine/DOI, 36.7 ± 0.1 °C. Prior to drug challenge there was no baseline difference found in body temperature between the treatment groups.
Chapter 4: Study 1: Role of dopaminergic receptors (D₁ and D₂) and serotonergic receptors (5HT₂) on MDMA and caffeine-induced body temperature changes.

Ketanserin pre-treatment: ANOVA of change in body temperature showed an effect of time [F(49,392) = 1.93, P<0.001], MDMA x time [F(49,392) = 15.88, P<0.001] and caffeine x time [F(49,392) = 2.17, P<0.001] only. Post hoc comparisons revealed that caffeine increased body temperature in ketanserin-treated animals 30 minutes following drug administration and for 30 minutes compared to the vehicle-treated group (P < 0.01). Administration of MDMA decreased body temperature 5 minutes following drug administration and for 40 minutes when compared to the vehicle-treated group (P<0.001). Caffeine did not alter MDMA-induced hypothermia in ketanserin-treated animals (Figure 4.1.7).

![Figure 4.1.7](image)

**Figure 4.1.7** Prior administration of ketanserin blocks the switch from MDMA-induced hypo- to hyperthermia following co-administration with caffeine. Baseline of 1 hour points averages prior any drug challenge were as follows (3 animals): Vehicle + Vehicle, 37.9 ± 0.4 °C; Caffeine + Vehicle, 36.7 ± 0.2 °C; Vehicle + MDMA, 36.8 ± 0.2 °C; Caffeine + MDMA, 37.2 ± 0.1 °C. Prior to drug challenge there was no baseline difference found in body temperature between the treatment groups.
Study 2: Effect of MDMA and caffeine on heart rate in rats following pre-treatment with SCH 23390, sulpiride and ketanserin. Does caffeine influence DOI/apomorphine-induced heart rate changes?

Heart rate was monitored for 1 hour pre- and for 4 hours post-challenge with MDMA (10 mg/kg), DOI (2 mg/kg) and apomorphine (1 mg/kg), alone or in combination with caffeine (10 mg/kg). Pre-treatments with SCH 23390 (1 mg/kg), sulpiride (100 mg/kg) and ketanserin (5 mg/kg) were administered 30 min prior to caffeine/MDMA challenge.

MDMA/Caffeine: ANOVA of change in heart rate showed an effect of MDMA [F(1,16) = 9.04, P = 0.008], caffeine [F(1,16) = 13.83, P = 0.001], time [F(49,784) = 7.14, P<0.001], MDMA x time [F(49,784) = 14.2, P<0.001], caffeine x time [F(49,784) = 2.67, P<0.001] only. Post hoc comparisons revealed that MDMA increased heart rate 2 hours and 20 minutes following drug challenge and for 50 minutes when compared to vehicle-treated controls (P<0.01). Co-administration with caffeine influenced MDMA-induced change in heart rate and provoked an increase in heart rate 1 hour and 15 minutes post-challenge and for the remainder of the trial when compared to caffeine or MDMA treatments alone (P<0.01). Caffeine treatment alone did not affect heart rate when compared to vehicle-treated controls (Figure 4.2.1).

Figure 4.2.1 Tachycardic response to MDMA following co-administration with caffeine. Values represent mean with standard error of the mean change from baseline of 6 animals. Baseline averages were as follows: Vehicle + Vehicle, 347 ± 1 bpm; Caffeine + Vehicle, 332 ± 12 bpm; Vehicle + MDMA, 352 ± 13 bpm; Caffeine + MDMA, 338 ± 8 bpm. Prior to drug challenge there was no baseline difference found in heart rate between the treatment groups.
Chapter 4: Study 2: Effect of MDMA and caffeine on heart rate in rats following pre-treatment with SCH 23390, sulpiride and ketanserin. Does caffeine influence DOI/apomorphine-induced heart rate changes?

SCH 23390 pre-treatment: ANOVA of change in heart rate showed an effect of caffeine \( [F(1,20) = 5.9, P = 0.024] \), time \( [F(49,980) = 3.54, P<0.001] \) and MDMA x time \( [F(49,980) = 5.19, P<0.001] \) only. Post hoc comparisons revealed that MDMA increased heart rate in SCH 23390 pre-treated animals 2 hours and 20, 35, 50 minutes, 3 hours and 3 hours and 5, 15, 40 minutes post-drug challenge when compared to vehicle-treated controls. Co-administration of caffeine with MDMA did not influence heart rate in SCH 23390 pre-treated animals compared to MDMA treatments alone (\( P<0.01 \)). Caffeine alone did not affect heart rate in SCH 23390 pre-treated animals when compared to their vehicle-treated counterparts (Figure 4.2.2).

Figure 4.2.2 Prior treatment with SCH 23390 attenuates the ability of caffeine to promote tachycardia following co-administration with MDMA. Values represent mean with standard error of the mean change from baseline of 6 animals. Baseline averages were as follows: Vehicle + Vehicle, 346 ± 7 bpm; Caffeine + Vehicle, 359 ± 14 bpm; Vehicle + MDMA, 372 ± 8 bpm; Caffeine + MDMA, 376 ± 10 bpm. Prior to drug challenge there was no baseline difference found in heart rate between the treatment groups.
Chapter 4: Study 2: Effect of MDMA and caffeine on heart rate in rats following pre-treatment with SCH 23390, sulpiride and ketanserin. Does caffeine influence DOI/apomorphine-induced heart rate changes?

**Sulpiride pre-treatment:** ANOVA of change in heart rate showed an effect of MDMA \([F(1,20) = 75.52, \ P<0.001]\), time \([F(49,980) = 13.32, \ P<0.001]\), MDMA x time \([F(49,980) = 10.42, \ P<0.001]\), caffeine x time \([F(49,980) = 3.35, \ P<0.001]\) and a MDMA x caffeine interaction \([F(49,980) = 6.42, \ P<0.001]\) only. Post hoc comparisons revealed that caffeine increased heart rate in sulpiride pre-treated animals 35, 40, 45, 50, 55 minutes, 1 hour 10 minutes and 1 hour 25 minutes following drug challenge when compared to vehicle-treated controls \((P<0.01)\). Treatment with MDMA increased heart rate in sulpiride pre-treated animals 25 minutes following MDMA administration and for the remainder of the trial when compared to vehicle-treated controls \((P<0.01)\). Caffeine decreased MDMA-induced tachycardia 1 hour and 5, 10 and 15 minutes following drug administration when compared to MDMA treatment alone \((P<0.01)\) (Figure 4.2.3).

![Graph showing heart rate changes](image)

*Figure 4.2.3 Prior treatment with sulpiride promotes tachycardia in response to MDMA administration alone or in combination with caffeine. Values represent mean with standard error of the mean change from baseline of 5-6 animals. Baseline averages were as follows: Vehicle + Vehicle, 381 ± 11 bpm; Caffeine + Vehicle, 391 ± 6 bpm; Vehicle + MDMA, 347 ± 8 bpm; Caffeine + MDMA, 381 ± 5 bpm. Prior to drug challenge there was no baseline difference found in heart rate between the treatment groups.*
Chapter 4: Study 2: Effect of MDMA and caffeine on heart rate in rats following pre-treatment with SCH 23390, sulpiride and ketanserin. Does caffeine influence DOI/apomorphine-induced heart rate changes?

Apomorphine: ANOVA of change in heart rate showed an effect of apomorphine \([F(1, 16) = 6, P = 0.02]\), time \([F(49,784) = 11.79, P<0.001]\), apomorphine x time \([F(49,784) = 1.72, P = 0.001]\) and caffeine x time \([F(49,784) = 2.09, P<0.001]\) only. Post hoc comparisons revealed that treatment with caffeine increased heart rate 55 minutes, 1 hour 10 and 15 minute following drug challenge and for 1 hour and 5 minutes when compared to vehicle controls \((P<0.01)\). Treatment with apomorphine tended to increase heart rate albeit non significantly when compared to vehicle controls whilst co-administration with caffeine did not alter this response (Figure 4.2.4).

![Figure 4.2.4 Effect of apomorphine, alone or in combination with caffeine, on heart rate. Values represent mean with standard error of the mean change from baseline of 5 animals. Baseline averages for heart rate were as follows: Vehicle + Vehicle, 350 ± 8 bpm; Caffeine + Vehicle, 342 ± 5 bpm; Vehicle + Apomorphine, 347 ± 14 bpm; Caffeine + Apomorphine, 353 ± 8 bpm. Prior to drug challenge there was no baseline difference found in body temperature between the treatment group.](image-url)
Chapter 4: Study 2: Effect of MDMA and caffeine on heart rate in rats following pre-treatment with SCH 23390, sulpiride and ketanserin. Does caffeine influence DOI/apomorphine-induced heart rate changes?

**DOI:** ANOVA of heart rate showed an effect of DOI [F(1, 8) = 9.52, P = 0.01], time [F(49,392) = 2.77, P<0.001] and DOI x time [F(49,392) = 2.77, P<0.001] only. Post hoc comparisons revealed that treatment with DOI decreased heart rate 10 minutes following drug administration and for 25 minutes when compared to vehicle controls (P<0.01). Caffeine alone did not alter heart rate compared to vehicle controls and did not influence the DOI-induced reduction in heart rate when compared to vehicle-treated controls (Figure 4.2.5).

![Figure 4.2.5 Effect of DOI, alone or in combination with caffeine, on heart rate. Values represent mean with standard error of the mean change from baseline of 3 animals. Baseline averages for heart rate were as follows: Vehicle + Vehicle, 369 ± 14 bpm; Caffeine + Vehicle, 369 ± 2 bpm; Vehicle + DOI, 376 ± 18 bpm; Caffeine + DOI, 385 ± 9 bpm. Prior to drug challenge there was no baseline difference found in body temperature, heart rate and activity counts between the treatment groups.](image-url)
Chapter 4: Study 2: Effect of MDMA and caffeine on heart rate in rats following pre-treatment with SCH 23390, sulpiride and ketanserin. Does caffeine influence DOI/apomorphine -induced heart rate changes?

**Apomorphine/DOI:** ANOVA of heart rate showed an effect of apomorphine/DOI [F(1, 19)= 14.58, P = 0.001], apomorphine/DOI x caffeine [F(1,19) = 29.06, P<0.001, time [F(49,931) = 3.05, P<0.001], apomorphine/DOI x time [F(49,931) = 3.38, P<0.001] and caffeine x time [F(49,931) = 1.94, P<0.001] only. Post hoc comparisons revealed that treatment with apomorphine/DOI reduced heart rate 10 minutes following drug administration and for 3 hours when compared to vehicle controls (P<0.01). Caffeine alone increased heart rate 10 minutes following drug administration and for the remainder of the trial when compared to vehicle controls (P<0.01) whilst, caffeine attenuated the reduction in heart rate associated with DOI/apomorphine 15 minutes following drug administration and for the remainder of the trial when compared to the DOI/apomorphine treatment alone (P<0.01) (Figure 4.2.6).

![Figure 4.2.6 Effect of apomorphine and DOI, alone or in combination with caffeine, on heart rate. Values represent mean with standard error of the mean change from baseline of 5-6 animals. Baseline averages for heart rate were as follows: Vehicle + Vehicle, 365 ± 3 bpm; Caffeine + Vehicle, 323 ± 14 bpm; Vehicle + Apomorphine/DOI, 344 ± 14 bpm; Caffeine + Apomorphine/DOI, 333 ± 16 bpm. Prior to drug challenge there was no baseline difference found in heart rate between the treatment groups.](image-url)
Chapter 4: Study 2: Effect of MDMA and caffeine on heart rate in rats following pre-treatment with SCH 23390, sulpiride and ketanserin. Does caffeine influence DOI/apomorphine-induced heart rate changes?

**Ketanserin pre-treatment:** ANOVA of change in heart rate showed an effect of MDMA \( [F(1, 8)= 6.83, P = 0. 031] \), MDMA x caffeine \( [F(1,8)= 8.13, P = 0.021] \), time \( [F(49,392) = 3.01, P<0.001] \) and MDMA x time \( [F(49,392) = 2.21, P<0.001] \) only. Post hoc comparisons revealed that MDMA increased heart rate at 35, 45 and 55 minutes and 2 hours for the remainder of the trial following drug administration compared to the vehicle-treated group (\( P< 0. 01 \)). Caffeine alone did not influence heart rate or MDMA-induced tachycardia (Figure 4.2.7).

**Figure 4.2.7** Prior administration of ketanserin blocks MDMA-induced reduction in heart rate following co-administration with caffeine. Values represent mean with standard error of the mean change from baseline of 3 animals. Baseline averages for heart rate were as follows: Vehicle + Vehicle, 395 ± 13 bpm; Caffeine + Vehicle, 343 ± 20 bpm; Vehicle + MDMA, 385 ± 13 bpm; Caffeine + MDMA, 384 ± 29 bpm. Prior to drug challenge there was no baseline difference found in heart rate between the treatment groups.
Study 3: Role of dopaminergic receptors \((D_1\) and \(D_2\)) and serotonergic \(5HT_2\) receptors on MDMA- and caffeine-induced locomotor changes.

Locomotor activity was monitored for 1 hour pre- and for 4 hours post-challenge with MDMA (10 mg/kg), apomorphine (1 mg/kg) and DOI (2 mg/kg) alone or in combination with caffeine (10 mg/kg). Pre-treatments with SCH 23390 (1 mg/kg), sulpiride (100 mg/kg) and ketanserin (5 mg/kg) were administered 30 min prior to caffeine/MDMA challenge.

MDMA/Caffeine: ANOVA of activity counts showed an effect of MDMA \([F(1,16) = 60.94, P<0.001]\), time \([F(49,784) = 8.7, P<0.001]\), MDMA x time \([F(49,784) = 4.13, P<0.001]\), caffeine x time \([F(49,784) = 2.16, P<0.001]\) and a caffeine x MDMA x time interaction \([F(49,784) = 1.54, P = 0.01]\). Post hoc comparisons revealed that MDMA treatment increased activity 30 minutes following drug challenge, which persisted until the end of the trial when compared to vehicle-treated controls. Co-administration with caffeine did not affect the locomotor response compared to treatment with MDMA alone. Caffeine treatment alone did not alter locomotor activity when compared to vehicle-treated controls (Figure 4.3.1).

![Figure 4.3.1 Locomotor response to MDMA following co-administration with caffeine](image)

Values represent mean with standard error of the cumulative activity counts of 6 animals. Baseline averages for activity value for the caffeine/MDMA experiment were as follows: Vehicle + Vehicle, 6.5 ± 3.2 cnts; Caffeine + Vehicle, 5.3 ± 1.3 cnts; Vehicle + MDMA, 4.5 ± 1.1 cnts; Caffeine + MDMA, 2.4 ± 1 cnts). Prior to drug challenge there was no baseline difference found in activity counts between the treatment groups.
**SCH 23390 pre-treatment:** Locomotor activity: ANOVA of activity counts showed an effect of time \([F(49,833) = 6.9, P<0.001]\), MDMA x time \([F(49,833) = 5.07, P<0.001]\), caffeine x time \([F(49,833) = 8.09, P<0.001]\) and caffeine x MDMA x time interaction \([F(49,833) = 3.84, P<0.001]\). Post hoc comparisons revealed that neither caffeine nor MDMA alone altered locomotor activity when compared to vehicle-treated controls. In addition, the combination of caffeine with MDMA did not alter locomotor activity in SCH pre-treated animals when compared to caffeine or MDMA treatments alone (Figure 4.3.2).

**Figure 4.3.2** Effect of MDMA and caffeine on locomotor activity in rats following pre-treatment with SCH 23390. Values represent mean with standard error of the cumulative activity counts of 5-6 animals. Baseline averages for activity values were as follows: Vehicle + Vehicle, 9.9 ± 0.5 cnts; Caffeine + Vehicle, 10.5 ± 3.3 cnts; Vehicle + MDMA, 14 ± 6.5 cnts; Caffeine + MDMA, 12.1 ± 2.8 cnts. Prior to drug challenge there was no baseline difference found in activity counts between the treatment groups.
Chapter 4: Study 3: Role of dopaminergic receptors (D₁ and D₂) and serotonergic 5HT₂ receptors on MDMA and caffeine-induced locomotor changes.

**Sulpiride pre-treatment:** ANOVA of activity showed effects of MDMA [F(1,20) = 311.88, P<0.001], MDMA x caffeine [F(1,20) = 29.74, P<0.001], time [F(49,980) = 73.67, P<0.001], MDMA x time [F(49,980) = 25.85, P<0.001], caffeine x time [F(49,980) = 7.03, P<0.001] and a MDMA x caffeine x time interaction [F(49,980) = 5.94, P<0.001]. Post hoc comparisons revealed that MDMA treatment increased activity 30 minutes following drug challenge, which persisted until the end of the trial when compared to vehicle-treated controls (P<0.01). Co-administration with caffeine did not affect the MDMA-induced locomotor response when compared to MDMA treatment alone. Caffeine alone increased locomotor activity 55 minutes and 1 hour post-drug administration (P<0.01) (Figure 4.3.3).

**Figure 4.3.3** Effect of MDMA and caffeine on locomotor activity in rats following pre-treatment with sulpiride. Values represent mean with standard error of the cumulative activity counts of 5-6 animals. Baseline averages for activity values were as follows: Vehicle + Vehicle, 21.2 ± 2.8 cnts; Caffeine + Vehicle, 16.6 ± 2.1 cnts; Vehicle + MDMA, 12 ± 3 cnts; Caffeine + MDMA, 13.4 ± 1.6 cnts. Prior to drug challenge there was no baseline difference found in activity counts between the treatment groups.
Chapter 4: Study 3: Role of dopaminergic receptors ($D_1$ and $D_2$) and serotonergic $5HT_1$ receptors on MDMA and caffeine-induced locomotor changes.

**Apomorphine:** ANOVA of locomotor activity showed an effect of apomorphine [$F(1, 16) = 11.66, P = 0.0035$], caffeine [$F(1, 16) = 26.45, P < 0.001$], time [$F(49, 784) = 34.91, P < 0.001$], apomorphine x time [$F(49, 784) = 6.82, P < 0.001$], caffeine x time [$F(49, 784) = 10.3, P < 0.001$] and a apomorphine x caffeine x time interaction [$F(49, 784) = 9.14, P < 0.001$]. Post hoc comparisons revealed that treatment with apomorphine increased locomotor activity 15 minutes following drug administration and for a period of 15 minutes when compared to vehicle controls ($P < 0.01$). Caffeine failed to influence locomotor activity alone or the locomotor response to apomorphine (Figure 4.3.4).

![Figure 4.3.4 Effect of apomorphine, alone or in combination with caffeine, on locomotor activity.](image)

**Figure 4.3.4** Effect of apomorphine, alone or in combination with caffeine, on locomotor activity. Values represent mean with standard error of the cumulative activity counts of 5 animals. Baseline averages for activity values were: Vehicle + Vehicle, 4.3 ± 1.2 cnts; Caffeine + Vehicle, 3 ± 1 cnts; Vehicle + Apomorphine, 4 ± 1 cnts; Caffeine + Apomorphine, 3.2 ± 1.4 cnts. Prior to drug challenge there was no baseline difference found in activity counts between the treatment groups.
**Chapter 4: Study 3: Role of dopaminergic receptors (D₁ and D₂) and serotonergic 5HT₂ receptors on MDMA and caffeine-induced locomotor changes.**

**DOI:** ANOVA of locomotor activity showed an effect of time [F(49,392) = 9.58, P<0.001], DOI x time [F(49,392) = 1.54, P<0.001] and a DOI x caffeine x time interaction [F(49,392) = 1.8 P = 0.001] only. Post hoc comparisons revealed that neither caffeine nor DOI influenced locomotor activity (Figure 4.3.5).

**Figure 4.3.5 Effect of DOI, alone or in combination with caffeine, locomotor activity.** Values represent mean with standard error of the cumulative activity counts of 3 animals. Baseline averages for activity values were as follows: Vehicle + Vehicle, 14 ± 2 cnts; Caffeine + Vehicle, 11 ± 2 cnts; Vehicle + DOI, 12 ± 1 cnts; Caffeine + DOI, 17 ± 3 cnts. Prior to drug challenge there was no baseline difference found in activity counts between the treatment groups.
Chapter 4: Study 3: Role of dopaminergic receptors (D₁ and D₂) and serotonergic 5HT₂ receptors on MDMA and caffeine-induced locomotor changes.

**Apomorphine/DOI**: ANOVA of locomotor activity showed an effect of apomorphine/DOI [F(1, 19) = 12.3, P = 0.002], caffeine [F(1,19) = 21.7], time [F(49,931) = 3.86, P<0.001], apomorphine/DOI x time [F(49,931) = 4.27, P<0.001] and caffeine x time [F(49,931) = 2.09, P = 0.008] only. Post hoc comparisons revealed that treatment with caffeine increased locomotor activity following drug challenge 2 hours and 5, 10, 15 minutes, 3 hours, 3 hours and 10, 45 minutes until the remainder of the trial when compared to vehicle controls (P<0.01). Treatment with apomorphine and DOI did not alter locomotor activity (Figure 4.3.6).

![Locomotor Activity Graph](image)

**Figure 4.3.6** Effect of apomorphine and DOI, alone or in combination with caffeine, on locomotor activity. Locomotor activity was monitored for 1 hour pre- and for 4 hours post-challenge with Apomorphine (10 mg/kg)/DOI (2 mg/kg) and caffeine (10 mg/kg), alone or in combination. Values represent mean with standard error of the cumulative activity counts of 5-6 animals. Baseline averages for activity values were as follows: Vehicle + Vehicle, 17.3 ± 3 cnts; Caffeine + Vehicle, 12 ± 2 cnts; Vehicle + Apomorphine/DOI, 11 ± 1.4 cnts; Caffeine + Apomorphine/DOI, 10.6 ± 1.5 cnts. Prior to drug challenge there was no baseline difference found in activity counts between the treatment groups.
Chapter 4: Study 3: Role of dopaminergic receptors (D₁ and D₂) and serotonergic 5HT₂ receptors on MDMA and caffeine-induced locomotor changes.

Ketanserin: ANOVA of change in locomotor activity showed an effect of MDMA x caffeine [F(1, 8) = 7.30, P = 0.027], time [F(49,392) = 4.16, P<0.001] and a MDMA x caffeine x time interaction [F(49,392) = 1.36, P = 0.05] only. Post hoc comparisons revealed that MDMA increased locomotor activity at 45, 50 and 55 minutes, 1 hour 40 and 45 minutes and 2 hours 45 and 50 minutes following drug administration and for 65 minutes compared to the vehicle-treated group (P<0.001). Co-administration of caffeine with MDMA did not affect locomotor activity when compared to the MDMA-treated group. Caffeine alone increased locomotor activity 30 minutes following drug administration and for the remainder of the challenge when compared to vehicle-treated controls (Figure 4.3.7).

Figure 4.3.7 Effect of MDMA and caffeine on locomotor activity in rats following pre-treatment with ketanserin. Values represent mean with standard error of the cumulative activity counts of 3 animals. Baseline averages for activity values were as follows: Vehicle + Vehicle, 14.6 ± 2.2 cnts; Caffeine + Vehicle, 10 ± 1.8 cnts; Vehicle + MDMA, 11 ± 2.5 cnts; Caffeine + MDMA, 8.1 ± 1.5 cnts Prior to drug challenge there was no baseline difference found in activity counts between the treatment groups.
4.4 DISCUSSION

In line with previous observations, the results show that caffeine provokes hyperthermia and tachycardia following co-administration with MDMA, but does not alter the locomotor stimulant effects of MDMA. In addition, the main outcomes of the current investigation are 1. Caffeine provokes a switch from D₂ receptor-mediated hypothermia to D₁ receptor-mediated hyperthermia following MDMA administration, 2. Dopamine D₁ but not D₂ receptor blockade attenuates the ability of caffeine to provoke tachycardia following MDMA administration, and 3. Dopamine D₁ but not D₂ receptor blockade attenuates MDMA-induced hyperactivity. Unsuccessful attempts were made to simulate the ability of caffeine to enhance MDMA-induced toxicity by substituting MDMA with the non-selective 5-HT and dopamine receptor agonists DOI and apomorphine respectively. However, pre-treatment with the 5-HT₂ receptor antagonist ketanserin blocked the ability of caffeine to provoke a switch from MDMA-induced hypothermia to hyperthermia, consistent with a role for 5-HT₂ receptors in the caffeine-mediated interaction with caffeine. Proposed mechanisms underlying these outcomes are outlined in the sections below.

Caffeine provokes a switch from dopamine D₂ receptor-mediated hypothermia to dopamine D₁ receptor-mediated hyperthermia following MDMA administration.

In rats MDMA provokes a biphasic change to core body temperature dependent on dose and environmental conditions (Dafters, 1995; Green et al., 2005). Dopamine and dopamine receptors are mediators of such changes where dopamine D₁ and D₂ receptor inhibition blocks MDMA-induced hyperthermia and hypothermia respectively. The results of the present investigation are consistent with this, where pre-treatment with sulpiride attenuated MDMA-induced hypothermia but failed to attenuate the ability of caffeine to promote MDMA-induced hyperthermia. As caffeine promotes a switch from hypo- to hyperthermia following MDMA administration to rats, and MDMA-induced hyperthermia may be blocked by dopamine D₁ receptor inhibition, it is conceivable that caffeine promotes a change from a dopamine D₂ to D₁ receptor-mediated response.
Chapter 4: Discussion

In support of this, MDMA-induced hypothermia was enhanced by prior treatment with SCH 23390. Pre-treatment with the dopamine D<sub>1</sub> receptor antagonist SCH 23390 blocked the ability of caffeine to promote a switch from hypo- to hyperthermia following MDMA administration. A modest attenuation by caffeine of MDMA-induced hypothermia was observed in these animals 2 hours following drug administration showing that caffeine can interact with the MDMA response, albeit to a much lesser extent, when dopamine D<sub>1</sub> receptors are blocked. To account for this, it is possible that the pharmacological effects of SCH 23390 may wane with time following administration or that over time the adenosine/PDE inhibitory properties of caffeine can moderate the hypothermia augmenting response to dopamine D<sub>1</sub> receptor blockade. Furthermore, an attenuation of the ability of caffeine to promote MDMA-induced hyperthermia was apparent in sulpiride pre-treated animals when compared to animals who received no pre-treatment. The fact that a switch from hypothermia to hyperthermia was not observed to the same extent may suggest that a high enough dose of sulpiride was not used. However there are numerous reports where doses of sulpiride 100 mg/kg and lower are effective in blocking dopamine D<sub>2</sub> receptor-mediated changes in body temperature and behaviour in rodents (Dias et al., 2006; Frantz et al., 1995; Maj et al., 1997; Nava et al., 2000). An alternative and more likely explanation is that dopamine D<sub>2</sub> receptor activation may also be necessary to enable a full manifestation of dopamine D<sub>1</sub>-mediated hyperthermia. Many behavioural and electrophysiological events associated with dopaminergic transmission have been associated with a concomitant activation of both dopamine D<sub>1</sub> and D<sub>2</sub> receptors indicative of a D<sub>1</sub>/D<sub>2</sub> receptor synergism (LaHoste et al., 2000). Despite the fact that dopamine D<sub>1</sub> and D<sub>2</sub>/D<sub>3</sub> synergism have been described in terms of the regulation of psychomotor behaviours and neuronal activity (Waddington et al., 1995; White et al., 1997) such synergy has not been elucidated to date with regard to the regulation of body temperature. Chaperon et al., (2003) described a probable cooperation between the two receptors as the dopamine D<sub>1</sub> antagonist SCH 23390 and L-741,626, a selective D<sub>2</sub> antagonist, reversed hypothermia induced by PD 128907, a dopamine D<sub>3</sub> receptor agonist, which was not altered by the selective D<sub>3</sub> antagonist A-437203. By contrast Salmi and co-workers (1993) demonstrated that hypothermia induced by D<sub>1</sub> receptor agonist A68930 was antagonised by SCH 23390 but not by the dopamine D<sub>2</sub>/D<sub>3</sub> antagonist raclopride while the opposite was reported for quinpirole-induced hypothermia (Salmi et al., 1993).
Chapter 4: Discussion

We recently provided evidence in support of a dopamine-related mechanism that may account for a switch from MDMA-induced hypo- to hyperthermia (Vanattou-Saifoudine et al., 2010). Specifically, both central catecholamine depletion and pre-treatment with the dopamine D₁ receptor antagonist SCH 23390 blocked MDMA-induced hyperthermia and the ability of caffeine to exacerbate MDMA-induced hyperthermia in group-housed rats. Moreover, catecholamine depletion or dopamine D₁ receptor blockade provoked a switch to hypothermia in response to MDMA. Dopamine has a greater affinity for dopamine D₂ and D₂-like receptors (Missale et al., 1998) and it is possible that when dopamine is depleted and there is insufficient release to provoke an overt dopamine D₁ receptor-mediated hyperthermic response to MDMA, a dopamine D₂-mediated hypothermia occurs instead. As MDMA induces the release of dopamine in the brain (Green et al., 2003) and caffeine has also been reported to influence central dopamine release (Cauli et al., 2005; Ferre, 2008), this may represent a mechanism whereby caffeine effects a change from a dopamine D₂ to a D₁ receptor-mediated response. As caffeine may interact with adenosine receptors to facilitate the release of dopamine, co-administration of caffeine with MDMA may provoke sufficient dopamine release to induce a switch from a D₂ receptor-mediated hypothermic response to a D₁ receptor-mediated hyperthermic response due to enhanced extracellular availability of dopamine. Previous experiments have shown that caffeine increases dopamine and glutamate release in the striatum via the blockade of inhibitory presynaptic adenosine A₁ receptors (Janusz et al., 2007; Okada et al., 1996; Quarta et al., 2004; Solinas et al., 2002). Postsynaptic mechanisms of caffeine may also be relevant where caffeine interacts with adenosine A₁ receptors and is therefore known to alter the affinity of dopamine for dopamine D₁ receptors with knock-on effects to intracellular cAMP signalling (Ferre et al., 1994; Neill et al., 2007). Further experimentation, however, is required to clarify such mechanisms.
Dopamine D₁ but not D₂ receptor blockade attenuates the ability of caffeine to provoke tachycardia following MDMA administration.

As a switch from D₂- to D₁-mediated responses is proposed to account for the actions of caffeine on MDMA-induced changes in core body temperature and dopamine D₁ and D₂ receptors are involved in the regulation of heart rate, it was conceivable that a similar mechanism was of relevance to the interaction observed between caffeine and MDMA on the heart rate response. Antagonism of dopamine D₁ receptors completely blocked the ability of caffeine to promote tachycardia following MDMA administration. It was also evident that prior treatment with SCH 23390 was capable of reversing the ability of MDMA to attenuate the transient increase in heart rate evident upon handling and drug administration. In this regard sympathomimetic stimulants such as MDMA or cocaine can produce a bradycardic-like response attributed to pressor-induced activation of the baroreceptor reflex and subsequent reduction in heart rate (Abrahams et al., 1996; Badon et al., 2002b; Knuepfer et al., 1992; O'Cain et al., 2000). By contrast, inhibition of dopamine D₂ receptors had little effect on the ability of caffeine to promote tachycardia following MDMA administration. Moreover, animals pre-treated with sulpiride showed a prolonged and persistent tachycardia following MDMA treatment alone. Thus, under dopamine D₂ receptor blockade the ability of MDMA to reduce heart rate is inhibited and a tachycardic response prevails. Such effects are consistent with the role of the dopamine D₂ receptor in the regulation of heart rate. Yoon and co-workers (1994) reported that an increase in heart rate induced by electrical stimulation in pithed rats was attenuated by the dopamine D₂ agonist lisuride which in turn was blocked by pretreatment with sulpiride. Further involvement of dopamine D₂ receptors in the regulation of heart rate was demonstrated by Chitravanshi and Calaresu (1992) where injection of dopamine into the right nucleus ambiguous localized in the lateral portion of the upper medulla provoked bradycardia, which was blocked by sulpiride in rats. Efferent projections from this nucleus include pre-ganglionic parasympathetic fibres that extend as cardioinhibitory neurons to the heart (Machado et al., 1988; Nosaka et al., 1979).

In a similar fashion to the mechanisms underlying the ability of caffeine to promote MDMA-induced hyperthermia, tachycardia observed following co-administration of caffeine with MDMA may be accounted for by a synergistic interaction between dopamine D₁ and D₂ receptors. In addition other neurotransmitters such as noradrenaline
may be involved. Dopamine acts directly on both dopaminergic D₁ and D₂ receptors and α and β adrenoceptors in the heart. Dopamine D₁ and D₂ receptors in the rat heart are localised in low abundance (Cavallotti et al., 2002; Ozono et al., 1996). Activation of D₁ receptors with fenoldopam and D₂ receptors with quinpirole induces a systemic vasodilatation which leads to a decrease in arterial pressure (Cavero et al., 1987) and corresponding compensatory increase in heart rate. It is therefore not unreasonable to suggest that dopamine D₁ and D₂ receptors might play a synergistic role in mediating the tachycardia associated with the co-administration of caffeine with MDMA. Both SCH 23990 and sulpiride influenced MDMA-induced changes in heart rate and caffeine provoked a switch to tachycardia. Sulpiride attenuates the tachycardia associated with the combined administration of caffeine and MDMA when compared to MDMA treatment alone. SCH 23390 pre-treatment attenuates MDMA-induced bradycardia. Such responses support a mechanism where both receptors need to be activated to induce the complete synergistic tachycardic response, such as the one observed following the co-administration of caffeine and MDMA in the absence of dopamine receptor antagonists. Other neurotransmitters such as noradrenaline might also be involved but further experiments need to be performed to clarify these mechanisms. The mechanism suggested is consistent with McNamara and co-workers (2007) who proposed that the ability of caffeine to promote tachycardia following MDMA administration to rats was most likely attributable to a sympathomimetic or central mechanism rather than a direct action of caffeine or MDMA on the heart.

Pre-treatment with SCH 23390, but not sulpiride, blocks MDMA-induced hyperactivity.

In line with our previous experiment, MDMA provokes hyperlocomotion which is not altered by caffeine (McNamara et al., 2007). Following SCH 23390 pre-treatment, hyperactivity induced by both MDMA and MDMA/caffeine treatments was attenuated, suggesting that MDMA-induced hyperlocomotion is at least partially mediated by dopamine D₁ receptors. A role for dopamine D₁ receptors in MDMA-induced hyperlocomotion has been reported by Benturquia and co-workers (2008) and others (Ball et al., 2003; Brown et al., 2007; Risbrough et al., 2006). Motor activity is controlled by dopaminergic transmission and dopamine release from presynaptic nerve
terminals. Dopamine inhibits its own release through dopamine D$_2$ autoreceptors resulting in a decrease of extracellular dopamine (Benoit-Marand et al., 2001; Cragg et al., 1997; Langer, 1997; Paul et al., 2002). It is then conceivable that antagonism of dopamine D$_2$ receptors will increase dopamine release and consequently activate postsynaptic dopamine D$_1$ receptors which are known to play an important role in locomotor activity regulation (Shieh et al., 1996; Vezina et al., 1991) and in locomotion induced by amphetamines (Hall et al., 2009a). The present results are consistent with such a mechanism as pre-treatment with sulpiride increased locomotion in rats treated with either MDMA alone or in combination with caffeine.

**Mechanisms mediating the interactive effects of caffeine with MDMA.**

Inhibition of adenosine A$_1$ and A$_2A$ receptors and phosphodiesterase may account for the ability of caffeine to influence dopaminergic transmission and dopamine-mediated effects on core body temperature and heart rate. In line with this, we have reported that the ability of caffeine to exacerbate MDMA-induced hyperthermia in group-housed animals involves a combination of adenosine A$_2A$ receptor antagonism and phosphodiesterase-4 (PDE-4) inhibition (Vanattou-Saffoudine et al., 2010). Adenosine A$_1$ receptors are widely distributed in the brain and play an important role in regulating dopamine release (Cauli et al., 2005; Fuxe et al., 1998; Jin et al., 1993; Neill et al., 2007; Quarta et al., 2004; Solinas et al., 2002; Wood et al., 1989). We have previously investigated the role of adenosine A$_1$ receptors in the modulation of MDMA-induced dopamine release from superfused striatal and hypothalamic tissue slices. DPCPX, an adenosine A$_1$ receptor antagonist like caffeine, enhanced MDMA-induced dopamine release from striatal tissue slices but attenuated the release from hypothalamic slices. Such results suggest that caffeine differentially regulates MDMA induced dopamine release in striatal and hypothalamic tissue slices, via inhibition of adenosine A$_1$ receptors. Adenosine A$_2A$ receptors are mainly localized in the striatum, nucleus accumbens and olfactory tubercles (Daval et al., 1991; Goodman et al., 1982; Jarvis et al., 1989b; Ongini et al., 1996; Parkinson et al., 1990). Adenosine A$_2A$ receptors have been found to be co-expressed and functionally coupled with dopamine D$_2$ receptors in the striatum and account for the ability of caffeine to influence striatal dopaminergic transmission (Ferre et al., 1992; Garrett et al., 1997; Pollack et al., 1995). As well as
being present in the central nervous system, both adenosine $A_1$ and $A_{2A}$ receptors are present in the heart, and their activation provokes a decrease or increase in heart rate, respectively. Such actions may underlie the mechanism(s) by which caffeine influences MDMA-induced changes in heart rate although further work is required to clarify these mechanisms.

**Role of 5-HT in MDMA and caffeine-induced hyperthermia and MDMA-induced bradycardia.**

Despite the fact that dopamine plays an important role in the ability of caffeine to exacerbate the toxicity of MDMA, a role for 5-HT, and especially 5-HT$_2$ receptors, cannot be ruled out. Co-administration of caffeine with either apomorphine or DOI alone did not influence their response on heart rate, body temperature and locomotor activity. However, co-administration of caffeine with both apomorphine and DOI provoked a reduction in the heart rate response and attenuation of the hypothermia in response to administration of the two agonists. Thus, in order for caffeine to effect an interaction with MDMA, both dopamine and 5-HT appear to play a vital role. In support of this, previous studies have reported that MDMA-induced dopamine release is 5-HT dependent (Koch et al., 1997; Green et al., 2003; Gudelsky et al., 2008). In line with these observations, we observed that the 5-HT$_2$ and non selective dopamine receptor agonists DOI and apomorphine respectively-induced hypothermia was attenuated by caffeine similar to the response obtained with combined treatment of caffeine and MDMA supporting a dual role for 5-HT and dopamine. Moreover, we have previously demonstrated (Vanattou-Saffoudine et al., 2010) that co-administration of caffeine with a combination of D-fenfluramine and D-amphetamine, but not following either agent alone, provokes an interaction similar to the co-administration of caffeine with MDMA. A role for 5-HT is also supported in the present study by evidence that pre-treatment with the preferential 5-HT$_2$ receptor antagonist ketanserin blocked the hyperthermic response to the co-administration of caffeine with MDMA and reduction in heart rate following MDMA administration. It is proposed that ketanserin may act to reduce MDMA-induced dopamine release, thereby preventing MDMA and caffeine-induced hyperthermia although further experiments are required to clarify this mechanism. By contrast, the present report suggests a role of 5-HT$_2$ receptors in regulating behavioural
responses to caffeine and MDMA, as pre-treatment with ketanserin enhances locomotor activity following caffeine or MDMA alone or in combination. Ketanserin is an antagonist of 5-HT$_2$ receptor with a higher affinity for 5-HT$_{2C}$ compared to 5-HT$_{2A}$ receptors. Hyperactivity produced by MDMA is reduced by 5-HT$_{2A}$ (Fletcher et al., 2002; Kehne et al., 1996) while 5-HT$_{2C}$ antagonists enhance it (Bankson et al., 2002; Fletcher et al., 2006).

In conclusion, the results of this study provide evidence for the participation of dopamine D$_1$ and D$_2$ receptors in the exacerbation by caffeine of MDMA-induced toxicity and suggest that caffeine promotes postsynaptic D$_{1/2}$ receptor-dependent responses. 5-HT$_2$ receptors are also implicated but are likely to play a role through the regulation of synaptic dopamine release. The interaction between caffeine and MDMA must be considered extremely pertinent in light of the unpredictable nature of Ecstasy-induced toxicity in humans. In addition, dopamine D$_1$ receptor blockade may represent a novel approach for treating individuals presenting with acute toxic reactions to Ecstasy with hyperthermia being the greatest contributor to a fatal outcome in the emergency room.
RESULTS
CHAPTER 5

Brain region specific modulatory actions of caffeine on MDMA "Ecstasy"-induced dopamine release: A role for adenosine A1 receptor blockade.
5.1 INTRODUCTION

Caffeine is the most widely consumed psychoactive substance. It is frequently consumed by recreational drug users with other psychostimulant drugs and may influence their toxicity but the mechanisms underlying the ability of caffeine to exacerbate MDMA-induced toxicity are not currently understood. In this regard we have recently reported in rats that depletion of central catecholamines but not serotonin attenuates MDMA-induced hyperthermia and its exacerbation by caffeine. In addition, prior treatment with the dopamine D₁ receptor antagonist, SCH 23390, attenuates the hyperthermic and tachycardic response associated with this drug combination (Vanattou-Saïfoudine et al., 2010).

As MDMA provokes the release of dopamine in the brain (Green et al., 2003), and caffeine has also been reported to influence central dopamine release (Cauli et al., 2005; Ferre, 2008), dopamine release may represent a mechanism whereby caffeine augments the acute toxicity of MDMA. Thus, the aim of this study was to determine the effect of caffeine on MDMA-induced dopamine release in superfused tissue slices pre-loaded with [³¹H] dopamine. This study was performed on tissue slices obtained from two brain regions implicated in the behavioural and physiological effects of MDMA: the striatum, a key region of the motor circuitry and harbor of nerve terminal dopamine release, and the hypothalamus where dopaminergic neurons important in thermoregulation are located (Fetissov et al., 2000; Hilaire et al., 2001; You et al., 2001).

There are biochemical mechanisms by which caffeine may interact with dopamine receptors or alter dopamine release. Specifically caffeine is a non selective adenosine A₁ and A₂A receptor antagonist (Fredholm et al., 1999; Nehlig, 1999; Ferre, 2008), and an antagonistic interaction between adenosine A₁ and dopamine D₁ receptors has been described in basal ganglia (striatum, globus pallidus and substantia nigra reticulata) and limbic regions (ventral pallidum and nucleus accumbens) (Ballarin et al., 1995; Ferre, 2008; Franco et al., 2007; Wood et al., 1989). Adenosine A₁ receptors, localized on the terminals of glutamatergic and dopaminergic neurons, can inhibit dopamine release in the brain particularly in the striatum (Borycz et al., 2007; Jin et al., 1993; O'Neill et al., 2007; Quarta et al., 2004).
Chapter 5: Introduction

As adenosine A₁ receptors are directly involved in regulating pre-synaptic dopamine release we determined if blockade of adenosine A₁ receptors might simulate the actions of caffeine on MDMA-induced dopamine release in tissue slices. Our results show regionally dependent effects of caffeine on MDMA-induced dopamine release which are simulated by application of the selective adenosine A₁ receptor antagonist DPCPX.
Chapter 5: Experimental Design

5.2 EXPERIMENTAL DESIGN

Tissue slice superfusion has been used to investigate the effects of D-amphetamine on dopamine release at a concentration of 10 μM (Herdon et al., 1985; Parker et al., 1986). Johnson and co-workers (1986) demonstrated that MDA, MDMA and related analogues provoked 5-HT release over a similar micromolar concentration range. Dopamine release induced by MDMA has also been investigated in superfused tissue slices over this concentration range (Fitzgerald et al., 1990) used 10 μM). Riegert and co-workers (2008) showed that MDMA (3 μM) enhanced the spontaneous outflow of dopamine from striatal slices. Based on the above, we tested concentrations of caffeine and MDMA over a micromolar range deemed physiologically relevant for determination of drug-induced release from tissue slices under superfusion conditions. Low micromolar concentrations failed to provoke dopamine release. Effective concentrations used between laboratories relate to the tissue slice preparation, the perfusion system and the superfusion conditions employed. As the superfusion technique is carried out under flow conditions ex vivo, it does not directly represent the intact physiological condition. Consequently concentrations of drug employed are an order of magnitude higher than those required to provoke dopamine release in vivo (see Methods section: Tissue slice superfusion technique).

Experiments were carried out as follows:

Study 1: Dose dependent effects of MDMA and caffeine on [³H] dopamine release from striatal and hypothalamic tissue slices.

Study 2: Effect of caffeine on MDMA-induced [³H] dopamine release from striatal and hypothalamic tissue slices

Study 3: Effect of DPCPX on MDMA-induced [³H] dopamine release from striatal and hypothalamic tissue slices
5.3 RESULTS

The results show a concentration dependent increase of \(^{3}H\) dopamine release from striatal and hypothalamic tissue slices following exposure to MDMA (30, 100 and 300 \(\mu\)M) or caffeine (300 \(\mu\)M). Furthermore, caffeine (100 \(\mu\)M) increased MDMA (30\(\mu\)M)-induced \(^{3}H\) dopamine release in the striatum while caffeine (100 \(\mu\)M) attenuated MDMA (100 \(\mu\)M)-induced \(^{3}H\) dopamine release in the hypothalamus. The adenosine receptor A\(_1\) antagonist DPCPX (30 \(\mu\)M) produced a caffeine-like effect on MDMA-induced dopamine release in both striatal and hypothalamic slices.

**Study 1: MDMA provokes \(^{3}H\) dopamine release and caffeine potentiates MDMA-induced \(^{3}H\) dopamine release from striatal tissue slices.**

To determine the most suitable concentration of MDMA required to induce \(^{3}H\) dopamine release from both striatal and hypothalamic slices, buffer containing various concentrations of MDMA (0, 30, 100 and 300 \(\mu\)M) was superfused onto striatal or hypothalamic slices. Similarly the effect of caffeine (0, 10, 30 and 100 \(\mu\)M) on \(^{3}H\) dopamine release was also determined in striatal and hypothalamic slices.

(a) **Caffeine provokes \(^{3}H\) dopamine release from striatal tissue slices.**

**Time course: ANOVA of \(^{3}H\) dopamine outflow** showed effects of caffeine \([F(3,16) = 5.18, P = 0.018]\), time \([F(16, 256) = 28.01, P<0.001]\) and a caffeine x time \([F(48, 256) = 2.23, P<0.001]\) interaction. Post hoc comparisons revealed caffeine (100 \(\mu\)M) induced dopamine release from striatal slices 20, 24, 28, 32 and 40 minutes following initial drug exposure (Figure 5.1.1).

**AUC: ANOVA of area under the curve** showed effects of caffeine on \(^{3}H\) dopamine release \([F(3, 16) = 4.56, P = 0.017]\). Post hoc comparisons revealed that caffeine (100 \(\mu\)M) increased the area under the curve when compared to vehicle-treated controls (Figure 5.1.1, inset).
Chapter 5: Study 1: MDMA provokes [\(^3\)H] dopamine release and caffeine potentiates MDMA-induced [\(^3\)H] dopamine release from striatal tissue slices.

![Graph showing fractional rate of [\(^3\)H] dopamine release](image)

**Figure 5.1.1** Caffeine (100 \(\mu\)M) induces [\(^3\)H] dopamine release from striatal slices. There was no difference in [\(^3\)H] dopamine outflow between the groups prior to drug exposure (T0 to T16). Values represent mean with standard error of the mean. The period of drug exposure is indicated by the bold line (T16 to T44). The AUC for the period of time tissue slices were exposed to the drugs is represented in the accompanying figure. * \(P<0.05\), **\(P<0.01\) vs. Vehicle Control. \(N=5\) animals per group.

(b) MDMA provokes $[^3]H$ dopamine release from striatal tissue slices

Time course: ANOVA of $[^3]H$ dopamine outflow showed effects of time $[F(16, 288) = 12.84, P<0.001]$ and a MDMA x time interaction $[F(48, 288) = 2.51, P<0.001]$. Post hoc comparisons revealed that MDMA (300 μM) induced dopamine release 16, 20, 24, 28 and 32 minutes following initial drug exposure when compared to vehicle-treated controls (Figure 5.1.2).

AUC: ANOVA of area under the curve showed effects of MDMA on $[^3]H$ dopamine release $[F(3, 18) = 3.42, P = 0.04]$. Post hoc comparisons revealed that MDMA (300 μM) increased the area under the curve when compared to vehicle-treated controls (Figure 5.1.2, inset).

Figure 5.1.2 MDMA (300 μM) induces $[^3]H$ dopamine release from striatal slices. There was no difference in $[^3]H$ dopamine outflow between the groups prior to drug exposure (T0 to T16). Values represent mean with standard error of the mean. The period of drug exposure is indicated by the bold line (T16 to T44). The AUC for the period of time tissue slices were exposed to the drugs is represented in the accompanying figure. * P<0.05 vs. Vehicle Control. N= 6 animals per group.

(c) Caffeine (100 μM) potentiates MDMA (30 μM)-induced $[^3]H$ dopamine release

Time course: ANOVA of $[^3]H$ dopamine outflow showed effects of caffeine [F(1,16) = 18.95, P<0.001], MDMA [F(1, 16) = 48.08, P<0.001], time [F(16, 256) = 55.59, P<0.001], caffeine x MDMA [F(1,16) = 5.52, P = 0.032], MDMA x time [F(16, 256) = 22.46, P<0.001], caffeine x time [F(16, 256) = 10.76, P<0.001] and a caffeine x MDMA x time interaction [F(16, 256) = 5.39, P<0.001]. Post hoc comparisons revealed that caffeine enhanced MDMA-induced dopamine release from striatal slices 20, 24, 28 and 32 minutes following initial drug exposure (Figure 5.1.3).

AUC: ANOVA of the area under the curve showed effects of MDMA [F(1, 16) = 11.15, P =0.004] and caffeine [F(1, 16) = 5.09, P = 0.038]. Post hoc comparisons revealed that caffeine in combination with MDMA provoked dopamine release when compared to MDMA- or caffeine-treated groups (Figure 5.1.3, inset).

Figure 5.1.3 Caffeine (100 μM) potentiates MDMA (30 μM)-induced $[^3]H$ dopamine release from striatal slices. There was no difference in $[^3]H$ dopamine outflow between the groups prior to drug exposure (T0 to T16). Values represent mean with standard error of the mean. The period of drug exposure is indicated by the bold line (T16 to T44). The AUC for the period of time tissue slices were exposed to the drugs is represented in the accompanying figure. + $P<0.05$ vs. Vehicle + MDMA. N= 5 animals per group.

Based on the responses obtained in study 1, appropriate concentrations of caffeine and MDMA were selected and the effect of caffeine (100 μM) on MDMA (30 μM)-induced $[^3]H$ dopamine release from hypothalamic slices was determined. As caffeine (100 μM) did not influence $[^3]H$ dopamine release from hypothalamic tissue slices in response to the application of MDMA (30 μM), a higher concentration of MDMA (100 μM) was also tested in combination with caffeine (100 μM).


Time course: ANOVA of $[^3]H$ dopamine outflow showed effects of time [$F(16, 224) = 23.11, P<0.001$] and caffeine x time interaction [$F(48, 224) = 3.26, P<0.001$]. Post hoc comparisons revealed that caffeine (100 μM) induced dopamine release from hypothalamic tissue slices 20, 24 and 28 minutes following initial drug exposure when compared to vehicle-treated controls (Figure 5.2.1).

AUC: ANOVA of area under the curve showed effects of caffeine on $[^3]H$ dopamine release [$F(3, 14) = 4.25, P = 0.025$]. Post hoc comparisons revealed that caffeine (100 μM) increased the area under the curve when compared to vehicle-treated controls (Figure 5.2.1, inset).
Chapter 5: Study 2: MDMA provokes [3H] dopamine release and caffeine attenuates 
MDMA-induced [3H] dopamine release from hypothalamic tissue slices

Figure 5.2.1 Caffeine (100 μM) induces [3H] dopamine release from hypothalamic slices. There was no difference in [3H] dopamine outflow between the groups prior to drug exposure (T0 to T16). Values represent mean with standard error of the mean. The period of drug exposure is indicated by the bold line (T16 to T44). The AUC for the period of time tissue slices were exposed to the drugs is represented in the accompanying figure. *P<0.05, **P<0.01 vs. Vehicle Control. N= 4-5 animals per group.

(b) MDMA provokes $[^3]H$ dopamine release from hypothalamic tissue slices.

Time course: ANOVA of $[^3]H$ dopamine outflow showed effects of MDMA [F(3, 14) = 33.61, P<0.001], time [F(16, 224) = 30.59, P<0.001] and MDMA x time interaction [F(48, 224) = 9.32, P<0.001]. Post hoc comparisons revealed that MDMA (100 µM) induced dopamine release from hypothalamic tissue slices 16, 20 and 24 minutes following initial drug exposure when compared to vehicle-treated controls. MDMA (300 µM) induced dopamine release 12, 16, 20, 24, 28, 32 and 36 minutes following initial drug exposure when compared to vehicle-treated controls (Figure 5.2.2).

AUC: ANOVA of the area under the curve showed effects of MDMA [F(3, 14) = 36.13, P<0.001]. Post hoc comparisons revealed that MDMA (100 µM and 300 µM) increased the area under the curve when compared to vehicle-treated controls (Figure 5.2.2, inset).

Figure 5.2.2 MDMA (100 and 300 µM) induces $[^3]H$ dopamine release from hypothalamic slices. There was no difference in $[^3]H$ dopamine outflow between the groups prior to drug exposure (T0 to T16). Values represent mean with standard error of the mean. The period of drug exposure is indicated by the bold line (T16 to T44). The AUC for the period of time tissue slices were exposed to the drugs is represented in the accompanying figure. *P<0.05, **P<0.01 vs. Vehicle Control. N= 4-5 animals per group.
Chapter 5: Study 2: MDMA provokes $[^{3}H]$ dopamine release and caffeine attenuates MDMA-induced $[^{3}H]$ dopamine release from hypothalamic tissue slices

(c) Caffeine (100 $\mu$M) attenuates MDMA-induced $[^{3}H]$ dopamine release from hypothalamic tissue slices.

Time course: ANOVA of $[^{3}H]$ dopamine outflow showed effects of MDMA [$F(1, 14) = 9.83$, $P = 0.0007$], MDMA x caffeine [$F(1, 14) = 7.35$, $P = 0.017$], time [$F(16, 224) = 8.71$, $P<0.001$], MDMA x time [$F(16, 224) = 2.58$, $P = 0.001$] and caffeine x MDMA x time [$F(16, 224) = 1.98$, $P = 0.016$] interactions. Post hoc comparisons revealed that MDMA induced dopamine release from hypothalamic slices 16, 20, 24 and 28 minutes following initial drug exposure when compared to vehicle-treated controls. Caffeine (100 $\mu$M) alone did not influence dopamine release but attenuated MDMA-induced dopamine release 20, 24 and 28 minutes following initial drug exposure when compared to MDMA-treated controls (Figure 5.2.3).

AUC: ANOVA of the area under the curve showed effects of MDMA [$F(1, 14) = 4.60$, $P = 0.05$], and a MDMA x caffeine interaction [$F(1, 14) = 9.65$, $P = 0.008$]. Post hoc comparisons revealed that MDMA (100 $\mu$M) induced dopamine release when compared to vehicle-treated controls. Caffeine (100 $\mu$M) did not influence dopamine release when compared to vehicle-treated controls. Co administration of caffeine with MDMA attenuated MDMA-induced dopamine release when compared to the MDMA-treated group (Figure 5.2.3, inset).

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**Figure 5.2.3** Caffeine (100 μM) attenuates MDMA (100 μM)-induced $[^3]H$ dopamine release from hypothalamic slices. There was no difference in $[^3]H$ dopamine outflow between the groups prior to drug exposure (T0 to T16). Values represent mean with standard error of the mean. The period of drug exposure is indicated by the bold line (T16 to T44. The AUC for the period of time tissue slices were exposed to the drugs is represented in the accompanying figure. **P<0.01 vs. Vehicle Control; + P<0.05, ++P<0.01 vs. Vehicle + MDMA. N= 4-5 animals per group.
Chapter 5: Study 3

Study 3: DPCPX simulates the effect of caffeine on MDMA-induced release of $[^3H]$ dopamine from striatal and hypothalamic tissue slices.

We further investigated if caffeine’s ability to modulate MDMA-induced dopamine release might be simulated by the selective high affinity antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). Buffer containing DPCPX (1 μM) alone or in combination with either MDMA (30 μM) or MDMA (100 μM) were superfused onto striatal and hypothalamic slices respectively. The concentration of DPCPX was selected from previous reports where DPCPX has been shown to be 3 times more potent than caffeine at the adenosine A₁ receptor (Fredholm et al., 1999).

(a) Striatum

Time course: ANOVA of $[^3H]$ dopamine outflow showed effects of MDMA $[F(1, 20) = 4.43, P = 0.048]$, DPCPX $[F(1, 20) = 10.97, P = 0.0035]$, time $[F(16, 320) = 11.92, P<0.001]$, MDMA x time $[F(16, 320) = 2.44, P = 0.0017]$ and DPCPX x time $[F(16, 320) = 6.46, P<0.001]$ interactions. Post hoc comparisons revealed that DPCPX in combination with MDMA provoked dopamine release from striatal slices 20, 24, 28, 32 and 36 minutes following initial drug exposure when compared to MDMA or DPCPX treatments alone (Figure 5.3.1).

AUC: ANOVA of the area under the curve showed effects of DPCPX (1 μM) $[F(1, 20) = 7.42, P = 0.013]$ and MDMA (30 μM) $[F(1, 20) = 3.74, P = 0.067]$ which was approaching significance. Post hoc comparisons revealed that co-administration of DPCPX with MDMA provoked dopamine release when compared to DPCPX or MDMA treatments alone (Figure 5.3.1, inset).
Chapter 5: Study 3: DPCPX simulates the effect of caffeine on MDMA-induced release of $[^3\text{H}]$ dopamine from striatal and hypothalamic tissue slices

Figure 5.3.1 DPCPX (1 μM) potentiates MDMA (30 μM)-induced $[^3\text{H}]$ dopamine release from striatal tissue slices. There was no difference in $[^3\text{H}]$ dopamine outflow between the groups prior to drug exposure (T0 to T16). Values represent mean with standard error of the mean. The period of drug exposure is indicated by the bold line (T16 to T44). The AUC for the period of time tissue slices were exposed to the drugs is represented in the accompanying figure. +P<0.05, ++P<0.01 vs. Vehicle + MDMA. N=6 animals per group.
Chapter 5: Study 3: DPCPX simulates the effect of caffeine on MDMA-induced release of $[^3\text{H}]$ dopamine from striatal and hypothalamic tissue slices

(b) Hypothalamus

Time course: ANOVA of $[^3\text{H}]$ dopamine outflow showed effects of MDMA [F(1, 19) = 14.74, P = 0.0011], DPCPX [F(1, 19) = 9.24, P = 0.0067], DPCPX x MDMA [F(1, 19) = 10.38, P = 0.0045], time [F(16, 304) = 9.41, P < 0.001], MDMA x time [F(16, 304) = 3.09, P < 0.001], DPCPX x time [F(16, 304) = 1.90, P = 0.019] and DPCPX x MDMA x time [F(16, 304) = 2.16, P = 0.0063] interactions. Post hoc comparisons revealed MDMA induced $[^3\text{H}]$ dopamine release 16, 20, 24, 28, 32 and 36 minutes following drug exposure when compared to vehicle-treated control. DPCPX (1 μM) alone did not influence dopamine release but it attenuated MDMA-induced dopamine release from hypothalamic slices 16, 20, 24, 28, 32 and 36 minutes when compared to MDMA treatment alone (Figure 5.3.2).

AUC: ANOVA of the area under the curve showed effects of MDMA (100 μM) [F(1, 19) = 8.78, P = 0.008] and an MDMA x DPCPX (1 μM) [F(1, 19) = 6.01, P = 0.024] interaction. Post hoc comparisons revealed that MDMA increased dopamine release when compared to vehicle-treated controls. DPCPX had no effect on dopamine release when compared to vehicle-treated controls. Co-administration of DPCPX with MDMA attenuated MDMA-induced dopamine release when compared to MDMA treatment alone (Figure 5.3.2, inset).
Chapter 5: Study 3: DPCPX simulates the effect of caffeine on MDMA-induced release of [³H] dopamine from striatal and hypothalamic tissue slices

Figure 5.3.2 DPCPX (1 μM) attenuates MDMA (100 μM)-induced [³H] dopamine release: from hypothalamic tissue slices. There was no difference in [³H] dopamine outflow between the groups prior to drug exposure (T0 to T16). Values represent mean with standard error of the mean. The period of drug exposure is indicated by the bold line (T16 to T44). The AUC for the period of time tissue slices were exposed to the drugs is represented in the accompanying figure. *P<0.05, **P<0.01 vs. Vehicle Control; +P<0.05, ++P<0.01 vs. Vehicle + MDMA. N= 5-6 animals per group
5.4 DISCUSSION

The current data demonstrates that caffeine can influence MDMA-induced dopamine release from striatal and hypothalamic tissue slices in a concentration and region dependent manner. When tested in combination, caffeine (100 μM) enhanced MDMA (30 μM)-induced dopamine release from striatal tissue slices. By contrast, caffeine (100 μM) failed to influence dopamine release from hypothalamic tissue slices when combined with MDMA (30 μM). In order to determine if caffeine might interact with MDMA-induced dopamine release in hypothalamic slices, a higher concentration of MDMA (100 μM) was tested. Caffeine (100 μM) attenuated MDMA (100 μM)-induced dopamine release from hypothalamic slices. To determine if adenosine A₁ receptors might be implicated in the ability of caffeine to influence MDMA-induced dopamine release, application of the adenosine A₁ receptor antagonist DPCPX produced caffeine-like actions in slices prepared from both regions. Based on these findings we propose that caffeine can effect an adenosine A₁ receptor dependent regulation of MDMA-induced dopamine release in a bi-directional fashion dependent on brain region.

Several previous reports have described MDMA induced dopamine release in superfused tissue slices and synaptosomal preparations (Fitzgerald et al., 1990; Fitzgerald et al., 1993b; Johnson et al., 1991; Johnson et al., 1986; Riegert et al., 2008; Schmidt et al., 1987; Steele et al., 1987) and in studies using in vivo intracranial microdialysis (Baumann et al., 2008; Benamar et al., 2008; Esteban et al., 2001; Gough et al., 1991; Sabol et al., 1998). Several previous investigations have also been carried out on the effects of caffeine (Bonanno et al., 2000; Borycz et al., 2007) on dopamine release from superfused tissue slices. These studies were performed on striatal, accumbal and hippocampal tissues but not on hypothalamic tissue. Previous experiments have shown that caffeine increases dopamine and glutamate release in the striatum via the blockade of inhibitory presynaptic adenosine A₁ receptors (Borycz et al., 2007; Okada et al., 1996; Quarta et al., 2004; Solinas et al., 2002). Such a mechanism is consistent with the observations of the present study and supports our contention that caffeine enhances MDMA-induced dopamine release in the striatum through an adenosine A₁ receptor mechanism. The effects of caffeine and DPCPX on MDMA-induced dopamine release
Chapter 5: Discussion

in the striatum may generalise to other amphetamines as others have reported adenosinergic modulation of methamphetamine-induced striatal dopamine release (Golembiowska et al., 1998) and methamphetamine-induced sensitization to striatal dopamine release (Shimazoe et al., 2000; Yoshimatsu et al., 2001).

The differential response obtained with caffeine on MDMA-induced dopamine release in hypothalamic tissue slices suggests that caffeine may influence dopamine release in this structure via an alternative mechanism involving adenosine A_1 receptors. Dopaminergic afferents and receptors are abundant in the hypothalamus and their neuroanatomical and neurochemical links with the regulation of body temperature, sleep regulation and sexual behaviour (Dominguez et al., 2005; Kelley et al., 2002; Kumar et al., 2007). Although adenosine receptors are distributed in many brain regions including the hypothalamus (Ferre et al., 2007c; Ochiishi et al., 1999), their presynaptic regulation of dopamine in addition to GABA and glutamate release, as described in the striatum, has not been reported in hypothalamus to date. Thus the mechanism that accounts for the ability of caffeine and DPCPX to attenuate MDMA-induced dopamine release in hypothalamic tissue slices is not as obvious as what is proposed in the striatum. Such effects might be accounted for by considering that adenosine A_1 receptor containing glutamatergic synapses indirectly modulate dopamine release (Borycz et al., 2007; Ciruela et al., 2006) and that glutamate and/or dopamine release within brain regions are under differential adenosinergic control as has been reported by Borycz and co-workers (2007) in sub-compartments of the striatum. Electrophysiological experiments in slices from the striatum, where the level of tonic inhibition by endogenous adenosine mediated by adenosine A_1 receptors was found to differ in different synaptic compartments by analyzing excitatory and inhibitory currents in the region, are further suggestive of this possibility (Brundege et al., 2002). Further work however is necessary to clarify mechanisms mediating adenosinergic modulation of dopamine release in the hypothalamus.

Opposite modulatory roles for adenosine A_1 and A_{2A} receptors on glutamate and dopamine release have been described in the shell of the nucleus accumbens (Quarta et
Similar opposing actions of adenosine receptor subtypes might play a role in the regulation of dopamine release in other brain regions where adenosine receptors are expressed including the hypothalamus, although such mechanisms have not been described to date. However, as the selective adenosine A₁ receptor antagonist provoked a similar response to caffeine in the hypothalamus, the effect of caffeine may be attributed to inhibition of adenosine A₁ receptors. Initially, higher concentrations of DPCPX (30μM) were tested in both striatal and hypothalamic tissue slices (Supplementary data 4 & 5) and despite decreasing the concentrations of DPCPX over 30 fold, the ability of DPCPX (1μM) to attenuate MDMA-induced dopamine release in hypothalamic tissue slices was preserved. The possibility that endogenous adenosine release may play a role in the effects observed cannot be discounted where adenosine produces a tonic stimulation of both A₁ and A₂A receptors. As adenosine A₁ receptors are known to inhibit A₂A receptors (Karcz-Kubicha et al., 2003; Okada et al., 1996; Quarta et al., 2004), A₁ receptor blockade may relieve such inhibition on A₂A mediated effects. Stimulation of adenosine A₂A receptors increases extracellular concentrations of dopamine and glutamate in the striatum (Golembiowska et al., 1997; Okada et al., 1996; Popoli et al., 1995). Should similar mechanisms exist in the hypothalamus; it is not unreasonable to suggest that the unopposed activation of adenosine A₂A receptors may account for the ability of DPCPX to attenuate MDMA-induced dopamine release in hypothalamic tissue slices. Such a mechanism may also be of relevance to the effects obtained with caffeine in light of the preferential affinity of caffeine for adenosine A₁ receptors (Fredholm et al., 1999; Nehlig, 1999).

As caffeine provokes a potentially lethal interaction with MDMA in vivo, the question arises as to the extent to which dopamine release may contribute to the toxicity. Our work to date has largely addressed mechanisms mediating the ability of caffeine to exacerbate MDMA-induced hyperthermia (McNamara et al., 2006; Vanattou-Saffoudine et al., 2010). With regard to body temperature regulation, a role for dopamine in the pre-optic and anterior hypothalamus (PO/AH) has been demonstrated (Clark et al., 1986; Cox et al., 1983; Hasegawa et al., 2000; Scott et al., 1984). Hasegawa et al. (2005) reported that the dual dopamine/noradrenaline reuptake inhibitor increases brain and core temperatures associated with an increase in the extracellular concentration of
dopamine in the PO/AH of freely moving rats. More recently, Benamar et al., (2008) reported an increase in extracellular dopamine in the PO/AH following MDMA administration to rats. This response was attenuated by pre-treatment with the selective dopamine D₁ receptor antagonist SCH 23390, which also attenuates the hyperthermic response associated with MDMA (Mechan et al., 2002). We have recently established that pre-treatment with SCH 23390 attenuates the ability of caffeine to exacerbate MDMA-induced hyperthermia (Vanattou-Saïfoudine et al., 2010). One mechanism, by which caffeine may potentiate dopamine-mediated changes in core body temperature, is through a potentiation of MDMA-induced dopamine release. Although such a mechanism seems of relevance in the striatum, caffeine, in fact, attenuates MDMA-induced dopamine release in hypothalamic tissue slices. Thus, presynaptic dopamine release is unlikely to account for the ability of caffeine to exacerbate MDMA-induced hyperthermia. Alternatively postsynaptic dopaminergic mechanisms need to be examined in order to account for the ability of caffeine to enhance MDMA-induced hyperthermia.

In conclusion, the results of this study show that caffeine differentially regulates MDMA-induced dopamine release from striatal and hypothalamic tissue slices and these effects are most likely mediated by adenosine A₁ receptor blockade. Whilst the ability of caffeine to enhance MDMA-induced dopamine release from striatal slices may play a role in toxicity observed in vivo, postsynaptic mechanisms should also be investigated to clarify the mechanisms by which caffeine exacerbates MDMA-induced toxicity.
RESULTS

CHAPTER 6

Dopamine mediated intracellular changes associated with the ability of caffeine to influence MDMA ("Ecstasy")-induced toxicity in rats.
6.1 INTRODUCTION

It is widely reported that the pharmacological actions of MDMA result in biogenic amine (5-HT and dopamine) release in several regions of the brain (Green et al., 2003). Caffeine has also been reported to induce an increase in the release of 5-HT and dopamine in the cortex, hippocampus and striatum of freely-behaving rats (Okada et al., 1996; 1997; Acquas et al., 2002). It is therefore conceivable that administration of caffeine could result in an augmentation of MDMA-induced 5-HT or dopamine release. As it has been previously demonstrated in Chapter 3, a direct role for 5-HT in the hyperthermic response that occurs following the co-administration of caffeine with MDMA is, however, unlikely. By contrast, an association between the hyperthermia observed following co-administration of caffeine and MDMA with dopamine has been shown. It is also noteworthy that co-administration of caffeine with amphetamine or cocaine leads to a dramatic increase in seizures and mortality in rats in comparison to administration of amphetamine or cocaine alone (Derlet et al., 1992). As amphetamine and cocaine increase extracellular dopamine, such interactions support a role for dopamine as an integral factor mediating severe adverse reactions associated with the concurrent use of caffeine.

In Chapter 4, we have discussed the role of both dopamine D₁ and D₂ receptor subtypes in MDMA-induced changes in body temperature. Co-administration of caffeine switches the hypothermic response to MDMA in individually housed animals to a profound hyperthermia (McNamara et al., 2006). Prior administration of the D₁ receptor antagonist SCH 23390 completely blocks this shift (Chapter 4). We therefore hypothesise that caffeine can override D₂ receptor-mediated hypothermia, and promote a switch to D₁-mediated hyperthermia.

The role of MDMA in rat locomotor activity has also been widely studied (see Chapter 5). MDMA is known to induce hyperlocomotion and produces behavioural characteristics of the serotonin syndrome (Callaway et al., 1990; Gold et al., 1988b; Green et al., 1995; Hiramatsu et al., 1989; Reveron et al., 2006; Spanos et al., 1989).
Chapter 6: Introduction

The serotonin syndrome is caused by an excess of intrasynaptic 5-HT which causes clinical/behavioural manifestations due to the action of 5-HT on its receptors (Gillman, 1999). MDMA induces both dopamine and 5-HT release which is greater for 5-HT when compared to monoamine release induced by amphetamine (Rothman et al., 2001). Furthermore, this pharmacological pattern is reflected by the psychobehavioural effects induced by Ecstasy (euphoria and hallucination) (Parrott, 2001) which do not occur in cocaine or amphetamine abusers. MDMA-induced locomotor activity has been observed in many studies, as might be expected of an amphetamine-like drug (Bankson et al., 2001; Callaway et al., 1992a; Callaway et al., 1992b; Gold et al., 1989; Yamamoto et al., 1988) which correlates with an increase in dopamine in the striatum (Hegadoren et al., 1995; Spanos et al., 1989). Peripheral and central administration of MDMA dose dependently enhanced locomotor activity in open field test (Bengel et al., 1998; Callaway et al., 1992a; Callaway et al., 1990; Dafters, 1994; Gold et al., 1989; Rempel et al., 1993; Scearce-Levie et al., 1999; Spanos et al., 1989). As 5-HT alone is not sufficient to increase locomotor activity (Aulakh et al., 1988), both 5-HT and dopamine are necessary, which is an important difference between MDMA- and amphetamine-induced locomotion (Bankson et al., 2001).

Locomotor activity in rats is increased after treatment with caffeine (Nehlig et al., 1992) albeit weaker when compared to cocaine and D-amphetamine (Antoniou et al., 1998). Dopamine has been proposed to account for the behavioural effects of caffeine as those are similar to the behaviours induced by drugs like cocaine and D-amphetamine which have a dopaminergic mechanism of action. Low to intermediate doses of caffeine increase locomotor activity (Antoniou et al., 1998). Selective dopamine D₁ and D₂ antagonists can block this effect. Blockade of A₂A receptors rather than A₁ participates through association with dopaminergic receptors in the stimulant effect of caffeine (Svenningsson et al., 1997). Furthermore, the psychostimulant effects of caffeine appear to be dependent on intact dopaminergic transmission as administration of reserpine blocks caffeine-induced locomotor activity (Ferre et al., 1991b; Waldeck, 1975; White et al., 1978). In addition, catecholamine depletion with alpha-methyl-para-tyrosine attenuates the locomotion induced by caffeine and amphetamine. Dopamine D₁ antagonists counteract the stimulant effect of caffeine which may be sufficient to prevent the increase in locomotion induced by activation of D₂ receptors (Braun et al., 1997).
Furthermore, in tolerant animals to caffeine-induced locomotor activity, cross tolerance to both dopamine D₁ and D₂ agonists has been observed (Garrett et al., 1994). Therefore, both dopaminergic subtypes are key players in caffeine-induced locomotion and tolerance to these effects.

Dopamine D₁ and D₂ receptors also have opposing actions at the biochemical level on the activity of adenylyl cyclase and protein kinase A (PKA). The dopamine and cAMP regulated phosphoprotein of 32 kDa (DARPP-32) is abundant in neurons which received dopaminergic input. Activation of PKA and the consequent phosphorylation of DARPP-32 on threonine 34 occur in response to dopamine acting on D₁ receptors. In contrast, activation of D₂ receptors results in inhibition of PKA activation, the activation of protein phosphatase 2B and the consequent dephosphorylation of DARPP-32. Phosphorylated DARPP-32 is a potent inhibitor of protein phosphatase-1 (Greengard et al., 1998). Thus the phosphorylation status of DARPP-32 provides an intra-neuronal marker of D₁/D₂ receptor activation, and may be useful to study the influence of caffeine on MDMA-induced intracellular changes associated with D₁/D₂ receptor activation.

c-fos was one of the first immediate early genes characterised and is commonly used due to its low basal expression making its up-regulation readily detectable (Farivar et al., 2004). As previously mentioned, dopamine, through dopamine D₁ receptors activation, stimulates adenylyl cyclase and therefore, increase the activity of PKA (Cooper et al., 2003), leading to cAMP response element binding protein (CREB) phosphorylation. Subsequently, p-CREB stimulates the transcription of c-fos via c-fos promoter which contains a cAMP response element (CRE) (Sng et al., 2004).

As discussed previously, there are biochemical mechanisms by which caffeine may interact with dopamine receptors or alter dopamine release. Specifically caffeine is an adenosine receptor antagonist (Nehlig, 1999; Fredholm et al., 1999), and also a weak inhibitor of cAMP phosphodiesterase (PDE). However, in the studies we have conducted to date, co-treatment with adenosine antagonists failed to provoke a caffeine-like
interaction with MDMA, indicating that blockade of adenosine receptors alone is unlikely to mediate caffeine's interaction with MDMA (Vanattou-Saïfoudine et al., 2010). Interestingly, we have found that concurrent administration of low doses of the cAMP PDE inhibitor rolipram and the adenosine A2A receptor antagonist SCH 58261, with MDMA, provokes a marked caffeine-like hyperthermic response. Whilst it has been reported that the inhibitory effect of caffeine on PDE is of little relevance at the concentrations of caffeine administered in vivo (Fredholm et al., 1999), based on these data, we suspect that caffeine's weak PDE inhibiting properties might well be relevant against a background of increased intracellular cAMP availability following MDMA-induced biogenic amine release in the brain.

In light of results to date, implying a role for dopamine D1 receptors and adenosine receptors/PDE, in mediating the ability of caffeine to exacerbate MDMA-induced hyperthermia, the current study sets out to determine if behavioural or core body temperature changes are associated with the activation of intracellular downstream targets, namely CREB, DARPP-32 and c-fos in the striatum and hypothalamus. The role of dopamine D1 receptors in mediating such changes was determined by pre-treatment with the dopamine D1 receptor antagonist SCH-23390. The experiments clarify if the effects of caffeine are mediated by postsynaptic mechanisms involving the dopaminergic system and, more specifically, if phosphorylation of DARPP-32 may be a useful intracellular marker that is predictive of a caffeine-induced switch from a D2 to D1 receptor-mediated phenotype, as previously suggested following MDMA administration to rats (Vanattou-Saïfoudine et al., 2010).

Thus, postsynaptic mechanisms which most likely involved an interaction between dopamine and adenosine receptors and/or phosphodiesterase (see Chapters 1 and 3), are responsible for the response to caffeine and MDMA.
6.2 EXPERIMENTAL DESIGN

Behaviours were scored by observation of the animal in their home cage environment. Rating scales were introduced for approximation of the 5-HT syndrome (Spanos et al., 1989) and amphetamine-induced behaviours (Mumford et al., 1979) following a challenge injection of caffeine, MDMA alone or in combination. Behavioural changes related to D-amphetamine and D-amphetamine with D-fenfluramine alone or in combination with caffeine were recorded in a similar fashion.

In the present study a combination of classification by observation and automatic recording was adopted as an appropriate way to study D-amphetamine-induced stereotyped and locomotor behaviours. Separate rating scales were used for quantifying stereotyped and locomotor behaviours so that these could be assessed independently. The most commonly used measures of stereotyped behaviours are based on time sampling and observational recordings which have been employed in previous investigations (Harkin et al., 2001; Harkin et al., 2000). As continuous recording of behaviour is difficult over extended periods especially when it is complex and rapidly changing, time sampling methods with interval recording provide appropriate estimates of the occurrences and duration of particular behaviours. The following were employed as rating scales to score the stereotyped behaviour and locomotor behaviours of the animals in their home cage.

For the locomotion, stereotypy and 5-HT loss related behaviours scorings, see the Methods chapter, Section: Behavioural assessments.

Animals were observed continuously following drug administration and a behavioural score assigned immediately preceding the temperature measurements. At the end of the trail, behavioural scores were totalled over the intervals comprising the 5 hour observation period.

Study 1: Influence of caffeine on MDMA-induced behaviour, hyperthermia and intracellular activation markers

Rats were treated with MDMA (15 mg/kg) and caffeine (10 mg/kg) alone or in combination and core body temperatures and behaviours were recorded as described
earlier. In a parallel set of animals, core body temperatures were recorded immediately prior to and 1 hour following caffeine/MDMA administration. In this set the animals were euthanized immediately after the temperature was recorded 1 hour following drug administration and their brains were rapidly removed. Striatum and hypothalamus were dissected out and processed for determinations of, phosphorylation of CREB, DARPP-32 by Western blotting and, expression of the immediate early gene \textit{c-fos} using Q-PCR as described earlier. 1 hour following drug administration coincides with peak effects on the behavioural and core body temperature response to the drugs and was therefore chosen as an appropriate time to determine the status of intracellular markers of activation (see \textbf{Methods}, section Western (immuno)blotting for CREB and DARPP-32 proteins and Real-time PCR).

\textit{Study 2: Effect of pre-treatment with the dopamine D\textsubscript{1} antagonist SCH 23390 on the intracellular changes associated with the combined administration of caffeine with MDMA.}

Rats were pre-treated with SCH 23390 (1 mg/kg) 30 minutes prior MDMA (15 mg/kg) and caffeine (10 mg/kg) alone or in combination. Core body temperatures were recorded immediately prior to and 1 hour following caffeine/MDMA administration. Animals were euthanized immediately after the temperature was recorded 1 hour following drug administration and phosphorylation of CREB, DARPP-32 by Western blotting and expression of the immediate early gene \textit{c-fos} using Q-PCR were measured in the striatum and in the hypothalamus as described in study 1.
6.3 RESULTS

Study 1: Caffeine enhances MDMA-induced hyperthermia and behaviour.

(a) Body temperature

ANOVA of body temperature showed effects of MDMA \([F(1,28) = 100.3, P<0.001]\), caffeine \([F(1,28) = 20.2, P = 0.0001]\), time \([F(7,196) = 168.1, P<0.001]\), MDMA x time \([F(7,196) = 21.9, P<0.001]\) and caffeine x time \([F(7, 196) = 4.83, P<0.001]\). Post hoc comparisons revealed that MDMA increased body temperature 0.5, 1, 2 and 3 hours following administration when compared to vehicle-treated controls. Caffeine alone did not significantly alter body temperature when compared to vehicle-treated controls. Caffeine increased MDMA-induced hyperthermia 0.5, 1, 1.5, 2 and 3 hours following drug administration compared to the MDMA alone treated group (Figure 6.1A).

With SCH 23390, three-way ANOVA of body temperature showed effects of MDMA \([F(1,28) = 21.4, P < 0.0001]\), time \([F(7,196) = 38.2, P < 0.0001]\), MDMA x time \([F(7,196) = 9.4, P < 0.0001]\) and a MDMA x caffeine x time interaction \([F(7,308) = 4.20, P<0.001]\). Pre-treatment with SCH 23390 blocked any increase in core body temperature following MDMA administration alone or in combination with caffeine. Post hoc comparisons revealed that MDMA induced hypothermia 1 hour following drug administration. Caffeine alone did not significantly alter body temperature when administered alone or in combination with MDMA-treated groups (Figure 6.1B).
Figure 6.1 SCH 23390 blocks the ability of caffeine to exacerbate MDMA-induced hyperthermia. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean with standard error of the mean of change in core body temperature in (A) control and (B) SCH 23390-treated animals. N= 5-8 animals per group. *P<0.01 vs. Vehicle Control; +P<0.01 vs. Vehicle + MDMA.
ANOVA of the locomotor activity showed effects of MDMA \(F(1,28) = 547.2, P<0.001\), caffeine \(F(1,28) = 483.7, P<0.001\), time \(F(4,112) = 213.1, P<0.001\), MDMA x time \(F(4,112) = 35.8, P<0.001\), caffeine x time \(F(4,112) = 35.6, P<0.001\) and MDMA x caffeine x time \(F(4,112) = 11.9, P<0.001\) interactions. Post hoc comparisons revealed that MDMA increased locomotor activity 1, 2, 3 and 4 hours following administration when compared to vehicle-treated controls. Caffeine alone significantly increased locomotion 1, 2, 3 and 4 hours following administration when compared to vehicle-treated controls. Caffeine potentiated MDMA-induced locomotion 2, 3 and 4 hours following drug administration when compared to the MDMA alone treated group (Figure 6.2.1).

**Figure 6.2.1 Caffeine potentiates MDMA-induced hyperlocomotion.** There was no difference in locomotor activity or stereotypy between the groups at T0 prior to challenge. Values represent mean with standard error of the mean. \(N=8\) animals per group. *\(P<0.01\) vs. Vehicle Control; +\(P<0.01\) vs. Vehicle + MDMA
(c) Stereotypic behaviours

ANOVA of the stereotypic behavior showed effects of MDMA \([F(1,28) = 1034.8, P<0.001]\), caffeine \([F(1,28) = 259.6, P<0.001]\), time \([F(4,112) = 100.2, P<0.001]\), MDMA x time \([F(4,112) = 23.9, P<0.001]\), caffeine x time \([F(4,112) = 6.6, P<0.001]\) and MDMA x caffeine x time \([F(4,112) = 5.5, P<0.001]\) interactions. Post hoc comparisons revealed that MDMA increased stereotypic behavior 1, 2 and 3 hours following administration when compared to vehicle-treated controls. Caffeine alone significantly increased stereotypy scores 1, 2 and 3 hour following the injection when compared to vehicle-treated controls. Caffeine potentiated MDMA-induced stereotypic behaviours 2, 3 and 4 hours following drug administration compared to the MDMA alone treated group (Figure 6.2.2).

![Figure 6.2.2 Caffeine potentiates MDMA-induced stereotypy. There was no difference in locomotor activity or stereotypy between the groups at T0 prior to challenge. Values represent mean with standard error of the mean. N= 8 animals per group. *P<0.01 vs. Vehicle Control; +P<0.01 vs. Vehicle + MDMA](image-url)
Chapter 6: Study 1: Caffeine enhances MDMA-induced hyperthermia and behaviour

(d) 5-HT syndrome

ANOVA of 5-HT score showed effects of MDMA [F(1,28) = 929.72, P < 0.0001], caffeine [F(1,28) = 150, P < 0.0001], MDMA x caffeine [F(1,28) = 150, P < 0.0001], time [F(4,159) = 128.50, P < 0.0001], MDMA x time [F(4,159) = 128.50, P < 0.0001], caffeine x time [F(4,112) = 26.52, P < 0.0001], and a MDMA x caffeine x time interaction [F(4,112) = 26.52, P < 0.0001]. Post hoc comparisons showed that MDMA increased 5-HT loss associated behaviors 1, 2 and 3 hours following drug administration when compared to vehicle-treated group. Caffeine alone did not influence these behaviors but co-administered with MDMA potentiated them 1, 2, 3 and 4 hours following drug administration when compared to MDMA-treated group (Figure 6.2.3).

**Figure 6.2.3** Caffeine potentiates MDMA-induced 5-HT syndrome. There was no difference in locomotor activity or stereotypy between the groups at T0 prior to challenge. Values represent mean with standard error of the mean. N= 8 animals per group. *P<0.01 vs. Vehicle Control; +P<0.01 vs. Vehicle + MDMA
Chapter 6: Study 2: Caffeine potentiates MDMA-induced phosphorylation of CREB but not DARPP-32 in the striatum

**Study 2: Caffeine potentiates MDMA-induced phosphorylation of CREB but not DARPP-32 in the striatum.**

ANOVA of change in body temperature showed effect of MDMA \([F(1,21) = 4.47, P = 0.04]\) and caffeine \([F(1,21) = 20.38, P < 0.001]\) only. Post hoc comparisons revealed that MDMA increased body temperature when compared to saline-treated group. Caffeine potentiated MDMA-induced hyperthermia when compared to the MDMA-treated group (Figure 6.3A).

ANOVA of p-CREB band density showed an effect of MDMA \([F(1,16) = 9.41, P = 0.008]\) and caffeine \([F(1,14) = 26.77, P < 0.001]\) only. Post hoc comparisons revealed that MDMA increased p-CREB when compared to saline controls. Caffeine potentiated the MDMA-induced increase in p-CREB when compared to the MDMA-treated group alone (Figure 6.3B).

ANOVA of CREB band density showed an effect of caffeine \([F(1,14) = 4.75, P = 0.04]\) only. Post hoc comparisons revealed that neither MDMA nor caffeine influenced CREB when compared to saline-treated controls (Figure 6.3C).

By contrast ANOVA of p-DARPP-32 or DARPP-32 band density did not show any effects of MDMA or caffeine (Figure 6.3D and E respectively).

ANOVA of cAMP concentrations showed an effect of MDMA \([F(1,18) = 5.63, P = 0.02]\) only. Post hoc comparisons revealed that neither MDMA nor caffeine influenced cAMP concentrations when compared to saline-treated group (Figure 6.3F).
Chapter 6: Study 2: Caffeine potentiates MDMA-induced phosphorylation of CREB but not DARPP-32 in the striatum

![Graph A](image)

![Graph B](image)

![Graph C](image)
Chapter 6: Study 2: Caffeine potentiates MDMA-induced phosphorylation of CREB but not DARPP-32 in the striatum

Figure 6.3 Caffeine potentiates the MDMA related increase in striatal p-CREB but not p-DARPP-32. Value represent mean with standard error of the mean of (A) temperature change and (F) cAMP concentrations. Values represent band density mean expressed as percentage of control with standard error of the mean of (B) p-CREB, (C) CREB, (D) p-DARPP-32, (E) DARPP-32. The western blot data are expressed as percentage of the control group. N= 4-8 animals per group. *P < 0.01 vs. Vehicle Control; +P < 0.01 vs. Vehicle + MDMA
Chapter 6: Study 3

Study 3 Combined treatments with caffeine and MDMA increases phosphorylation of CREB and DARPP-32 in the hypothalamus.

ANOVA of p-CREB band density showed an effect of MDMA \([F(1,16) = 18.41, P < 0.001]\) only. Post hoc comparisons revealed that whilst neither treatment alone significantly influenced p-CREB when compared to vehicle-treated controls, combined treatment of caffeine with MDMA, increased p-CREB in MDMA-treated animals when compared to the caffeine nor MDMA alone treated groups (Figure 6.4A).

ANOVA of CREB band density showed effect of caffeine \([F(1,26) = 5.65, P = 0.02]\) and caffeine x MDMA interaction \([F(1,26) = 4.36, P = 0.04]\). Post hoc comparisons revealed that neither MDMA nor caffeine influenced CREB when compared to vehicle-treated controls. Similarly caffeine failed to influence CREB in combination with MDMA (Figure 6.4B).

ANOVA of p-DARPP-32 band density showed an effect of caffeine \([F(1,28) = 3.86, P = 0.05]\) and MDMA \([F(1,28) = 15.19, P < 0.001]\) only. Post hoc comparisons revealed that, whilst neither treatment alone significantly influenced p-DARPP-32 compared to vehicle-treated controls, combined treatment of caffeine with MDMA increased p-DARPP-32, when compared to the caffeine or MDMA alone treated animals (Figure 6.4C).

ANOVA of DARPP-32 band density showed an effect of MDMA \([F(1,28) = 6.52, P = 0.01]\) only. Post hoc comparisons revealed that neither MDMA nor caffeine influenced DARPP-32 when compared to vehicle-treated group. Similarly caffeine failed to influence DARPP-32 in combination with MDMA (Figure 6.4D).

ANOVA of cAMP concentrations showed an effect of MDMA \([F(1,18) = 5.63, P = 0.02]\) only. Post hoc comparisons revealed that neither MDMA nor caffeine influenced cAMP concentrations when compared to vehicle-treated group (Figure 6.4E).
Chapter 6: Study 3: Combined treatment with caffeine and MDMA increases phosphorylation in hypothalamic CREB and DARPP-32
Chapter 6: Study 3: Combined treatment with caffeine and MDMA increases phosphorylation in hypothalamic CREB and DARPP-32

Figure 6.4 Co-administration of caffeine and MDMA provokes an increase in hypothalamic p-CREB and p-DARPP-32. Values represent mean band density expressed as percentage of control with standard error of the mean of (A) p-CREB, (B) CREB, (C) p-DARPP-32, (D) DARPP-32 and (E) cAMP concentrations. The western blot data are expressed as percentage of the control group. N= 4-8 animals per group. +P<0.01 vs. Vehicle + MDMA

193
Chapter 6: Study 4

Study 4: Caffeine potentiates MDMA-induced c-fos expression in striatum but not in the hypothalamus.

ANOVA of fold change in striatal c-fos expression showed an effect of caffeine \([F(1,21) = 4.47, P = 0.04]\) and MDMA \([F(1,21) = 20.38, P < 0.001]\) only. Post hoc comparisons revealed that MDMA increased c-fos expression when compared to vehicle-treated controls. Caffeine enhanced MDMA-induced c-fos expression when compared to the MDMA alone treated group (Figure 6.5A).

ANOVA of fold change in hypothalamic c-fos expression showed an effect of MDMA \([F(1,18) = 14.82, P < 0.001]\) only. Post hoc comparisons revealed that MDMA increased c-fos expression when compared to the vehicle-treated group. Caffeine had no effect alone nor did it significantly influence the MDMA-induced change (Figure 6.5B).
Chapter 6: Study 4: Caffeine potentiates MDMA-induced c-fos expression in striatum but not in the hypothalamus

Figure 6.5 MDMA-induced c-fos expression is potentiated by the co-administration of caffeine in the striatum but not the hypothalamus. Values represent mean with standard error of the mean of fold change in c-fos expression in the (A) striatum and (B) hypothalamus. N= 8 animals per group. *P<0.01 vs. Vehicle Control; +P<0.01 vs. Vehicle + MDMA.
Study 5: Pre-treatment with SCH 23390 blocks proteins phosphorylation induced by caffeine and MDMA in the striatum and hypothalamus.

ANOVA of change in body temperature showed effect of MDMA [F(1,13) = 19.17, P<0.001] only. Post hoc comparisons revealed that MDMA decreased body temperature when compared to vehicle-treated group. Caffeine alone did not influence body temperature when compared to vehicle-treated group (Figure 6.6).

Figure 6.6 SCH 23390 pre-treatment reduces body temperature in MDMA-treated group and blocks caffeine potentiation. Values represent mean band density with standard error of the mean of temperature change. N= 5 animals per group. *P<0.01 vs. Vehicle control.
Striatum

ANOVA of p-CREB band density showed an effect of MDMA x caffeine \( [F(1,12) = 6.49, P = 0.02] \) only. When compared to Figure 5A, SCH-23390 pre-treatment blocks MDMA-induced CREB phosphorylation and its potentiation by caffeine (Figure 6.7B).

ANOVA of CREB band density and post hoc comparison showed no effect of either caffeine, MDMA or the combination (Figure 6.7C).

ANOVA of p-DARPP-32 (Figure 6.7D) and DARPP-32 (Figure 6.7E) bands density did not show any MDMA or caffeine effects.
Figure 6.7 SCH 23390 blocks MDMA-induced striatal p-CREB alone or in combination with caffeine. Values represent mean band density expressed as percentage of control with standard error of the mean of (B) p-CREB, (C) CREB, (D) p-DARPP-32 and (E) DARPP-32. The western blot data are expressed as percentage of the control group. N= 5 animals per group.
Hypothalamus

ANOVA of p-CREB band density showed an effect of MDMA \([F(1,12) = 5.37, P = 0.03]\) only. When compared to Figure 6A, SCH-23390 pre-treatment blocks caffeine action on CREB phosphorylation in MDMA-treated animals when compared to MDMA-treated group (Figure 6.8A).

ANOVA of CREB band density and post hoc comparison showed no effect of either caffeine, MDMA or the combination (Figure 6.8B).

ANOVA of p-DARPP-32 band density showed an effect of MDMA x caffeine \([F(1,12) = 4.17, P = 0.06]\) only. When compared to Figure 6C, SCH-23390 blocked the increase of DARPP-32 phosphorylation in MDMA-treated animals by caffeine when compared to MDMA-treated animals (Figure 6.8C).

ANOVA of DARPP-32 band density showed no effect of either caffeine, MDMA or the combination (Figure 6.8D).
Chapter 6: Study 5: Pre-treatment with SCH 23390 blocks proteins phosphorylation induced by caffeine and MDMA in the striatum and hypothalamus

**A**

![Graph](image)

Caffeine (10mg/kg) -  +  -  +  
MDMA (15mg/kg)  -  -  +  +  

**B**

![Graph](image)

Caffeine (10mg/kg) -  +  -  +  
MDMA (15mg/kg)  -  -  +  +  

β-actin  
p-CREB  

CREB  
β-actin
Figure 6.8 SCH 23390 blocks the increase in hypothalamic p-CREB and p-DARPP-32 associated with the co-administration of caffeine with MDMA. Values represent mean band density expressed as percentage of control with standard error of the mean of (A) p-CREB (B) CREB (C) p-DARPP-32 and (D) DARPP-32. The western blot data are expressed as percentage of the control group. N= 5 animals per group.
Chapter 6: Study 6

Study 6: Pre-treatment with SCH 23390 blocks the intracellular effects-induced by caffeine and MDMA, and associated with c-fos, in the striatum and hypothalamus.

ANOVA of striatal c-fos fold change showed effect of caffeine \( [F(1,12) = 2.57, P = 0.13] \) only. Compared to Figure 6.7A, post hoc comparisons revealed that SCH 23390 pre-treatment blocks MDMA-induced c-fos expression when compared to vehicle-treated group and its potentiation by caffeine when compared to MDMA-treated group (Figure 6.8A).

ANOVA of hypothalamic c-fos fold change showed effect of MDMA x caffeine \( [F(1,13) = 7.47, P = 0.17] \) only. Compared to Figure 6.7B, post hoc comparisons revealed that SCH 23390 pre-treatment blocks MDMA-induced c-fos expression when compared to vehicle-treated group (Figure 6.8B).
Chapter 6: Study 6: Pre-treatment with SCH 23390 blocks the intracellular effects-induced by caffeine and MDMA, and associated with c-fos, in the striatum and hypothalamus.

**Figure 6.9** SCH 23390 blocks the increase in striatal and hypothalamic c-fos expression associated with the co-administration of caffeine with MDMA. Values represent mean fold change in expression with standard error of the mean of c-fos expression in (A) striatum and (B) hypothalamus. N= 5 animals per group.
**6.4 DISCUSSION**

The results of this study show an augmentative effect of caffeine (10 mg/kg) on MDMA (15 mg/kg)-induced behavioural changes including locomotor activity, stereotypic behaviour and behaviours associated with the 5-HT syndrome. Assessment of intracellular activation markers showed that caffeine enhances the phosphorylation of CREB and the expression of c-fos following MDMA administration in both striatum and hypothalamus. Similarly phosphorylation of DARPP-32 is enhanced but this change is restricted to the hypothalamus suggesting that, there are regional differences in the activation of this marker. These changes occurred, in tandem, with the ability of caffeine to promote MDMA-induced hyperthermia. Pre-treatment with the dopamine D₁ antagonist SCH-23390 blocked the ability of caffeine to enhance MDMA-induced hyperthermia, and to affect intracellular changes in response to MDMA administration, consistent with a role for D₁ receptors in the caffeine mediated responses.

Proposed mechanisms underlying these outcomes are discussed further in the sections below.

**Caffeine potentiates MDMA-induced behaviours.**

In the current investigation administration of MDMA to rats provoked hyperactivity and 5-HT syndrome, as previously described by others (Gold et al., 1988a; Hiramatsu et al., 1989; Shankaran et al., 1999; Spanos et al., 1989). As caffeine has a profound effect on MDMA-induced changes in core body temperature and heart rate (McNamara et al., 2006; McNamara et al., 2007), it was conceivable that caffeine would potentiate MDMA-induced behaviours. Previously however, McNamara and co-workers (2006) reported by contrast to the effects on body temperature, that co-administration of caffeine did not alter the locomotor stimulant effects or 5-HT syndrome associated with MDMA or MDA. This observation suggested that the drug interaction could not be clearly defined by behavioural observation. However in the present investigation, with some refinement of the behavioural scoring technique, a behavioural interaction between caffeine and MDMA was observed. Characterisation of the behavioural response revealed a response comprising stereotypical behaviours, commonly observed following amphetamine administration. In further behavioural tests, we examined the effects of
caffeine (10 mg/kg) in combination with D-amphetamine (1 mg/kg) and observed that, caffeine did not significantly influence D-amphetamine-induced locomotion or stereotyped behaviors. Co-treatment of caffeine with a combination of D-amphetamine (1 mg/kg) and the selective 5-HT releasing agent D-fenfluramine (5 mg/kg) however, provoked an increase in behaviors, when compared to animals treated with D-amphetamine and D-fenfluramine alone (Supplementary data 6 & 7). Such a response is reminiscent of the ability of caffeine to enhance the hyperthermia associated with combined administration of D-amphetamine and D-fenfluramine, by comparison to either of these agents alone, and indicates that both 5-HT and dopamine are important contributors to the particular behavioral interactions between caffeine and MDMA. Changes, that occur in locomotor activity following the administration of MDMA, are related to changes in 5-HT and dopaminergic activity in mesolimbic and nigrostriatal dopamine pathways and their terminal regions, the nucleus accumbens and the striatum (Bast et al., 2002; Baumann et al., 2008; Bubar et al., 2004; Hall et al., 2009b). It was therefore of interest, to determine if caffeine could influence markers of intraneuronal signalling, subsequent to dopamine receptors activation following MDMA administration, in the hypothalamus (through association with changes in core body temperature) and the striatum (key structure in mediating the behavioural response to MDMA).

Caffeine enhances MDMA-induced activation of CREB, DARPP-32 and c-fos: a role for dopamine D<sub>1</sub> receptors

As previously reported, MDMA provoked an increase in core body temperature and this response was potentiated by co-administration with caffeine. Prior administration of SCH 23390 blocked MDMA-induced hyperthermia and its exacerbation by caffeine, consistent with a role for dopamine D<sub>1</sub> receptors in the interaction. These results are consistent with those previously reported where SCH-23390 blocked MDMA-induced hyperthermia (Mechan et al., 2002b) and its exacerbation by caffeine (Vanattou et al., 2010). Associated with these changes and the aforementioned changes in behavior, caffeine was found to potentiate MDMA-induced phosphorylation of CREB and expression of c-fos in the striatum. However, no changes were observed in the
phosphorylation of DARPP-32, following MDMA administration alone or in combination with caffeine. By contrast, caffeine was observed to potentiate MDMA-induced phosphorylation of CREB and DARPP-32 in addition to the expression of *c-fos* in the hypothalamus. Activation of intracellular markers CREB, DARPP and *c-fos* reflects the activation of dopamine D$_1$ receptors which couple positively to intracellular cAMP signalling and the activation of PKA. In this regard, a role for dopamine D$_1$ receptors is confirmed as these changes were also attenuated by prior treatment with SCH-23390.

Our results following MDMA administration are consistent with those reported by others. Phosphorylation of DARPP-32 is induced by several psychostimulants, including cocaine and amphetamine, and regulated by several neurotransmitters (Nairn *et al.*, 2004). Martinez-Turrillas and co-workers (2006) have shown that MDMA increases p-CREB in both cortex and hippocampus in animals, which relates to the expression of brain derived neurotrophic factor (BDNF). Others have reported an induction of p-CREB, following systemic amphetamine administration in rats (Dalley *et al.*, 1999; Konradi *et al.*, 1996; Liu *et al.*, 1996). As CREB phosphorylation, in turn, has been reported to be regulated by dopamine and dopamine D$_1$ receptor activation (Hyman *et al.*, 1995), and the ability of caffeine to influence MDMA has been proposed to be dopamine dependent, it is conceivable that caffeine potentiates MDMA-induced CREB phosphorylation in the striatum and hypothalamus via a dopamine D$_1$ dependent mechanism.

DARPP-32 appears to play an important role in the stimulant effect of caffeine. Genetic depletion of DARPP-32 shows a decrease in caffeine effect on motor activity, results similar to the use of adenosine A$_{2A}$ antagonist (Lindskog *et al.*, 2002). In addition, the psychomotor effect of caffeine depends on its ability to antagonise adenosine A$_{2A}$ receptors transmission in striatopallidal neurons (Fisone *et al.*, 2004). Blockade of these receptors reduces basal cAMP production and therefore, inhibits phosphorylation of DARPP-32 at Thr$^{34}$ (Andersson *et al.*, 2005) and increases phosphorylation at Thr$^{75}$ (Lindskog *et al.*, 2002). In the present investigation, a differential response in p-DARPP
was obtained between the striatum, where no change was found following MDMA administration alone or in combination with caffeine, and the hypothalamus, where caffeine was found to enhance p-DARPP following MDMA administration. These results are in accordance with the results presented in Chapter 3, where dopamine release in both striatum and hypothalamus was not regulated in a similar fashion. In the striatum, adenosine A1 receptors regulate dopamine release following administration of caffeine. Moreover, adenosine A1 receptors negatively couple with dopamine D1 receptors; thus, these mechanisms could account for the increase in MDMA-induced CREB phosphorylation by caffeine. In the hypothalamus, pre-synaptic regulation of either dopamine or glutamate release by adenosine receptors has not been reported. However, A1 receptors play an important role in dopamine release in the MDMA and caffeine interaction within this brain region (see Chapter 3). DARPP-32 has been found in the mediodasal hypothalamus (Hokfelt et al., 1988) and is mostly present on dopaminceptive neurons bearing the D1 receptor subtype and in tanycytes within the hypothalamus. Auger et al., in 2001, demonstrated that treatment with oestradiol increases hypothalamic DARPP-32 phosphorylation two fold compared to the control with no change in the cerebral cortex. As p-DARPP-32 immunoreactivity has been observed in the preoptic area and ventromedial hypothalamus where dopamine D1 receptor binding (Boyson et al., 1986), D1 receptor immunoreactivity (Huang et al., 1992; Levey et al., 1993) and D1 mRNA (Fremeau et al., 1991; Weiner et al., 1991) have also been localised, phosphorylation of DARPP-32 observed after treatment with both MDMA and caffeine in the present study, might be due to an indirect influence of adenosine A1 receptors (see Chapter 3 for the key role of this receptor subtype). However, as A2A receptors have been identified in the ventrolateral preoptic area of the hypothalamus and are important in caffeine effect on MDMA-induced hyperthermia (see Chapter 1), further work will be necessary to investigate their role in p-DARPP-32 expression.

Expression of c-fos, a well established marker for cellular activation, has been shown to be increased in several brain areas especially brain areas in rats such as the striatum following MDMA administration (Erdtmann-Vourliotis et al., 1999; Erdtmann-Vourliotis et al., 2000). MDMA has been associated with a localised but widespread
induction of \(c-fos\) mRNA within areas such as the striatum and paraventricular area of the hypothalamus (Stephenson et al., 1999). Other psychostimulants, which enhance central dopamine release, have been reported previously to increase the expression of \(c-fos\) via dopamine \(D_1\) receptor activation (Canales et al., 2000; Graybiel et al., 1990; Lin et al., 1996; Moratalla et al., 1992; Young et al., 1991). Caffeine administration has previously shown to induce an increase in \(c-fos\) expression within the striatum via its antagonism of \(A_1\) receptors (Dassesse et al., 1999). However, these authors administered a dose of caffeine 10-times higher (100 mg/kg, i.p.) that the dose used in the present thesis, which may account for this increase of expression, as the striatum is a rich dopaminergic cerebral region and expression of immediate early genes can be modulated by manipulation of dopaminergic transmission. A pivotal role for \(D_1\) receptors signalling pathway has been confirmed in the present investigation, as pre-treatment with SCH 23390 totally blocked this upregulation of \(c-fos\). When taken together, the results of the present study support a dopamine \(D_1\) receptor dependent regulation of \(c-fos\) expression following MDMA administration and its enhancement by caffeine in both striatum and hypothalamus.

Co-administration with caffeine potentiated MDMA-induced behaviours and hyperthermia which was associated with increased p-CREB, p-DARPP-32 and \(c-fos\) expression in the hypothalamus and with increased p-CREB and \(c-fos\) in the striatum, when compared to either treatment alone. SCH 23390 blocked MDMA-induced hyperthermia and its exacerbation by caffeine and attenuated the changes in p-CREB, p-DARPP and \(c-fos\) in the striatum and hypothalamus consistent with a dopamine \(D_1\) receptor related mechanism, convergent on intracellular cAMP intracellular signalling mediating the acute toxicity associated with the combined treatment of caffeine and MDMA. If the interaction we observed in this rodent model generalises to humans, such a pattern of recreational drug use could have serious acute and long-term health consequences for recreational “Ecstasy” users. Targeting the dopamine \(D_1\) pathway may represent a way to treat patients presenting with acute and potentially life threatening toxicity associated with MDMA.
CHAPTER 7
GENERAL DISCUSSION
7.1 Introduction

Many studies have been conducted in order to elucidate the mechanisms of action of the substituted amphetamines, and to understand why certain people are less or more sensitive to their toxic effects. MDMA, a major component of Ecstasy tablets, is a widespread drug of abuse, particularly among young people, and is perceived as a "soft and safe" drug, due to a low incidence of serious adverse effects on users. However, it has been classified as class A in the United Kingdom and is known to induce many adverse effects. Another issue raised by the abuse of MDMA is that, recreational users are often polydrug abusers which might contribute to MDMA-associated toxicity. Of recent concern is the trend for users to combine the ingestion of MDMA and caffeine within the same drug taking episode, intentionally or inadvertently. This happens through the presence of large proportions of caffeine present in "Ecstasy" tablets and on account of freely available caffeinated beverages, including "energy" drinks such as Red Bull. Therefore, the influence of caffeine on MDMA-associated toxicity has been investigated in recent years. Recently, caffeine has been found to be lethal when co-administered to rats with MDMA or its metabolite, MDA. Sublethal doses of both MDMA and MDA induce an exaggerated and acute hyperthermia, and 5-HT loss when administered with repeated doses; these effects are potentiated by caffeine (McNamara et al., 2006). For that reason, it is essential to fully understand the interaction between caffeine and MDMA especially if these findings can be extended to humans. In fact, one of the major features of MDMA use in humans and administration in rodents is hyperthermia, which is, for recreational users, due to impaired thermogulation and dependent on ambient temperature and on the environment where the drug is consumed. McNamara et al., (2007) have also demonstrated that, in singly-housed animals, MDMA induces hypothermia and bradycardia, an effect that is reversed by co-administration of caffeine. Thus, a dual dopaminergic theory has been proposed, where the physiological effects of MDMA, in singly-housed animals, mainly involve dopamine D2 receptors and can be reversed by caffeine, via activation of a dopamine D1 pathway.
7.2 Mechanisms of the interaction

Overview of the results (see Schematic below)

The aim of the studies, presented in this thesis, was to elucidate the mechanisms mediating the acute interaction between caffeine and MDMA in rats. Two experimental models were used for this purpose: first, a group-housed model, simulating the environment in which recreational users are known to consume Ecstasy (i.e. dancing clubs or crowded environments). Caffeine potentiated MDMA-induced hyperthermia and subsequently, was observed to enhance the behavioural effects of MDMA, the activation of intracellular targets (that is protein phosphorylation and immediate early gene expression), and the effects of MDMA on central dopamine release. Second, a singly-housed model has also been used to confirm the interaction between caffeine and MDMA, and provided advance evidence in support of a dopaminergic mechanism and the role of the dopaminergic receptors subtypes D₁ and D₂. Finally, the experiments have demonstrated that the interaction between MDMA and caffeine is unique due to a co-dependency on the stimulation of both 5-HT and dopaminergic transmission.

As MDMA is known to influence both serotonergic and dopaminergic neurotransmission, this was the initial focus of investigation. Surprisingly, depletion of 5-HT in the brain, although recognised as the main neurotransmitter target of MDMA, did not alter MDMA-induced hyperthermia or its exacerbation by caffeine while catecholamine depletion did. However, further agonist/antagonist experiments confirmed that the involvement of 5-HT in this interaction could not be ruled out. Pretreatment with ketanserin or prazosin blocked MDMA-induced hyperthermia and its exacerbation by caffeine. Furthermore, caffeine induced lethality and hyperthermia when co-administered with D-fenfluramine and D-amphetamine together, but not when co-administered with either of these drugs alone. Similarly, caffeine provoked hyperthermia in animals treated with both apomorphine and DOI but not with either agent alone.
In a second set of studies, the role of caffeine was investigated. As caffeine, at the dose used, antagonises adenosine A_1 receptors, several antagonists were tested but failed to simulate the effects of caffeine on MDMA-induced hyperthermia, even when combined with phosphodiesterase inhibitors. However, co-administration of a PDE-4 inhibitor, rolipram, with a selective A_2A antagonist, SCH 58261, provoked a similar caffeine effect on MDMA. As adenosine receptors are negatively coupled with dopamine receptors, it is possible that caffeine enhances dopaminergic neurotransmission, thereby potentiates MDMA-induced hyperthermia. In addition, PDE inhibition is known to increase the intracellular concentration of cAMP. It was then conceivable that the interaction between caffeine and MDMA may influence postsynaptic cAMP-dependent intracellular changes.

In the striatum, MDMA-induced phosphorylation of CREB was increased by caffeine, while either drug alone or in combination did not have any effects on the phosphorylation of DARPP-32. In the hypothalamus, the phosphorylation of CREB and DARPP-32 were increased only when caffeine and MDMA were co-administered. Furthermore, the present results suggest a downstream intracellular activation of the immediate early gene, c-fos. Pre-treatment with SCH-23390 blocked these intracellular changes induced by MDMA, alone or in combination with caffeine, suggesting mechanisms driven by dopamine D_1 activation. Moreover, this could be due to the difference in body temperature induced by SCH 23390 which promotes hypothermia in MDMA-treated animals. The role of dopamine and especially dopamine D_1 receptors in MDMA-induced behavioral and physiological changes (Benamar et al., 2008; Brown et al., 2007; Green et al., 2005; Hall et al., 2009a), together with the activation of transcription factors and immediate early genes (Acquas et al., 2007; Shirayama et al., 2000) is supported by numerous reports.

Dopamine plays a role in mediating the acute pharmacological effects of both MDMA and caffeine. With regard to its modulating influence, caffeine, by its indirect action on adenosine receptors, increases dopaminergic transmission and increases bodily and mental capacity especially by eliminating fatigue symptoms in humans. In addition, the
reinforcing effect of caffeine associated with adenosine receptors have been observed in animal models (El Yacoubi et al., 2000; Halldner et al., 2004) and suggests that caffeine may lead to behavioural sensitization. Furthermore, sensitization or tolerance, following habitual use of caffeine, could influence the habitual use and response to MDMA or other psychostimulant drugs. In fact, caffeine, cocaine and amphetamine each induce dopamine release in the shell of the nucleus accumbens (Solinas et al., 2002), the main mechanism proposed to be involved in the rewarding and motor-activation effects induced by these psychostimulants (Pontieri et al., 1995; Wise et al., 1987). The mesocortical limbic dopaminergic pathway (containing ventral tegmental area, nucleus accumbens, amygdala and prefrontal cortex) is important in behavioural sensitization and as co-treatment with caffeine and MDMA has been observed to be mediated, at least in part, by dopamine and leads to an increase in p-CREB and/or p-DARPP expression in the striatum and the hypothalamus, it is therefore of interest that caffeine may also influence the reinforcing effects of MDMA. In fact, dopamine D₁ receptors are very important in behavioural changes after psychostimulant treatment and DARPP-32 expression has been found to be increased after acute stimulation with cocaine or methamphetamine (Nishi et al., 2000). CREB phosphorylation and expression of IEGs such as c-fos, jun and delta-fos b have been observed in behaviour-sensitised animals due to D₁ receptors supersensitivity occurring in the nucleus accumbens (Carrie et al., 2003; Nestler, 2002; Nestler et al., 1997).

It is possible that the ability of caffeine to interact with MDMA may generalise to other drugs which increase dopamine transmission in the brain. In certain instances, such drugs are used clinically, including apomorphine; a dopamine agonist used to treat the “on-off” effects associated with L-dopa treatment of Parkinson’s disease, D-amphetamine, a dopamine releaser used in the treatment of attention deficit hyperactivity disorder and narcolepsy, and buproprion, a dopamine reuptake inhibitor used in the treatment of depression and facilitation of smoking cessation. Well-known dopaminergic drugs of abuse such as cocaine can easily be ingested with caffeine and potentially result in increased toxicity. Currently, there is a lack of information regarding the influence of caffeine on the toxicity of such drugs. Generalised mechanisms underlying the ability of caffeine to influence the response and use of other psychostimulants are outlined below:
Figure 7.1 Schematic representation of the MDMA and caffeine interaction. Group housed model: (1) MDMA induces dopamine release and interacts with dopamine D₁ and D₂ receptors which are activating or inhibiting adenylyl cyclase respectively. MDMA stimulates 5-HT release and indirectly interacts with 5-HT₂ receptors, known to increase dopamine release. (2) Downstream, following receptor activation, MDMA leads to an activation of the PKA signalling pathway and induction of the expression of c-fos (3) Co-administration with caffeine antagonises adenosine A₂A receptors, which are physiologically negatively coupled with dopamine D₂ receptors. Therefore, caffeine increases dopaminergic transmission. In addition, by its inhibition of phosphodiesterase 4, caffeine prevents cAMP metabolism and increases the activity of the PKA pathway. Finally, co-administration of caffeine with MDMA increases (4) the phosphorylation of CREB in the striatum and (5) phosphorylation of CREB and DARPP-32 in the hypothalamus. This cascade of reactions signals the convergent inter and intracellular points at which caffeine and MDMA interact and which may account for the profound behavioural, physiological and neurochemical changes characteristic of this drug interaction. Singly-housed
model: (6) MDMA induces dopamine release which activates dopamine D₂ receptors. Co-administration with caffeine switches from a dopamine D₂ to activation/promotion of a dopamine D₁ pathway (7).

Caffeine and cocaine

With the exception of some clinical cases where accidental death happened after co-ingestion of cocaine with caffeine (Caughlin et al., 1993), neither animal nor human studies have reported that co-ingestion or co-administration of caffeine and cocaine is lethal, unless very high doses of both drugs are used (Derlet et al., 1992). However, intoxication with cocaine is one the main cause of drug-related deaths reported by US physicians, due to its cardiovascular toxicity. In fact, cocaine is a very powerful stimulant that can induce myocardial infarction and arrhythmia, a consequence of vasoconstriction of the coronary artery and accelerated atherosclerosis (Phillips et al., 2009). Even if an interaction between caffeine and cocaine has not been fully detailed in human studies, many animal studies have shown a relationship between the effects of caffeine and cocaine, with caffeine acting to potentiate the stimulatory effects of cocaine (Gasior et al., 2000; Schenk et al., 1990). Caffeine also increases the discriminative effects of cocaine by partially substituting the cocaine-discriminative stimulus in rats trained to discriminate cocaine from saline (Gauvin et al., 1989; Harland et al., 1989; Young et al., 1998). In addition, it potentiates the reinforcing effects of cocaine: self-administration of cocaine was induced more rapidly and the cocaine-induced dopamine release was greater compared to non caffeine-treated animals (Horger et al., 1991; Kuzmin et al., 2000; Schenk et al., 1999; Schenk et al., 1994). Self et al., (1996) showed that pretreatment with caffeine reinstated extinguished cocaine self administration, emphasising the fact that caffeine could reinstore drug seeking behavior even after a long period of abstinence. While Budney et al., in 1993, showed that, among cocaine abusers, daily caffeine intake is higher than among persons without any drug abuse history, Liguori et al., (1997) demonstrated that caffeine did not produce a cocaine like effect and did not increase the desire for cocaine in ex-coffee consumers. They also demonstrated however that oral caffeine increases cocaine-appropriate responding in humans trained to discriminate cocaine. In conclusion, as the acute cardiotoxic effects of caffeine are well known with an acute rise in blood pressure, increase in arterial stiffness and impaired endothelium dependent vasodilatation (Riksen et al., 2009), the co-
ingestion of cocaine and caffeine should be taken seriously as a potential threat particularly to the cardiovascular system.

**Caffeine and amphetamine**

Similar to cocaine in its effects, amphetamine is a longer-lasting psychostimulant which induces mainly dopamine release (Kuczenski *et al*., 1997a; Kuczenski *et al*., 1997b; Kuczenski *et al*., 1997c; Rothman *et al*., 2006; Sulzer *et al*., 1995; Zolkowska *et al*., 2006) but also interacts with serotonin and noradrenaline (Florin *et al*., 1994; Jones *et al*., 1999; Rothman *et al*., 2001). Amphetamine is known to increase wakefulness and diminish appetite and fatigue in humans, and is also widely used in the athletic domain to increase the tolerance to anaerobic metabolism (Docherty, 2008). Long term abuse of amphetamine has been shown to induce profound behavioral changes, hypertension and nerve damage in humans (George, 2000). Most of the studies performed in rodents demonstrate that caffeine potentiates amphetamine-induced locomotor activity (Cauli *et al*., 2003; Celik *et al*., 2006; Palmatier *et al*., 2003; Simola *et al*., 2006). In 2006, researchers from the University of Florida showed that some high energy drinks exceeded the maximum recommended limit of caffeine, which could be a risk factor for vulnerable individuals and inducing important serious health effects such as anxiety and heart palpitation (McCusker *et al*., 2006). It is possible that such drinks, sometimes described as “highly vitalizing”, resulting in increased metabolism and performance enhancement, may cause several health disorders when taken with amphetamine, especially following long-term consumption.

**Caffeine and some clinically used drugs examples.**

Apomorphine is an example of a dopamine agonist used as a treatment in both Parkinson’s and Alzheimer’s disease due to its dopaminergic stimulant properties in the brain. Studies performed here with apomorphine showed that caffeine did not influence the physiological effects of this drug. However, when co-administered with a 5-HT₂ receptor agonist, caffeine reduced the increased heart rate associated with both drugs and attenuated their hypothermic response. This further supports a role not only for
dopamine but also for 5-HT, in the mechanism of action of caffeine. Although D-amphetamine has been misused as a recreational stimulant drug, it has been employed for many clinical applications including the treatment of attention deficit hyperactivity disorder, depression and obesity. In a similar way to apomorphine however, caffeine does not seem to influence the physiological effects of this drug unless it is co-administered with a 5-HT releasing agent. Caffeine can easily be ingested with either recreational stimulants or clinically used therapeutic drugs that interact with the dopaminergic and/or 5-HT neurotransmitter systems in the brain, and could therefore increase their toxicity and safety in humans.

7.3 Limitations of the study

The role of dopamine has been profoundly investigated in this thesis but several findings indicate that 5-HT is also a key player in this MDMA and caffeine interaction as suggested by the DOI/apomorphine and D-amphetamine/D-fenfluramine studies and the ketanserin pre-treatment in individually housed animals. While the role of 5-HT2 receptors has been explored, analysis of the eventual role of 5-HT release in caffeine and MDMA interaction will be of interest. Furthermore, in catecholamine depleted animals, consequences on the behaviours in both MDMA- and MDMA/caffeine- treated animals will be important to further analyse the role of catecholamines in locomotion, stereotypy and 5-HT syndrome related behaviours. Finally, an important question related to tolerance or sensitization developed or not to the effects of caffeine following repeated administration should be addressed. As caffeine is most often consumed on a regular habitual basis, this should confirm if tolerance or sensitization develops to the effects of caffeine following chronic administration. Both tolerance and sensitization to the psychomotor response to caffeine and cross sensitization with other psycho-stimulants have been reported to occur in rats. This experiment is necessary to clarify the effects of chronic exposure to caffeine on MDMA-related changes to body temperature and behaviours.
7.4 Future Studies

Immediate Early genes

MDMA influences immediate early gene expression such as c-fos but also egr-1, egr-3 (Salzmann et al., 2003). The cocaine- and amphetamine-regulated transcript (CART) is also a good marker of cAMP/PKA signaling pathway. Activation of CART is directly associated with psychostimulant addiction (Vicentic et al., 2007). CART modulates mesolimbic dopaminergic transmission and alters psychostimulant associated reinforcing behaviours. Therefore, it would be necessary to investigate the expression of different IEGs to determine the influence of caffeine on MDMA-induced expression changes to pertinent genes of relevance to recreational substance abuse.

As transcriptional regulation of c-fos has been related to Ca\(^{2+}\) intracellular concentrations (Hardingham et al., 1998), it would be of interest to investigate the possible role of Ca\(^{2+}\) in the interaction between caffeine and MDMA. MDMA has previously been shown to interact with protein kinase C (PKC), and a reduction in plasmalemmal and vesicular dopamine transport following administration, was attenuated by the administration of PKC inhibitors (Hansen et al., 2002). Caffeine has also been linked to PKC with a study reporting PKC activity involvement in caffeine-mediated glutamatergic release through A\(_1\) receptors, which thereon affects dopamine release (Wang, 2007). As well as this, dopamine D\(_1\)-type receptor stimulation has been linked to Ca\(^{2+}\)-dependent signalling via G\(_q\)-coupled release of Ca\(^{2+}\) from intracellular stores (Bergson et al., 2003), implicating Ca\(^{2+}\) as another avenue of interest, regard intracellular mechanisms.

Repeated administration effects

The studies here were carried out after a single administration of either caffeine or MDMA. However, caffeine can be consumed on a daily basis by humans and long-term exposure could sensitise recreational abusers to the effects of MDMA. After repeated doses of MDMA, cardiotoxicity (Badon et al., 2002b), decreased social interaction (Clemens et al., 2007), increase locomotor activity and decrease attention (Piper et al., 2005) have been reported in animal studies. Behavioural and physiological effects of
General Discussion

caffeine have also been reported to change following repeated administration. It would be therefore be interesting to investigate whether chronic caffeine administration would alter the behavioural, physiological and intracellular effects observed following acute or long-term MDMA treatment. Sensitization or tolerance following habitual use of caffeine could enhance or reduce the interactions observed with MDMA.

Dopamine / 5-HT release

As the role of adenosine $A_1$ receptors have been explored in MDMA- and caffeine-induced dopamine release, it would be of interest to investigate the role of other receptors such as $A_{2A}$ receptors which have been shown to play a central role in the effects of caffeine in group-housed animals. Moreover, MDMA acts indirectly on both dopaminergic $D_1$ and $D_2$ receptors. Their activation could be associated with changes in the availability of extracellular dopamine. In addition caffeine, by its action on both adenosine $A_1$ and $A_{2A}$ receptors may influence both $D_1$ and $D_2$ receptors (as they are negatively regulate by adenosineric receptors) and their synergistic actions could lead to intracellular, cellular, physiological and behavioural interactions.

Another aspect of MDMA is its ability to induce 5-HT release and since the contribution of this neurotransmitter has been observed in the MDMA and caffeine interaction, the release of presynaptic 5-HT following MDMA and caffeine treatment could also be explored in the striatum and the hypothalamus.

7.5 Summary of the results and conclusion

The exclusive interaction between caffeine and MDMA is a very serious issue particularly in light of the unpredictable effects of Ecstasy in users. The results of this thesis elucidate how the combination with caffeine greatly increases the toxicity of this substituted amphetamine as measured by lethality, increased body temperature and increased heart rate and included the participation of dopamine release and dopamine receptor activation, phosphodiesterase inhibition and antagonism of adenosine receptors. These pharmacological targets may represent novel therapeutic strategies for patients presenting with acute ecstasy intoxication.
APPENDICES
Prior administration of the D₁ receptor antagonist SCH-23390 (1mg/kg) blocks the interaction between caffeine and D-fenfluramine with D-amphetamine. There was no difference in body temperature between the groups at T0 prior to challenge. N = 8 animals per group. Values represent mean with standard error of the mean. * P < 0.05 vs. Vehicle Control; + P < 0.01 vs. D-amphetamine/D-fenfluramine treated group.
Pretreatment with sulpiride (70mg/kg) promotes hyperthermia in response to MDMA administration alone. The ability of caffeine to potentiate MDMA-induced hyperthermia is not apparent. Values represent mean with standard error of the mean change from baseline of 5-6 animals. Baselines of 1 hour points average prior any drug challenge were as follows: Vehicle + Vehicle, 36.9 ± 0.2 °C; Caffeine + Vehicle, 36.9 ± 0.1 °C; Vehicle + MDMA, 36.6 ± 0.1 °C; Caffeine + MDMA, 36.7 ± 0.14 °C. Prior to drug challenge there was no baseline difference found in body temperature between the treatment groups.
Pre-treatment with prazosin failed to influence the ability of caffeine to attenuate MDMA-induced hypothermia. There was no difference in body temperature between the groups at T0 prior to challenge. Values represent mean of 7-8 rats with standard error of the mean. *P<0.01 vs. Vehicle control, + P<0.01 vs. Vehicle + MDMA.
DPCPX (30 μM) potentiates MDMA (30 μM)-induced [³H] dopamine release from striatal tissue slices. Values represent mean with standard error of the mean. +P<0.05 vs. Vehicle + MDMA. N= 6 animals per group.
**DPCPX (30 μM) attenuates MDMA (30 μM)-induced [³H] dopamine release from hypothalamic tissue slices.** Values represent mean with standard error of the mean. +P<0.05 vs. Vehicle + MDMA. N= 6 animals per group.
Caffeine potentiates locomotor behaviour induced by the combination D-amphetamine/D-fenfluramine. There was no difference in body temperature between the groups at T0 prior to challenge. N = 8 animals per group. Values represent mean with standard error of the mean. *P < 0.001 vs. Vehicle Control; +P < 0.001 vs. Vehicle + D-amphetamine/D-fenfluramine.
Caffeine potentiates stereotyped behaviour induced by the combination D-amphetamine/D-fenfluramine. There was no difference in body temperature between the groups at T0 prior to challenge. N = 8 animals per group. Values represent mean with standard error of the mean. *P < 0.001 vs. Vehicle Control; +P < 0.001 vs. Vehicle + D-amphetamine/D-fenfluramine.
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250


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PUBLICATIONS
&
PRESENTATIONS


Vanattou-Saïfoudine N and Harkin A. (2010). Caffeine promotes a switch from dopamine D2 to D1 receptor mediated heart rate, body temperature and behavioural responses to MDMA (“Ecstasy”) administration to rats. Accepted in Psychopharmacology (April 2010).


PRESENTATIONS


N. Vanattou-Saïfoudine, A. Harkin (2008) A lethal combination of caffeine and MDMA (“Ecstasy”) is reproduced with D-amphetamine and D-fenfluramine but not with D-amphetamine or D-fenfluramine alone. Published in Journal of Psychopharmacology 22(5); A65, TF05. Poster presentation at the British Association for Psychopharmacology Summer Meeting, Harrogate, United Kingdom, July 2008.
